Systems Biology Perspectives on Calcium Signaling and DNA Repair

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Abstract

This work deals with two paradigms of cellular signaling: a change in the intracellular Ca\(^{2+}\) concentration represents often one of the first steps in the transduction of incoming stimuli; the ability to properly respond to external injuries and repair damaged DNA is an essential component for life. The approaches used to investigate Ca\(^{2+}\) signaling and DNA repair bear common points. Mathematical models are used to assess system properties, such as efficiency in the signal processing, specificity of the response, and design principles behind the different pathways. A close collaboration with experimentalist made it possible to directly compare model predictions and experimental data, and to test different mechanistic hypothesis.

The first part of this thesis focuses on the phosphoinositide-dependent Ca\(^{2+}\) signaling. Hormones that act through the Ca\(^{2+}\) releasing messenger, inositol 1,4,5-trisphosphate (IP\(_3\)), cause intracellular Ca\(^{2+}\) oscillations, which have been ascribed to Ca\(^{2+}\) feedbacks on the IP\(_3\) receptor. Recent studies have shown that IP\(_3\) levels oscillate together with the cytoplasmic Ca\(^{2+}\) concentration. To investigate the functional significance of this phenomenon, mathematical models of the interaction of both second messengers have been developed. The models account for both positive and negative feedbacks of Ca\(^{2+}\) on the phosphoinositide metabolism, mediated by Ca\(^{2+}\) activation of phospholipase C and IP\(_3\) 3-kinase, respectively. The theoretical analysis shows that each of these Ca\(^{2+}\) feedbacks substantially expands the range of oscillation frequencies of a core oscillator based on Ca\(^{2+}\) and IP\(_3\)R dynamics, compared to Ca\(^{2+}\) fluctuations obtained with clamped IP\(_3\) (Chapter 2). The action of the feedbacks depends on the turnover rate of IP\(_3\). To shape the oscillations, positive feedback requires fast IP\(_3\) turnover, whereas negative feedback requires slow IP\(_3\) turnover. This suggests to perturb the IP\(_3\) turnover in order to study the feedbacks present (Chapter 3): Whereas increasing the IP\(_3\) turnover by overexpressing IP\(_3\) metabolizing enzymes gives no information on the underlying feedbacks, slowing the IP\(_3\) dynamics with an IP\(_3\) binding protein can reveal positive feedback. This theory has been tested in chinese hamster ovary cells by transiently expressing an IP\(_3\) binding protein. The overexpression of this fusion protein exerted a dose-dependent suppression of agonist-induced Ca\(^{2+}\) oscillations that is consistent with an oscillator model including positive feedback of Ca\(^{2+}\) on IP\(_3\) generation. This prominent role of the IP\(_3\) dynamics in modulating Ca\(^{2+}\) oscillations demanded a more deeper analysis of the fate of the IP\(_3\) precursor, phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)).
To this end, a detailed model for the phosphoinositide pathway based on measured metabolite concentrations has been developed (Chapter 4). The model illustrates the importance of futile (de)phosphorylation cycles for regenerating PIP$_2$ during stimulation, an essential property to support long-lasting Ca$^{2+}$ signals. Alternatively, when futile cycling is weak, the presence of positive feedback of Ca$^{2+}$ on the IP$_3$ production also allows to regenerate the PIP$_2$ pool. Taken together, the experimental data and theoretical analysis presented in this thesis indicate that IP$_3$ oscillations are an essential component of the Ca$^{2+}$ oscillator, they are physiologically important for supporting the efficient frequency encoding of hormone dose and the long-lasting Ca$^{2+}$ signals observed in many cell types.

The second part of the thesis is devoted to nucleotide excision repair (NER). It is a versatile DNA repair mechanism that can remove different type of lesions, such as UV light induced pyrimidine dimers and bulky adducts caused by chemical agents. It requires the concerted action of many different proteins that assemble at sites of damaged DNA. Despite our detailed biochemical knowledge about NER, many questions concerning its dynamic behavior remain unanswered, in particular, it is controversial whether repair factors are assembled sequentially or in a random way at the site of damage, or whether they exist as a holocomplex. Therefore, to understand the mechanisms underlying the protein assembly and the performance of repair, a mathematical model, delineating hallmarks and general characteristics of NER, has been developed. First, the binding and dissociation kinetics of repair factors are related to the structural properties of the system, such as the sequential order in which the factors enter repair (Chapter 7). Second, using in vivo kinetic data for the recruitment of three different protein factors at local damaged nuclei, the model parameters are determined and the dynamic behavior of the repair process is scrutinized in detail. The observed saturation of NER is predicted to rely on the high engagement of the recognition factor in repair. Furthermore, the model predicts a quiescent phase, where the amounts of factors involved in repair remains high and constant for a long time period, a forecast that has been validated experimentally. The theoretical analysis of repair performance indicates that a sequential assembly process is remarkably advantageous in terms of repair efficiency and can show a marked selectivity for the damaged substrate (Chapter 8). Alternative mechanisms for repairosome formation, including random assembly and preassembly, can readily become kinetically unfavorable. Based on the model, new experiments are proposed to gain further insight into the early and late steps of this complex process and to critically test model predictions (Chapter 10). Taken together, this thesis provides a kinetic framework for NER and rationalizes why many processes within the nucleus show a sequential assembly strategy.
Zusammenfassung


den beschädigten Substrat sehr vorteilhaft sein kann (Kapitel 8). Alternative Mechanis-
men für die Bildung des Repairosoms, wie z.B. zufällige Bindung oder Preassemblierung,
können schnell kinetisch unvorteilhaft werden. Anhand des Modells wurden neue Exper-
mente vorgeschlagen, um die ersten und letzten Schritte des Reparaturprozesses besser
zu verstehen und Modellvorhersagen kritisch zu testen (Kapitel 10). Diese Arbeit hilft
die kinetischen Randbedingungen für NER zu verstehen und macht ersichtlich, warum
viele Prozesse, die im Zellkern stattfinden, eine sequentielle Strategie verfolgen.
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Part I

Dynamics of Calcium and IP$_3$: Feedbacks, Oscillations, and Futile Cycling
Chapter 1

Introduction to the phosphoinositide and calcium signaling

One of the essential cellular functions is the ability to sense the external environment and respond appropriately to incoming informations. To perform these tasks, the cellular plasma membrane is equipped with a palette of different protein receptors. A signaling cascade can be initiated by the binding of ligand to these receptors and, eventually, the desired response, such as changes in the metabolic activity, expression of specific genes, and secretion of signaling molecules, is obtained.

Over the years many molecular players in the signaling cascades have been identified and the emerging picture is a very dynamic one. It turns out that the information resides both in the timing of the signals as well as in the downstream targets of the receptors. To finely control the time course of the target proteins a series of self-regulatory mechanisms, such as activatory and inhibitory feedbacks, have evolved. Mathematical modeling has proved a useful tool for the quantitative understanding of the dynamics of signaling cascades. It allowed, among other things, to test specific mechanistic hypothesis and experimental protocols, and rationalize why certain pathway features can be (dis)advantageous for the signaling efficiency.

In this part of my thesis, I concentrate on the phosphoinositide-dependent Ca$^{2+}$ signaling, a pathway which is of central importance because many cellular processes are controlled by Ca$^{2+}$ ions and phosphoinositide derivates. In the mid of the eighties, the discovery that the cytoplasmic Ca$^{2+}$ concentration changes periodically [195], was an additional indication that, together with at that time already known glycolytic and
cAMP oscillations [47, 126], biochemical rhythms are a frequent phenomena in life. Since then, oscillations in several signaling pathways have been found (e.g. circadian rhythms, p53 oscillations, mitotic oscillations) and to understand their molecular basis both experimental as well as theoretical approaches have been used (see e.g. [19, 48, 88, 90]).

Below, I will briefly summerize some of the known experimental facts in the phosphoinositide-dependent Ca$^{2+}$ signaling and point towards the still controversial questions after 20 years of research.

1.1 Signal transduction via phosphoinositides and calcium

After binding of ligand to the plasma membrane receptor, and activation of their proximate effector (e.g. the $G_{\alpha}$ subunit of trimeric G-proteins), the next steps in the signaling cascade often involve enzymes which generate lipid-derived second messengers. This relays the information arriving at the plasma membrane into the inner of the cell.

Phosphoinositides are undoubtedly one the most prominent source for lipid-derived second messengers, they comprise a family of eight species, which differ in their combination of phosphate groups at the inositol ring. Among these phosphatidylinositol-4,5-bisphosphate (PIP$_2$) represents a focal point in phosphoinositide-dependent signaling [31, 188]. PIP$_2$ itself is involved in the regulation of the actin cytoskeleton and membrane trafficking [77, 105]. Its phosphorylation by phosphatidylinositol 3-kinase generates phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$), a lipid which is responsible for the recruitment and eventually activation of a variety of PH-domain containing proteins at the inner surface of the cell membrane. These proteins have been found to be involved in cytoskeleton remodeling, mitogenesis, cell growth, and survival [176, 178]. The hydrolysis of PIP$_2$ by phospholipase C (PLC) produces two important second messengers inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). The former is a potent activator of intracellular Ca$^{2+}$ channels, the IP$_3$-receptors, the latter activates protein kinase C (PKC). A quick look through any physiology book show that both Ca$^{2+}$ and PKC are involved in several cellular processes such as secretion of neurotransmitters, activation of transcription factors, and energy metabolism. Ca$^{2+}$ can bind directly to proteins and so regulate their activity, or act via the protein calmodulin. Calmodulin is found as part of larger proteins or freely diffusing and changes its conformation upon Ca$^{2+}$ binding.
1.2. Mechanisms underlying Ca\(^{2+}\)/IP\(_{3}\) oscillations

The intracellular Ca\(^{2+}\) concentration is tightly regulated by a combination of Ca\(^{2+}\) channels and pumps. In a resting cell, the Ca\(^{2+}\)-ATPases in the ER and plasma membrane keep the Ca\(^{2+}\) concentration in the cytoplasm low (∼100 nM), compared to the concentration in the ER (∼20 µM) and the Ca\(^{2+}\) concentration outside the cell (∼1 mM). Binding of agonist to its receptor at the plasma membrane activates PLC, which produces IP\(_{3}\). This messenger in turn activates the IP\(_{3}\)-receptor Ca\(^{2+}\) channels (IP\(_{3}\)R) located in the ER membrane and causes their opening and an outflow of Ca\(^{2+}\) into the cytoplasm. The increase in cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{c}\)) further activates the IP\(_{3}\)R, a phenomenon called calcium induced calcium release (CICR). This leads to an explosive rise of [Ca\(^{2+}\)]\(_{c}\) to values up to ∼1 µM.

In a multitude of cell types, one does not observe a simple increase of [Ca\(^{2+}\)]\(_{c}\) to a new steady state but a sequence of Ca\(^{2+}\) spikes, or Ca\(^{2+}\) oscillations. The strength of the extracellular stimulus is encoded primarily in the frequency of the Ca\(^{2+}\) oscillations, which increases with the degree of stimulation. For example, in rat hepatocytes, the periods of Ca\(^{2+}\) oscillations range over one order of magnitude, from above 250 s for low concentrations of hormones, such as vasopressin and noradrenalin, to about 30 s for higher hormone doses [138]. Ca\(^{2+}\) signals usually originate from a specific site in the cell and propagate through the cell as intracellular Ca\(^{2+}\) waves [9, 86, 139]. The signals can also propagate between neighboring cells, these intercellular Ca\(^{2+}\) waves have been observed in several cell preparations, which maintain a certain functional integrity. The propagation can rely on the secretion of extracellular messengers/hormones as well as on the diffusion of messengers via gap junctions (see e.g. [118, 121, 122, 137, 172, 202]).

1.2 Mechanisms underlying Ca\(^{2+}\)/IP\(_{3}\) oscillations

A long-standing question has been whether the oscillations are generated by the cellular Ca\(^{2+}\) transporters and channels themselves or whether they originate upstream in the signal transduction machinery, between hormone binding to its receptor and the activation of Ca\(^{2+}\) fluxes. It has been proposed that the periodic release of Ca\(^{2+}\) ions from the endoplasmic reticulum (ER) can be brought about through the regulatory properties of the IP\(_{3}\) receptor (IP\(_{3}\)R), the main type of ER calcium release channel in non-excitable cells [10, 51, 101, 203]. Mathematical models have demonstrated how fast activation and delayed inhibition of the IP\(_{3}\)R by cytoplasmic Ca\(^{2+}\) can drive repetitive Ca\(^{2+}\) spiking [36, 80, 144]. In these models, IP\(_{3}\) is required to initially open the IP\(_{3}\)R and sensitize the channel toward feedback activation by cytoplasmic calcium. Therefore, Ca\(^{2+}\) oscillations can occur when IP\(_{3}\) concentration is held at a constant value. However, models
based on a simple description of the IP$_3$R dynamics generally produce [Ca$^{2+}$]$_c$ oscillations with short periods (≈ 10-60 sec) and, thus do not reproduce the long inter-spike intervals observed experimentally. Long-period oscillations have been obtained when additional mechanisms, such as the regulation of IP$_3$R by phosphorylation, stochastic gating phenomena or slow calcium buffer, are included [41, 102, 153].

Recently, it has become possible to monitor IP$_3$ changes in intact cells. These experiments have shown that, for some of the agonists used, the IP$_3$ concentration is highly dynamic and can oscillate together with cytoplasmic calcium [61, 112, 171, 201]. This raises the intriguing possibility that a coupled IP$_3$-Ca$^{2+}$ oscillator may generate long-period oscillations and underlie the efficient frequency-encoding of the hormone dose.

The existence of both positive and negative feedbacks of Ca$^{2+}$ on IP$_3$ metabolism could mediate fluctuations in cellular IP$_3$ levels. The production of IP$_3$ is catalyzed by a diverse family of phosphoinositide-specific PLC isoforms [135]. All PLC isoforms are activated by Ca$^{2+}$ ions, although their sensitivities to [Ca$^{2+}$] vary greatly [55, 134]. This feedback can have an important role in Ca$^{2+}$ wave propagation [33, 65, 177, 181]. IP$_3$ is removed by either phosphorylation or dephosphorylation through IP$_3$ 3-kinase (IP$_3$3K) or IP$_3$ 5-phosphatase (IP$_3$5P), respectively. IP$_3$ removal by IP$_3$3K is activated by Ca$^{2+}$ [22, 111, 194]. Moreover, it has also been suggested that protein kinase C (PKC), which is activated by receptor-mediated increases in Ca$^{2+}$ and diacylglycerol, may inhibit IP$_3$ production by inactivating agonist receptors [25, 112].

1.3 Research objectives

It is presently not clear, which of the above described Ca$^{2+}$ feedbacks on the IP$_3$ metabolism influences the Ca$^{2+}$ dynamics, and what effects they will have on the Ca$^{2+}$ oscillations and on the phosphoinositide metabolism as a whole.

Previous mathematical models have shown that IP$_3$-mediated Ca$^{2+}$ release coupled to Ca$^{2+}$-activated PLC can generate oscillations, without any requirement of IP$_3$R regulation by Ca$^{2+}$ [25, 104]. These models have been criticized because in some cell types Ca$^{2+}$ oscillations can also be elicited when the [IP$_3$] is clamped or its non-metabolizable analogs are used [51, 100, 185]. The incorporation of Ca$^{2+}$-activation of PLC into a Ca$^{2+}$ oscillator model based on the above-described IP$_3$R properties has been reported to modulate Ca$^{2+}$ oscillations [28], while the inclusion of IP$_3$3K has been found to have practically no effect [34, 38]. The complete absence, in all these models, of the reactions leading to the IP$_3$ precursor PIP$_2$ does not allow to fully appreciate the implications of
1.3. Research objectives

Ca\textsuperscript{2+} feedbacks on the phosphoinositide metabolism. The few models that have been developed for this pathway [106, 140, 197] omit some of the essential reactions and metabolites. Thus the dynamic view of the whole phosphoinositide metabolism is only partial.

In the present work, I have carried out a systematic modeling study of the interaction between cellular Ca\textsuperscript{2+} transports and phosphoinositide metabolism. In a first model I include the dynamics of IP\textsubscript{3}, Ca\textsuperscript{2+}, and IP\textsubscript{3}R and take into account positive and negative feedback of Ca\textsuperscript{2+} on the IP\textsubscript{3} metabolism. These are mediated by Ca\textsuperscript{2+} activation of IP\textsubscript{3} generation through PLC and Ca\textsuperscript{2+} activation of IP\textsubscript{3} removal by IP\textsubscript{3}3K, respectively. To better understand the role of oscillations in the phosphoinositide metabolism, I developed, based on the measured metabolite concentrations, a detailed model for this pathway and coupled it to the Ca\textsuperscript{2+} dynamics. With these models following questions have been addressed:

(i) Do the Ca\textsuperscript{2+}-dependent feedback mechanisms on the phosphoinositide metabolism serve a physiological role?

(ii) Which requirements have to be met by the Ca\textsuperscript{2+} and the phosphoinositide pathway for these feedbacks to be effective?

(iii) Is there a way to distinguish, experimentally, between the different feedbacks?

(iv) Which are the design principles of the phosphoinositide pathway and how does it respond to activation of PLC and periodic modulation of the Ca\textsuperscript{2+} concentration?

I begin by analyzing the role of Ca\textsuperscript{2+} feedbacks on the phosphoinositide metabolism in modulating the properties of a core oscillator based on Ca\textsuperscript{2+} and IP\textsubscript{3}R dynamics, in particular, under which conditions these feedbacks can modify the frequency encoding of the hormone signal. The model analysis indicated which are the critical parameters that could be modified experimentally in order to identify the type of feedbacks present (Chapter 2). I, therefore, simulated two experimental protocols: The overexpression of IP\textsubscript{3} metabolizing enzymes and the expression of IP\textsubscript{3} binding proteins. The predictions of the latter experiment were compared to experiments in a mammalian cell line (Chapter 3). In the last chapter, I studied the phosphoinositide metabolism in detail and investigated, which reactions control the PIP\textsubscript{2} pool during stimulation, and how the whole metabolism is influenced by Ca\textsuperscript{2+} feedbacks (Chapter 4).

This theoretical work has been done in close collaboration with the group of Prof. A.-P. Thomas and Prof. L.D. Gaspers (University of Medicine and Dentistry of New Jersey, Newark, USA), which provided the experimental data presented here.
Chapter 2

Ca\textsuperscript{2+} feedbacks on IP\textsubscript{3} improve frequency encoding of the stimulus

2.1 Materials and methods

2.1.1 Mathematical model

The model accounts for the formation and degradation of IP\textsubscript{3}, the main Ca\textsuperscript{2+} fluxes across the ER and plasma membrane, and the IP\textsubscript{3}R dynamics (see Fig. 2.1). It is formulated in terms of four variables: the IP\textsubscript{3} and Ca\textsuperscript{2+} concentration in the cytoplasm, \( p \) and \( c \), respectively; the calcium concentration in the ER stores, \( s \); and the fraction of IP\textsubscript{3}R that have not been inactivated by Ca\textsuperscript{2+}, \( r \).

**IP\textsubscript{3} dynamics:** IP\textsubscript{3} is produced by PLC, whose activity depends on agonist dose and Ca\textsuperscript{2+}. The Ca\textsuperscript{2+} sensitivity of PLC can be modeled by [11]

\[
 v_{PLC} = V_{PLC} \frac{c^2}{K_{PLC}^2 + c^2}.
\]  
(2.1)

The maximal rate \( V_{PLC} \) depends on the agonist concentration, whereas \( K_{PLC} \) characterizes the sensitivity of PLC to Ca\textsuperscript{2+}. IP\textsubscript{3} is removed through dephosphorylation by IP\textsubscript{3} 5-phosphatase (IP\textsubscript{3}5P) and phosphorylation by IP\textsubscript{3} 3-kinase (IP\textsubscript{3}3K) which is modeled as

\[
 v_{deg} = v_{5P} + v_{3K} = \left( k_{5P} + k_{3K} \frac{c^2}{K_{3K}^2 + c^2} \right) p,
\]  
(2.2)

where \( k_{5P} \) and \( k_{3K} \) are the IP\textsubscript{3} dephosphorylation and phosphorylation rate constants, respectively. The Ca\textsuperscript{2+} dependence of the IP\textsubscript{3}3K is described by a Hill function with the...
Figure 2.1: Interactions between Ca\textsuperscript{2+} transport processes and IP\textsubscript{3} metabolism included in the model. The solid and dashed arrows indicate transport/reaction steps and activations, respectively. The bold quantities indicate the model variables: IP\textsubscript{3}, cytoplasmic IP\textsubscript{3}; Ca(cyt), free cytoplasmic Ca\textsuperscript{2+}; Ca(ER), free Ca\textsuperscript{2+} in the ER; IP\textsubscript{3}R\textsubscript{a}, active conformation of the IP\textsubscript{3}R. The other abbreviations denote: IP\textsubscript{3}R\textsubscript{i}, inactive conformation of the IP\textsubscript{3}R; \(v_{rel}\), rate of Ca\textsuperscript{2+} release through the IP\textsubscript{3}R; \(v_{serca}\), active Ca\textsuperscript{2+} transport into the ER; \(v_{inac}\) and \(v_{rec}\) rate of Ca\textsuperscript{2+} induced IP\textsubscript{3}R inactivation and recovery rate, respectively; \(v_{PLC}\), production rate of IP\textsubscript{3}; \(v_{5P}\) and \(v_{3K}\) rates of IP\textsubscript{3} dephosphorylation and phosphorylation, respectively; \(v_{in}\) and \(v_{out}\), rates of Ca\textsuperscript{2+} influx and extrusion across the plasma membrane, respectively.

Half-saturation constant \(K_{3K}\) \cite{22}. According to Fink et al. \cite{43} and Sims and Albritton \cite{150} one can assume that the two enzymes are not saturated with IP\textsubscript{3}, justifying the linear rate law.

For the purpose of the subsequent analysis, we write the balance equation for the IP\textsubscript{3} concentration in the following form

\[
\frac{dp}{dt} = v_{PLC} - v_{deg}
= \frac{1}{\tau_p} \left[ V_{PLC} \frac{c^2}{K_{PLC}^2 + c^2} - \left( \eta \frac{c^2}{K_{3K}^2 + c^2} + (1 - \eta) \right) p \right],
\]

where I introduce the characteristic time of IP\textsubscript{3} turnover

\[
\tau_p = 1/(k_{3K} + k_{5P}),
\]
and the ratio of the maximal IP$_3$K rate to the total maximal degradation rate of IP$_3$

$$\eta = \frac{k_{3K}}{(k_{3K} + k_{3P})}. \quad (2.5)$$

The strength of the positive feedback will be tuned by changing $K_{PLC}$ (the Ca$^{2+}$ sensitivity of PLC), and the strength of the negative feedback will be tuned by changing $\eta$ (the relative expression level of IP$_3$3K). Although both feedbacks can be present simultaneously, it is useful to first analyze them separately. Therefore, I define the "positive-feedback model" in which PLC is sensitive to Ca$^{2+}$ ($K_{PLC} > 0$) and IP$_3$3K is not expressed ($\eta = 0$), and the "negative-feedback model" in which IP$_3$3K is present ($\eta > 0$) and PLC is assumed insensitive to physiological Ca$^{2+}$ changes ($K_{PLC} = 0$). Note that the rescaled maximal PLC activity $V_{PLC} = V_{PLC}\tau_p$ equals the steady-state concentration of IP$_3$ that would be attained in the absence of positive and negative feedbacks.

**IP$_3$R and Ca$^{2+}$ dynamics:** Ca$^{2+}$ release flux through the IP$_3$R is modeled according to Li and Rinzel [91]

$$v_{rel} = \left( k_1 \left( r \frac{c}{K_a + c} \frac{p}{K_p + p} \right)^3 + k_2 \right) (s - c). \quad (2.6)$$

The model by Li and Rinzel is a simplification of the De Young and Keizer model which considered eight different states for each IP$_3$R subunit [28]. The cubic term represents the open probability of the IP$_3$R, which is assumed to open when 3 or 4 subunits of the tetrameric IP$_3$R are in the gating state. The gating state is attained when IP$_3$ and Ca$^{2+}$ bind to their respective activating binding sites, which accounts for the sensitization by IP$_3$ and calcium induced calcium release (CICR), and the inactivating binding site is devoid of Ca$^{2+}$. Whereas the first two events are assumed to be fast, allowing for a quasi-steady state approximation, the inhibition occurs on a slower time scale. The fraction of IP$_3$Rs that have not yet been inactivated by Ca$^{2+}$ follows

$$\frac{dr}{dt} = v_{rec} - v_{inac} = \frac{1}{\tau_r} \left( 1 - r \frac{K_i + c}{K_i} \right) \quad (2.7)$$

where $\tau_r = 1/k_{rec}$ gives the characteristic time of receptor recovery after inactivation.

The rate equation for active transport of Ca$^{2+}$ across the ER membrane by the sarco endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) follows Lytton et al. [97]

$$v_{serca} = V_{serca} \frac{c^2}{K_{serca}^2 + c^2}. \quad (2.8)$$
Chapter 2. Frequency encoding of the stimulus

The rate equation for the active transport of Ca$^{2+}$ by the plasma membrane Ca$^{2+}$-ATPase (PMCA) follows Camello et al. [15]

$$v_{out} = \varepsilon V_{pm} \frac{c^2}{K_{pm}^2 + c^2}.$$  

The calcium influx includes a leak into the cell and a stimulation dependent influx which is assumed proportional to the maximal PLC activity

$$v_{in} = \varepsilon (v_0 + \phi V_{PLC}).$$

The dimensionless parameter $\varepsilon$ measures the relative strength of the plasma membrane fluxes, which is known to be cell-type specific. With these rate expressions the balance equation for cytoplasmic Ca$^{2+}$ reads

$$\frac{dc}{dt} = v_{rel} - v_{serca} + v_{in} - v_{out}$$

$$= \left( k_1 \left( r \frac{c}{K_a + c K_p + p} \right)^3 + k_2 \right) (s - c) - V_{serca} \frac{c^2}{K_{serca}^2 + c^2}$$

$$+ \varepsilon \left( v_0 + \phi V_{PLC} - V_{pm} \frac{c^2}{K_{pm}^2 + c^2} \right).$$  \hspace{1cm} (2.8)

I first carry out the model analysis for the simpler case that the plasma membrane fluxes are negligible. By setting $\varepsilon = 0$ the total Ca$^{2+}$ concentration in the cell is conserved and can be expressed as $c_{tot} = c + \beta s$, where $\beta$ is the ratio of effective cytoplasmic volume to effective ER volume (both accounting for Ca$^{2+}$ buffering). Therefore, I can insert for the ER calcium in Eq. (2.8) $s = (c_{tot} - c)/\beta$. In the presence of plasma-membrane fluxes ($\varepsilon > 0$) this conservation no longer holds and a kinetic equation for $s$ must be added:

$$\frac{ds}{dt} = \frac{1}{\beta} \left[ V_{serca} \frac{c^2}{K_{serca}^2 + c^2} - \left( k_1 \left( r \frac{c}{K_a + c K_p + p} \right)^3 + k_2 \right) (s - c) \right].$$  \hspace{1cm} (2.9)

The meaning and numerical values of the kinetic parameters are given in Tab. 2.1. The half-activation constant of PLC and IP$_3$3K were taken from [11] and [22], respectively. The IP$_3$ degradation rate constants were chosen in accordance to [43, 150]. In the positive-feedback model (Ca$^{2+}$ activation of PLC), the parameters for the Ca$^{2+}$ transport processes and the IP$_3$R were taken from [91]. In the negative-feedback model (Ca$^{2+}$ activation of IP$_3$3K), no substantial effect of the IP$_3$3K on the oscillations is obtained with these parameters. However, for different parameters (as given) the IP$_3$3K effects
2.1. Materials and methods

Reference parameter values

<table>
<thead>
<tr>
<th></th>
<th>Positive feedback</th>
<th>Negative feedback</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IP&lt;sub&gt;3&lt;/sub&gt; dynamics parameters:</strong></td>
<td></td>
<td></td>
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<tr>
<td>Half-activation constant of IP&lt;sub&gt;3&lt;/sub&gt;K, $K_{3K}$</td>
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<tr>
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<tr>
<td>Half-activation constant of PLC, $K_{PLC}$</td>
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<tr>
<td><strong>Ca&lt;sup&gt;2+&lt;/sup&gt; transport and structural parameters:</strong></td>
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<tr>
<td>Ratio of effective volumes ER/cytosol, $\beta$</td>
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<td>Maximal SERCA pump rate, $V_{serca}$</td>
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<td>Half-activation constant, $K_{serca}$</td>
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<tr>
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<td>Total Ca&lt;sup&gt;2+&lt;/sup&gt; concentration (for $\varepsilon = 0$), $c_{tot}$</td>
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<td><strong>IP&lt;sub&gt;3&lt;/sub&gt;R parameters:</strong></td>
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<td></td>
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<td>Maximal rate of Ca&lt;sup&gt;2+&lt;/sup&gt; release, $k_1$</td>
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Table 2.1: The table gives the reference parameter set. Changes are indicated in the respective figure legends. The maximal rate of PLC, $V_{PLC}$, is taken as the stimulation-dependent control parameter.
were pronounced. The differences in the Ca\(^{2+}\) fluxes between the two models can be accounted for by variations in the expression of the involved proteins. The differences in the Ca\(^{2+}\) binding properties to the activating site of the IP\(_3\)R can be due to differences in the expression of IP\(_3\)R subtypes, with IP\(_3\)R subtype 1 having a higher Ca\(^{2+}\) dissociation constant for the activating site than subtypes 2 and 3 [99, 129]. Numerical solutions of the differential equations system Eqs. (2.3), (2.7)–(2.9) were obtained using a 4th order Runge-Kutta algorithm implemented in XPPAUT (http://www.math.pitt.edu/bard/xpp/xpp.html). Bifurcation analysis were done using the program AUTO2000 [29]. Calcium waves with the local kinetics given by Eqs. (2.3), and (2.7)–(2.9) and diffusion of Ca\(^{2+}\) and IP\(_3\) in the cytoplasm were calculated numerically with a finite-difference Crank-Nicholson scheme (see Appendix F). Wavespeeds for solitary waves were evaluated using AUTO2000.

### 2.1.2 Experimental procedures

**Cell culture:** Chinese Hamster Ovary cells (CHO) were cultured in Ham’s F-12 media supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were seeded onto poly-D-lysine coated glass coverslips (25 mm) and maintained in culture until 70-80% confluent prior to experimental protocols.

**Plasmid construction and transfection protocols:** The cDNA encoding 620 amino acids of the N-terminal rat type 1 IP\(_3\) receptor [13] was ligated in-frame to the C-terminus of the enhanced green fluorescent protein gene in the plasmid pEGFP-C1 (Clontech, Palo Alto CA, USA) to generate the plasmid pEGFP-LBD. Cell cultures were transiently transfected with either pEGFP-LBD (EGFP-LBD) or pEGFP-C1 (EGFP) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocols. Agonist-evoked [Ca\(^{2+}\)]\(_c\) responses were recorded in transfected cultures following a 16-48 hour incubation period.

**Imaging measurements of [Ca\(^{2+}\)]\(_c\) and fluorescent proteins:** Calcium imaging experiments were performed in an HEPES-buffered physiological saline solution (HBSS) comprised of (in mM): 25 HEPES (pH 7.4 at 37 °C), 121 NaCl, 5 NaHCO\(_3\), 4.7 KCl, 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 2.0 CaCl\(_2\), 10 glucose, 0.1 sulphobromophthalein and 0.25% (w/v) fatty acid-free BSA. Cell cultures were loaded with fura-2/AM by incubation with 5 \(\mu\)M fura-2/AM plus Pluronic F-127 (0.02% v/v) for 20–40 min in HBSS. The cells were washed and transferred to a thermostatically regulated microscope chamber (37
2.2 Frequency encoding and period control

2.2.1 Positive- and negative-feedback models exhibit frequency encoding of agonist dose

In many cell types, one observes a strong dependence of the frequency of Ca\(^{2+}\) oscillations on the dose of the applied receptor agonist, while the oscillation amplitude remains nearly constant [138]. A stepwise increase of the agonist concentration is accompanied by a prompt rise of the oscillation frequency. In the model, the maximal rate of PLC, \(V_{PLC}\), is a measure of agonist concentration. The positive-feedback model, with Ca\(^{2+}\) activation of PLC, and the negative-feedback model, with Ca\(^{2+}\) activation of IP\(_3\)K, were subjected to stepwise increases in \(V_{PLC}\). The responses are shown in Fig. 2.2A, B, respectively, with the time points of increase indicated by arrowheads. Both models exhibit a large range of oscillation frequencies with little change in \([Ca^{2+}]_c\) amplitude (Fig. 2.2A, B; top traces). The pronounced increase in the rate of spiking with increasing stimulus is the hallmark of the experimentally observed frequency encoding. For very large stimuli, a plateau of elevated \([Ca^{2+}]_c\) is reached, again in agreement with experimental data.

The \([Ca^{2+}]_c\) oscillations in both models consist of a series of sharp spikes with baseline interludes (Fig. 2.2A, B). The peak values of \([Ca^{2+}]_c\) and \([IP_3]\) occur nearly at the same time. The shape of the \([IP_3]\) oscillations in the two models is different. In the positive-
Chapter 2. Frequency encoding of the stimulus

Figure 2.2: Agonist-induced IP$_3$ and Ca$^{2+}$ oscillations in the positive- and negative-feedback models. (A) Positive-feedback model with Ca$^{2+}$ activation of PLC. Changes in [Ca$^{2+}$]$_c$, [IP$_3$] and in the fraction of active receptors (upper, middle and lower panels) after stepwise increases in the agonist concentration (arrowheads), modeled by an increase in the maximal rate of PLC ($V_{PLC} = 0.3$ µM/s for $t < 100$ with successive increases to 0.787, 1, 1.5 and 2.5 µM/s). (B) Negative-feedback model with Ca$^{2+}$ activation of IP$_3$3K. The response is shown for a step-protocol with $V_{PLC} = 0.1$ nM/s for $t < 100$, followed by increases to 0.45, 2.5, 5.8 and 10 nM/s. (C) Bifurcation diagram for the positive-feedback model; shown are the maxima and minima of the [Ca$^{2+}$]$_c$ oscillations (thick lines) and the [Ca$^{2+}$]$_c$ steady states (thin lines) as a function of the stimulus ($V_{PLC}$). Solid and dashed lines indicate stable and unstable states, respectively. HB, Hopf bifurcation; HC, homoclinic bifurcation; SN, saddle node bifurcation; FB, saddle node of periodics. (D) Bifurcation diagram for the negative-feedback model. PD, period doubling; TR, torus bifurcation. Between PD and HB$_1$ as well as between TR and FB there exist complex oscillations (omitted for clarity). The parameter values are listed in Table 2.1.

feedback model, [IP$_3$] exhibits baseline-separated spikes (Fig. 2.2A). In contrast, in the negative-feedback model, [IP$_3$] follows a zig-zag pattern: the occurrence of a [Ca$^{2+}$]$_c$ spike leads to an abrupt decrease in [IP$_3$], after which [IP$_3$] slowly builds up again over the whole oscillation period (Fig. 2.2B). The IP$_3$R activities show similar dynamics in
2.2. Frequency encoding and period control

The behavior of the two model systems for different stimulation strengths can be summarized in bifurcation diagrams (Fig. 2.2C, D), where the steady states of $[\text{Ca}^{2+}]_e$ and the maxima and minima of $[\text{Ca}^{2+}]_e$ oscillations as a function of $V_{\text{PLC}}$ have been computed. For very low PLC activity, both models show a stable steady state of low $[\text{Ca}^{2+}]_e$; similarly, an elevated $[\text{Ca}^{2+}]_e$ plateau is reached at relatively high PLC activity (these stable steady states are indicated by thin solid lines). For an intermediate range of $V_{\text{PLC}}$, the steady states are unstable (thin dashed lines). In these regions, both models exhibit oscillations ($[\text{Ca}^{2+}]_e$ maxima and minima in stable oscillations are depicted by thick solid lines). The oscillations arise either via Hopf bifurcations (HB), or, in the case of the positive-feedback model, also by a homoclinic bifurcation (HC). Further bifurcations are indicated and referred to in the figure legend. In particular, a homoclinic bifurcation is associated with the existence of multiple steady states, which arise through saddle-node bifurcations (Fig. 2.2C, SN). Such multistationarity is typical for models that neglect the plasma-membrane fluxes of calcium; this point will be discussed in more detail below. In the negative-feedback model, there are two regions near the Hopf bifurcations HB$_1$ and HB$_2$ (prior to the point PD and after the point TR in Fig. 2.2D) where irregular and bursting oscillations are observed. Because these two regions are extremely narrow compared to the total stimulation range in the negative-feedback model, our focus will be on the regular oscillations.

In the two bifurcation diagrams, one notices that the values required for oscillations are considerably smaller in the negative-feedback model than in the positive-feedback model. This is primarily a consequence of the different feedback mechanisms. First, in the positive-feedback model the actual PLC activity is Ca$^{2+}$-dependent and, therefore, $V_{\text{PLC}}$ is lower than at resting $[\text{Ca}^{2+}]_e$. Second, owing to the calcium activation of IP$_3$3K in the negative-feedback model, the IP$_3$ degradation rate at resting $[\text{Ca}^{2+}]_e$ is much smaller than in the positive-feedback model, requiring a smaller rate of IP$_3$ production to raise the [IP$_3$] and induce oscillations.

2.2.2 The wide range of oscillation periods is due to interactions of IP$_3$ and Ca$^{2+}$ dynamics

To elucidate whether the IP$_3$ dynamics participate in frequency encoding, I compared the oscillation periods in models without any Ca$^{2+}$ feedback on IP$_3$ (resulting in a constant [IP$_3$]) and in the two feedback models (with [IP$_3$] oscillations).

I begin by discussing the positive-feedback model, where the Ca$^{2+}$ activation of
an agonist-dependent PLC is considered. The strength of the positive feedback can be tuned by changing the value of the $\text{Ca}^{2+}$ activation constant, $K_{PLC}$. For $K_{PLC}$ being much lower than the basal $[\text{Ca}^{2+}]_c$, PLC is always saturated with $\text{Ca}^{2+}$ and its activity is independent of variations in $[\text{Ca}^{2+}]_c$. In particular, by setting $K_{PLC} = 0$ positive feedback will effectively be eliminated. This model with constant $[\text{IP}_3]$ shows fast calcium oscillations with a period of 10-15 s (Fig. 2.3A, dashed line). Introducing positive feedback by setting $K_{PLC} > 0$ causes oscillations with long periods at low stimulation. The frequency encoding of the stimulus becomes very pronounced when the sensitivity of PLC to changes in $[\text{Ca}^{2+}]_c$ is just above basal $[\text{Ca}^{2+}]_c$ (Fig. 2.3A, solid lines, $K_{PLC} = 0.1$ and 0.2 $\mu$M).

For the negative-feedback model, I recall that there are two removal pathways for IP$_3$, one catalyzed by the $\text{Ca}^{2+}$-insensitive IP$_3$ 5-phosphatase (IP$_3$5P) and the other by the $\text{Ca}^{2+}$-activated IP$_3$ 3-kinase (IP$_3$3K). The strength of the negative feedback is modified by taking different concentration ratios of IP$_3$5P and IP$_3$3K. The feedback strength is expressed by the ratio of the maximal IP$_3$3K rate to the total degradation rate of IP$_3$; $\eta = k_{3K}/(k_{3K} + k_{5P})$, where $k_{3K} + k_{5P}$ has been kept constant in the following calculations. The oscillation periods in the absence of negative feedback ($\eta = 0$), and therefore constant $[\text{IP}_3]$, are shown in Fig. 2.3B (dashed line). When $\eta$ is sufficiently high, the negative feedback has a pronounced effect on the range of oscillations periods; for approx. $\eta \geq 0.6$ there is an increase in the period range (Fig. 2.3B, solid lines).

Note that the two models without feedback are not identical in their ability to frequency encode the stimulus (compare Fig. 2.3A and B). Simulations indicate that the IP$_3$3K has an impact on the oscillation period only when the $\text{Ca}^{2+}$ fluxes between ER and cytoplasm are comparatively slow and the IP$_3$R is less sensitive to $\text{Ca}^{2+}$ activation. To expose the period effect of the negative feedback, different parameter values than in the positive-feedback model has been chosen (see Tab. 2.1). The differences in sensitivity towards the PLC activity observed in the two graphs have been explained above (Section 2.2.1).

In the positive-feedback model, arbitrarily long periods can be obtained (exceeding the 200 s shown), which are due to the onset of the oscillations via a homoclinic bifurcation (see also Fig. 2.2C). In the model, the homoclinic bifurcation specifically occurs when the plasma-membrane fluxes of $\text{Ca}^{2+}$ are neglected, which is a valid simplification for many cell types in which the contribution of these fluxes to $\text{Ca}^{2+}$ oscillations is small [154]. The more general case when the plasma-membrane $\text{Ca}^{2+}$ fluxes are included has also being studied ($\varepsilon > 0$, see materials and methods Section 2.1). Then there is a unique steady state and the homoclinic bifurcation no longer exists. Nevertheless, long-
2.2. Frequency encoding and period control

Figure 2.3: Frequency encoding of agonist stimulus. (A) Positive feedback. Oscillation periods observed at different stimulation strengths (varying $V_{PLC}$). Increasing the half-saturation constant of PLC for Ca$^{2+}$, $K_{PLC}$, from 0 (no functional positive feedback) to 0.2 µM (functional feedback) greatly enhances frequency encoding. (B) Negative feedback. Increasing the amount of IP$_{3}$K relative to IP$_{3}$5P ($\eta$) enhances frequency encoding. (C, D) The feedback effects shown in A and B are preserved when plasma-membrane fluxes of Ca$^{2+}$ are included ($\varepsilon = 1$). (E) Range of oscillation periods, $\Delta T = T_{\text{max}} - T_{\text{min}}$, in the presence (+) and absence (w/o) of positive feedback for two different strengths of the plasma-membrane Ca$^{2+}$ fluxes ($\varepsilon = 1, 4$). (F) Range of oscillation periods in the presence (−) and absence (w/o) of negative feedback for two different strengths of the plasma-membrane Ca$^{2+}$ fluxes ($\varepsilon = 1, 4$).

period oscillations are present (Fig. 2.3C). Importantly, the dependence of the period range of the oscillations on $K_{PLC}$ remains very similar.

The plasma-membrane fluxes of Ca$^{2+}$ have also been introduced into the negative-feedback model (Fig. 2.3D). One observes a similar picture as without plasma membrane fluxes, provided that the plasma-membrane fluxes are comparatively moderate. How-
ever, when the plasma-membrane Ca\textsuperscript{2+} fluxes are large enough, the effect of IP\textsubscript{3}3K on the oscillation period practically disappears. In contrast, the period behavior in the positive-feedback model is less affected by changes in the magnitude of the plasma-membrane Ca\textsuperscript{2+} fluxes. To show this, I evaluated the range of oscillation periods $\Delta T = T_{\text{max}} - T_{\text{min}}$, where $T_{\text{max}}$ and $T_{\text{min}}$ are the maximal and minimal period that are obtained for low and high stimulation, respectively, for two different strengths of the plasma-membrane Ca\textsuperscript{2+} fluxes ($\varepsilon = 1$ and $\varepsilon = 4$). The positive-feedback model exhibits in both cases a much larger period range than the corresponding model without feedback (Fig. 2.3E). In contrast, the increase of the period range through negative feedback is only seen when the plasma-membrane Ca\textsuperscript{2+} fluxes are comparatively small, $\varepsilon = 1$ (Fig. 2.3F).

In summary, both positive and negative feedbacks of Ca\textsuperscript{2+} on IP\textsubscript{3} may serve a physiological role by greatly enhancing the range of frequency-encoding of the agonist stimulus. The frequency-encoding supported by the positive feedback is more robust against variations in the kinetic parameters of the Ca\textsuperscript{2+} transport processes.

### 2.2.3 The two feedback-models respond differently to changes in the IP\textsubscript{3} turnover time

In the model simulations, I have noticed that the characteristic time of IP\textsubscript{3} turnover $\tau_p$ has a decisive impact on the Ca\textsuperscript{2+}-IP\textsubscript{3} oscillators. The measured IP\textsubscript{3} turnover times span a relatively wide range, from 0.1 to $> 10$ s depending on cell type and experimental conditions [43, 150]. One finds that fast IP\textsubscript{3} turnover ($\tau_p \approx 0.1$–2 s) is associated with long oscillation periods in the positive-feedback model. Conversely, the negative-feedback model exhibits long-period oscillations when the IP\textsubscript{3} turnover is comparatively slow ($\tau_p \approx 10$–15 s).

Insight into the origin of this difference between the two models can be gained by looking at the time courses of the model variables. In the positive feedback model, fast IP\textsubscript{3} turnover ($\tau_p = 0.1$ s) yields high-amplitude oscillations in $[\text{Ca}^{2+}]_c$ and $[\text{IP}_3]$ (Fig. 2.4A left panel, solid and dashed lines, respectively). $[\text{Ca}^{2+}]_c$ and $[\text{IP}_3]$ rise simultaneously, and IP\textsubscript{3}-induced Ca\textsuperscript{2+} release and Ca\textsuperscript{2+}-activated IP\textsubscript{3} production coincide. After termination of the $[\text{Ca}^{2+}]_c$ spike, $[\text{IP}_3]$ returns quickly to a basal level, because Ca\textsuperscript{2+}-activated IP\textsubscript{3} production has ceased and IP\textsubscript{3} degradation is fast. Also, the IP\textsubscript{3}R close efficiently after the spike (Fig. 2.4A, dotted line showing the fraction of open IP\textsubscript{3}R). For slow IP\textsubscript{3} turnover ($\tau_p = 15$ s) $[\text{IP}_3]$ does not sufficiently decline after the $[\text{Ca}^{2+}]_c$ spike, leading to an increased basal opening of the IP\textsubscript{3}R, lower ER Ca\textsuperscript{2+} store loading and, consequently, much less pronounced $[\text{Ca}^{2+}]_c$ spikes (Fig. 2.4A, right panel).
Figure 2.4: IP$_3$ turnover time controls feedbacks. (A) Positive-feedback model. Dynamics of [Ca$^{2+}$]$_c$, [IP$_3$] and the fraction of open IP$_3$R (solid, dashed and dotted lines, respectively) during an oscillation period; the fraction of open IP$_3$R is given by $r_c p/(c + K_c(p + K_p))^3$ (see Eq. (2.6)). Fast IP$_3$ turnover yields a pronounced spike (left panel, $\tau_p = 0.1$ s), while slow IP$_3$ turnover supports only a small-amplitude response (right panel, $\tau_p = 15$ s). (B) The negative-feedback model shows the opposite behavior, a small-amplitude response for fast IP$_3$ turnover (left panel, $\tau_p = 0.1$ s) and a sharp spike for slow IP$_3$ turnover (right panel, $\tau_p = 15$ s). (C) Positive-feedback model. Bifurcation diagram showing the maxima and minima of the [Ca$^{2+}$]$_c$ oscillations as a function of the stimulus for different values of the IP$_3$ turnover. The bifurcation diagrams for different values of $\tau_p$ are compared by plotting them against the product $\overline{V}_{PLC} = V_{PLC} \tau_p$; in this way, the steady-state concentrations of Ca$^{2+}$ and IP$_3$ are identical for a given $\overline{V}_{PLC}$ (solid and dashed lines indicate stable and unstable states respectively; the stability of the steady state is shown for $\tau_p = 15$ s). Both amplitude and range of stimuli leading to oscillations increase with faster IP$_3$ turnover. (D) The corresponding bifurcation diagrams for the negative-feedback model show the opposite behavior. The amplitude and range of stimuli leading to oscillations increase with slow IP$_3$ turnover. (E, F) Dependence of frequency encoding on IP$_3$ turnover in the positive- and negative-feedback models, respectively. $T_{max}$ and $T_{min}$ are the largest (for low stimulation) and smallest (for high stimulation) oscillation period.
Not only the spike characteristics but also the oscillation period and the range of agonist stimuli that give oscillations are affected by the IP$_3$ half-life. For fast IP$_3$ turnover, oscillations in the positive-feedback model are observed over a wide range of stimuli (Fig. 2.4C, $\tau_p = 0.1$ s). Slower IP$_3$ turnover leads to reduced oscillation ranges and also much smaller amplitudes (Fig. 2.4C, $\tau_p = 5$ and 15 s). Importantly, also the capacity for frequency encoding of the stimulus, as measured by the period range of oscillations, is high when the IP$_3$ turnover is fast (Fig. 2.4E).

In the negative-feedback model, changing the time scale of IP$_3$ turnover has the opposite effect. When IP$_3$ turnover is fast ($\tau_p = 0.1$ s), a rise in $[\text{Ca}^{2+}]_c$ triggers, via activation of IP$_3$3K, a pronounced decrease in $[\text{IP}_3]$, which in turn limits further Ca$^{2+}$ release (Fig. 2.4B, left panel). Therefore, the $[\text{Ca}^{2+}]_c$ spikes are relatively small and $[\text{IP}_3]$ shows strong variations (solid and dashed lines, respectively). When the IP$_3$ lifetime is larger ($\tau_p = 15$ s), $[\text{IP}_3]$ remains at a relatively high level throughout and the $[\text{Ca}^{2+}]_c$ spikes are accordingly more pronounced (Fig. 2.4B, right panel). Moreover, for slow IP$_3$ degradation, the range of stimuli where oscillations occur is larger (Fig. 2.4D). The capacity for frequency encoding as measured by the range of oscillation periods, $\Delta T$, strongly increases with the IP$_3$ half-life (Fig. 2.4F). This finding agrees with the frequent observation that negative feedback is more prone to oscillate when the controlled variable (here IP$_3$) responds slowly.

To summarize, frequency encoding in the two feedback models poses opposite requirements on IP$_3$ turnover: positive and negative feedbacks are efficient frequency modulators when the IP$_3$ turnover is fast and slow, respectively. The critical IP$_3$ lifetimes estimated in the model indicate that both cases could be realized physiologically.
2.2.4 Period control is shared by all processes

The calculations have shown that the inclusion of IP$_3$ dynamics strongly alters the frequency properties of the oscillator and, particularly, leads to long-period oscillations. I have therefore quantified the control of the IP$_3$ dynamics and the other processes present in the model on the oscillation period. To this end, the following sensitivity measure has been used

$$C_i = \frac{\tau_i}{T} \frac{\partial T}{\partial \tau_i} \quad \text{for } i = er, pm, p, r \quad (2.10)$$

which is referred to as period control coefficients (see also [192]). The $C_i$ set the change of the oscillation period $T$ in proportion to the change in the characteristic time $\tau_i$ of an individual process $i$. I analyzed the control of the following processes: IP$_3$ metabolism (with $\tau_p$ as defined in Eq. (2.4)), the IP$_3$R dynamics (with $\tau_r$ as defined in Eq. (2.7)), Ca$^{2+}$ transport across the ER membrane (achieved by scaling $v_{rel} - v_{serca}$ with $1/\tau_{er} = V_{serca}/K_{serca}$ in Eqs. (2.8) and (2.9)), and Ca$^{2+}$ transport across the plasma membrane (achieved by scaling $v_{in} - v_{out}$ with $1/\tau_{pm} = \varepsilon V_{pm}/K_{pm}$ in Eq. (2.8)). A positive period control coefficient implies that a slowing of the respective process (i.e. increase in $\tau_i$) raises the period. At any point, the period control coefficients sum up to unity, $C_p + C_r + C_{er} + C_{pm} = 1$, so that each coefficient quantifies the relative contribution of a single process to the oscillation period [57].

The control coefficients were calculated for various levels of stimulation in the positive and negative feedback models. Because these levels correspond to different oscillation periods, we can plot the $C_i$ against the period. Fig. 2.5A, B depict the result for the model without plasma-membrane fluxes of Ca$^{2+}$ ($\varepsilon = 0$). The two feedback models yield a similar picture. The control is distributed between the dynamics of IP$_3$, Ca$^{2+}$ and IP$_3$R. In long-period oscillations, the IP$_3$ turnover has the leading control (dot-dashed lines). The IP$_3$R dynamics contributes more significantly to setting the period of fast oscillations, especially in the positive-feedback model (dotted lines).

The distribution of control becomes more complex when the plasma-membrane fluxes of Ca$^{2+}$ are included (Fig. 2.5C, D; $\varepsilon = 1$), although, interestingly, the plasma-membrane fluxes exert very little period control themselves (dashed lines). There are several notable features. First, the fast oscillations in the positive-feedback model are no longer dominated by the IP$_3$R dynamics. There is even a rather counter-intuitive behavior at intermediate periods where acceleration of the IP$_3$R dynamics would result in a slowing of the oscillations ($C_r < 0$). Second, the overall tendency that the IP$_3$ dynamics are more relevant for slow oscillations is preserved. However, the dynamics of ER Ca$^{2+}$ release and refilling now play a more pronounced role in setting the period (solid lines).
Figure 2.5: Control coefficients for the oscillation period. (A, B) Positive- and negative-feedback models, respectively, in the absence of Ca\(^{2+}\) fluxes across the plasma membrane (\(\varepsilon = 0\)); control coefficients of Ca\(^{2+}\) exchange across the ER membrane (\(C_{er}\), solid line), IP\(_3\) metabolism (\(C_p\), dash-dotted line) and IP\(_3\)R dynamics (\(C_r\), dotted line) as function of the period of the oscillations. A positive period control coefficient signifies that a slowing of the corresponding process increases the oscillation period. (C, D) show the period control coefficients in the positive- and negative-feedback models, respectively, in the presence of plasma-membrane fluxes of Ca\(^{2+}\) (\(\varepsilon = 1\)). The dashed line indicates the control exerted by Ca\(^{2+}\) exchange across the plasma membrane (\(C_{pm}\)).

This quantification of period control reveals that no process can be singled out as a unique period controlling factor. Depending on the oscillation mechanism and the reference period, the IP\(_3\) turnover, the ER Ca\(^{2+}\) fluxes and the IP\(_3\)R dynamics can all exert strong control.
Chapter 3

How to distinguish Ca$^{2+}$ feedbacks on IP$_3$ metabolism

The previous analysis has shown that oscillation mechanisms involving Ca$^{2+}$-activated PLC or IP$_3$3K are sensitive to the time scale of IP$_3$ turnover. This offers the possibility to experimentally interfere with the oscillation mechanism by perturbing the IP$_3$ turnover. First, I will present the predictions obtained from the mathematical models and then compare them to experiments where an IP$_3$ binding molecule has been overexpressed in a mammalian cell line.

3.1 Model predictions

3.1.1 Accelerating IP$_3$ turnover by overexpressing IP$_3$ metabolizing enzymes

The overexpression of IP$_3$ metabolizing enzymes would accelerate the IP$_3$ turnover and also decrease the [IP$_3$] (see Eqs. (2.3)–(2.5)). The decrease in the concentration of IP$_3$ can then be compensated for by an increase in the agonist dose. I first tested whether an increase in the Ca$^{2+}$-insensitive IP$_3$-metabolizing enzyme, IP$_3$ 5-phosphatase (IP$_3$5P), would interfere with oscillation mechanisms (i) without Ca$^{2+}$ feedback on IP$_3$, (ii) with positive feedback, or (iii) with negative feedback. In the absence Ca$^{2+}$ feedbacks, implying constant [IP$_3$], oscillations can be abolished by elevating IP$_3$5P levels and recovered by an increase in $V_{PLC}$ (agonist dose). I found that the same holds in the presence of Ca$^{2+}$ activated PLC (results not shown). This is in line with the observation that the positive-feedback model functions with fast IP$_3$ dynamics. A similar behavior is
predicted by the negative-feedback model. As shown in Fig. 3.1A, B for two increasing stimulation levels (indicated by arrows), Ca\(^{2+}\) oscillations in the presence of IP_35P are lost for low stimulation and recovered for high stimulation. By simulating a large number of such cases, one finds that the oscillations are generally faster after introducing IP_35P into the model. This is mainly due to the diminished role of IP_33K, which was crucial in setting the long periods, compared to IP_35P (corresponding to a decrease in \(\eta\), Eq. (2.5), see Fig. 2.3). However, this predicted acceleration of the oscillations by IP_35P would be difficult to identify experimentally.

Next I tested the effect of increasing the concentration of IP_33K, which introduces or enhances the negative feedback of Ca\(^{2+}\) on IP_3. Fig. 3.1C depicts typical [IP_3] and [Ca\(^{2+}\)]_c oscillations observed before and after introducing IP_33K in the positive-feedback model (solid and dashed lines, respectively). The amplitude of the oscillations with IP_33K is substantially reduced. Interestingly, although the average IP_3 concentration is lower after introduction of IP_33K the frequency of the oscillations is increased. This effect is observed throughout the stimulation range and is summarized in Fig. 3.1D. Here the longest and shortest periods of the oscillations (obtained at low and high stimulation, respectively) are plotted against the rate constant of IP_33K (\(k_{3K}\)). In particular, the long period oscillations progressively disappear as the concentration of IP_33K increases. Very high levels of IP_33K are predicted to abolish the [Ca\(^{2+}\)]_c oscillations completely (indicated by the dotted line in Fig. 3.1D). Within this parameter range, the oscillations cannot be recovered by increasing the stimulus. A qualitatively very similar behavior was observed in the absence of Ca\(^{2+}\) activated PLC and in the negative-feedback model (results not shown).

The diminished amplitude of the oscillations and the eventual loss of oscillations are specifically obtained by increasing the IP_33K concentration and are not found with an increase in IP_35P. However, in both cases the predicted experimental observations do not depend strongly on whether any feedback mechanism, negative or positive, was present prior to the perturbation. Thus, accelerating IP_3 turnover by overexpressing IP_3 metabolizing enzymes is a poor strategy to verify the existence of Ca\(^{2+}\)-dependent feedback mechanisms.

The effects of overexpressing the Ca\(^{2+}\)-insensitive IP_35P can always be compensated by raising the agonist stimulus to keep the IP_3 level high. Overexpression of IP_33K is predicted to lead to small-amplitude Ca\(^{2+}\) spikes and, at high concentration, abolish the oscillations completely. This scenario is independent of whether (positive or negative) IP_3 feedbacks were present before the overexpression. Therefore, these experiments do not allow one to draw conclusions on the state of the system prior to perturbation.
3.1. Model predictions

Figure 3.1: Acceleration of IP$_3$ turnover by overexpressing IP$_3$-metabolizing enzymes. (A) Negative-feedback model without IP$_3$ 5-phosphatase ($k_{5P} = 0$, $\eta = 1$). At 10 sec and 500 sec $V_{PLC}$ is increased from 0 to 0.3 and 11 nM/s, respectively (arrowheads). (B) Same model with overexpressed IP$_3$5P, modeled as an increase in the IP$_3$ dephosphorylation rate constant $k_{5P} = 0.1/s$ ($\eta = 0.5$). The oscillations are abolished for low stimulation but reappear at high stimulation. (C) Effect of expressing the Ca$^{2+}$ dependent IP$_3$ 3-kinase in the positive-feedback model. [IP$_3$] and [Ca$^{2+}$]$_c$ oscillations are shown in the absence ($k_{3K} = 0$, solid lines) and presence of IP$_3$3K ($k_{3K} = 2/s$, dashed lines) for $V_{PLC} = 0.4 \mu M/s$. The amplitude of the oscillations and the period decrease after introduction of IP$_3$3K. (D) Frequency encoding in the positive-feedback model decreases as the concentration of IP$_3$3K increases (increase in $k_{3K}$). Plotted are the longest ($T_{max}$, circles) and shortest ($T_{min}$, squares) oscillation periods, obtained for low and high stimulation, respectively. For high levels of IP$_3$3K, no oscillations are found ($k_{3K} > 2.593/s$, dotted vertical line). Parameters as in Tab. 2.1, except in C and D $K_{PLC} = 0.1 \mu M$.

### 3.1.2 Slowing IP$_3$ turnover with an IP$_3$ buffer

A stronger result is obtained if the IP$_3$ turnover is slowed by introducing IP$_3$-binding proteins (IP$_3$ buffers) into the cell. To be specific, I assume a monovalent IP$_3$ buffer with on and off rate constants $k_{on}$ and $k_{off}$, respectively. The IP$_3$ balance equation
(Eq. (2.3)) is then modified to

\[
\frac{dp}{dt} = v_{\text{PLC}} - v_{\text{deg}} - k_{\text{on}}(B - C)p + k_{\text{off}}C \quad (3.1)
\]

\[
\frac{dC}{dt} = k_{\text{on}}(B - C)p - k_{\text{off}}C, \quad (3.2)
\]

where \( p \) as before stands for [IP\(_3\)], \( B \) denotes the total concentration of the introduced IP\(_3\) buffer, and \( C \) is the concentration of occupied IP\(_3\) buffer. If the binding of IP\(_3\) to the buffer is fast compared to the IP\(_3\) degradation rate, the amount of occupied buffer will be in equilibrium with [IP\(_3\)]. This implies \( C = Bp/(KB + p) \), where \( KB = k_{\text{off}}/k_{\text{on}} \) is the dissociation constant. Summing Eqs. (3.1)-(3.2) and using the equilibrium relation for \( C \), one obtains for the dynamics of unbound IP\(_3\)

\[
\frac{dp}{dt} = \frac{1}{\tau'_p} \left[ V_{\text{PLC}} \frac{c^2}{K^2_{\text{PLC}} + c^2} - \left( \eta \frac{c^2}{K^2_{\text{IP3}} + c^2} + (1 - \eta) \right) p \right] \quad (3.3)
\]

with the modified characteristic time of IP\(_3\) turnover

\[
\tau'_p = \tau_p \left( 1 + \frac{BK_B}{(KB + p)^2} \right). \quad (3.4)
\]

The IP\(_3\) buffer creates an additional bound pool of IP\(_3\) that is assumed to be protected from degradation. Then the build-up of free IP\(_3\) after PLC activation is delayed, because the buffer binding sites also need to be filled. Similarly, the decay of free [IP\(_3\)] is slowed, because IP\(_3\) molecules dissociate from the buffer as cellular [IP\(_3\)] decreases. Precisely these two effects are accounted for by the modified time constant \( \tau'_p \), which increases with the buffer concentration (Eq. (3.4)). Note that the balance between the rates of IP\(_3\) production and degradation is unaffected by the buffer. In particular, the IP\(_3\) buffer would not change the steady-state concentration of free IP\(_3\) attained when \( v_{\text{PLC}} = v_{\text{deg}} \). This remains true also with more detailed models for the IP\(_3\) metabolism, where the dynamics of its precursor PIP\(_2\) has been taken into account. Only when the intracellular total inositol is conserved does one observe a small decrease in the steady-state [IP\(_3\)] in the presence of IP\(_3\) buffer (see Section 4.4). However, in experiments inositol is present in the culture medium so that the intracellular inositol pool is probably continuously refilled.

Introducing an exogenous IP\(_3\) buffer into a core Ca\(^{2+}\) oscillator model operating with constant PLC activity and Ca\(^{2+}\)-insensitive degradation (such as the models discussed

\[1\] The cell contains endogenous IP\(_3\) binding sites, such as the IP\(_3\)R. They have already been accounted for by the characteristic time of IP\(_3\) turnover, \( \tau_p \), defined in the absence of the exogenous IP\(_3\) buffer.
above in the absence of Ca\(^{2+}\) feedbacks on IP\(_3\), will delay the rise in [IP\(_3\)] after PLC activation. However, eventually the same steady-state concentration of free [IP\(_3\)] would be reached as without buffer. Therefore, Ca\(^{2+}\) oscillations may set in with an increased latency, while spike shape and period would be unaffected by the presence of the IP\(_3\) buffer.

When introducing the IP\(_3\) buffer into the positive- and negative-feedback models, one finds that for low concentrations of IP\(_3\) buffer (0 < B < 10 \(\mu\)M for \(K_B = 0.13 \mu\)M) the oscillations persist in both models. However, depending on which type of Ca\(^{2+}\) feedback is present, IP\(_3\) buffer affects the kinetic properties of the \([Ca^{2+}]_c\) oscillations in very different ways. In the positive-feedback model, the IP\(_3\) buffer slows the Ca\(^{2+}\) responses. The rate of \([Ca^{2+}]_c\) rise in an individual spike is predicted to be decreased by the buffer in a dose-dependent manner (Fig. 3.2A, solid line). Another characteristic property of \([Ca^{2+}]_c\) oscillations is the wave speed, that is, the velocity at which a calcium spike propagates through the cell. To evaluate the effect of an IP\(_3\) buffer on wave propagation, diffusion of Ca\(^{2+}\) and IP\(_3\) in the cytoplasm was added to the model. The resulting reaction-diffusion equations were solved numerically on a one-dimensional domain. The propagation speed of a Ca\(^{2+}\) spike shows a very similar behavior as the rate of \([Ca^{2+}]_c\) rise: it decreases strongly as a function of IP\(_3\) buffer concentration in the positive-feedback model (Fig. 3.2B, solid line). In contrast, in the negative-feedback model, the IP\(_3\) buffer will cause hardly perceptible increases in the rate of Ca\(^{2+}\) rise (Fig. 3.2A, dashed line) and the wave speed (Fig. 3.2B, dashed line). These two properties are well suited for experimental measurements, because they have been found to be remarkably constant in cells not perturbed by IP\(_3\) buffer [137, 169].

For higher concentrations of IP\(_3\) buffer (B > 20 \(\mu\)M), the differences between the positive- and negative-feedback models are even more pronounced. In the positive-feedback model, the IP\(_3\) buffer completely abolishes the \([Ca^{2+}]_c\) oscillations, and instead a single slow \([Ca^{2+}]_c\) transient is observed (Fig. 3.2C). In the negative-feedback model, the \([Ca^{2+}]_c\) oscillations persist even for very high concentrations of IP\(_3\) buffer, although the oscillation period is increased (Fig. 3.2D).

Because IP\(_3\) buffering causes the strongest effects in the positive-feedback model, this case will be analyzed in more detail. Fig. 3.3A summarizes the results by showing the regions of oscillatory behavior as a function of the stimulus (\(V_{PLC}\)) and the concentration of exogenous IP\(_3\) buffer. Four different regions can be distinguished. In region I, the IP\(_3\) buffer slows the oscillations with respect to the rise time and propagation speed (see solid lines in Fig. 3.2A, B). In region II, high enough IP\(_3\) buffer concentrations abolish the oscillations (shown in Fig. 3.2C). In region III, IP\(_3\) buffer can cause more
Figure 3.2: Slowing of the IP$_3$ turnover with an IP$_3$ buffer. (A) The maximal rate of [Ca$^{2+}$]$_c$ rise during a Ca$^{2+}$ spike decreases as a function of IP$_3$ buffer concentration in the positive-feedback model (solid line), while it is barely affected in the negative-feedback model (dashed line). The results are shown for $V_{PLC} = 3$ µM/s (positive feedback) and 0.4 nM/s (negative feedback); similar results are obtained for other values. (B) The intracellular wave speed decreases as a function of IP$_3$ buffer concentration in the positive-feedback model (solid line), while it is barely affected in the negative-feedback model (dashed line). A solitary Ca$^{2+}$ wave is initiated by a local increase in [IP$_3$]; Ca$^{2+}$ and IP$_3$ diffuse in the cytoplasm with diffusion constants of 20 and 280 µm$^2$/s, respectively [4]; $V_{PLC} = 0.976$ µM/s, (positive feedback) and 0.217 nM/s (negative feedback). (C) High IP$_3$ buffer concentration abolishes oscillations in the positive-feedback model ($B = 50$ µM, $V_{PLC} = 1.6$ µM/s). (D) Oscillations persist in the presence of IP$_3$ buffer in the negative-feedback model ($B = 50$ µM, $V_{PLC} = 0.6$ nM/s). In all panels the IP$_3$ buffer dissociation constant is $K_B = K_p = 0.13$ µM. For the positive-feedback model $\varepsilon = 2, \tau_p = 1$ s, $\tau_r = 6$ s, $\nu_0 = 0.002$ µM/s, $\phi = 0.001$ s. For the negative-feedback model $\varepsilon = 0.1$. Other parameters are listed in Tab. 2.1.

complex oscillations, such as the bursting oscillations shown in Fig. 3.3B. For a large range of stimuli, the oscillations disappear when sufficiently high amounts of IP$_3$ buffer are added (transition into region II). However, for strong stimulation, there can be an additional domain, region IV (Fig. 3.3A). Here, the oscillations persist even at very high
Figure 3.3: Complex responses to an IP$_3$ buffer in the positive-feedback model. (A) Bifurcation diagram showing the region of oscillations as function of stimulus ($V_{PLC}$) and IP$_3$ buffer concentration (gray-shaded area; the solid lines indicate the locus where the steady state becomes unstable via a Hopf bifurcation). In region I, regular oscillations have a decreased rate of [Ca$^{2+}$]$_c$ rise with increased [IP$_3$ buffer] as shown in Fig. 3.2A. In region II, the IP$_3$ buffer abolishes the Ca$^{2+}$ oscillations completely, as shown in Fig. 3.2C. In region III, bursting [Ca$^{2+}$]$_c$ oscillations are observed (the lower boundary of this region is determined by a period doubling bifurcation, dotted line). An additional region IV is indicated. It is characterized by oscillations persisting even at high [IP$_3$ buffer]. The parameters are as in Fig. 3.2, with $\varepsilon = 2$. When the strength of the Ca$^{2+}$ plasma-membrane fluxes is increased ($\varepsilon = 10$), regions III and IV disappear (inset). (B) Example of bursting oscillations observed in region III (top panel, control without IP$_3$ buffer; bottom panel, [IP$_3$ buffer] = 10 $\mu$M; $V_{PLC} = 1.6$ $\mu$M/s). (C) Example of oscillations in region IV ([IP$_3$ buffer] = 100 $\mu$M; $V_{PLC} = 5$ $\mu$M/s), which are characterized by high frequency and low amplitude.

IP$_3$ buffer concentration but have strongly diminished amplitude (Fig. 3.3C). Note that in the presence of sufficiently high IP$_3$ buffer, only fast oscillations (at high stimulation) can be retained. The long-period oscillations, which depend on Ca$^{2+}$ feedback on PLC are invariably abolished by the IP$_3$ buffer.

Whether regions III and IV exist, depends on the kinetic parameters of the Ca$^{2+}$ fluxes. The inset in Fig. 3.3A shows a situation where the Ca$^{2+}$ fluxes across the plasma membrane are increased 5-fold. Then only regions I and II remain, and sufficiently high buffering of IP$_3$ always suppresses oscillations. Closer analysis revealed that if the system can oscillate for constant [IP$_3$] then IP$_3$ buffer never completely abolish the oscillations and a region IV exists (see also below).
3.1.3 A general property for systems with feedbacks on IP$_3$ metabolism

The model predictions described in the previous sections do not appear to be crucially dependent on the specific choice for the mathematical model. I tested four different models and obtained qualitatively similar results [25, 69, 147, 151] (see Appendix A). This indicates that there is a general property behind our observations. Here I will show, using a simple and analytical approach, how slowing or accelerating the IP$_3$ turnover affect differently systems with positive or negative feedback of Ca$^{2+}$ on IP$_3$ metabolism.

A simplified model for coupled Ca$^{2+}$-IP$_3$ oscillations contains three variables (see Fig. 2.1): cytoplasmic and total calcium concentration $c$ and $z$ ($c_{tot}$ in the previous model), and the IP$_3$ concentration $p$. For presentation purposes, I assume that the (in)activation of the IP$_3$R by Ca$^{2+}$ and IP$_3$ is fast compared to other processes. The higher dimensional model can be treated in an analogous way. The Ca$^{2+}$ concentration in the ER is related to the total concentration by $s = (z - c)/\beta$. The time changes of the three variables are described by

$$\frac{dc}{dt} = f(c, z, p); \quad \frac{dz}{dt} = g(c); \quad \text{and} \quad \frac{dp}{dt} = w(c, p). \quad (3.5)$$

The functions $f(c, z, p)$ and $g(c)$ describe the transport fluxes of cytoplasmic Ca$^{2+}$ across the ER and plasma membrane, $w(c, p)$ the production and degradation of IP$_3$. For realistic functions $f$, $g$ and $w$ the existence of a limit cycle must generally be shown numerically. However, the local stability properties of the steady state give us a hint. One can show that, due to the Ca$^{2+}$ transport across the plasma membrane, there is a unique steady state $(\bar{c}, \bar{z}, \bar{p})$ [144]. Therefore, changes in stability of this steady state are likely to be connected with a Hopf bifurcation and the birth/death of a limit cycle. Generally, if the steady state is unstable, it is to be expected that the trajectories move toward a stable limit cycle. Note that a stable limit cycle and a stable steady state can coexist. The following analysis can make no predictions in this regard.

I will focus on how the IP$_3$ dynamics can contribute in destabilizing or stabilizing the steady state and so possibly generate or abolish Ca$^{2+}$ oscillations. The local stability of the steady state is determined by the Jacobian:

$$J = \begin{pmatrix} f_c & f_z & f_p \\ g_c & 0 & 0 \\ w_c & 0 & w_p \end{pmatrix}. \quad (3.6)$$

The subscripts denote the partial derivatives at the steady state, e.g. $f_c = \frac{\partial f}{\partial c} \big|_{(c, z, p)}$. 

3.1. Model predictions

The gating properties of the IP$_3$R and the properties of the Ca$^{2+}$ transport processes allow to fix the signs of some of the terms in the Jacobian

- $f_z > 0$, the Ca$^{2+}$ concentration in the cytoplasm increases when the total concentration increases.

- $f_p > 0$, an increase in IP$_3$ causes the opening of the IP$_3$R and outflow of Ca$^{2+}$ from the ER into the cytoplasm.

- $g_c < 0$, a high Ca$^{2+}$ concentration in the cytoplasm favors the transport of Ca$^{2+}$ outside the cell.

- $w_p < 0$, IP$_3$ is degraded proportional to its concentration.

The sign of $w_c$ is positive when Ca$^{2+}$ activates the IP$_3$ production (positive feedback), negative in the presence of Ca$^{2+}$ dependent degradation of IP$_3$ (negative feedback), and vanishes in the absence of feedbacks of Ca$^{2+}$ on IP$_3$. When accelerating the IP$_3$ dynamics, e.g. by overexpressing IP$_3$ metabolizing enzymes, $w_c$ and $w_p$ increase in magnitude without changing sign. Conversely, slowing the IP$_3$ dynamics, e.g. by expressing an IP$_3$ binding protein, decreases $w_c$ and $w_p$.

The stability of the steady state is determined using the Routh-Hurwitz criterion, which states that all Eigenvalues of the Jacobian have negative real parts when

$$
\begin{align*}
    a_0 &= f_z g_c w_p \\
    a_1 &= -f_z g_c - f_p w_c + f_c w_p \\
    a_2 &= -f_c - w_p \\
    a_3 &= a_2 a_1 - a_0
\end{align*}
$$

are all positive. Due to the previous assumptions, $a_0 > 0$. The other terms simplify when the IP$_3$ dynamics is fast compared to the other processes $w_p, w_c \gg 1$, then $a_1 \approx -f_p w_c + f_c w_p$ and $a_2 \approx -w_p$. When the IP$_3$ dynamics is slow $w_p, w_c \ll 1$, then $a_1 \approx -f_z g_c$ and $a_2 \approx -f_c$.

I compare the behavior of a positive-feedback model ($w_c > 0$), a negative-feedback model ($w_c < 0$), and a model without feedback of Ca$^{2+}$ on IP$_3$ ($w_c = 0$) when the steady-state is identical in all three models. Equal $[\text{IP}_3]$ can be obtained by adjusting e.g. the stimulus dependent IP$_3$ production. Compared to the case without feedback, the IP$_3$ production should be higher with positive feedback model and lower with negative feedback (see also the detailed model). The cytoplasmic and total Ca$^{2+}$ concentrations are identical in all three models when the plasma membrane fluxes are unaffected by
Chapter 3. How to distinguish Ca\(^{2+}\) feedbacks on IP\(_3\) metabolism

<table>
<thead>
<tr>
<th>If (f_c &gt; 0)</th>
<th>IP(_3) dynamics</th>
<th>(a_0)</th>
<th>(a_1)</th>
<th>(a_2)</th>
<th>(a_3)</th>
<th>stability</th>
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<tr>
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<td>±</td>
<td>±</td>
<td>−</td>
<td>unstable</td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>slow dynamics</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>unstable</td>
<td></td>
</tr>
<tr>
<td>fast dynamics</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>unstable</td>
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</tr>
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<td>−</td>
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<tr>
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<td>±</td>
<td>+</td>
<td>±</td>
<td>stable/unstable</td>
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Table 3.1: Stability properties of the steady state when \(f_c > 0\) in a model without feedbacks, positive or negative feedback of Ca\(^{2+}\) on IP\(_3\). Shown are also the signs of the Routh-Hurwitz coefficients. In the negative feedback model, for fast IP\(_3\) dynamics, the steady state is stable when \(f_p w_c - f_c w_p < 0\).

the stimulus. An equal steady state allows to easily compare the three models. Then the partial derivatives, except of course \(w_c\) and \(w_p\), are identical for the three cases considered.

Depending on the magnitude of the positive feedback of Ca\(^{2+}\) on the IP\(_3\)R and on the transport processes that remove Ca\(^{2+}\), \(f_c\) can be either positive or negative; both cases will be discussed. The stability properties of the steady state for \(f_c > 0\) are summarized in Tab. 3.1. If the system can oscillate in the absence of Ca\(^{2+}\) feedbacks on the IP\(_3\) metabolism, i.e. the steady state is unstable, slowing the IP\(_3\) dynamics does not stabilize the system, i.e. abolish the oscillations, in either positive- or negative-feedback models. Overexpressing the enzyme IP\(_3\)3K which corresponds to accelerating the IP\(_3\) dynamics in the negative-feedback model, can stabilize the steady state and so may suppress the oscillations.

The stability properties of the steady state for \(f_c < 0\) are summarized in Tab. 3.2. When the steady state is stable in the absence of feedbacks, the presence of a positive feedback of Ca\(^{2+}\) on IP\(_3\) can destabilize the system and so generate oscillations. The conditions that must be fulfilled is a sufficiently fast IP\(_3\) dynamics and \(f_p w_c - f_c w_p > 0\). In the last formula one recognizes that the mutual activation of IP\(_3\) and Ca\(^{2+}\), \(f_p w_c\), needs to be strong compared to processes responsible for the clearance of Ca\(^{2+}\) and the degradation of IP\(_3\), which enter \(f_c w_p\). Slowing of the IP\(_3\) dynamics stabilizes the system,
3.1. Model predictions

If $f_c < 0$

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<th>$a_1$</th>
<th>$a_2$</th>
<th>$a_3$</th>
<th>stability</th>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>stable</td>
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<tr>
<td>fast dynamics</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>stable/unstable</td>
</tr>
<tr>
<td>negative feedback, $w_c &lt; 0$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>any dynamics</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>stable</td>
</tr>
</tbody>
</table>

Table 3.2: Stability properties of the steady state when $f_c < 0$. Shown are also the signs of the Routh-Hurwitz coefficients. For fast IP$_3$ dynamics and in the presence of Ca$^{2+}$ activation of IP$_3$ production the steady state is unstable when $f_p w_c - f_c w_p > 0$.

and so abolishes the oscillations. The presence of a negative feedback does not affect the stability of the steady state and so may not give rise to Ca$^{2+}$ oscillations.

These conclusions are independent of specific expressions for the rates $f$, $g$ and $w$. In particular, slowing of the IP$_3$ dynamics may unmask a critical role of positive feedback of Ca$^{2+}$ on IP$_3$ in the oscillatory mechanism, and is predicted to cause a transition from oscillatory to non-oscillatory behavior.
3.2 Expression of an IP₃ buffer suppresses Ca²⁺ oscillations

The analytical and numerical results obtained so far indicate that the expression of an IP₃ buffer can be used to identify the dominating Ca²⁺ feedback on IP₃ metabolism. The most significant difference in the responses of a system with positive feedback and a system with negative feedback is that agonist-induced oscillations always persist in the latter case whereas they can be abolished in the former.

To examine the model predictions experimentally, a molecular IP₃ buffer developed in the laboratory of A.P. Thomas (Newark, USA) has been used. It consists of the N-terminal ligand binding domain of rat type 1 IP₃R linked to enhanced green fluorescent protein (EGFP-LBD; [46], Gaspers et al. in preparation). Chinese hamster ovary (CHO) cells were transiently transfected with EGFP or EGFP-LBD, then challenged with submaximal and maximal ATP concentrations. The subsequent [Ca²⁺]ᵢ responses were monitored via changes in the fura-2 fluorescence ratio. EGFP fluorescence was utilized to distinguish transfected from non-transfected cells in a given field of view and to estimate the intracellular concentration of the transgene (see materials and methods, Section 2.1).

The addition of low ATP concentrations elicited periodic [Ca²⁺]ᵢ spikes in > 85% of the CHO cells expressing EGFP (Fig. 3.4A, B) or non-expressing cells from cultures transfected with EGFP-LBD (not shown). Agonist-evoked [Ca²⁺]ᵢ oscillations required functional ER Ca²⁺ stores (i.e. thapsigargin-sensitive), but ceased abruptly upon removing extracellular Ca²⁺ suggesting that plasma membrane Ca²⁺ fluxes are relatively strong in this cell type (not shown). In both EGFP-expressing and non-expressing cells, the [Ca²⁺]ᵢ increase immediately following agonist challenge was more prolonged and the rate of Ca²⁺ rise faster than subsequent [Ca²⁺]ᵢ oscillations (Fig. 3.4A, C). No systematic differences were evident in agonist sensitivity or the pattern of the [Ca²⁺]ᵢ spiking between EGFP expressing and non-expressing cells suggesting that neither EGFP nor the transfection reagents per se had significant effects on Ca²⁺ signaling. By contrast, the presence of EGFP-LBD had a dose-dependent effect on the agonist dependent Ca²⁺ oscillations in CHO cells (Fig. 3.4A). High levels of EGFP-LBD expression correlated with a loss of repetitive [Ca²⁺]ᵢ spiking and the appearance of low amplitude [Ca²⁺]ᵢ increases (Fig. 3.4A, B). Moreover, EGFP-LBD expression significantly slowed the rate of [Ca²⁺]ᵢ rise (Fig. 3.4C; p < 0.01) and significantly broadened the width of the [Ca²⁺]ᵢ spike (Fig. 3.4D; p < 0.05) compared to EGFP expressing cells.
3.2. Expression of an IP₃ buffer suppresses Ca²⁺ oscillations

Figure 3.4: Effects of an IP₃ buffer on ATP-evoked [Ca²⁺]c oscillations in CHO cells. Cells (n = 5 independent cultures) were transiently transfected with pEGFP-LBD (EGFP-LBD) or pEGFP-C1 (EGFP) and imaged as described in Section 2.1. (A) Typical ATP-evoked [Ca²⁺]c spikes in CHO cells expressing EGFP or different levels of EGFP-LBD. (B) Cells expressing EGFP-LBD were arbitrarily divided into low (489 ± 66 units; n = 18 cells) or high (3170 ± 480 units; n = 27 cells) categories using a cutoff of 1000 fluorescence intensity units. The estimated mean EGFP-LBD concentration was 6 ± 0.8 or 38 ± 6 µM, respectively. Truncated spikes are low amplitude [Ca²⁺]c oscillations similar to those shown in the bottom traces of panel A. (C) Initial rates of [Ca²⁺]c rise.
Chapter 3. How to distinguish Ca\textsuperscript{2+} feedbacks on IP\textsubscript{3} metabolism

For these data, only EGFP (n = 52 cells) or EGFP-LBD (n = 20 cells) expressing cells, where low ATP challenge (0.5 or 1 µM) evoked at least 3 sequential baseline-separated Ca\textsuperscript{2+} spikes, have been analyzed. This was observed predominately in cells expressing low levels of EGFP-LBD and, thus, probably the actions of IP\textsubscript{3} buffering on the kinetics of [Ca\textsuperscript{2+}]\textsubscript{c} oscillations is underestimated.

According to the theoretical results, the disappearance of the oscillations and a slowing of the Ca\textsuperscript{2+} rise suggests that IP\textsubscript{3} oscillations driven by positive feedback of Ca\textsuperscript{2+} on IP\textsubscript{3} production are involved in this system. We have simulated the positive-feedback model with relatively strong plasma-membrane Ca\textsuperscript{2+} fluxes as observed in CHO cells. At high concentrations of IP\textsubscript{3} buffer (Fig. 3.4E), the model exhibits single transients (for lower agonist dose) and repetitive truncated spikes (for high agonist dose). Both responses closely resemble the experimentally observed patterns in cells expressing high amounts of EGFP-LBD. The Ca\textsuperscript{2+} oscillations at lower concentrations of IP\textsubscript{3} buffer (Fig. 3.4F) exhibit a broadening of the individual spikes, which is very similar to the experimental observation in cells expressing low amounts of EGFP-LBD. Also the observed decrease in the rate of Ca\textsuperscript{2+} rise is reproduced by the model (data similar to Fig. 3.2). A model with negative feedback could account for none of the experimental findings (see Fig. 3.2).

Figure 3.4 (continued): (D) The width of the [Ca\textsuperscript{2+}]\textsubscript{c} spike were determined at half peak height. (E,F) Positive-feedback model, Ca\textsuperscript{2+} activation of PLC. (E) Simulations with different IP\textsubscript{3} buffer concentrations (as indicated) show a good agreement with the EGFP-LBD experimental data in A. An increase in ATP is simulated by an increase in the maximal activity of PLC (arrowheads). (F) One also observes a significant increase in spike width. To match the Ca\textsuperscript{2+} oscillations in CHO cells, all variables have been slowed by a factor 10, reference parameter set as in Fig. 3.2. In (E) \( V_{PLC} = 0.125, 0.2, 0.4 \mu\text{M/s} \). Initial condition at \( V_{PLC} = 0.05 \mu\text{M/s} \). In (F) \( V_{PLC} = 0.2 \mu\text{M/s} \).
Chapter 4

Towards an integrative model for the phosphoinositide metabolism

In the previous chapters, I was able to show how the dynamics of inositol 1,4,5-trisphosphate (IP$_3$) plays a central role in modulating Ca$^{2+}$ signals. The models for the Ca$^{2+}$ dynamics used here, and most of published models (see reviews [144, 152]), consider production and degradation of IP$_3$ but neglect the reactions leading to its precursor phosphatidylinositol-4,5-bisphosphate (PIP$_2$). The mathematical models tacitly assume that the PIP$_2$ level remains constant during stimulation. However, this is in contradiction to experimental evidence in several cell lines where PIP$_2$ and its precursor phosphatidylinositol-4-phosphate (PIP) clearly decrease upon stimulation with hormones [116, 167, 189, 197]. This raises the question on how production and degradation of PIP$_2$ are balanced in order to maintain, during stimulation, the observed elevated cytoplasmic IP$_3$ concentration. A long-lasting IP$_3$ signal is the *conditio sine qua non* for long-lasting [Ca$^{2+}$]$_e$ oscillations measured in cells such as hepatocytes.

After shortly reviewing the reactions involved in the phosphoinositide pathway and the available data, I will discuss several alternative mathematical models. The experimental data allow to estimate kinetic parameters and so to make predictions on duration and amplitude of IP$_3$ signals, and on the contribution of the different reactions. If the PIP$_2$ production flux is independent of PIP$_2$ and stimulus, an increase in phospholipase C (PLC) activity can only lead to a transient increase in IP$_3$. The estimated maximal duration of this transient is far below the duration of agonist dependent Ca$^{2+}$ signals. To generate a long-lasting increase in IP$_3$ concentration the PIP and PIP$_2$ phosphatase, two enzymes, which counteract phosphatidylinositol (PI) 4-kinase and PIP 5-kinase, are required. This regulatory advantage is gained at the cost of higher energy consumption,
necessary to maintain the futile phosphorylation-dephosphorylation cycles. I finally show, by coupling the Ca\(^{2+}\) and IP\(_3\) metabolism models, how Ca\(^{2+}\) feedbacks on the IP\(_3\)/phosphoinositide metabolism can give long-lasting oscillating Ca\(^{2+}\) signals without the requirement of futile cycles.

4.1 The phosphoinositide pathway

4.1.1 Structure

Phosphatidylinositol phosphates are composed of a membrane-associated phosphatidic acid group and a glycerol moiety that is linked to a phosphorylated inositol head group (Fig. 4.1A). The main pathway for their production and degradation is shown in Fig. 4.1B. In this pathway one can differentiate between metabolites bound to cellular membranes and freely diffusing.

![Figure 4.1](image_url)

Figure 4.1: (A) Scheme of PIP\(_2\) which can be hydrolyzed by phospholipase C to IP\(_3\) and DAG. (B) Main pathway of phosphoinositides synthesis and degradation. Inositol is phosphorylated/dephosphorylated at different positions (indicated by the number in brackets). DAG is first phosphorylated to phosphatidic acid (PA) before binding to CTP to form CDP-DAG.

Several isoforms of phospholipase C (PLC) are activated by external stimulation [135], they catalyze hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) to diacylglycerol (DAG) and the freely diffusing inositol 1,4,5-trisphosphate IP\(_3\). In a series
4.1. The phosphoinositide pathway

of phosphorylation and dephosphorylation reactions IP$_3$ is freed of all its phosphates to finally obtain inositol. On the other branch of the pathway, DAG is activated by covalent binding to the nucleotide CTP. Under action of PI synthase CDP-DAG and inositol are consumed to form phosphatidylinositol. The phosphorylation of its inositol moiety by PI 4-kinase generates PIP, which is further phosphorylated by PIP 5-kinase to PIP$_2$. Phosphorylation of PIP and PI is counteracted by PIP$_2$ 5-phosphatase and PIP 4-phosphatase, respectively. The production of CDP-DAG is also opposed by two phosphatases.

The pathway depicted in Fig. 4.1B is the main synthesis and degradation pathway, however one should keep in mind that there are side branches which can be regulated by external stimuli [18, 53, 178]. For instance, it has been reported that PIP$_2$ can be produced by 5-phosphorylation of PI and subsequent 4-phosphorylation of PI-5-phosphate [18, 128]. PIP$_2$ can be further phosphorylated to PI (3,4,5) trisphosphate by the phosphatidylinositol 3-kinase (cf. [178]). The substrates necessary for the synthesis of PI, inositol and DAG, can also come from side branches of the metabolism. For instance inositol can be synthesized from different sugars (e.g. glucose-6-phosphate), DAG can be obtained from the hydrolysis of other phosphoinositides, e.g. phosphatidylcholine or phosphatidylethanolamine (see also the KEGG database http://www.genome.jp/kegg/pathway.html [78]). The contribution of these different branches to the homeostasis of the phosphoinositides has not been quantified in detail. Therefore, for the modeling I will not take them into account.

4.1.2 Quantitative data

Measurements of pathway metabolites were done principally in rat hepatocytes and mouse neuroblastoma cell lines with radioactively labeled PI, PIP, PIP$_2$ and inositol phosphates. A detailed summary of experimental results is given in Tab. 4.1. The relative amounts of the three phosphoinositides are consistent in the different cell lines and experiments. The plasma membrane density of PI ([PI]) is much higher than PIP density ([PIP]) and PIP$_2$ density ([PIP$_2$]), [PIP] is slightly lower than [PIP$_2$]. The fractional changes after stimulation are also similar in the different experiments. [PI] remains nearly constant, whereas [PIP] and [PIP$_2$] decrease by a factor of $\sim 2$. In contrast the absolute values often differ by one order of magnitude. For IP$_3$ a similar trend is observed. The reported absolute concentrations in the unstimulated cells strongly varies between cell lines and experiment, but the increase observed after stimulation is about 10 fold. The changes in phosphoinositides concentrations last for the total duration of
Table 4.1: Concentrations of phosphoinositides, DAG, IP3 and IP2 products in unstimulated and stimulated cells. The other cells studied are smooth muscle cells, and Xenopus Oocytes. \[197\]. IP2 represents the sum of different inositol-phosphates (IP1, IP2, IP3).

<table>
<thead>
<tr>
<th></th>
<th>Unstimulated cells</th>
<th>Hormone stimulated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td>96.5%</td>
<td>94.32%</td>
</tr>
<tr>
<td>PIP</td>
<td>1.33%</td>
<td>2.53%</td>
</tr>
<tr>
<td>PIP2</td>
<td>2.08%</td>
<td>3.14%</td>
</tr>
<tr>
<td>DAG</td>
<td>–</td>
<td>212077</td>
</tr>
<tr>
<td>IP3</td>
<td>–</td>
<td>0.1</td>
</tr>
<tr>
<td>IP3x</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

In [43, 197] effect of hormone is transient (<150 s). In [42, 51] IP3 is directly increased. Given is the lowest IP3 concentration leading to a Ca2+ signal.

Other cells studied are smooth muscle cells, and Xenopus Oocytes. \[197\]. IP2 represents the sum of different inositol-phosphates (IP1, IP2, IP3). In part of the literature phosphoinositides quantities are given in amount/mg cell protein. These values are converted into densities (molecules/\(\mu m^2\)).

Concentrations of phosphoinositides, DAG, IP3 and IP2 products in unstimulated and stimulated cells. The other cells studied are smooth muscle cells, and Xenopus Oocytes. \[197\]. IP2 represents the sum of different inositol-phosphates (IP1, IP2, IP3).
4.1. The phosphoinositide pathway

<table>
<thead>
<tr>
<th>Half-lifes (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte</td>
</tr>
<tr>
<td>IP$_3$</td>
</tr>
<tr>
<td>IP$_4$</td>
</tr>
<tr>
<td>IP$_x$</td>
</tr>
<tr>
<td>DAG</td>
</tr>
</tbody>
</table>

$^\dagger$ for high and low [Ca$^{2+}$]$_c$, respectively.

Table 4.2: Half-lifes of the PLC reaction products. IP$_x$ stands for the sum of inositol phosphates (i.e. IP$_3$, IP$_4$, IP$_2$ ...). Half-life is determined after $1/e$ decay.

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Half-lifes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>Stimulated</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>0.15 µM, $Y_1$</td>
</tr>
<tr>
<td>IP$_3$ products</td>
<td>–</td>
</tr>
<tr>
<td>PI molecules/µm$^2$</td>
<td>12015, $X_1$</td>
</tr>
<tr>
<td>PIP molecules/µm$^2$</td>
<td>3223, $X_2$</td>
</tr>
<tr>
<td>PIP$_2$ molecules/µm$^2$</td>
<td>4000, $X_3$</td>
</tr>
</tbody>
</table>

Table 4.3: Reference concentrations and half-lifes in unstimulated and stimulated cells used for the modeling. Shown are also the notations used in the mathematical models.

stimulation (>15 min [189], >30 min [168], >60 min [116]). In some cases, one observes for [PIP$_2$] and [PIP] a slow (>30 min) recovery to basal levels [116]. Only in bradykinin stimulated neuroblastoma cells do the [IP$_3$] and [PIP$_2$] changes last for less than 120 s [43, 197]. The metabolites half-lifes are also a source of valuable informations (Tab. 4.2). There is strong variation between cell types, but for one cell type the experimental values are similar. Typically, the half-life of DAG and IP$_3$ products is about 10–20 times longer than that of IP$_3$.

Reference values which are used for estimating the model parameters are listed in
Tab. 4.3. The differences pointed previously may be accounted for by (i) increasing all concentrations by the same factor, and by (ii) changing the life time of IP$_3$. In (i) the values of most of the parameters remain unchanged as these are determined by the density ratios and the degradation rate constant of IP$_3$ (see below). In (ii) all parameters are rescaled by the same factor.

### 4.2 The phosphoinositide pathway as a linear chain

Phosphorylation of PI and PIP is counteracted by the PIP phosphatase and the PIP$_2$ phosphatase, respectively (Fig. 4.1B). These phosphorylation-dephosphorylation cycles are so called futile cycles. Futile because the ATP consumed to maintain a certain net production rate of IP$_3$ is higher in their presence. I will show how this apparently unfavorable property allows for a flexible control of cytosolic IP$_3$ concentration.

#### 4.2.1 The model

The mathematical treatment of the phosphoinositide pathway is simplified by following assumptions:

- Inositol and CDP-DAG do not change during stimulation so that phosphatidylinositol is produced by the PI synthase at a constant rate. PI is removed by PI 4-kinase mediated phosphorylation and by an additional path. This additional degradation path reveals essential for controlling [IP$_3$]. It can be equated with 5- and/or 3-phosphorylation by PI 5-kinase and 3-kinase, respectively [6, 128].

- Degradation of IP$_3$ is described by one reaction, the fate of IP$_3$ products is not specifically modeled.

- Reaction rates depend linearly on the concentrations. Thus, the enzymes are assumed to be unsaturated with substrate.

The first two points lead to the linear chain depicted in Fig. 4.2. The latter one allows for a precise estimation of kinetic parameters. Further in the text some of these assumptions will be relieved, and their role in IP$_3$ homeostasis will be discussed.
The phosphoinositide pathway as a linear chain

4.2. The phosphoinositide pathway as a linear chain

The system of equations governing the time evolution of PI, PIP and PIP$_2$ plasma membrane densities is

\[
\begin{align*}
\frac{dX_1}{dt} &= v - \kappa X_1 - k_1 X_1 + k_{-1} X_2, \\
\frac{dX_2}{dt} &= k_1 X_1 - k_{-1} X_2 - k_2 X_2 + k_{-2} X_3, \\
\frac{dX_3}{dt} &= k_2 X_2 - k_{-2} X_3 - k_3 X_3.
\end{align*}
\]

(4.1)

(4.2)

(4.3)

For the cytosolic IP$_3$ concentration one has

\[
dY_1 = k_3 f X_3 - k_4 Y_1.
\]

(4.4)

$X_1$, $X_2$, $X_3$ are the plasma membrane densities of PI, PIP, and PIP$_2$, respectively, $Y_1$ is the cytosolic concentration of IP$_3$, $v$ is the PI synthase rate, $\kappa$ the rate constant of the additional PI degradation path. $k_1$ and $k_2$ are phosphorylation rate constants characterizing respectively PI 4-kinase and PIP 5-kinase. $k_3$ characterizes the activity of PLC, $k_4$ the degradation of IP$_3$. The rate constants of PIP and PIP$_2$ dephosphorylation are $k_{-1}$ and $k_{-2}$, respectively. The factor $f = A_{pm}/(V_{cyt} N_A)$ in Eq. (4.4) (membrane surface $A_{pm}$ to cytoplasmic volume $V_{cyt}$ and Avogadro constant $N_A$) accounts for a reaction occurring at the plasma membrane, but which releases its product in the cytosol.

In steady state ($dX_i/dt = 0$, $i = 1, \cdots, 3$; $dY_1/dt = 0$) the concentrations are given by

\[
\begin{align*}
Y_1 &= \frac{V_m f k_3}{k_4 k_3 + K_d}, \\
X_3 &= V_m \frac{1}{k_3 + K_d}, \\
X_2 &= \frac{V_m k_{-2} + k_3}{k_2 k_3 + K_d}, \\
X_1 &= \frac{V_m (k_{-1} + k_2) k_3 + \frac{k_{-1} k_{-2}}{k_{-1} + k_2}}{k_1 k_2 + k_3 + K_d}.
\end{align*}
\]

(4.5)

(4.6)

(4.7)

(4.8)

I introduced the maximal production rate through the linear chain $V_m$ and the half-saturation rate constant $K_d$

\[
\begin{align*}
a) \quad V_m &= \frac{vk_1 k_2}{k_1 k_2 + \kappa (k_{-1} + k_2)}, \quad \text{and} \quad b) \quad K_d &= \frac{\kappa k_{-1} k_{-2}}{k_1 k_2 + \kappa (k_{-1} + k_2)}. \quad (4.9)
\end{align*}
\]

Using the previous relations some of the parameters can be expressed as function of the known metabolite concentrations when the cell is unstimulated. These are denoted
by \(X_1, X_2, X_3\), and \(Y_1\) (see Tab. 4.3). One obtains for the PI synthase rate

\[
v = \kappa X_1 + \frac{Y_1 k_4}{X_1 f}, \tag{4.10}
\]

for the phosphorylation rate constants

\[
k_1 = k_1 \frac{X_2}{X_1} + \frac{Y_1 k_4}{X_1 f}, \tag{4.11}
\]
\[
k_2 = k_2 \frac{X_3}{X_2} + \frac{Y_1 k_4}{X_2 f}, \tag{4.12}
\]

and for the basal PLC activity

\[
k_3 = k_4 \frac{Y_1}{X_3}. \tag{4.13}
\]

If the phosphoinositides distribution during stimulation is known, the dephosphorylation rate constants can be determined from

\[
k_{-1} = f_c \frac{k_4 (Y_1 X_1 - Y_1 X_1^*)}{f(X_2 X_1^* - X_2 X_1)} \tag{4.14}
\]
\[
k_{-2} = f_c \frac{k_4 (Y_1 X_2 - Y_1 X_2^*)}{f(X_3 X_2^* - X_3 X_2)} \tag{4.15}
\]

Where * denotes the steady state concentrations during stimulation, which is at a PLC rate constant of \(k_3 = \frac{Y_1}{fX_3} k_4\). Similarly for the 3/5 phosphorylation of PI one has

\[
\kappa = f_c \frac{k_4 (Y_1^* - Y_1)}{f(X_1^* - X_1)}. \tag{4.16}
\]

The parameter \(f_c\) is introduced to tune the contribution of futile cycling to the pathway. By changing \(f_c\), the reference unstimulated steady state remains unaffected (see Section 4.6).
### Parameter values

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Without phosphatases</th>
<th>With phosphatases</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC, $k_3$</td>
<td>$2.38 \times 10^{-3}$/$s$</td>
<td></td>
</tr>
<tr>
<td>PI 4-kinase, $k_1$</td>
<td>$0.794 \times 10^{-3}$/$s$</td>
<td>$0.0173$/$s$</td>
</tr>
<tr>
<td>PIP 5-kinase, $k_2$</td>
<td>$2.96 \times 10^{-3}$/$s$</td>
<td>$0.7135$/$s$</td>
</tr>
<tr>
<td>PIP phosphatase, $k_{-1}$</td>
<td>0</td>
<td>$0.0616$/$s$</td>
</tr>
<tr>
<td>PIP$<em>2$ phosphatase, $k</em>{-2}$</td>
<td>0</td>
<td>$0.5725$/$s$</td>
</tr>
<tr>
<td>IP$_3$ degradation, $k_4$</td>
<td></td>
<td>$0.15$/$s$</td>
</tr>
<tr>
<td>PI synthase rate, $v$</td>
<td>546.28 molecules/$\mu m^2$/s</td>
<td></td>
</tr>
<tr>
<td>PI 5/3-kinase, $\kappa$</td>
<td>$0.04467$/$s$</td>
<td></td>
</tr>
<tr>
<td>†Inositol phosphates degradation, $k_5$</td>
<td>$0.015$/$s$</td>
<td></td>
</tr>
<tr>
<td>†PI synthase rate constant, $k_6$</td>
<td>$0.725$/$s$</td>
<td></td>
</tr>
<tr>
<td>†Total inositol, $Y_t$</td>
<td>$70.8 \times 10^6$ molecules</td>
<td></td>
</tr>
<tr>
<td>$f = A_{pm}/(V_{cyl}N_A)$</td>
<td></td>
<td>$2.358 \times 10^{-3}$ $\mu M$$\mu m^2$</td>
</tr>
</tbody>
</table>

† The parameters $k_5$ and $k_6$, and total inositol amount are used in Section 4.3.

Table 4.4: Second column: Parameters are determined from the unstimulated reference state (Tab. 4.3) and Eqs. (4.10)–(4.13). Third column: Parameters are determined from the steady state in unstimulated and stimulated cells and Eqs. (4.10)–(4.13), (4.14)–(4.15). Agonist increases the PLC rate constant $k_3$, the reference stimulated steady state is obtained for $k_3 = 0.0662$/$s$. The half-life of IP$_3$ and inositol phosphates gives $k_4$ and $k_5$, respectively (see Tab. 4.3). From [81] $A_{pm} = 3550$ $\mu m^2$ and $V_{cyl} = 2.5$ pl which yields the coefficient $f$.

#### 4.2.2 Transient [IP$_3$] increase

External stimulation is known to activate PLC and so to increase the rate of PIP$_2$ hydrolysis, $k_3$. According to Eqs. (4.5) and (4.9) this may lead to a prolonged increase in [IP$_3$] if and only if the two phosphatases and the additional PI degradation path are active. Given that any of these processes is missing, that is either $k_{-1}$, $k_{-2}$ or $\kappa$ are zero (Figs 4.3A, 4.4A, D), the steady-state [IP$_3$] does not change with an increase in PLC
activity (half-saturation rate constant $K_d$ vanishes, Eq. (4.9b)). It holds

$$ Y_1 = \frac{V_m f}{k_4} = \frac{v k_1 k_2 f}{k_4 (k_1 k_2 + \kappa (k_{-1} + k_2))} = \Upsilon_1. $$

Accordingly, the stationary [IP$_3$] is controlled by processes which are not supposed to be activated during stimulation. An increase in PLC rate constant produces a transient elevation in [IP$_3$], which will inevitably return to its basal level (Fig. 4.3B). On the other hand [PIP$_2$] decreases and reaches a lower steady state during stimulation (Eq. (4.6) and Fig. 4.3C). Also [PIP] sinks if [PIP$_2$] phosphatase is active (Eq. (4.7), Fig. 4.4C), whereas [PI] decreases only when both phosphatases are present (Eq. (4.8), Fig. 4.4F).

The transient in [IP$_3$] is accompanied by few [Ca$^{2+}$]$_c$ spikes (Fig. 4.3D). These signals are often ascribed to desensitization of agonist receptors, by e.g. internalization or covalent modifications [26, 75]. In the light of IP$_3$ metabolism, the depletion of PIP$_2$ is intrinsically a desensitization mechanism. Indeed, increasing the activity of PLC transiently “resensitizes” the system and produces additional spikes (Fig. 4.3D, arrows). Information on which type of cells show transient signals of this type can be gained when the duration of these is compared to the duration of Ca$^{2+}$/IP$_3$ signals observed in experiments. The definitions proposed by Heinrich et al. (2002) [58] prove useful for this comparison. The mean signal duration is defined by

$$ \theta = 2 \int_0^\infty t^2 \Delta Y_1(t) dt - \left( \int_0^\infty t \Delta Y_1(t) dt \right)^2, \quad (4.18) $$

where $\Delta Y_1(t) = Y_1(t) - \Upsilon_1$. Only high enough IP$_3$ concentrations sensitize the IP$_3$-receptors and generate [Ca$^{2+}$]$_c$ signals, thus the second important quantity is the mean amplitude which is defined by

$$ \alpha = \frac{\int_0^\infty \Delta Y_1(t) dt}{\theta}. \quad (4.19) $$

A graphical representation of these two measures is shown in Fig. 4.3B.

I calculated the duration and amplitude of the signal when the system is in the reference unstimulated state, that is Eqs. (4.10)–(4.13) must be fulfilled. As described in Appendix B.1 when PIP$_2$ phosphatase is inactive ($k_{-2} = 0$) one derives for the signal duration

$$ \theta = 2 \sqrt{\frac{1}{k_3^2} + \frac{1}{k_4^2}}, \quad (4.20) $$

and for the IP$_3$ signal amplitude

$$ \alpha = \frac{\Upsilon_1 \left( \frac{1}{k_3} - \frac{1}{k_5} \right)}{\theta}. \quad (4.21) $$
4.2. The phosphoinositide pathway as a linear chain

![Diagram of the phosphoinositide pathway]

**Figure 4.3:** Transient increase in \([\text{IP}_3]\). (A) The three depicted pathways do not have the PIP\(_2\) phosphatase. They all exhibit the same phosphoinositide kinetics (see B and C) after stimulation of PLC. (B) Changes in \([\text{IP}_3]\) after agonist mediated activation of PLC. Shown are the signaling length \(\theta\) and amplitude \(\alpha\) (Eqs. (4.18)–(4.19)). (C) PIP\(_2\) density (solid line) decreases during stimulation, whereas PI and PIP densities (dashed lines) remain constant. Changes are relative to the unstimulated state. (D) \([\text{Ca}^{2+}]_c\) oscillations, grey line, cease when the \([\text{IP}_3]\), black line, falls below a threshold value. Increasing the stimulus transiently resensitizes the system to agonist. Parameters listed in Tab. 4.4, (B), (C) PLC rate constant \(k_3\) is set to 0.007/s, in (D) at time indicated by arrows \(k_3 = 0.007, 0.014\) and 0.062/s. Model for the \([\text{Ca}^{2+}]\) dynamics given by Eqs. (2.7)–(2.9). Parameter listed in Tab. 2.1 left column, no positive feedback \((K_{PLC} = 0)\).

where \(k_3^* \geq k_3\) is the rate constant of stimulated G-protein bound PLC and \(\overline{Y}_1\) is the steady state unstimulated \([\text{IP}_3]\).

The above formulas demonstrate that only processes which are directly connected to \([\text{IP}_3]\), that is PLC and \([\text{IP}_3]\) degradation, govern its time evolution. In particular, PIP phosphatase alone does not affect the kinetics of \([\text{IP}_3]\) (see Fig. 4.3A). Stronger stimulation has opposing effects on amplitude and duration of the \([\text{IP}_3]\) signal. For a high activity of the PLC \((k_3^* \gg k_3, k_4)\), the \([\text{IP}_3]\) transient has the shortest duration and the largest...
amplitude

\[ \theta(k_3^* \gg k_3, k_4) = \frac{2}{k_4}, \]

\[ \alpha(k_3^* \gg k_3, k_4) = \frac{\gamma_1 k_4}{2k_3}, \]

whereas for weak stimulation, \( k_3^* \approx k_3 \), the signal has a maximal duration, but vanishing amplitude

\[ \theta(k_3^* \approx k_3) = 2\sqrt{\frac{1}{k_3^2} + \frac{1}{k_4^2}}, \] \hspace{1cm} (4.22)

\[ \alpha(k_3^* \approx k_3) = 0. \]

With the parameter values from Tab. 4.4 one obtains an IP\(_3\) signal duration which does not exceed 14 min (Eq. (4.22)). This value represents the upper limit for the IP\(_3\) signal duration. To generate a \([\text{Ca}^{2+}]_c\) signal, \([\text{IP}_3]\) should be high enough to activate the IP\(_3\)R leading inevitably to shorter signals. For example, with an IP\(_3\) signal amplitude of 0.15 \(\mu\)M the signal is not longer than 5 min. The presence of PIP\(_2\) phosphatase alone does not allow for a steady increase in \([\text{IP}_3]\) after PLC activation, however, it changes the signal duration and amplitude. One can show that in the presence of PIP\(_2\) phosphatase \((k_{-2} \neq 0, k_{-1} = 0 \text{ and } \kappa = 0)\) the signal duration increases, whereas the signal amplitude is only slightly modified (see Appendix B.1). For \( k_3^* \rightarrow k_3 \) and large \( k_{-2} \) the signal duration is maximized and reaches 25 min. With a signal amplitude of 0.15 \(\mu\)M the duration does not exceed 15 min\(^1\). Thus, the presence of PIP\(_2\) phosphatase makes the phosphoinositide pathway more efficient by prolongating the IP\(_3\) signal. The signal duration further increases when both phosphatases are active and the additional PI degradation path is absent \((k_{-1}, k_{-2} \neq 0 \text{ and } \kappa = 0)\). One obtains a maximal duration of \(\sim 1\) hour, and \(\sim 20\) min with a signal amplitude of 0.15 \(\mu\)M. To illustrate these properties, I plotted in Fig. 4.4E the transients obtained in the three cases discussed above for an amplitude of 0.15 \(\mu\)M.

To summarize, the phosphoinositide pathway shown in Fig. 4.2 without one of the phosphatases or without the additional PI degradation pathway can only give rise to transient IP\(_3\) signals. This may underly the \([\text{Ca}^{2+}]_c\) dynamics in cells with short-lasting \([\text{Ca}^{2+}]_c\) signals. For all cases discussed, the estimated duration of IP\(_3\) transients with non-vanishing amplitude does not exceed 30 minutes. In hepatocytes, \([\text{Ca}^{2+}]_c\) oscillations can last for more than 90 min, indicating long-lasting IP\(_3\) signals ([138], L.D. Gaspers, \footnote{The signal duration is maximized by varying the stimulated PLC rate constant \(k_3^*\) with the constraint of fixed signal amplitude. This problem is solved using the method of Lagrange-multipliers.}.)
4.2. The phosphoinositide pathway as a linear chain

Figure 4.4: The transient IP$_3$ signal lasts longer when PIP$_2$ phosphatase is active. (A) The two pathways show identical kinetics (same reference unstimulated state). (B) [IP$_3$] after agonist mediated activation of PLC. (C) [PIP$_2$] (solid line) and [PIP] (dashed line) decrease, whereas [PI] (dot-dashed line) remains constant. (D) Both phosphatases are active, but PI is degraded by PI 4-kinase only. (E) [IP$_3$] after activation of PLC (solid black line). For comparison the shorter lasting IP$_3$ transients in the absence of PIP$_2$ phosphatase (blue line, same as B) or PIP phosphatase (red line, same as Fig. 4.3). (F) All three phosphoinositides decrease. Parameters given in Tab. 4.4, $k_3 = 0.007/s$; in (B),(C) $k_{-1} = 0$, $k_{-2} = 0.1/s$, $k_2 = 0.127/s$; in (E), (F) $\kappa = 0$, $v = 9.5$ molecules/µm$^2$/s, $k_{-1} = 0.1/s$, $k_{-2} = 0.1/s$, $k_1 = 0.0276/s$, $k_2 = 0.127/s$.

personal communication). The observed [IP$_3$] increase in hepatocytes lasts for more than 60 min [116]. These results indicate that at least in such cell type all processes depicted in Fig. 4.2 must be active. The phosphatases play a determinant role in controlling the [IP$_3$] dynamics (see Section 4.5). Alternatively, as suggested by Xu and coworkers [197],
the kinases themselves could be activated during stimulation (see discussion in Chapter 5).

### 4.2.3 Steady increase in \([\text{IP}_3]\) obtained with futile cycling

As pointed out in the previous section, activation of PLC leads to a long-lasting increase in \([\text{IP}_3]\) when the two phosphatases and the PI 3/5-kinases are active (Eq. (4.5) and (4.9)). The rise in \([\text{IP}_3]\) is accompanied by a decrease in the plasma membrane density of the three phosphoinositides (Fig. 4.5B). The depicted curves closely match the experimental measurements from Willars and coworkers [189]. Also the kinetics are in agreement with the experimental data (Fig. 4.5C, D). One notices that the initial inflection observed in the data is not reproduced by the model. Willars and coworkers suggested that this inflection is due to agonist receptor desensitization. The insets of Fig. 4.5C and D show that implementing this hypothesis, by assuming a PLC rate constant which relaxes in time to a lower value (see Figure 4.5 legend), improves the accordance between data and model.

For strong stimulation, \(k_3 \gg K_d\), the \([\text{IP}_3]\) reaches a maximal value \(Y_{1m}\), which represents the upper limit in \([\text{IP}_3]\) attained with the phosphoinositide pathway. Using Eq. (4.5) and Eqs. (4.10)–(4.12) one obtains

\[
Y_{1m} = Y_1 + \frac{fX_3k_1k_2}{k_4 \left( k_{-1} \left( \kappa + \frac{k_4Y_1}{X_1} \right) + k_{-2} \left( \frac{X_3}{X_2} + \frac{k_2X_3Y_1}{X_1X_2} \right) + \frac{k_{-1}X_2}{X_1} + \kappa k_2 \frac{X_1}{X_2} + \frac{k_2X_1}{X_1X_2} \right)}.
\]

(4.23)

Accordingly, any \([\text{IP}_3]\) can be reached provided that the activity of both phosphatases and PI 3/5-kinases are sufficiently high (large \(k_{-1}, k_{-2}, \) and \(\kappa\)). This implies increased phosphorylation of phosphoinositides and so additional energy consumption. The ATP hydrolysis rate is obtained straightforward from

\[
v_{\text{ATP}} = f \left[ (\kappa + k_1)X_1 + k_2X_2 \right].
\]

(4.24)

For example, in the unstimulated state the phosphoinositide pathway uses 7.18 µM/s ATP. With a cytosolic ATP concentration of \(\sim 1\) mM [3], the total pool of ATP cycles in approx. 2.5 min only to maintain the phosphoinositide pathway.

### 4.3 Conserved inositol/DAG moieties

One of the assumptions in the previous models was that the concentrations of the PI precursors inositol and CDP-DAG remain constant. However, closer inspection of
4.3. Conserved inositol/DAG moieties

Figure 4.5: Steady increase in [IP$_3$]. (A) Reaction scheme. (B) Steady state [PI] (dot-dashed line), [PIP] (dashed line), [PIP$_2$] (solid line), and [IP$_3$] (grey line) as function of agonist stimulus ($k_3$, Eqs. (4.5)–(4.7)). Changes are relative to the unstimulated state. (C) Kinetics of [IP$_3$] in model and experiment (squares). (D) Kinetics of [PI], [PIP], [PIP$_2$] in model and experiments (triangles, squares, circles, respectively). Experimental data are from [189]. At time $t = 0$, the PLC rate constant is set to $k_3 = 0.0662$/s. Better agreement with the data is obtained by assuming partial desensitization of the agonist receptor (insets in C and D). This is modeled by taking a time dependent PLC activity $k_3 = 0.0662(1 + e^{-t/\tau})$/s where $\tau = 100$ s is the characteristic time of receptor desensitization [75]. The PLC rate constant decreases exponentially from 0.1324/s to 0.0662/s. Parameters listed in Tab. 4.4, right column.

Fig. 4.1B indicates that during stimulation their production rate may change due to accumulation of IP$_3$ and DAG. I will show here how this may feeds forward and regulate the [IP$_3$].

If there are no additional production and degradation pathways for inositol and DAG, as shown in Fig. 4.1, or if these are slow compared to the depicted processes, then the total amount of inositol and DAG can be considered constant during stimulation. For example, total inositol consists of free inositol plus PI, PIP, PIP$_2$, IP$_3$, and the IP$_3$ products. For clarity, the mathematical model is posed in terms of a conserved total
Figure 4.6: Total amount of inositol is conserved. The above reactions are included in the model. An IP₃ buffer can bind reversibly to IP₃ and form the complex Y₆ (see Section 4.4).

inositol concentration. CDP-DAG is assumed, as before, to remain constant during stimulation. In the scheme of Fig. 4.6, the IP₃ degradation reactions are lumped into one reaction which leads to an intermediary inositol phosphate pool with concentration Y₂. A second reaction produces inositol, with concentration Y₃, which is used to synthesize PI. The time changes in X₁, Y₂ and Y₃ are given by

\[
\frac{dX_1}{dt} = \frac{k_6 Y_3}{f} - k_1 X_1 + k_{-1} X_2
\]

\[
\frac{dY_2}{dt} = k_3 Y_1 - k_5 Y_2, \quad \frac{dY_3}{dt} = k_5 Y_2 - k_6 Y_3
\]

The model equations for the other metabolites remain unchanged (Eqs. (4.1)–(4.4)).

The conservation relation for the inositol moiety, given as the number of molecules per cell, reads

\[
Y_i = A_{pm} (X_1 + X_2 + X_3) + V_{cyt} N_A (Y_1 + Y_2 + Y_3), \quad (4.25)
\]

where \(A_{pm}\) and \(V_{cyt}\) are the plasma membrane area and the cytosolic volume, respectively. One obtains for the steady state [IP₃]

\[
Y_1 = \frac{Y_i}{V_{cyt} N_A k_4 Q}, \quad (4.26)
\]

with the following denominator

\[
Q = \sum_{i=1}^{6} \frac{1}{k_i} + \frac{k_{-1}}{k_1 k_2} + \frac{k_{-2}}{k_2 k_3} + \frac{k_{-1} k_{-2}}{k_1 k_2 k_3}. \quad (4.27)
\]

For the steady state of the other metabolites one has

\[
X_1 = \frac{Y_i}{A_{pm}} \left( \frac{1}{k_1} + \frac{k_{-1}}{k_1 k_2} + \frac{k_{-1} k_{-2}}{k_1 k_2 k_3} \right) \frac{1}{Q}, \quad (4.28)
\]
Conservation of the inositol moiety obviates for additional degradation paths for PI (3/5 phosphorylation), making the system more energy efficient. Although PLC activation can always produce an increase in [IP₃], the experimental data suggests futile phosphorylation cycles in order to explain (i) the strong increase in [IP₃], and (ii) the decrease in PI, PIP, and PIP₂ densities during stimulation. Indeed, after introducing the two phosphatases according to Eqs. (4.14)–(4.15) the model reproduces these experimental facts. The model simulations are nearly indistinguishable from the simulations shown in Fig. 4.5. Using a similar mathematical treatment, the same conclusions are derived when the DAG moiety is conserved or both total DAG and inositol are conserved (not shown).
4.4 Effect of an IP$_3$ buffer on the [IP$_3$] in the absence of Ca$^{2+}$ feedbacks

The derived phosphoinositide pathway models allow to answer whether the effects of the IP$_3$ buffer on the Ca$^{2+}$ signals, in particular abolishment of [Ca$^{2+}$]$_c$ oscillations (see section 3.2), could be ascribed to depletion of IP$_3$?

I have shown that this is not the case when the phosphoinositide metabolism is reduced to IP$_3$ production and degradation. The IP$_3$ buffer slows the kinetics of IP$_3$, but does not change its steady state. The same result is obtained with the more detailed linear chain model, which accounts for the synthesis of the IP$_3$ precursor PIP$_2$ (Section 4.2). Only if the inositol moiety is conserved an IP$_3$ buffer modifies the steady state [IP$_3$]. This is because sequestration of IP$_3$ in a non-metabolizable pool effectively decreases the total amount of available inositol.

To assess the magnitude of such an effect, I rewrite the conservation relation for the inositol moiety in the presence of the IP$_3$ buffer (see Fig. 4.6)

$$Y_t = A_{pm}(X_1 + X_2 + X_3) + V_{cyt}N_A(Y_1 + Y_2 + Y_3 + Y_b). \tag{4.31}$$

In steady state the concentration of IP$_3$ bound to buffer is given by $Y_b = B_tY_1/(Y_1 + K_B)$ where $B_t$ is the total concentration of IP$_3$ buffer and $K_B$ its dissociation constant. One derives the implicit relation

$$Y_1(B_t) = \frac{Y_t}{V_{cyt}N_A k_4 Q} - \frac{B_t Y_1(B_t)}{Y_1(B_t) + K_B k_4 Q} \tag{4.32}$$

It is straightforward to solve the corresponding quadratic equation and obtain the IP$_3$ concentration. One finds that the [IP$_3$] monotonically decreases with the IP$_3$ buffer dissociation constant $K_B$ and reaches a minimum for $K_B = 0$ (Fig. 4.7). Therefore, following inequality for the ratio of [IP$_3$] with and without IP$_3$ buffer holds

$$1 \geq \frac{Y_1(B_t = 30)}{Y_1(B_t = 0)} \geq 1 - V_{cyt}N_A B_t Y_t. \tag{4.33}$$

The above formula shows that the decrease in [IP$_3$] due to buffering crucially depends on the total amount of cellular inositol $Y_t$. The reported phosphoinositides concentrations varied by a factor of 10 (see Section 4.1.2), thus the total inositol $Y_t$ can be estimated to lie between 70 and 700 million molecules/cell. With the maximal IP$_3$ buffer concentration used in experiments of about 30 µM one obtains

$$1 > \frac{Y_1(B_t = 30)}{Y_t(B_t = 0)} \geq 0.36 \text{ to } 0.93. \tag{4.34}$$
4.5. Perturbing the phosphoinositide pathway

Figure 4.7: Changes in steady state \([\text{IP}_3]\) as function of buffer dissociation constant \(K_B\). Shown is \([\text{IP}_3]\) in resting and stimulated cells when total inositol \(Y_t\) is 70 (solid line) or 700 (dashed line) million molecules. The decrease in \([\text{IP}_3]\) due to \(\text{IP}_3\)-buffering is more pronounced at low total inositol (solid line).

Therefore, for strong stimulation, the \([\text{IP}_3]\) reaches 0.54–1.39 \(\mu\)M. It has been shown that in this range the \([\text{IP}_3]\) is high enough to induce \([\text{Ca}^{2+}]_c\) oscillations [51]. Thus, even for this extreme case, where conservation of inositol and strong binding of the buffer to \(\text{IP}_3\) are assumed, the effect of \(\text{IP}_3\) buffering on the \([\text{Ca}^{2+}]_c\) dynamics cannot be ascribed to depletion of \(\text{IP}_3\).

4.5 Perturbing the phosphoinositide pathway

To verify the model predictions, with respect to the contribution of the different enzymes to the \(\text{IP}_3\) dynamics, one could inhibit or activate some of the kinases and phosphatases (with RNAi for instance cf. [98]). The effect of such perturbations can be analyzed using the metabolic control analysis. I focus on the control exerted by the different reactions on the \([\text{IP}_3]\). The unstimulated state is used as reference state. For example, the control coefficient of PLC on the \([\text{IP}_3]\) is defined by

\[
C_{k_3} = \frac{k_3}{k_3 + K_d} \cdot \frac{\partial Y_t}{\partial k_3},
\]

where the overline indicates the unstimulated steady state. This coefficient, gives the fractional changes in \([\text{IP}_3]\) after an increase in the rate constant of the PLC mediated reaction, \(k_3\). A positive (negative) control coefficient implies an increase (decrease) in \([\text{IP}_3]\).

For the linear chain model one obtains

\[
C_{k_3} = \frac{K_d}{k_3 + K_d},
\]

indicating that PLC exerts no control on the \([\text{IP}_3]\) if \(K_d\) vanishes. This occurs when the activities of the phosphatases and/or the PI 3/5-kinases are weak (Eq. (4.9)). Con-
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\[ C_v = 1 \]
\[ C_{k_1} = 0.982 \]
\[ C_{k_2} = 0.937 \]
\[ C_{k_3} = 0.933 \]
\[ C_{k_{-2}} = -0.933 \]
\[ C_{k_{-1}} = -0.937 \]
\[ C_\kappa = -0.982 \]

\[ C_{k_6} = 0.0006 \]
\[ C_{k_4} = -0.996 \]
\[ C_{k_5} = 0.032 \]

Figure 4.8: Control coefficients of the indicated reactions on the [IP$_3$] in the linear chain model (A) and in the model with conservation of the inositol moiety (B).

versely, when these enzymes are highly active $K_d$ is large and the control exerted by PLC is maximized. The expressions for the other control coefficients are listed in Tab. 4.5 left column. PLC, PI 4-kinase, PIP 5-kinase, and PI synthase have always positive control on [IP$_3$], indicating that activation of these enzymes increases the [IP$_3$]. On the other hand, phosphatases and the PI 3/5-kinases have negative control. The expressions in Tab. 4.5, yield a ranking in the control, which is valid for any parameter values

\[ |C_v| = |C_{k_4}| > |C_{k_1}| > |C_\kappa| > |C_{k_2}| = |C_{k_{-1}}| > |C_{k_3}| = |C_{k_{-2}}|. \]  

(4.36)

Accordingly, PLC exerts the weakest control on the [IP$_3$] and the IP$_3$ degradation the strongest. For the estimated parameters the control appears, however, to be evenly distributed between all processes and the differences between absolute control coefficients are small (Fig. 4.8A).

Control can distribute differently when the total amount of inositol is conserved. For
4.5. Perturbing the phosphoinositide pathway

the PLC reaction one has

\[
C_{k_3} = \frac{\overline{X}_3 A_{pm}}{Y_t} \left( 1 + \frac{k_{-2}}{k_2} + \frac{k_{-1} k_{-2}}{k_1 k_2} \right). 
\]

(4.37)

The control of PLC on the \([IP_3]\) is proportional to the relative amount of PIP2, \(\overline{X}_3/Y_t\), and increases in the presence of phosphatases. If PLC is the bottleneck reaction in the phosphoinositide pathway, because of a low rate constant, the inositol moiety accumulates in form of PIP2 (\(\overline{X}_3 \rightarrow Y_t/A_{pm}\)). Then, even in the absence of phosphatases, the control exerted by the PLC can be large and an increase in \(k_3\) produces significant changes in the \([IP_3]\). The control coefficient of the other reactions also increases when the inositol moiety accumulates in form the reaction substrate (Tab. 4.5, right column). An exception is made by the IP3 degradation. The control exerted by its rate constant \(k_4\) is negative and approaches zero with accumulation of its substrate IP3. Using the formulas given in Tab. 4.5 one derives following relations, which hold independently of the parameter choice,

\[
|C_{k_2}| > |C_{k_{-1}}| \quad \text{and} \quad C_{k_2} + C_{k_{-1}} = 1 \quad (4.38)
\]

\[
|C_{k_3}| > |C_{k_{-2}}| \quad \text{and} \quad C_{k_3} + C_{k_{-2}} = 1. \quad (4.39)
\]

Which reaction has the strongest control depends on the distribution of phosphoinositides in the resting state. According to the reported distribution, inositol accumulates primarily as PI, so that inositol phosphates degradation and the PI synthase have practically no control on the \([IP_3]\). The highest absolute control is exerted by the IP3 degradation, followed by the PLC reaction (Fig. 4.8B). Note that due to the low levels of PIP2, the control exerted by the PLC in the absence of phosphatases is low (\(C_{k_3} = 0.2\), compared to 0.933 with phosphatases)

In both the linear model and the model with conservation of inositol, inhibition of the phosphatases should produce a significant increase in the IP3 concentration. The changes in the \([IP_3]\) are predicted to be similar to changes obtained after hormone addition (i.e. activation of PLC).
Chapter 4. Model for the phosphoinositide metabolism

Control coefficients

<table>
<thead>
<tr>
<th>Process</th>
<th>Linear model</th>
<th>Conservation of inositol</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{k_3}$ PLC</td>
<td>$\frac{K_d}{k_3 + K_d}$</td>
<td>$\frac{X_3 A_{pm}}{Y_t} \left(1 + \frac{k_{-2}}{k_2} + \frac{k_{-1} k_{-2}}{k_1 k_2}\right)$</td>
</tr>
<tr>
<td>$C_{k_4}$ IP$_3$ degradation</td>
<td>$-1$</td>
<td>$-1 + \frac{Y_1 V_{cyt} N_A}{Y_t}$</td>
</tr>
<tr>
<td>$C_{k_1}$ PI 4-kinase</td>
<td>$1 - \frac{V_m k_3}{v k_3 + K_d}$</td>
<td>$\frac{X_1 A_{pm}}{Y_t} \frac{k_2 k_3 + k_{-1}(k_3 + k_{-2})}{k_2 k_3 + k_{-1}(k_3 + k_1 k_{-2})}$</td>
</tr>
<tr>
<td>$C_{k_{-1}}$ PIP phosphatase</td>
<td>$-\frac{K_d(1 + k_3/k_{-2})}{k_3 + K_d}$</td>
<td>$-\frac{X_2 A_{pm}}{Y_t} \frac{k_1}{k_1}$</td>
</tr>
<tr>
<td>$C_{k_2}$ PIP 5-kinase</td>
<td>$\frac{K_d(1 + k_3/k_{-2})}{k_3 + K_d}$</td>
<td>$\frac{X_2 A_{pm}}{Y_t} \left(1 + \frac{k_{-1}}{k_1}\right)$</td>
</tr>
<tr>
<td>$C_{k_{-2}}$ PIP phosphatase</td>
<td>$-\frac{K_d}{k_3 + K_d}$</td>
<td>$-\frac{X_3 A_{pm}}{Y_t} \left(\frac{k_{-2}}{k_2} + \frac{k_{-1} k_{-2}}{k_1 k_2}\right)$</td>
</tr>
<tr>
<td>$C_{k_5}$ inositol phosphates</td>
<td>0</td>
<td>$\frac{Y_2 V_{cyt} N_A}{Y_t}$</td>
</tr>
<tr>
<td>deg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{v}, C_{k_6}$ PI synthase</td>
<td>1</td>
<td>$\frac{Y_3 V_{cyt} N_A}{Y_t}$</td>
</tr>
<tr>
<td>$C_{\kappa}$ PI 3/5-kinase</td>
<td>$-1 + \frac{V_m k_3}{v k_3 + K_d}$</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.5: Control of the different enzymatic reactions on the IP$_3$ concentration. Control coefficients are calculated for the linear model (Eq. (4.5)) and the model with conservation of the inositol moiety (Eq. (4.26)).
4.6 Positive feedback of Ca\textsuperscript{2+} on IP\textsubscript{3} obviates for futile cycling

The mathematical modeling of the phosphoinositide pathway indicates that long-lasting non-oscillating IP\textsubscript{3} signals are obtained in the presence of phosphatases, and such futile cycles demand increased energy (Eq. (4.24)). However, we learned that to induce [Ca\textsuperscript{2+}]\textsubscript{c} oscillations the [IP\textsubscript{3}] must not remain constant. In Chapter 2, I demonstrated how the mutual interaction of oscillating [Ca\textsuperscript{2+}]\textsubscript{c} and [IP\textsubscript{3}] brings about a variety of dynamic behaviors and how several experimental facts could be explained by this interaction. The theoretical results put forward a possible physiological role for [IP\textsubscript{3}] oscillations namely a better frequency encoding of the external stimulus. In this last section, I will show how Ca\textsuperscript{2+} feedbacks on IP\textsubscript{3} metabolism relax the necessity for futile cycling and allow to economize cellular energy.

For the mathematical treatment, I will use the linear chain phosphoinositide metabolism model (Section 4.2). Ca\textsuperscript{2+} feedback on IP\textsubscript{3} production mediated by activation of PLC is taken into account by modifying the equation for the [IP\textsubscript{3}]

$$\frac{dY_1}{dt} = k_3 \left( \mu \frac{c^2}{K_{PLC}^2 + c^2} + 1 - \mu \right) f X_3 - k_4 Y_1. \quad (4.40)$$

Here $\mu$ is the fraction of Ca\textsuperscript{2+} activated to Ca\textsuperscript{2+} insensitive PLC and $c$ the [Ca\textsuperscript{2+}]\textsubscript{c}. For the [Ca\textsuperscript{2+}]\textsubscript{c} dynamics, the model with delayed IP\textsubscript{3}R receptor inactivation is used (Eqs. (2.7)–(2.9)).

In the absence of phosphatases and PI 3/5-kinase, activation of the Ca\textsuperscript{2+} insensitive PLC induces only a few transients in [Ca\textsuperscript{2+}]\textsubscript{c} and [IP\textsubscript{3}] because [PIP\textsubscript{2}] depletes (Fig. 4.9A). On the contrary, activation of the Ca\textsuperscript{2+} sensitive PLC gives rise to sustained [Ca\textsuperscript{2+}]\textsubscript{c} oscillations (Fig. 4.9B). The rationale for this is that during the interspike interval, characterized by low [Ca\textsuperscript{2+}]\textsubscript{c}, PLC is less active so that the PIP\textsubscript{2} hydrolysis rate is low and PIP\textsubscript{2} can regenerate. Mutual activation of IP\textsubscript{3} production and Ca\textsuperscript{2+} release brings the [Ca\textsuperscript{2+}]\textsubscript{c} to the threshold for calcium induced calcium release. The IP\textsubscript{3} necessary to sensitize the IP\textsubscript{3}R towards Ca\textsuperscript{2+} is supplied by the newly regenerated pool of PIP\textsubscript{2}. The explosive increase in [Ca\textsuperscript{2+}]\textsubscript{c} is accompanied by PLC activation which causes concomitant [IP\textsubscript{3}] increase and [PIP\textsubscript{2}] decrease. Thereafter, the [Ca\textsuperscript{2+}]\textsubscript{c} spike is brought to an end by several complementary processes such as IP\textsubscript{3}R inactivation, depletion of PIP\textsubscript{2}, depletion of the ER Ca\textsuperscript{2+} store. The cycle begins anew with regeneration of the PIP\textsubscript{2} pool. The resulting oscillations have the typical properties for an oscillation mechanisms that include positive feedback of Ca\textsuperscript{2+} on IP\textsubscript{3}, namely long-period oscillations.
Figure 4.9: In the absence of futile cycling and 
Ca$^{2+}$ -activated PLC, increase in the 
PLC rate constant $k_3$ produces a [Ca$^{2+}$]c transient and PIP$_2$ depletion (black and blue line, 
respectively). However, with Ca$^{2+}$ activated PLC (B), [Ca$^{2+}$]c oscillates due to transient 
regeneration of the PIP$_2$ pool. (C) Oscillatory domain (shadowed region) in the absence of 
Ca$^{2+}$-feedback on PLC. The ATP-hydrolysis rate increases when the kinase rate constants 
are increased due to higher phosphatases activity (futile cycling). (D) The oscillatory region is 
considerably enlarged when Ca$^{2+}$ activation of PLC is present. Diamond and square in C and 
D indicate parameters for panel A and B, respectively. The linear model without conservation 
of inositol is used, similar results are obtained with conservation of inositol (not shown). PIP 
and PIP$_2$ phosphatase as well as PI 3/5-kinases are gradually introduced by increasing $f_c$ in 
Eqs. (4.14)–(4.16) from 0 to 1. The ATP-hydrolysis rate $v_{ATP}$ is determined from Eq. (4.24). 
Parameters: (A) $f_c = 0$, $\mu = 0$, $k_3 = 0.25$/s; (B) $f_c = 0$, $\mu = 1$, $k_3 = 0.25$/s; (C) $\mu = 0$, (D) 
$\mu = 1$. In all simulations $k_4 = 1$/s, all rate constants are determined from Eqs. (4.11)–(4.16) 
and are accordingly higher than listed in Tab. 4.4. Parameters for the Ca$^{2+}$ dynamics as given 
in Tab. 2.1, positive feedback.
and a marked frequency encoding of the stimulus (data not shown, see examples in
Chapter 2).

Introducing phosphatases and the PI 3/5-kinase, rescues the pathway with Ca\(^{2+}\)
insensitive PLC with respect to stimulus induced \([\text{Ca}^{2+}]_c\) oscillations. This leads to a
higher ATP consumption, because the phosphoinositide concentrations must be main-
tained by increasing the phosphorylation rates. I recall that the rate of ATP hydrolysis
in the phosphoinositide pathway is given by

\[
v_{\text{ATP}} = f [(\kappa + k_1)X_1 + k_2X_2].
\]

(4.41)

In Fig. 4.9 I plotted the oscillatory region as function of \(v_{\text{ATP}}\) and of the stimulus de-
pendent PLC rate constant \(k_3\) (shadowed region). When futile cycling is strong enough,
so that the \([\text{IP}_3]\) attains the threshold for activating the \(\text{IP}_3\)R, \([\text{Ca}^{2+}]_c\) oscillations are
observed also in the system without Ca\(^{2+}\) feedback on PLC. However, as shown in
chapter 2, such a pure Ca\(^{2+}\)/\(\text{IP}_3\)R oscillator has fast \([\text{Ca}^{2+}]_c\) oscillation which only poorly
frequency-encode the stimulus. Positive feedback of Ca\(^{2+}\) on \(\text{IP}_3\) production, in addition
of enhancing the ability to frequency encode the stimulus, allows for \([\text{Ca}^{2+}]_c\) oscillations
in the complete absence of futile cycling and so economizes cellular energy, and greatly
expands the parameter domain where \([\text{Ca}^{2+}]_c\) oscillations are observed (Fig. 4.9D com-
pare to C).

In both systems, one notices that for intermediary ATP hydrolysis rates \([\text{Ca}^{2+}]_c\), os-
ccillations persist for high PLC rate constants (Fig. 4.9C, D). In experiments, the typical
hallmark of high agonist dose is the elevated steady cytoplasmic Ca\(^{2+}\) concentration
at high agonist dose [170]. This difference between model and experiment is readily
explained by the fact that the maximal \([\text{IP}_3]\) reached within this parameter region,
is below the threshold value where the \(\text{IP}_3\)R are continuously open and the elevated
\([\text{Ca}^{2+}]_c\) steady state is stable (Eq. (4.23)). In principle, for strong stimulation, processes
other than high \([\text{IP}_3]\) may bring the oscillations to an end. These are, for example,
stimulus dependent Ca\(^{2+}\) entry from the external medium or agonist receptor desensi-
tization [26, 75, 149], both processes can be easily taken into account in the model (see
e.g. Chapter 2).

From the different isoforms of PLC, some are activated at physiological \([\text{Ca}^{2+}]_c\),
whereas some are activated at much lower concentrations than basal \([\text{Ca}^{2+}]_c\), and so are
insensitive to \([\text{Ca}^{2+}]_c\) changes [135]. To evaluate their contribution, the strength of the
positive feedback is tuned by varying the fraction \(\mu\) of Ca\(^{2+}\) sensitive to Ca\(^{2+}\) insensitive
PLC (see Eq. 4.40), then, the ATP hydrolysis rate over which activation of PLC induces
long-lasting \([\text{Ca}^{2+}]_c\) oscillations is computed. The results are summarized in Fig. 4.10.
Figure 4.10: Presence of positive feedback of Ca\(^{2+}\) on IP\(_3\) production improves energy efficiency. In the shadowed region activation of PLC (\(k_3\) increase) induces [Ca\(^{2+}\)]\(_c\) oscillations. The more Ca\(^{2+}\) activated PLC the less futile (de)phosphorylation cycling is needed. When only Ca\(^{2+}\) dependent PLC is present futile cycling is not necessary for an oscillating [Ca\(^{2+}\)]\(_c\) signal. For parameters below the dashed line introducing an IP\(_3\) buffer quenches the oscillations completely.

The solid line divides the parameter space into two regions: in the shadowed region, feedback strength and futile cycling are high enough, so that activation of PLC brings the [IP\(_3\)] above the threshold to sensitize the IP\(_3\)R and so generates [Ca\(^{2+}\)]\(_c\) oscillations; below the black solid line, activation of PLC induces only a few [Ca\(^{2+}\)]\(_c\) transients.

These results give an appealing interpretation for the experiments performed with an [IP\(_3\)] clamped at a fixed value. In some cell types, including hepatocytes, one observes [Ca\(^{2+}\)]\(_c\) oscillations during such experimental protocol [16, 51, 164, 204]. This suggests that [Ca\(^{2+}\)]\(_c\) oscillations can occur at a constant [IP\(_3\)]. On the other hand, the experiments with the IP\(_3\) buffer ascribe a central role to IP\(_3\) oscillations in controlling [Ca\(^{2+}\)]\(_c\) oscillations. According to the theoretical results, Ca\(^{2+}\) feedback on PLC may be essential to regenerate the PIP\(_2\) pool, and this positive feedback is impaired when the IP\(_3\) dynamics is slowed after expressing an IP\(_3\) buffer. Therefore, if futile cycling is weak, below the dashed line in Fig. 4.10, the IP\(_3\) buffer quenches agonist induced
oscillations because the PIP\(_2\) pool depletes. However, if PIP\(_2\) hydrolysis is bypassed, by supplying IP\(_3\) externally, the cell regains the ability to generate \([\text{Ca}^{2+}]_c\) oscillations. This shows that the necessity for [IP\(_3\)] oscillations in agonist mediated [Ca\(^{2+}\)]\(_c\) signaling and the ability to oscillate for clamped [IP\(_3\)] are not mutually exclusive.
Chapter 5

Discussion part I: Calcium and IP$_3$ dynamics

This work represents a contribution for the understanding of the phosphoinositide metabolism in general and the IP$_3$ mediated Ca$^{2+}$ signals in particular. The increasing evidence that hormone evoked periodic Ca$^{2+}$ spiking can be accompanied by oscillations of the Ca$^{2+}$-releasing second messenger IP$_3$ [61, 112, 161, 171, 201], raises the questions of (i) the underlying mechanisms of IP$_3$ oscillations, (ii) their potential functional role, and (iii) the prerequisites of the phosphoinositide metabolism for generating long-lasting IP$_3$ signals and oscillations. The theoretical analysis and experiments presented here provide insight in all three issues.

Several processes could be involved in the generation of IP$_3$ oscillations. Feedbacks of IP$_3$ and the second product of the PLC reaction, diacylglycerol, on PLC and upstream agonist receptor/G-protein could produce IP$_3$ oscillations without involvement of Ca$^{2+}$ [25, 62]. Alternatively, feedbacks on IP$_3$ metabolism may be mediated by Ca$^{2+}$, resulting in coupled IP$_3$–Ca$^{2+}$ oscillators [28, 34, 83, 104]. In this work, the focus was put on the latter type of feedback oscillators because they can naturally account for the experimental observations of (i) Ca$^{2+}$ oscillations at clamped IP$_3$ concentration and (ii) coupled IP$_3$ and Ca$^{2+}$ oscillations. I considered prototypical positive and negative feedbacks of Ca$^{2+}$ ions on IP$_3$ metabolism: Ca$^{2+}$ activation of PLC and Ca$^{2+}$ activation of IP$_3$ 3-kinase, respectively.
Physiological role of IP$_3$ oscillations

Frequency encoding

The incorporation of feedbacks of Ca$^{2+}$ on IP$_3$ into a core Ca$^{2+}$ oscillator model based on the regulatory properties of the IP$_3$ receptor can greatly expand the sensitivity of signal transduction to the hormonal stimulus. The presence of either feedback increases the range of agonist concentrations where one observes Ca$^{2+}$ oscillations and enhances the ability to frequency-encode the agonist dose. Mathematical models indicate that a large range in stimulus dependent frequencies permits a more precise and selective decoding of the Ca$^{2+}$ oscillations by Ca$^{2+}$-regulated processes (Salazar et al., submitted; [37, 145]). The Ca$^{2+}$ feedbacks on the IP$_3$ metabolism represent a possible mechanism for the generation of robust long-period oscillations. This is likely to be an important component of frequency-modulated Ca$^{2+}$ signals, since physiological responses are controlled in this lower frequency range [30, 52].

Energy efficiency

I was able to show, that coupling the phosphoinositide metabolism to the Ca$^{2+}$ dynamics using the positive feedback of Ca$^{2+}$ on the IP$_3$ production, greatly enhances the energy efficiency of the Ca$^{2+}$/IP$_3$ signaling pathway (Section 4.6). This is because such a feedback allows the regeneration the PIP$_2$ pool in the interspike intervals, and so reduces the necessity of futile phosphorylation/dephosphorylation cycles in the phosphoinositide metabolism. One of the interesting results was that IP$_3$ oscillations may be the only way for generating long-lasting Ca$^{2+}$ oscillations in vivo. This occurs if the activities of the PIP 4-phosphatase and the PIP$_2$ 5-phosphatase are not strong enough, so that activation of PLC does not lead to the [IP$_3$] necessary to sensitize the IP$_3$R. In this case, an IP$_3$ buffer will inevitably abolish the [Ca$^{2+}$]$_c$ oscillations. The necessity for PIP$_2$ regeneration and IP$_3$ oscillations can be bypassed by supplying IP$_3$ externally. The [Ca$^{2+}$]$_c$ can then oscillate at clamped [IP$_3$] as observed in experiments.

These properties do not appear to rely on the precise choice of the model for the Ca$^{2+}$/IP$_3$R dynamics. Indeed, the models by Sneyd et al. (2004) [154], Tang and Othmer (1992) [163], or Dupont and Goldbeter (1993) [35], implemented with Ca$^{2+}$ activation of PLC and the phosphoinositide metabolism, gave qualitatively similar results (results not shown). The essential component is the positive feedback of Ca$^{2+}$ on the IP$_3$ production. With the negative feedback model, which describes the Ca$^{2+}$ dependent IP$_3$ degradation, the PIP$_2$ pool does not regenerate in the interspike intervals. Therefore,
in the negative feedback model, I could not find sustained Ca\(^{2+}\) oscillations when futile cycling is weak (not shown).

**Coordination of intercellular Ca\(^{2+}\) signals**

An additional possible role for IP\(_3\) oscillations is to facilitate the coordination of intercellular Ca\(^{2+}\) signals. In the intact liver, cells can communicate with their neighbors by diffusion of small molecules through intercellular channels (gap junctions). This is a premise for a coordinated response to the external hormonal stimulation, where one observes repetitive Ca\(^{2+}\)-waves propagating through a large number of cells [113, 137]. If the gap-junctional coupling is disrupted, the coordinated intercellular waves are abolished and the cells oscillate independently with different oscillation frequencies. This heterogeneity between cells is supposed to arise from intercellular variations in the number of agonist-receptors, and so in the amount of produced IP\(_3\) [20, 172].

In models without feedbacks of Ca\(^{2+}\) on IP\(_3\), intercellular diffusion of Ca\(^{2+}\) alone is sufficient to account for the coordinated response observed in an heterogeneous population of cells coupled by gap junctions [63, 64]. For this, the gap-junctional Ca\(^{2+}\) permeability must be higher than a critical value. When IP\(_3\) does not oscillate its intercellular diffusion smooths out the differences between cells, and so decreases the value of the critical Ca\(^{2+}\) gap-junctional permeability. I have previously shown together with T. Höfer that the intercellular diffusion of IP\(_3\) alone cannot actively couple cells [123]. In fact, if the Ca\(^{2+}\) permeability is too low, one observes a coordinated response which lasts only for a limited number of spikes [36].

*In vivo*, the theoretically estimated gap-junctional permeability of Ca\(^{2+}\), necessary for the intercellular coordination, may not be attained because Ca\(^{2+}\) is strongly buffered in the cytoplasm [187]. The fact that, due to Ca\(^{2+}\) feedbacks, IP\(_3\) oscillates, and that IP\(_3\) is only weakly buffered (its diffusion is 10-20 times faster than the diffusion of Ca\(^{2+}\) [4]), now makes IP\(_3\) a promising candidate for the active synchronization of Ca\(^{2+}\) oscillations in coupled cells. This notion is supported by recent theoretical results (Politi et al., in press; [196]).

**Properties of Ca\(^{2+}\)/IP\(_3\) oscillators**

For the positive feedback to modulate the oscillation properties, the Ca\(^{2+}\) sensitivity of PLC needs to be only somewhat above basal [Ca\(^{2+}\)]\(_c\) (\(K_{PLC} = 0.1-0.2 \ \mu M\)). Such values are in agreement with experimental data [11]. This feedback delays the onset of
the $\text{Ca}^{2+}$ spike, because both $[\text{IP}_3]$ and $[\text{Ca}^{2+}]_c$ must rise to a certain level for triggering explosive opening of the IP$_3$R. In this way, long oscillation periods arise for low levels of stimulation, while for strong stimuli the high IP$_3$ level obviates the need for additional $\text{Ca}^{2+}$ activation of PLC. I have here specifically assumed that $\text{Ca}^{2+}$ and agonist act on the same isoform of PLC (e.g. PLC$\beta$). However, similar results were obtained in a model variant where the isoform PLC$\delta$, which is a $\text{Ca}^{2+}$-sensitive but agonist-insensitive PLC isoform, is also included (data not shown).

Negative feedback exerts control on the $\text{Ca}^{2+}$ oscillations when IP$_3$ removal takes place predominantly via IP$_3$3K rather than by the IP$_3$5P (> 60% of the removal flux at high $[\text{Ca}^{2+}]_c$ carried by IP$_3$3K). Long oscillation periods are generated when the $[\text{IP}_3]$ drops during the $\text{Ca}^{2+}$ spike due to IP$_3$3K activation and subsequently recovers slowly to the level needed to activate the IP$_3$R. I have found that this mechanism requires a finely tuned interplay between IP$_3$ metabolism and $\text{Ca}^{2+}$ fluxes. This sensitivity may explain the discrepancy to the work of Dupont and Erneux [34], who reported only small effects of IP$_3$3K on $[\text{Ca}^{2+}]_c$ oscillation periods. In contrast, the effects of the positive feedback were found to be very robust with respect to the properties of the core $\text{Ca}^{2+}$ oscillator.

The different modes of action of positive and negative feedbacks are reflected by opposing requirements on the lifetime of IP$_3$. In the case of positive feedback, IP$_3$ turnover must be fast to support long-period oscillations, allowing (i) for coincidence of $\text{Ca}^{2+}$ and IP$_3$ spikes and (ii) for the rapid removal of IP$_3$ in the wake of a spike. In the case of negative feedback, slow IP$_3$ turnover is required for the slow recovery of IP$_3$ levels in between spikes. In different cellular systems, either one of the IP$_3$ feedbacks could play a significant role in controlling $\text{Ca}^{2+}$ oscillations, depending primarily on the underlying turnover rate of IP$_3$. However, they cannot be expected to act synergistically.

The theory can be used to estimate, whether IP$_3$ oscillations, generated by positive feedback of $\text{Ca}^{2+}$ on IP$_3$, can play a significant role in modulating $\text{Ca}^{2+}$ oscillations. If the $\text{Ca}^{2+}$ oscillations have a high frequency and the IP$_3$ turnover time is below the time in between spikes, one expects the amplitude of the IP$_3$ oscillations to be very small. These small amplitude IP$_3$ oscillations will not modulate the $[\text{Ca}^{2+}]_c$ oscillations. Thus, a system with fast $\text{Ca}^{2+}$ oscillations and positive feedback behaves as it would have a constant $[\text{IP}_3]$. This is in agreement with recent experimental and theoretical results. The results by Sneyd and coworkers indicate that in airway smooth muscle cells, a cell type which show comparatively fast oscillations, the $\text{Ca}^{2+}$ oscillations do not depend on IP$_3$ oscillations (frequencies of 0.2–0.5 spikes/s). On the contrary, in the slower oscillating pancreatic acinar cells the authors found that IP$_3$ oscillations affect
the Ca^{2+} oscillations (frequencies 0.03–0.04 spikes/s) [155].

**Identification of Ca^{2+} feedbacks on IP_{3}**

A critical question is the experimental identification of the mechanisms that drive IP_{3} oscillations. The theoretical analysis showed that slowing the IP_{3} turnover by means of an IP_{3} buffer can be used to distinguish between the two feedback mechanisms. I demonstrated that in models without Ca^{2+} feedbacks on IP_{3}, an IP_{3} buffer will have practically no effect on the steady state IP_{3} concentration and so on the Ca^{2+} oscillations. An IP_{3} buffer can quench the oscillations generated by an IP_{3}–Ca^{2+} oscillator based on positive feedback of Ca^{2+} on IP_{3}, but not by one based on negative feedback. The analytical results indicate that the weak response of the negative-feedback oscillator to IP_{3} buffering could be a general property not limited to a mechanism operating through IP_{3} 3-kinase. Indeed, very similar results are obtained with an alternative mechanism acting through PKC-dependent inactivation of the agonist receptors (Gaspers et al., submitted).

To test this theoretical prediction, the ligand binding domain of the type 1 IP_{3}R has been expressed in a mammalian cell line. This construct acts as an IP_{3} buffer. The observed dose-dependent suppression of Ca^{2+} oscillations demonstrates that the IP_{3} dynamics play a critical role in the oscillator mechanism. Moreover, the detailed agreement between the experimental data and the simulations of the positive-feedback model is consistent with a coupled IP_{3}–Ca^{2+} oscillator based on Ca^{2+} activation of PLC.

The model suggests a further experiment to strengthen the theoretical and experimental results. The Ca^{2+} oscillations, which have been suppressed by slowing the IP_{3} dynamics with an IP_{3} buffer, should be rescued by injecting the IP_{3} metabolizing enzyme IP_{3} 5-phosphatase and stimulating with higher agonist doses. The rationale for this is that the IP_{3} 5-phosphatase will fasten the IP_{3} turnover, and so allow for the [IP_{3}] to decrease in the wake of a Ca^{2+} spike.

**Key features of the phosphoinositide metabolism**

In this work, I developed one of the first detailed mathematical models for the phosphoinositide pathway. The model was a compromise between completeness and experimentally available data for the different reactions and metabolites. These data were used to estimate the kinetic parameters and to assess the contribution of the different reactions
to the IP₃ homeostasis.

To generate, after PLC activation, a sustained increase in [IP₃], the PIP 4-phosphatase and PIP₂ 5-phosphatase are essential. Furthermore, PI must be metabolized at least by two different enzymes (the PI 4-kinase and e.g. the PI 3-kinase). In the absence of one of these enzymes, the increase in [IP₃] is transient and lasts not longer than 30 minutes. To account for the experimental data, the two phosphatases must have similar activities as their opposing kinases (PI 4-kinase and PIP 5-kinase, respectively). This is reflected in the strong control the phosphatases exert on the [IP₃] and the elevated energy consumption. These results do not depend on the assumed linear reaction rate laws. The description of the enzyme activities with more realistic reversible Michaelis-Menten rate laws led to similar conclusions (see Appendix B.3).

I already discussed how the presence of Ca²⁺ activated PLC can rescue Ca²⁺ signaling when the dephosphorylation rates are low. A second and simple way to obtain a long-lasting IP₃ signal is the upregulation of the producing enzymes. The model predicts that, in order to supply PIP₂ for maintaining an elevated IP₃ flux, a stimulus dependent increase in the synthesis rate of PI is required. Furthermore, the PI 4-kinase and PIP 5-kinase rates must also be stimulus dependent, in order to reproduce the experimental data (see Appendix B.2). There are indications that this could be the case. PIP 5-kinase and PI 4-kinase have been shown to be activated after binding of ligand to tyrosine kinase receptors or G-protein coupled receptors (see review [188] and [21, 143, 173, 197]). The PI-transfer protein, a protein responsible for the transfer of PI from the ER membrane to the plasma membrane, has been shown to be activated by GTP binding proteins, and is required for the epidermal growth factor dependent signaling (see review by [72] and [24, 79]).

In the last years, the increased attention devoted to the phosphoinositide metabolism, revealed how complex and intricated can be the regulation and signaling of such a pathway. The quantitative data that will soon be available for the other metabolites will allow to develop more detailed models and make predictions on how the different signaling pathways controlled by the phosphoinositides interact.
Part II

DNA Repair: a Paradigm for Nuclear Processes
Chapter 6

Introduction to DNA repair and experimental methods

The DNA is continuously damaged by different endogenous and exogenous agents. Thermal fluctuations break about 5000 purine bases per day (spontaneous *depurination*), similarly spontaneous *deamination* of cytosine to uracyl occurs at a frequency of 100 bases/cell/day [3]. Reactive oxygen species and radiation alter the base pairing ability of the DNA and produce double strand-breaks at a frequency of $\sim 1$ million bases/cell/day [49]. These high mutational rates would be disastrous in the absence of DNA repair.

There are several DNA repair mechanisms that reduce the mutations per cell generation below 1 base pair change per $10^9$ base pairs [3]. One distinguishes five main repair pathways:

1. **Direct reversal** converts modified nucleotides back to their original structure without removing them from the sugar-phosphate backbone. This repair mechanism often uses only one protein to perform repair. For example, in many species, except placental mammal, UV-light induced damages are removed by a photolyase (cf. [3]).

2. **Recombinational repair** uses the recombination machinery and the information of the sister chromatin to repair double-strand breaks generated by high-energy radiation or during mitotic recombination (cf. [82]).

3. **Mismatch repair** removes replication errors (cf. [84]).

4. **Base excision repair** removes spontaneous deaminations, oxidative and alkylation damages (cf. [7]).
5. *Nucleotide excision repair* (NER) removes helix-distorting lesions such as bulky adducts, cisplatin and UV-light induced damages (cf. [27]).

Repair in the four latter pathways is a multiprotein process. The involved proteins are thought to assemble at the site of damage and perform the basic tasks of recognition and excision of the damage, synthesis of the correct nucleotide sequence and ligation of the nick.

DNA damage and repair are interconnected with several other processes. When DNA lesions occur at cell cycle checkpoints the *cell cycle* is arrested to prevent cells from replicating damaged DNA. If repair is completed the cell cycle may resume, otherwise when the DNA is damaged beyond repair cells may initiate the signaling cascade which leads to regulated cell death, that is *apoptosis*. *Transcription* is blocked after DNA damage and there is a specific repair pathway for removing lesions on transcribed genes. Furthermore, some of the proteins used for repair can be engaged in other processes in the absence of damage (e.g. transcription, replication, cell cycle regulation [27]). Mutations in enzymes involved in repair can lead to severe disorders. For example Xeroderma Pigmentosum (XP) is a disease related to NER. The patients suffer from extreme sun-sensitivity and an increased incidence of skin cancer.

The specific role of DNA repair as a junction of replication, recombination, and transcription, and the shared mechanisms to these DNA related processes makes it an ideal starting point for their comprehension.

### 6.1 Nucleotide Excision Repair

Nucleotide excision repair (NER) is a versatile DNA repair mechanism for eliminating helix-distorting lesions throughout the genome. Its most abundant substrates are UV-induced photoproducts, mainly cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) (see Fig. 6.1). For their removal, placental mammals are fully dependent on functional NER [45]. NER eliminates DNA injuries employing a multi-step strategy that requires the combined action of at least 25 different polypeptides that detect the lesion and excise a 24-32 residue oligonucleotide from the damaged strand, after which the replication machinery fills in the gap [27].

There are two distinct NER pathways: transcription-coupled NER (TC-NER) and global genome NER (GG-NER). TC-NER removes lesions from the transcribed strand of active genes. Damage recognition occurs at RNA polymerases that are blocked at DNA lesions, and is mediated by the TC-NER-specific protein cokayne syndrome group A
6.1. Nucleotide Excision Repair

Figure 6.1: UV-light induces dimerization of pyrimidine nucleotides (thymine and cytosine) and produces 6-4 photoproducts (6-4PPs) and cyclobutane pyrimidine dimers (CPDs). The helix distortion caused by a 6-4PP is more pronounced then the distortion caused by a CPD.

and B, and XPA binding protein 2 proteins [54, 110]. After recognition, the subsequent steps in TC-NER are thought to be the same as for GG-NER. TC-NER is crucial for cell survival after DNA damage, but the vast majority of genomic lesions are eliminated by GG-NER.

Seven protein complexes are found to be important for functional GG-NER. Below, I summerize briefly the known function of the different protein complexes.

### XPC-HR23B

There is increasing evidence that the heterodimer XPC-HR23B (183 kDa) plays a central role in damage recognition [158]. Its binding to DNA causes a slightly opening of the DNA helix and appears to be sufficient for the recruitment of transcription factor II H (TFIIH) [40, 76, 165, 180, 199]. The XPC subunit is thought to play the active part in NER [133, 199], whereas the HR23B subunit is involved in protecting XPC from proteosomal degradation [114]. XPC-HR23B has a high affinity for a broad spectrum of DNA lesions such as UV-light induced lesions (6-4 PP and CPD) and cisplatinum adducts. To a lower extent it binds to small bubbles on the DNA devoid of lesions and to non-damaged DNA. This has lead to the hypothesis that XPC-HR23B continuously probes the DNA for lesions.
UV-DDB

The role of the UV-damaged DNA binding factor (UV-DDB, 176 kDa) is still controversial. UV-DDB has a high affinity for both 6-4PP and CPD and has been suggested to be the recognition factor. However, it is essential for repairing CPD but not for repairing 6-4PP [73]. Recent studies indicate that UV-DDB assists XPC in repair by monoubiquitinating it. In this case, it is not a signal for targeting XPC to the proteasome but on the contrary reduces XPC degradation rate by increasing its affinity for HR23B [114]. Furthermore, ubiquitination leads to an increased affinity of XPC for damaged and non-damaged DNA [159]. This increased affinity may be needed to recognize CPDs.

TFIIH

Transcription factor II H (TFIIH) is a holocomplex of considerable size (450 kDa) containing up to nine subunits. As its name indicates TFIIH is also involved in transcription with RNA polymerase I and II [44, 74]. TFIIH is recruited at the site of damage after binding of XPC-HR23B and is responsible for strand separation 10-12 nucleotides around the lesion [40]. This is mediated by its bidirectional ATP-dependent helicase activities [141, 142]. TFIIH bound to DNA may serve as the platform for recruiting the other repair factors which interact with TFIIH [5].

XPA

Xeroderma Pigmentosum group A protein (XPA) (30 kDa) has also been suggested to be involved in the first step of NER [92], possibly in combination with RPA [131] (see below). Although XPA shows high affinity for damaged DNA in vitro [183], in vivo experiments indicate that recruitment of XPA needs functional XPC-HR23B [180]. Because a DNA-XPC-HR23B-TFIIH complex may form on undamaged DNA, due to the affinity of XPC-HR23B to this substrate, the role of XPA is thought to be that of a “damage verificator” which prevents erroneous repair of undamaged DNA (cf. review by [166]). The binding of XPA may stabilize the DNA-XPC-HR23B-TFIIH complex, induce further opening of the DNA by stimulating the helicase activity of TFIIH [190], and allow for the recruitment of other repair factors. Indeed, the 5’ endonuclease ERCC1-XPF can only be recruited to the damage in the presence of XPA [180].
6.2. Experimental methods used to study NER

RPA

Replication protein A (RPA, 121 kDa) has a very high affinity for single stranded DNA (ssDNA) and protects it from misfolding and unwanted endonucleases. For the NER process it has been suggested that RPA acts as damage detector [131]. However, RPA most probably binds after TFIIH when the two ssDNA have been formed by the helicases of TFIIH. RPA appears to be necessary for the correct positioning of the two endonucleases XPG and ERCC1-XPF and is an essential component for the resynthesis of the DNA after incision. Initially XPA and RPA were supposed to exist as a dimer in the absence of damage [56]. However, recent experiments indicate that RPA can bind to damaged DNA in the absence of XPA [127, 179]. Besides being involved in NER the RPA is used in many processes that have ssDNA as intermediates (replication, recombination, and other repair pathways).

XPG

The 133 kDa XPG is a 3’ endonuclease which shows interactions with TFIIH in vitro [5]. The presence of XPG in the dual-incision complex is necessary for ERCC1-XPF to bind and cleave the 5’ ssDNA [184].

ERCC1-XPF

The heterodimer ERCC1-XPF (136 kDa) is a 5’ endonuclease. Several reports suggest that this factor is probably the last factor recruited for completing the dual-incision complex [40, 180].

Resynthesis and ligation

After dual incision the DNA must be synthesized and ligated. The proteins involved are PCNA, which acts as a loading platform for DNA polymerases δ and ε. After resynthesis DNA ligase I is thought to ligate the nick. Most of the proteins involved in this process are the same as for replication.

6.2 Experimental methods used to study NER

To facilitate the understanding of my work I give a short overview of the experimental methods used to investigate NER. NER is initiated by irradiating cell nuclei with UV-
A: Global damage

B: FRAP

![Diagram](image)

Figure 6.2: Global damage experiments. (A) The whole nucleus is damaged at once. (B) Fluorescence recovery after photobleaching (FRAP) is used to estimate immobile fractions and diffusion coefficients.

light. One differs between global damage, where the whole nucleus is irradiated, and local damage, where only part of the nucleus is irradiated. Generally, one takes cells where a repair factor is completely absent or malfunctioning (e.g. XP-cells), this mutation is then rescued with a fluorescent protein tagged version of the missing repair factor (e.g. XPC-GFP). The survival of the recombinant cells after UV-irradiation show that they have a functioning NER similar to wild-type cells (i.e. cells with no mutations in NER). Cells with the fluorescent construct are used for life-cell imaging. Alternatively, antibodies specific for the repair factors specific are used.

### 6.2.1 Global damage experiments

In global damage experiments the whole nucleus is irradiated with UV-C light (Fig. 6.2A). Typical doses to observe saturation are around 8 J/m$^2$. At these doses $1.3-8 \times 10^5$ CPDs and $0.38-1.8 \times 10^5$ 6-4PP are generated [120, 175, 198]. With fluorescently labeled proteins and photobleaching methods the diffusional properties of the repair factors prior and after irradiation are measured (see below). Furthermore, the fraction of proteins immobilized on the DNA after damage induction can be obtained, this is the fraction of proteins involved in repair. A summary of the experimental data is given in Tab. 6.1. The listed values for the immobile fractions are for saturating doses of UV light (8-10 J/m$^2$). Experiments show that a further rise in the UV dose did not produce measurable increases in the immobile fractions.
6.2. Experimental methods used to study NER

### Global damage experiments

<table>
<thead>
<tr>
<th>Factor</th>
<th>Immobile fraction</th>
<th>Diffusion coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>XPC-HR23B [66]</td>
<td>40%</td>
<td>1 μm²/s</td>
</tr>
<tr>
<td>TFIH [67]</td>
<td>40%</td>
<td>6±0.2 μm²/s</td>
</tr>
<tr>
<td>XPA [127]</td>
<td>35%</td>
<td>13±2 μm²/s</td>
</tr>
<tr>
<td>ERCC1-XPF [71]</td>
<td>35%</td>
<td>15±5 μm²/s</td>
</tr>
</tbody>
</table>

Table 6.1: Fraction of repair factors involved in repair after global irradiation of cell nuclei (saturating dose) and diffusion coefficients. The diffusion coefficients are determined in non-irradiated cells using FRAP (see below).

#### 6.2.2 Local damage experiments

In these experiments, only a small part (∼1/7-1/10 of the nuclear volume) of the nucleus is irradiated. The typical dose of UV-C light used is much higher than for global damage experiments (∼100 J/m² inducing 1.6-3.2×10⁵ CPDs and 4.8-1.3×10⁵ 6-4PP [120, 175, 198]). Starting, prior to damage, with an homogeneous distribution of fluorescently labeled repair protein, the fluorescence in the local damaged area increases over time (Fig. 6.3A). This is a hallmark that a repair factor binds to damaged DNA.

To quantify the fraction of proteins involved in repair, one takes the difference between fluorescence in the local damaged area and in a control region of equal size (area 1 and 2 in Fig. 6.3A), and normalizes this value with the total fluorescence of the nucleus. This procedure is necessary as it is not possible to distinguish between fluorescence emitted from freely diffusing and DNA bound factors. The recruitment process as a whole can then be characterized by the accumulation time (see Tab. 6.2).

The mean time a repair factor resides in the locally damaged area is monitored using photobleaching methods, this time is a measure on how long a protein resides in the repair complex (see below and Fig. 6.4). A summary of the residence time experiments is given in Tab. 6.2. The listed values are for a saturating dose. Experiments show that a further increase in UV dose, e.g. from 100 to 200 J/m², does not produce faster accumulation nor an increase in immobile fractions (M. Luijsterburg and M. Moné, unpublished data). Local damage experiments combined with immunofluorescence techniques can be used to study if a factor binds when one of the other factors is absent (due to a mutation). This gives a hint on the mutual dependencies between factors. The results are summarized in Tab. 6.3.

Although measurements used in this work are performed in different cell lines (ham-
Figure 6.3: Local damage experiments (A) Only a small part of the nucleus is damaged. The right panel shows the accumulation of XPC-GFP in the local damaged area (arrows). Bar represents 10 µm. (B) The recruitment of repair factors leads to an increase in fluorescence in the damaged area. Shown is \( \frac{F_{l1}(t) - F_{l2}(t)}{F_{lN}(t)} \), the difference between fluorescence in the local damaged area 1 \( F_{l1}(t) \) and in a control region 2 \( F_{l2}(t) \) normalized with the total fluorescence in the nucleus \( F_{lN}(t) \) (solid line). The maximal immobile fraction is indicated by the dashed line.

ster and human cell lines), one can assume that they represent the same process. That is, if one could measure simultaneously all factors in one cell, the data should be similar as the data obtained from the independent measurements in different cell lines. This is justified by two observations: (i) Survival after UV irradiation is the same in all transfected cells and in wild-type cells [67, 71, 127]; (ii) The accumulation kinetics appear to be surprisingly similar between hamster and human. The dynamics of XPG recruitment was found to be the same in both cell types (M. Luijsterburg and R. van Driel, unpublished data).

### 6.2.3 Photobleaching methods

Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are reviewed in Houtsmuller and Vermeulen [70]. Their applications to the repair process are described by Hoogstraten et al. (2002) and Rademakers et al. (2003) [67, 127]. Here I give a brief overview.
6.2. Experimental methods used to study NER

Local damage experiments

<table>
<thead>
<tr>
<th>Factor</th>
<th>Accumulation time ($t_{1/2}$)</th>
<th>Immobile fraction</th>
<th>Residence time</th>
</tr>
</thead>
<tbody>
<tr>
<td>XPC-HR23B $^1$ [66, 124]</td>
<td>195 (150) sec</td>
<td>13 ± 3.3%</td>
<td>44 ± 10 sec</td>
</tr>
<tr>
<td>TFIH [67, 107]</td>
<td>140 (115) sec</td>
<td>14.8 ± 1.1%</td>
<td>102 ± 5 sec</td>
</tr>
<tr>
<td>XPG$^1$</td>
<td>200 (200) sec</td>
<td>9.1 ± 1.8%</td>
<td>–</td>
</tr>
<tr>
<td>XPA$^1$ [127]</td>
<td>810 (600) sec</td>
<td>7.4%</td>
<td>160 ± 22 sec</td>
</tr>
<tr>
<td>ERCC1-XPF [107]</td>
<td>75 (65) sec</td>
<td>13.4 ± 1.7%</td>
<td>144 ± 27 sec (g.d.)</td>
</tr>
</tbody>
</table>

$^1$ Luijsterburg et al. in preparation.

g.d.: data from global damage experiments.

Table 6.2: The accumulation kinetics from local damage experiments can be summarized using the accumulation times, second column, defined in 7.2.2. In brackets is the $t_{1/2}$ time to attain half the plateau. Third column gives the fraction of repair factors in the local damaged area, i.e. factors involved in repair. The fourth column gives the mean time a factor is involved in repair, the residence time, as defined in Appendix D.1 (see also Fig. 6.4).

<table>
<thead>
<tr>
<th>Immunolabeling</th>
<th>XPC-HR23B</th>
<th>TFIH(XPB)</th>
<th>XPA</th>
<th>RPA</th>
<th>XPG</th>
<th>ERCC1-XPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆XPC</td>
<td>n.b.</td>
<td>n.b.</td>
<td>n.b.</td>
<td>n.b.</td>
<td>n.b.</td>
<td>n.b.</td>
</tr>
<tr>
<td>∆XPA</td>
<td>b.</td>
<td>b.</td>
<td>n.b.</td>
<td>b.</td>
<td>b.</td>
<td>n.b.</td>
</tr>
<tr>
<td>∆XPF</td>
<td>–</td>
<td>–</td>
<td>b.</td>
<td>–</td>
<td>b.</td>
<td>n.b.</td>
</tr>
<tr>
<td>∆XPG</td>
<td>–</td>
<td>–</td>
<td>b.</td>
<td>b.</td>
<td>n.b.</td>
<td>b.</td>
</tr>
</tbody>
</table>

Table 6.3: Summary of the results from immunolabeling experiments [127, 179, 180]. A mutant ∆ has a malfunction/absence in one of the repair factors (first column). In this mutant background one repair factor is immunolabeled (first rows). b.: Binding of a labeled factor in a specific mutant background. n.b.: no binding is observed. A minus (–) is used when no experimental data were available in that combination.

In FRAP a small area of the nucleus is bleached and the recovery to steady state is monitored (Fig. 6.2B). The time needed for recovering depends on the diffusion properties of the fluorescently labeled repair factor. Using computational methods one can estimate the diffusion coefficient [157]. If part of the repair factors are bound to DNA, because they are involved in repair, then the FRAP curve does not fully recover. The difference between the FRAP curve prior and post UV irradiation gives the fraction of
proteins involved in repair.

In FLIP a small strip at the opposite pole of the nucleus from the local damage is continuously bleached (Fig. 6.4A, region 2). After few seconds there no more fluorescence is emitted outside the local damaged area. When a factor dissociates from the repair complex it can freely diffuse in the nucleus and eventually becomes bleached in region 2. Thus, the mean time to completely lose fluorescence in the local damaged area quantifies the mean time a protein is involved in repair, this time is referred to as the residence time (Fig. 6.4B). To minimize the contribution of diffusion to the residence time a combination of FRAP and FLIP has been developed [70]. In combined FRAP-FLIP experiments the bleaching occurs only for a few seconds. The time to reestablish the initial fluorescence difference between the local damaged area and a control region is the residence time.

6.3 Research objectives

Although many details on the biochemistry of NER are known, there are several questions that are not completely resolved:

(i) How do the repair factors assemble? Do they assemble sequentially or randomly at the site of damage, or does a fully pre-assembled holocomplex perform repair?
(ii) Which are the processes that limit repair? Can we assess the efficiency of NER in removing lesions?

(iii) How are the factor released after repair? Do they leave the DNA simultaneously or sequentially?

Mathematical models can help in address these questions and first attempts to quantitatively model DNA repair have been done for another repair pathway (base excision repair [156]). I developed a mathematical model for NER that accounts for the sequential assembly of NER proteins at the sites of DNA damage, their release, and the removal of the lesions as proposed by recent experiments [1, 67, 127, 180]. I explored the general characteristics of the sequential assembly mechanism and derived analytical expressions, which describe repair factors recruitment and release kinetics at the damaged sites. These expressions have been used to draw general conclusions on how the structural properties of NER, i.e. assumed binding and release order, determines the repair factor kinetics (Chapter 7). For the identification of the model parameters, I used the assembly kinetics of XPC-HR23B, TFIH, and ERCC1-XPF at the local damaged nuclear area [107, 124]. The direct comparison of model and data revealed to what extent the model assumptions, deduced from several experimental facts, are in agreement with the observed kinetic data (Section 7.3). The developed model is used to assess the efficiency and specificity of repair in relation to the system parameters, i.e. reaction rates, concentrations, and number of repair factors (Chapter 8). For this, measures that quantifies these two properties have been defined and applied to the model. In the literature, beside sequential assembly, other mechanisms have been proposed for assembling the repairosome. The efficiency of the alternative pathways are compared to the efficiency of the sequential pathway. The critical reviewing of the theoretical and experimental results (Chapter 9) suggested several experiments to better understand the initial and final steps in NER (Chapter 10)

This theoretical work has been done in close collaboration with the group of Prof. Roel van Driel, University of Amsterdam, The Netherlands. Within this group, most of the experiments presented in this work have been done by Dr. Martijn Moné.
Chapter 7

A sequential assembly strategy in NER

7.1 The mathematical model

7.1.1 Model description

The mathematical model takes into account six separate events: a recognition binding step, four intermediate binding steps, and a DNA repair plus dissociation step (Fig. 7.1). It assumes a strict sequential, reversible binding of NER factors to a damaged DNA site and their subsequent simultaneous release after a successful repair event. Repair is initiated by the recognition of the damage by XPC-HR23B, its binding leads to the intermediary complex $S_1$ (DNA/XPC-HR23B). The binding of the other four repair factors occurs sequentially in the order as shown in Fig. 7.1 (according to [180]). The binding of ERCC1-XPF to the intermediary complex $S_4$ completes the assembly process, generating the full pre-incision complex (PC). Subsequent dual endonuclease incision of the damaged strand, dissociation of the DNA damage and of the protein factors, and the filling of the gap by the replication machinery are treated as a single reaction: the repair step.

The concentrations of the free damage $S_0$ and the intermediary complexes $S_i$ are
governed by

\[
\frac{dS_0}{dt} = V_{\text{dam}} - \tilde{k}_1 S_0 F_1 + k_{-1} S_1 \tag{7.1}
\]

\[
\frac{dS_i}{dt} = \tilde{k}_i S_{i-1} F_i - k_{-i} S_i - \tilde{k}_{i+1} S_i F_{i+1} + k_{-(i+1)} S_{i+1} \tag{7.2}
\]

\[
\frac{dS_n}{dt} = \tilde{k}_n S_{n-1} F_n - k_{-n} S_n - k_R S_n \tag{7.3}
\]

Equation (7.2) applies for \(i = 1, \ldots, n-1\), where \(n\) is the number of different repair factors. The \(F_i\)'s are the concentrations of the free factors, \(\tilde{k}_i\) and \(k_R\) denote the binding rate constant and the rate constant of the repair step, respectively, \(k_{-i}\) is the dissociation rate constant of the \(i\)th repair factor from its corresponding substrate. \(V_{\text{dam}}\) denotes the rate at which lesions are formed.

For the analysis of NER efficiency it is useful to define the total concentration of DNA lesions

\[
D_T = \sum_{i=0}^{n} S_i \tag{7.4}
\]

From Eqs. (7.1)–(7.3) it follows that

\[
\frac{dD_T}{dt} = V_{\text{dam}} - V_R. \tag{7.5}
\]

Here, I introduced the rate at which damaged sites are removed \(V_R = k_R S_n\).

The total concentrations \(T_i\) of the factors are

\[
T_i = F_i + I_i \tag{7.6}
\]

where

\[
I_i = \sum_{j=0}^{n} S_j. \tag{7.7}
\]

is the concentration of the \(i\)th factor engaged in repair, and therefore immobilized on the DNA. From the previous formula follows that the more upstream in the chain a factor is recruited, the higher its immobilized concentration

\[
I_i > I_j, \text{ for } j > i \tag{7.8}
\]

During the times span of the experiments (~10 min), the total concentration of XPC-HR23B, and probably of the other repair proteins, remains constant [2, 114]. Thus, the repair system is fully described by Eqs. (7.1)–(7.3) where \(F_i = T_i - I_i\).

NER proteins also have an affinity for undamaged DNA (e.g. XPC-HR23B) and several repair factors are involved in processes besides repair (e.g. TFIIH in transcription)
7.1. The mathematical model

\[ F_1, F_2, F_3, F_4, F_5 \]
\[ S_0 \quad S_1 \quad S_2 \quad S_3 \quad S_4 \quad S_5 \quad \text{Repaired DNA} \]

Free DNA lesion

Figure 7.1: Scheme of the basic model showing the sequential binding of five different protein factors to a damaged DNA site and their simultaneous release after repair.

[3, 60, 67, 133]. As illustrated in Appendix C.1 the presence of undamaged binding sites reduces the free factor concentrations available for repair. One can show that

\[ F_i = \frac{(T_i - I_i)}{\beta_i}, \]

where the coefficients \( \beta_i \) are referred to as buffering coefficients. Their values are directly proportional to the amount of undamaged binding sites (\( \beta_i = 1 \) without undamaged binding sites; \( \beta_i > 1 \) otherwise). The model equations that account for the presence of undamaged binding sites read

\[ \frac{dS_i}{dt} = k_iS_{i-1}(T_i - I_i) - k_{-i}S_i - k_{i+1}S_i(T_{i+1} - I_{i+1}) + k_{-(i+1)}S_{i+1}. \] (7.9)

The effective binding rate constants are defined by \( k_i = \tilde{k}_i/\beta_i \). The above formula shows that the presence of undamaged binding sites slows the formation of intermediary complexes and thus of the PC.

The model accounts for a situation where DNA damage is evenly distributed throughout the nucleus. In this case, there are no spatial gradients in the factor concentrations. If a high concentration of damage sites is induced locally, it may be necessary to include the nuclear diffusion of the factors. This is taken into account when directly comparing the model simulations with the data from local damage experiments (Section 7.3, page 96).

7.1.2 Damage generation

At low UV light intensities the amount of damages increases linearly with the UV dose. I will assume that the linear dependency remains valid for the high UV doses (>30 J/m²) used in local damage experiments. From [175, 198] one obtains\(^1\)

\[
\text{Concentration of 6-4PP [\(\mu\text{M}\)] = } \frac{57218 \times UV \times f}{V_d N_A} = 0.3166 \times UV
\] (7.10)

\(^1\)In the initial phase of repair, mainly 6-4PPs are recognized and removed by NER, therefore, the other type of damage induced with UV (CPD) is not considered at this stage.
The UV light intensity is given in J/m$^2$. $f$ is the fraction of damaged nucleus ($\approx 1/7$), $V_d = fV_n$ is the volume of the local damaged area, with a nuclear volume $V_n = 0.3$ pl, $N_A$ is the Avogadro’s constant. The linear dependency of the number of damaged sites on the UV light intensity has several implications for the interpretation of the data and for the model predictions. This caveat will be discussed in more detail in Chapter 10.

7.2 General properties of the sequential model

7.2.1 Saturation and maximal repair rate

In experiments, the amount of factors involved in repair saturates with increasing UV light intensity. Also, the kinetics of accumulation in the local damaged area does not become faster for increasing dose (cf. Section 6.2, page 79). These two facts indicate that NER is saturated. With the model one can try to provide a mechanistic explanation. For example, saturation can merely be due to the fact that not enough damages have been induced. However, this option will be neglected for the moment, because I assumed that the number of damages increases with the UV light Eq. (7.10) (see Chapter 10, where alternatives have been modeled).

The second possibility is that the recognition factor becomes fully involved in repair. In the model, increasing the initial damage concentration causes additional involvement of factors in repair as long as repair complexes can be initiated. How many repair complexes can be initiated depends on the available free diffusing molecules of the first factor. Saturation at high UV light doses can be identified with depletion of the free diffusing pool of the first factor ($F_1 \approx 0$); in this situation, damages are removed with a maximal rate $V_R = V_{\text{max}}$ (see Appendix C.4). This result is comparable to enzyme kinetics, where at high substrate concentrations the enzyme reaches its maximal rate because all the catalytic sites are occupied by substrate.

7.2.2 Time hierarchy of binding and release: analytical results

Before proceeding with the direct comparison of model and data (as done in Section 7.3, page 96), I will derive analytical expressions, which relate the structure of the model to the kinetics of the repair proteins. I will present two measures, the accumulation and the residence time, that characterize the factor kinetics at the local damage.

For better illustrating these two measures, I plotted, qualitatively, the type of experimental data obtained from local damage experiments (Fig. 7.2A). At $t = 0$ the
7.2. General properties of the sequential model

Figure 7.2: Accumulation and residence times. (A) Qualitative data as would be obtained from local damage experiments (simulation). After damage, at \( t = 0 \), one observe accumulation of the repair factor in the locally damaged area. The accumulation time \( \alpha_4 \) characterizes its recruitment kinetics. As soon as a plateau is reached, bleaching outside the local damaged area is initiated (FLIP). The residence time \( \rho_4 \) quantifies the dissociation kinetics from the repairosome. (B) During accumulation, all reactions before and after binding of a factor (bold faced) contribute to its recruitment kinetics. In FLIP experiments, the observed loss in fluorescence is proportional to the dissociation rate from the repairosome. This is because, the fluorescence of the free diffusing molecules is continuously quenched.

nucleus is damaged locally, this initiates an increase in fluorescence in the local area (the concentration of proteins involved in repair rises). Within 2–5 min, the fluorescence does not change any further and a plateau is attained. This marks the end of a factor accumulation/recruitment phase, whereof the kinetics is characterized by the accumulation time \( \alpha_i \) (Fig. 7.2A). During the plateau phase, where the release of factors is balanced by new factors entering repair, bleaching of an area outside the local damage is initiated (FLIP). First all the free diffusible factors are bleached, so that only factors bound to damage are visible. Then, as soon as a factor leaves the repairosome, it diffuses away from the local damaged area and eventually becomes bleached to. Because new proteins entering repair are not fluorescent, one observes a continuous decrease of the fluorescence intensity in the local damaged area. This kinetics is directly proportional to repair factors leaving the repairosome and is characterized by the mean residence time \( \rho_i \) (Fig. 7.2A).

In the model, when the spatial gradients are neglected, the accumulation time of the \( i \)th factor simply reads

\[
\alpha_i = \frac{\int_0^{t_p} [I_i(t_p) - I_i(t)] dt}{I_i(t_p) - I_i(0)},
\]

(7.11)
where \( t_p \) is the time point when the plateau is reached. A similar definition is used for the mean residence time

\[
\rho_i = \frac{\int_0^\infty [I_i(t) - I_i(\infty)] \, dt}{I_i(0) - I_i(\infty)}.
\]

(7.12)

Note that in the latter equation, the time point \( t = 0 \) is set when bleaching is initiated (for a general definition see Appendix D.1). Both the accumulation and residence time can be computed from experimental data and allow for a quantitative comparison of model and experiment.

**Accumulation time:** At low damage, the amount of factors immobilized on the DNA is small compared to their total amount \((T_i - I_i) \approx T_i\); therefore, the system of differential equations describing NER can be linearized. Analytical expressions for the accumulation times are derived assuming that the free damage \( S_0 \) does not significantly decrease during the recruitment of repair factors (Appendix D.2). When binding is irreversible \((k_{-i} = 0)\), the accumulation time of the \( i \)th factor reads

\[
\alpha_i = \frac{\sum_{j=i+1}^{n+1} \frac{1}{\kappa_j} \left( \sum_{l=2}^{j} \frac{1}{\kappa_l} \right)}{\sum_{j=i+1}^{n+1} \frac{1}{\kappa_j}},
\]

(7.13)

where the \( \kappa_i = k_i T_i \) are the first order rate constants for the transitions between intermediary complexes. In this compact notation, the repair rate constant \( k_R \) is denoted by \( \kappa_{n+1} \).

For example, the accumulation time of the last factor is \((n = 5)\)

\[
\alpha_5 = \frac{1}{\kappa_2} + \frac{1}{\kappa_3} + \frac{1}{\kappa_4} + \frac{1}{\kappa_5} + \frac{1}{\kappa_6}.
\]

(7.14)

One recognizes that not only the binding of the 5th factor itself, but also processes occurring prior and after incorporation of this factor, that is binding of other repair factors and repair/release, respectively, contribute to its accumulation time. An exception is the binding of the first factor, which does not influence the accumulation kinetics \((\kappa_1 \text{ does not enter Eq. (7.14)})\). In fact, due to the approximations made, the binding of the first factor only contributes in setting the immobilized concentrations at the end of the accumulation phase.

The same properties are found for the accumulation time of all other factors. Furthermore, using Eq. (7.13), one can show that the accumulation time of factors that
bind later in the pathway is higher than of early binding factors

\[ \alpha_j > \alpha_i, \quad \forall j > i. \]  (7.15)

Also for reversible binding does relation (7.15) hold true (see Appendix D.2.3). When the initial damage is high one can harness the fact that the first factor is nearly completely immobilized \((T_1 \approx I_1)\). Assuming that the other factors are only weakly immobilized \((T_i - I_i \approx T_i, \ i > 1)\), allows to compute the accumulation times (see Appendix D.3.2). One can show that, also in this case, relation (7.15) holds true.

Therefore, for these simplified cases with linear rate laws, the recruitment kinetics of a factor reflects its binding position. Early binding factors reach the plateau faster than later binding factors. Deviation from this order, may indicate that (i) the binding position of a factor is different, or that (ii) the structure of the NER pathway is more complicated than assumed. In particular, release may not occur simultaneously (see Section 10.3, page 134). For the complete model, when free factor concentrations do change in time, general conclusions cannot be derived. As we will see, when customizing the model parameters to the data, the analytical results represent a guideline but not the full answer.

In the expressions derived above, I neglected the diffusion of repair factors from the non-damaged area to the damaged area, which is a valid approximation for most of the factors who diffuse fast compared to the measured accumulation times (see Tables 6.1, 6.2, and Appendix D.1). Diffusion slows down the recruitment of repair factors and this is of particular interest for the first factor. The binding rate of the first factor is proportional to the amount of damages, and, at saturating UV dose, the number of damaged sites is in large excess, therefore, the first factor will quickly bind to the DNA as soon as it reaches the damaged area. Thus, the accumulation time of the first factor at saturating conditions, is predicted to be mainly influenced by its diffusional properties and not by its binding/dissociation characteristics. This point is illustrated in Section 7.3, where the model parameters have been estimated from the experimental data using a NER model that includes repair factor diffusion.

**Residence time:** As described above, the residence time of a factor \(i\) quantifies the mean time it remains at the damaged site. In experiments, this is determined from the kinetics of the local fluorescence intensity loss\(^2\), which is initiated by photobleaching of the free diffusing proteins (FLIP, see Fig. 7.2). In the model, bleaching corresponds to

\(^2\)In combined FRAP-FLIP experiments bleaching occurs only for a short time. However, in the framework of the model FRAP-FLIP and FLIP experiments give similar residence times (not shown).
$F_i(t > 0) = 0$, so that only the dissociation of the fluorescent factor from the repair complexes is observed, but not its binding. Because experiments are performed after a plateau in the immobile and free fraction of the factors has been reached, it can be assumed that the free factor concentrations remain constant during bleaching. For irreversible binding ($k_{-i} \approx 0$), one derives following residence times (cf. Appendix D.2.4)

$$
\rho_i = \frac{1}{k_R} + \sum_{j=i+1}^{n} \frac{1}{k_j F_j} \left(1 - \frac{I_j(0)}{I_i(0)}\right) \quad \text{and} \quad \rho_n = \frac{1}{k_R},
$$

(7.16)

where $I_j(0)$ is the concentration of the $j$th factor involved in repair at the beginning of bleaching ($t = 0$).

Equation (7.16), shows that the residence time is determined by the repair step, which is the step where the factor dissociates from the repairosome, and the transition from one intermediary complex to another (Fig. 7.2B, bleaching). Furthermore, due to relation $I_j(0) > I_i(0)$ ($j > i$), valid in a sequential assembly and simultaneous release mechanism (Eq. (7.8)), one obtains

$$
\rho_j < \rho_i \quad \text{for} \quad \forall j > i
$$

(7.17)

This formula indicates that there is a strict relation between binding position and residence time. Early-binding factors reside longer at the site of damage than later-binding factors. The reason is that they have to pass a larger number of intermediate complexes before being released at the end of the pathway. When factors bind reversibly, the dissociation of a factor from its intermediary complex also influences its residence time. One can show that relation (7.17) holds true if the factors bind equally well to their respective substrate $\kappa_i = \kappa$ and $k_{-i} = k_{-}$, $i \leq n$ (see Appendix D.2.4).

This hierarchy in the residence times can break when the binding properties of repair factors to their respective substrate differ. Then, an early binding factor may reside for a shorter time in the repair complex than a late binding factor. This occurs, for example, when the intermediary complex formed by the binding of the factor of interest (e.g. complex $S_4$ in Fig. 7.2B, bottom panel) is unstable, that is the probability to dissociate just after binding is high. Then, the repair factor primarily leaves the damaged site by dissociating from its intermediary complex.

If the residence times of early binding factors is lower than of those which bind later, the presented theoretical analysis indicates that (i) factor release is not simultaneous; for example, the early binding factor is not needed for completion of the repair complex and leaves the repairosome at an early stage (see Section 8.3.2). (ii) Intermediary complexes containing the early binding factor are unstable, so that the repair factor dissociates
before the repair complex is completed (see Appendix D.2.4). A direct comparison between the residence times estimated in the model and the experimental values is presented in Section 7.3.3, page 102.
Chapter 7. A sequential assembly strategy in NER

7.3 Comparison of experimental data and model predictions

7.3.1 Recruitment of repair factors to the damage

Data acquisition and treatment: The data used for the development of the model were the accumulation kinetics in local damaged nuclei of XPC-GFP, XPB-GFP (a subunit of TFIIIH), and ERCC1-GFP. These proteins have been expressed respectively in human XPC-deficient, human XPB-deficient, and CHO ERCC1-deficient cells and rescue the defects in NER [107, 124]. The immobile fractions as determined by FRAP in global damage experiments are used to set the plateau values of TFIIIH and ERCC1-XPF [67, 71] (cf. Section 6.2). This is because FRAP is presumed to be a more reliable method for measuring immobile fractions, and with large excess of damaged sites, the maximal immobile fractions in local and global damage experiments should be approximately the same (see Chapter 10). To set the plateau level of XPC-HR23B I proceeded differently. According to the model, at saturation, the first factor will be fully engaged in repair (cf. Section 7.2.1). However, with FRAP only a maximum of $\sim 40\%$ of XPC-HR23B was found to be immobilized at high UV doses [66]. A possible explanation is that only a subpopulation of XPC-HR23B is functional in NER, e.g. because not properly folded. Therefore, in the model, I assume that at the high UV dose used here this subpopulation is $\sim 100\%$ involved in repair. For a more exhaustive discussion on this point I refer the reader to the Chapter 9 and 10.

Irreversible binding: The experimental data obtained from the local damage experiments are shown in Fig. 7.4A-C. One notices that at the saturating UV dose used (120 J/m$^2$), the accumulation kinetics of XPC-HR23B is slow. The model presented previously, where diffusion of the factors is not explicitly considered, would predict, at such large damage loads, a much faster accumulation of the first factor (not shown). I postulated that the observed accumulation kinetics of XPC-HR23B may be due to its slow diffusion from the non-damaged to the damaged area. Thus, to simulate the accumulation experiments and account for the spatial gradients arising after local generation of damages, I included nuclear diffusion of the free factors in the NER model; furthermore, the experimental procedure, necessary for the estimation of the immobile fractions, is explicitly modeled (see Appendix E).

The NER model, including diffusion of the repair factors, is fitted to the accumulation data. In a first step, the binding reactions are assumed irreversible ($k_{-i}$). This
Figure 7.3: Estimation of confidence intervals and parameter dependencies. (A) Parameter distribution of the binding rate constants (shown for TFIH, $k_2$) obtained by fitting the model to bootstrapped data (black bars). The 90% confidence interval is calculated by integrating the cumulative distribution (solid line). (B) Most parameters can be estimated independently. (C) The unbuffered binding rate constant for XPC-HR23B $k_1$ has high variance and positively correlates with the buffering coefficient $\beta_1$.

simplification is justified by the fact that XPC-HR23B readily binds to damaged DNA in vitro, with an equilibrium constant of 0.5 to 5 nM [8, 60]. One can estimate that the probability for XPC-HR23B to dissociate from the damaged site before TFIH binds is very low (< 1%). Thus, XPC-HR23B is committed to repair as soon as it binds to the damage. Assuming similar dissociation constants for the other factors leads to analogous conclusions. Hence, the contribution of the dissociation events to the repair process is expected to be small.

The fitting yields the binding rate constants for all NER factors and an effective diffusion coefficient for XPC-HR23B. The effective diffusion coefficients of the other factors are taken from literature (Tab. 7.2, set 1). I recall that the effective diffusion coefficients account for the impairment in diffusion caused by binding of repair factors to non-damaged sites (see Appendix E). The 90% confidence intervals were estimated using bootstrap [39, 125]. Briefly, I generated synthetic data by drawing for each time point of the original data set so many data points as the original set (drawing with replacement). The model is then fitted to this synthetic data set. The operation is repeated so many times as necessary to obtain a smooth parameter distribution. From this distribution, it is straightforward to calculate the 90% confidence interval (Fig. 7.3A). One can also assess how good the parameters can be determined independently from each other. For nearly all parameters I found no correlation to another parameter, Fig. 7.3B. An exception is the positive correlation observed between the XPC-HR23B unbuffered
Figure 7.4: (A-C) Accumulation in the UV damaged area of XPC-HR23B, TFIIH and ERCC1-XPF (average of 3, 10 and 9 experiments, respectively). Red and black lines are model predictions using parameter set 1 and 2, irreversible binding Tab. 7.2, respectively (model with free factor diffusion, Appendix E.1). In simulations, DNA repair is initiated by 38 µM damages in one-seventh of the nuclear volume. (D-E) Simulated immobilized fractions using, respectively, set 1 and 2. In (E) kinetics of ERCC1-XPF ($F_3$) is shorter than of XPC-HR23B ($F_1$) and TFIIH ($F_2$). Immobilization of the putative limiting factor $F_3$ is more rapid and complete than for the other factors. The sum of least squares is 100.7 and 49.73 for set 1 and 2, respectively. According to a $\chi^2$ test, the observed values can be explained by the noise in the data ($p < 0.01$). A likelihood ratio test indicates that parameter set 2 is significantly better in reproducing the data than parameter set 1 [109, 125].

The model with the estimated parameters properly reproduces the recruitment kinetics for both XPC-HR23B and TFIIH (Figs. 7.4A and B, respectively: red curves). It predicts an effective diffusion coefficient of 0.1–0.3 $\mu$m$^2$/s for XPC-HR23B, which accounts for its slow accumulation in the locally damaged nuclear area. This value is remarkably low, as a diffusion coefficient of $\sim 15$ $\mu$m$^2$/s is expected based on its molec-
ular size and assuming free diffusion. The low effective diffusion coefficient indicates that a large fraction (\(\sim 98\%\)) of XPC-HR23B is transiently immobilized (see Appendix C.1 and E.1). This notion is supported by the fact that XPC-HR23B has a significant affinity for undamaged DNA [148], and by recent experimental data on mobility and nuclear distribution of XPC-HR23B in vivo [66]. Using the same parameter set that is adequate for XPC-HR23B and TFIIH I found that the predicted accumulation kinetics of ERCC1-XPF is slightly slower compared to its measured behavior (Fig. 7.4C; red line; predicted accumulation time 110 s, compared to a measured value of 75 s). In fact, the binding phases of all NER factors have similar kinetics (Fig. 7.4D). In agreement with the analytical results (Section 7.2.2), the tendency is that early binding factors accumulate faster than late binding factors.

For each repair factor, the experimentally reported concentration values vary by a factor of 2–3 [5, 174, 191] and this may affect the model predictions. Therefore, I tested whether variations in the total concentrations could improve the agreement between the ERCC1-XPF simulated and measured accumulation data. The model predicts that the assembly kinetics of XPC-HR23B, TFIIH, and ERCC1-XPF can all be reproduced faithfully if one of the NER factors that binds after TFIIH, but prior to ERCC1-XPF, is present at a significantly lower concentration than assumed in parameter set 1 (Tab. 7.2). Figures 7.4A–C (black lines) show a model simulation where the total concentration of the third factor (in my model XPG) is estimated numerically to be three to four times lower than the concentration of the other NER factors (Tab. 7.2, set 2). Due to its low concentration, a large fraction of this protein becomes involved in DNA repair (compare the curves for \(F_3\) in Fig. 7.4D and E), and will therefore become "limiting" for the accumulation of ERCC1-XPF. In contrast, the initial accumulation of both XPC-HR23B and TFIIH is unaffected.

**Reversible binding:** A factor, after binding of TFIIH, that is highly immobilized during repair, has not been yet identified experimentally. It is now important to understand, whether the prediction of a limiting factor is related to the irreversibility assumption, or whether it is a result of the model structure (sequential binding and simultaneous release). I therefore tested the performance of the complete model, with reversible binding of the factors, against the data.

The fitting yields binding and dissociation rate constants of the factors (Tab. 7.3, set 3). There is only a slight improvement in the quality of the fit (Fig. 7.5A-C, red curves), and the obtained results are similar to the irreversible model (Fig. 7.4A-C, red curves). In particular, the fast kinetics of ERCC1-XPF is only partially reproduced. However, if
the total factor concentrations are also fitted numerically (Tab. 7.3, set 4), the agreement between model and data considerably increases, and also the accumulation kinetics of ERCC1-XPF is reproduced faithfully (Fig. 7.5A-C, black curves). Interestingly, except for XPC-HR23B, the immobile fractions are all below 80% (Fig. 7.5E). This indicates that when the binding of downstream factors to their intermediary complex is strongly reversible (see Tab. 7.3), the accumulation of XPC-HR23B, TFIIH, and ERCC1-XPF can all be reproduced without invoking the existence of a limiting factor. However, as shown in Section 7.3.3, the presence of dissociation events prior the repair step dramatically modifies how long the factors reside at the damaged site. One obtains large differences in the simulated and experimentally measured residence times.

7.3.2 Long-time behavior

I wanted to test whether the model could correctly reproduce the kinetic behavior over longer time periods. To this end, the ERCC1-XPF immobilization has been measured
over several hours. In experiment and model, one can distinguish a long plateau phase during which the amount of immobilized ERCC1-XPF remained approximately constant (Fig. 7.6; diamonds: experimental data; solid line: simulation). The model predicts that during this phase the number of damaged DNA sites decreases with a nearly constant and maximal rate $V_{\text{max}}$ (dashed line). Finally, in the model and experiment, one observes a "release" phase where the damage-bound protein fraction gradually decreases\(^3\). A plateau, followed by a monophasic decrease in the fraction bound to the DNA has been measured for other repair factors (M. Luijsterburg, personal communication).

The time course shown in Fig. 7.6, with its long plateau phase, indicates that the repair system is indeed saturated. The model predicts that: (i) a similar time course is expected for the other repair factors, and (ii) if the initial damage concentration is increased, the characteristics of both the assembly and the "release" phase remain unaffected; only the duration of the plateau phase changes, proportionally to the amount of initial damage (Fig. 7.6: dotted line).

\(^3\)Only in the experiment shown in Fig. 7.6 does one observe a third phase (for $t > 4000$ s). During this phase the bound fraction decreases more slowly. Such a marked biphasic behavior is not observed in the model.
### Residence times (in sec)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Experiment</th>
<th>Set 1</th>
<th>Set 2</th>
<th>Set 3</th>
<th>Set 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>XPC-HR23B</td>
<td>∼ 44</td>
<td>166.6</td>
<td>221.6</td>
<td>222.4</td>
<td>884.3</td>
</tr>
<tr>
<td>TFIIH</td>
<td>∼ 102</td>
<td>147.1</td>
<td>196.1</td>
<td>153.9</td>
<td>142.9</td>
</tr>
<tr>
<td>XPG</td>
<td>∼</td>
<td>143.7</td>
<td>142.8</td>
<td>62.4</td>
<td>6.9</td>
</tr>
<tr>
<td>XPA</td>
<td>∼ 160</td>
<td>143.4</td>
<td>142.8</td>
<td>10.4</td>
<td>3.7</td>
</tr>
<tr>
<td>ERCC1-XPF</td>
<td>∼ 144</td>
<td>142.8</td>
<td>142.8</td>
<td>2.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 7.1: Residence times as obtained from experiments and with the mathematical model using the different parameter sets (Tab. 7.2–7.3). In the model the residence times have been calculated for saturating conditions as described in Appendix D.3.3.

#### 7.3.3 Life time of repair factors in the repairosome

The residence times represent a source of independent data against which the developed model can be tested. In Tab. 7.1 I listed the experimental data together with the model results for the four different parameter sets. When binding is irreversible (set 1 and 2), the calculated residence times of TFIIH, XPA, and ERCC1-XPF are in the range of the reported values. This is not the case when binding is reversible (set 3 and 4). One finds, for XPA and ERCC1-XPF, residence times which are far below the measured values. This is due to the fact that, for these factors, it is likely to leave the DNA before finishing repair (large dissociation rate constants \( k_{-4} \) and \( k_{-5} \), Tab. 7.3). For example, the residence time of ERCC1-XPF reads

\[
\rho_5 = \frac{1}{k_{-5} + k_R}.
\]  

(7.18)

According to the estimated parameters \( k_{-5} \gg k_R \), so that the dissociation reaction is the main process which determines the residence time of ERCC1-XPF.

A second feature, observed for all parameter sets, is that the residence time of XPC-HR23B is far above its experimentally obtained residence time. I will show, in Section 8.3.2, how a low residence time of XPC-HR23B may be accounted for by the fact that the last step of repair is performed in the absence of XPC-HR23B. This allows for XPC-HR23B to leave the repairosome at an earlier time point.
7.3.4 Concluding remarks

To summarize, the developed model performs well for most of the experimental data available. The few differences between model predictions and experimental data are not due to the choice of parameters, as different parameter sets and situations have been tested, but rather indicate that some of the assumptions that entered the model may need to be reviewed. In particular, relaxing the assumption of simultaneous release of the factors may bring to the fore other dynamic properties of NER. This will be discussed in detail in Chapter 10.
### Table 7.2: The nuclear volume is 0.3 pl. In set 1 total concentrations are based on published data [5], whereas in parameter set 2 the concentration of the 3rd factor is estimated numerically. The repair rate constant $k_R$ corresponds to the inverse of the residence time measured for ERCC1-XPF [71]. Due to the lack of kinetic data for the 3rd and 4th factor, I took $k_3 = k_4 = k_5$. The bold faced parameters are estimated by fitting the model depicted in Fig. 7.1 with free factor diffusion (Appendix E) to the accumulation curves shown in Fig. 7.4A–C. Binding is irreversible ($k_{-i} = 0$). Numbers in brackets give the 90% confidence intervals as determined with bootstrapping. The diffusion coefficients of the free TFIH, XPA, and ERCC1-XPF ($\tilde{D}_2$, $\tilde{D}_4$ and $\tilde{D}_5$ respectively) and their buffering coefficients ($\beta_2$, $\beta_4$ and $\beta_5$ respectively) were taken from literature [67, 71, 127]. The diffusion coefficients of the free XPC-HR23B and XPG proteins ($\tilde{D}_1$ and $\tilde{D}_2$ respectively) were estimated according to their molecular weights. For XPG there are no indications that it binds to undamaged DNA ($\beta_3 = 1$).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Set 1</th>
<th>Set 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total concentrations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_1, T_2$</td>
<td>0.44, 0.6 $\mu$M</td>
<td>0.44, 0.6 $\mu$M</td>
</tr>
<tr>
<td>$T_3$</td>
<td>0.44 $\mu$M</td>
<td>0.1959 $\mu$M (0.191–0.258)</td>
</tr>
<tr>
<td>$T_4, T_5$</td>
<td>1.1, 0.55 $\mu$M</td>
<td>1.1, 0.55 $\mu$M</td>
</tr>
<tr>
<td>Effective binding rate constants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_1$</td>
<td>$0.0118/\mu$M/s (0.01-0.088)</td>
<td>$0.0054/\mu$M/s (0.0046-0.01)</td>
</tr>
<tr>
<td>$k_2$</td>
<td>$0.0697/\mu$M/s (0.065-0.085)</td>
<td>$0.0631/\mu$M/s (0.059-0.09)</td>
</tr>
<tr>
<td>$k_3, k_4, k_5$</td>
<td>$0.373/\mu$M/s (0.282-0.427)</td>
<td>5/\mu$M/s (0.85–93.77)</td>
</tr>
<tr>
<td>Repair rate constant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_R$</td>
<td>0.007/s</td>
<td>0.007/s</td>
</tr>
<tr>
<td>Diffusion coefficients</td>
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<td></td>
</tr>
<tr>
<td>$\tilde{D}_1, \tilde{D}_2, \tilde{D}_3, \tilde{D}_4, \tilde{D}_5$</td>
<td>15, 6, 15, 15, 15 $\mu$m$^2$/s</td>
<td>15, 6, 15, 15, 15 $\mu$m$^2$/s</td>
</tr>
<tr>
<td>Buffering coefficients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>90 (87.4-136.4)</td>
<td>60 (47.53-92.82)</td>
</tr>
<tr>
<td>$\beta_2, \beta_3, \beta_4, \beta_5$</td>
<td>1.2, 1, 1, 1</td>
<td>1.2, 1, 1, 1</td>
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<tr>
<td>Effective diffusion coefficients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$D_1$</td>
<td>$0.16 \mu$m$^2$/s (0.11-0.171)</td>
<td>$0.25 \mu$m$^2$/s (0.16-0.31)</td>
</tr>
<tr>
<td>$D_2, D_3, D_4, D_5$</td>
<td>5, 15, 15, 15 $\mu$m$^2$/s</td>
<td>5, 15, 15, 15 $\mu$m$^2$/s</td>
</tr>
</tbody>
</table>
7.3. Comparison of experimental data and model predictions

---

**Model parameter and values: reversible binding**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Set 3</th>
<th>Set 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total concentrations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_1$</td>
<td>0.44 $\mu$M</td>
<td>1.1 $\mu$M (0.897-1.1)</td>
</tr>
<tr>
<td>$T_2$</td>
<td>0.6 $\mu$M</td>
<td>1.07 $\mu$M (0.764-1.1)</td>
</tr>
<tr>
<td>$T_3$, $T_4$, $T_5$</td>
<td>0.44, 1.1, 0.6 $\mu$M</td>
<td>0.44, 0.44, 0.44 $\mu$M</td>
</tr>
<tr>
<td><strong>Effective binding rate constants</strong> ($k_i = \tilde{k}_i/\beta_i$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_1$</td>
<td>0.0078/µM/s (0.0071-0.0084)</td>
<td>0.0043/µM/s (0.0039-0.0046)</td>
</tr>
<tr>
<td>$k_2$</td>
<td>0.0783/µM/s (0.062-0.143)</td>
<td>0.0155/µM/s (0.0135-0.023)</td>
</tr>
<tr>
<td>$k_3, k_4, k_5$</td>
<td>5/µM/s (0.91-9.7)</td>
<td>5/µM/s (4.8-5.2)</td>
</tr>
<tr>
<td><strong>Dissociation rate constants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{-1}$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$k_{-2}$</td>
<td>0.0762/s (0.02-0.6513)</td>
<td>0.0138/s (0.012-0.036)</td>
</tr>
<tr>
<td>$k_{-3}, k_{-4}, k_{-5}$</td>
<td>0.463/s (0.12-0.714)</td>
<td>0.85/s (0.66-0.9)</td>
</tr>
</tbody>
</table>

Table 7.3: NER model with reversible binding and free factor diffusion (Appendix E). Bold faced parameters are fitted to the accumulation curves in Fig. 7.4A-C, number in brackets give the 90% confidence interval. Due to the lack of kinetic data for the 3rd and 4th factor, I assumed binding and dissociation rate constants equal to the rate constants of the 5th factor. Parameters which are not listed are as given in Tab. 7.2, set 1. In parameter set 4 also the total factor concentrations are estimated numerically. The total concentration of the 3rd, 4th, and 5th factor are assumed to be the same.
Chapter 7. A sequential assembly strategy in NER
Chapter 8

Efficiency of sequential NER and pathway alternatives

8.1 Repair efficiency

Repair is considered to be efficient if the DNA damage is rapidly removed. The mean repair time, needed to remove a certain amount of initial damaged DNA sites, is defined by

\[ \tau_R = \frac{1}{D_T(0)} \int_0^\infty D_T(t) dt \]  

(8.1)

where \( D_T(t) \) denotes the damage concentration in time. The value of the repair time for the local damage experiments is indicated by an arrow in Fig. 7.6 (\( \tau_R = 2161 \) s).

Efficient repair is characterized by a low \( \tau_R \). To assess how the system parameters affect repair efficiency, I define the following control coefficients

\[ C_{\tau R}^p = \frac{p}{\tau_R} \frac{\partial \tau_R}{\partial p}, \]

(8.2)

where \( p \) denotes one of the rate constants or one of the factor concentrations [57]. Parameters that strongly affect repair efficiency will have high absolute values for their corresponding control coefficients. Two cases can be distinguished, a low and a high initial damage, for which analytical expressions of the repair time are derived.

8.1.1 Low initial damage

In this case, the immobile fraction of the factors is low (\( T_i - I_i \approx T_i \)). With the so linearized system one can calculate the repair time analytically (Appendix D.2.2). If
Control on the repair time: low damage

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Set 1</th>
<th>Set 2</th>
<th>Set 3</th>
<th>Set 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1T_1$</td>
<td>-0.5167</td>
<td>-0.7113</td>
<td>-0.601</td>
<td>-0.4157</td>
</tr>
<tr>
<td>$k_2T_2$</td>
<td>-0.0642</td>
<td>-0.0447</td>
<td>-0.0472</td>
<td>-0.1331</td>
</tr>
<tr>
<td>$k_3T_3$</td>
<td>-0.0163</td>
<td>-0.0017</td>
<td>-0.005</td>
<td>-0.0322</td>
</tr>
<tr>
<td>$k_4T_4$</td>
<td>-0.0065</td>
<td>-0.0003</td>
<td>-0.0071</td>
<td>-0.0737</td>
</tr>
<tr>
<td>$k_5T_5$</td>
<td>-0.0131</td>
<td>-0.0006</td>
<td>-0.057</td>
<td>-0.1816</td>
</tr>
<tr>
<td>$k_R$</td>
<td>-0.3832</td>
<td>-0.2414</td>
<td>-0.3508</td>
<td>-0.461</td>
</tr>
<tr>
<td>$k_{-1}$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$k_{-2}$</td>
<td>0</td>
<td>0</td>
<td>0.0031</td>
<td>0.0146</td>
</tr>
<tr>
<td>$k_{-3}$</td>
<td>0</td>
<td>0</td>
<td>0.0025</td>
<td>0.0305</td>
</tr>
<tr>
<td>$k_{-4}$</td>
<td>0</td>
<td>0</td>
<td>0.0065</td>
<td>0.0722</td>
</tr>
<tr>
<td>$k_{-5}$</td>
<td>0</td>
<td>0</td>
<td>0.0561</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Reference repair time

| $\tau_R$ (sec) | 372.77 | 591.71 | 484.68 | 508.54 |

Table 8.1: Control coefficients of the reaction rate constants on the repair time as obtained from the four parameter sets. Note that the repair times (last row) has been calculated for low damage conditions, a case that most likely reproduce the situation in nature (e.g. sun exposure). In experiments a high initial damage has been used.

binding is irreversible, $k_{-i} = 0$, the repair time reads

$$\tau_R = \sum_{i=1}^{n+1} \frac{1}{\kappa_i},$$

(8.3)

where $\kappa_i = k_iT_i$ and $\kappa_{n+1} = k_R$ ($i = 1, \cdots, n$).

Clearly, all binding reactions and the repair step contribute to the repair time. The control coefficients

$$C_{\tau_R}^{\kappa_i} = -\frac{1}{\tau_R\kappa_i},$$

(8.4)

reveal that the strongest influence on the repair time is exerted by the slowest step. For low damage concentrations, the recognition and the repair step, because of their low rate constants, are the principal processes that limit repair (Tab. 8.1, set 1 and 2).

The presence of dissociation events prior to the repair step ($k_{-i} \neq 0$) changes the
repair time

\[ \tau_R = \sum_{i=1}^{n+1} \frac{1}{\kappa_i} + \sum_{i=0}^{n-1} \sum_{j=1}^{n-i} \frac{\prod_{l=j}^{i+j} k_{-l}/\kappa_l}{\kappa_{i+j+1}}. \]  

(8.5)

If the stability of the intermediary complexes is decreased by increasing the dissociation rate constants, the repair efficiency is impaired (repair time rises). For the estimated parameters, one finds, as for irreversible binding, that the recognition and repair steps exert the highest control on the repair time (Tab. 8.1, set 3 and 4).

Assuming that the factors bind equally well to their respective substrate (\( \kappa_i = \kappa \) and \( k_{-i} = k_− \) for \( i \leq n \)) simplifies the repair time as follows

\[ \tau_R = \sum_{i=0}^{n} \left( \frac{k_−}{\kappa} \right)^i \left( \frac{1}{\kappa_R} + \frac{n-i}{\kappa} \right). \]  

(8.6)

The quantity \( k_−/\kappa = k_−/(kT) \) represents the dissociation constant of a factor to its intermediary complex. Equation (8.6) brings to light a condition for NER to be efficient in removing DNA lesions: as NER involves a large number of proteins (\( n \geq 5 \))\(^1\), these proteins must be sufficiently affine to their substrate (\( k_−/\kappa \leq 1 \)). The importance of this condition is illustrated in Fig. 8.1, when \( k_−/\kappa \leq 1 \) the repair time increases only moderately with the number of factors, whereas for \( k_−/\kappa > 1 \) the rise is very pronounced. Already for five repair factors the differences between these two cases can be considerable. According to the estimated parameters, the condition \( k_{-i}/\kappa_i < 1 \) is fulfilled for nearly all factors (except the 2nd factor in set 3), indicating that NER, for the number of required factors, is a rather efficient pathway (Tab. 7.3).

### 8.1.2 High initial damage

If a large number of lesions is introduced at \( t = 0 \), it is not possible to calculate \( \tau_R \) explicitly. An approximation for the time changes of the damage concentration is given by (Appendix C.4)

\[ \frac{dD_T}{dt} = -V_R \approx -V_{max} \frac{D_T}{D_T + K_d}, \]  

(8.7)

where \( V_{max} \) is the maximal repair rate and \( K_d \) is the damage concentration at which lesions are removed at half-maximal rate (\( V_R = V_{max}/2 \)). Using Eq. (8.7) one derives (Appendix D.2.2)

\[ \tau_R \approx \frac{1}{2} D_T(0)V_{max} + \frac{K_d}{V_{max}}. \]  

(8.8)

\(^1\)In the model, I considered the five most prominent repair factors, however, there are indications that at least two additional proteins, RPA and UV-DDB, are also involved in repair [59, 108, 119, 159]
Figure 8.1: Low initial damage: Repair time as function of the number of repair factors, Eq. (8.6). The dissociation rate constant $k_\text{m}$ is increased from 0.1 (square), to 0.2 (circle) and 0.3 (triangle). The latter case is considerably less efficient in removing damages. The other parameters are $k_R = 0.007$ and $\kappa = kT = 0.2/s$.

Which indicates that the repair time increases linearly with the initial damage concentration $D_T(0)$. For the control coefficients one obtains

$$C_{p}^{rR} = \frac{2K_d}{2K_d + D_T(0)} C_{p}^{K_d} - C_{p}^{V_{\text{max}}}, \tag{8.9}$$

where $C_{p}^{V_{\text{max}}}$ and $C_{p}^{K_d}$ are the control coefficients of parameter $p$ on the maximal repair rate $V_{\text{max}}$ and the half-saturation constant of repair $K_d$, respectively (defined in analogy to Eq. (8.2)). These two coefficients are specific to the repair system and are independent of the inflicted damage. At a saturating damage concentration ($D_T(0) \gg K_d$), only parameters affecting $V_{\text{max}}$ will influence the repair time, $C_{p}^{rR} = C_{p}^{V_{\text{max}}}$.  

The control coefficients shown in Tab. 8.2 demonstrate that each reaction step contributes to the repair time. Increasing the total concentrations of early binding NER proteins appears to have the strongest effect on repair efficiency (largest positive control on $V_{\text{max}}$). In contrast, for the rate constants, the largest positive effect on $V_{\text{max}}$ is exerted by the repair rate constant. This is because the repair step is relatively slow compared to the time needed to assemble the PC. Conversely, changes in the binding rate constant of the first NER factor ($k_1$) have no effect on $V_{\text{max}}$ (control coefficient equals zero, Tab. 8.2). The rationale for this is that, under saturating conditions, the first factor will be completely involved in repair. Increasing $k_1$, on the other hand, has a strong negative effect on $K_d$, making the system more proficient for repairing low amounts of DNA damage (cf. to the previous section). This analysis, valid for small parameter changes, pinpoints steps that are likely to have the strongest influence on
8.1. Repair efficiency

Control Coefficients for the Maximal Repair Rate and the Half-Saturation Constant

<table>
<thead>
<tr>
<th>XPC-HR23B</th>
<th>TFFIIH</th>
<th>XPG</th>
<th>XPA</th>
<th>ERCC1-XPF</th>
<th>Repair step</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control on $V_{max}$ by binding rate constants and total factor concentrations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{k}^{V_{max}}$</td>
<td>0 ($k_1$)</td>
<td>0.145 ($k_2$)</td>
<td>0.071 ($k_3$)</td>
<td>0.016 ($k_4$)</td>
<td>0.046 ($k_5$)</td>
</tr>
<tr>
<td>$C_{T}^{V_{max}}$</td>
<td>0.612 ($T_1$)</td>
<td>0.329 ($T_2$)</td>
<td>0.221 ($T_3$)</td>
<td>0.021 ($T_4$)</td>
<td>0.091 ($T_5$)</td>
</tr>
<tr>
<td>Control on $K_d$, the half-saturation constant of repair</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{k}^{K_d}$</td>
<td>-0.632 ($k_1$)</td>
<td>0.086 ($k_2$)</td>
<td>0.068 ($k_3$)</td>
<td>0.01 ($k_4$)</td>
<td>0.04 ($k_5$)</td>
</tr>
<tr>
<td>$C_{T}^{K_d}$</td>
<td>-0.148 ($T_1$)</td>
<td>0.322 ($T_2$)</td>
<td>0.279 ($T_3$)</td>
<td>0.017 ($T_4$)</td>
<td>0.1 ($T_5$)</td>
</tr>
</tbody>
</table>

Table 8.2: The term in brackets refers to the parameter for which the control coefficient has been calculated (using parameter set 1, Table 1). One has $V_{max} = 1.92$ nM/s and $K_d = 0.52$ µM. These two values are in agreement with the experimentally estimated values of 1.03–2.24 nM/s for $V_{max}$ and $\sim 1$ µM for $K_d$ [71, 175].

repair efficiency.

Figures 8.2A and B analyze the effect of large changes in the system parameters on repair efficiency, characterized by the repair time $\tau_R$. Interestingly, changing the value of one of the binding rate constants over a 25-fold range has little or no effect on repair time (Fig. 8.2A). Also increasing the concentration of just one of the protein factors (Fig. 8.2B) has only a limited influence on repair efficiency. The rationale for this is that after an increase in rate, achieved by making one step faster (e.g. by increasing the concentration of one of the protein factors), the repair process as a whole will be limited by one of the other reaction steps. On the other hand, changes in the repair rate constant $k_R$ appear to significantly modulate the repair time (Fig. 8.2B). This is because the repair/release step is slow compared to the other steps, thereby suppressing the availability of free repair proteins.

This analysis reveals an important property of sequential assembly systems: No single component uniquely controls the repair process. There are some processes that seem to have a larger control. I have shown that the last step in the model depicted in Fig. 7.1 has a stronger control on repair efficiency compared to processes characterizing the assembly of the pre-incision complex. In order to verify this hypothesis experimentally it would be useful to measure the maximal rate of damage removal ($V_{max}$) for different factor concentrations. The results of the simulations are shown in Fig. 8.2C. Different from the previous case, rising the concentrations of all factors simultaneously strongly increases $V_{max}$, resulting in a more efficient repair. The relationship between $V_{max}$ and
Figure 8.2: Repair efficiency for large parameter changes. An increase in efficiency corresponds to a decrease in repair time $\tau_R$. (A) Fold changes in $\tau_R$ due to changes in each of the individual rate constants. $\tau_R$ is calculated from Eq. (8.1) for an initial damage of 4 $\mu$M given to the whole nucleus (i.e. $7.2 \times 10^5$ lesions). Fold changes are relative to reference parameter values $k^{ref}$ and reference repair time ($\tau_R^{ref} = 20.56$ min) obtained from set 1, Tab. 7.2. (B) Fold changes in $\tau_R$ due to changes of each of the total NER factor concentrations. (C) Changes in maximal repair rate $V_{max}$ due to variations in total concentrations of all NER factors simultaneously. The total concentrations are increased or decreased by an equal factor. With a slow repair step ($k_R = 0.001/s$, upper dashed line, or 0.007/s, solid line), $V_{max}$ depends nearly linearly on the factor concentrations (first order reaction). For a fast repair step ($k_R = 0.7/s$, lower dashed line), where repair is limited by the assembly of the proteins, the dependency on factor concentrations is of second order. The values for $V_{max}$ have been normalized. Similar results are obtained for parameter set 2 to 4 (Tab. 7.2 and 7.3).

the protein concentrations depends on the process that limits repair. If repair is limited by the rate of the last step, the relation between factor concentrations and $V_{max}$ is ap-
proximately linear (Fig. 8.2C, upper dashed and solid line). On the other hand, if repair is limited by the rate of PC assembly, \( V_{max} \) depends on protein factor concentrations as in a second order reaction (Fig. 8.2C, lower dashed line). This result can be proved analytically for the case of irreversible binding \((k_{-i} = 0)\) and equal total concentrations \(T\) (see Appendix C.4). When the limiting process is the repair step, i.e. \( k_i \gg k_R \),

\[
V_{max} \approx k_R T. \tag{8.10}
\]

When repair is limited by the assembly of the factors, i.e. \( k_i \ll k_R \),

\[
V_{max} \approx k_n \lambda_{n-1} T^2, \tag{8.11}
\]

which is second order in \(T\). The constant \( \lambda_{n-1} \) depends on all binding rate constants and the repair rate constant (cf. Appendix C.4, page 172).

The values of \( V_{max} \) and \( K_d \) as well as of the repair time can be measured experimentally (e.g. [71, 175]). Therefore, several of the model predictions presented here, concerning the perturbation of the factor concentrations, could be tested experimentally.

### 8.1.3 Relation between repair efficiency and accumulation kinetics

NER is a versatile repair mechanism that recognizes several type of lesions. It is interesting to understand, whether the factor recruitment kinetics can shed light on which type of lesion is repaired and on how efficiently this lesion is removed.

To be specific, I assume that the binding properties of the repair factors to the different type of lesions are not equal. How the accumulation times, characterizing the recruitment kinetics, change for variations in the binding and dissociation rate constants is plotted in Fig. 8.3. I recall that a decrease (increase) in the binding (dissociation) rate constant goes hand in hand with a rise in the repair time, and so a less efficient repair. Surprisingly, the accumulation times can display a maximum as function of the rate constants (Fig. 8.3). This is a counterintuitive result, one would expect that the better a type of damage is repaired, the faster the factors are recruited (low accumulation time).

The theoretical analysis shows that the accumulation kinetics of factors to the local damage is not always correlated with repair efficiency. Recruitment of factors to lesions to which they are less affine, for example due to higher dissociation rate constants, could proceed faster than the recruitment to lesions which are more efficiently repaired (low dissociation rate constants).
Figure 8.3: The accumulation times are calculated for high damage (see Appendix D.3.2) as function of the binding rate constants (r.c.) $k_i$ (A) or the dissociation rate constants $k_{-i}$ (B). The numbers indicate the repair factor for which the accumulation time is plotted. The rate constants are varied simultaneously by the same coefficient. The reference parameter set is given in Tab. 7.2, set 1. The dissociation rate constants are equal for all factors $k_{-i} = k_{-} = 0.1/s$. These calculations have been performed for the linearized system ($T_i - I_i \approx T_i$, $i > 1$), similar results are obtained for the complete model and for the other parameter sets (not shown).

### 8.2 Selectivity of NER

The previous analysis demonstrated that repair efficiency decreases with the number of repair factors (the repair time increases, Eq. (8.6)). Therefore, with respect to efficiency, it would be better to perform the repair task with only one protein. In fact, some organisms use a photolyase for removing 6-4PPs and one for removing CPDs [3]. Which are then the advantages of using a multitude of proteins for performing repair?

NER has a wide range of substrates that all cause distortions of the DNA helix. This fact indicates how unlikely it is to have an extremely high affinity just for a specific substrate, as this would impair the ability to recognize the other type of lesions. It has been shown, in vitro, that the differences in NER protein affinity between damage and undamaged DNA are small. For example, the affinity of XPC and UV-DDB for non-damaged DNA is only slightly lower than for thymine dimers. Thymine dimers, however, are still repaired efficiently by NER [132], whereas undamaged DNA, which can have spontaneous helix distortions, is excised to a much lesser extent by the endonucleases [14]. The problem one is faced to, is to minimize excision of undamaged DNA, an energy consuming process and possible source of mutations [49], when the affinity to lesions is
only slightly higher than to non-damaged sites. In the following, I will analyze how the usage of several proteins in NER can resolve this problem.

Let assume two types of substrates for NER: The lesions and some slight helix distortions. The two substrates differ in their affinity for the repair factors. A system is highly selective, if it repairs the damage much faster than the excision of non-damaged sites. I define the selectivity of repair by

\[
\sigma = \frac{\tau_R^* - \tau_R}{\tau_R^*}. \tag{8.12}
\]

where \(\tau_R^*\) is mean time needed to excise non-damaged sites. It is reasonable to assume that the recognition and repair of damages occurs faster than the excision of non-damaged sites, thus \(\tau_R^* > \tau_R\). The selectivity \(\sigma\) varies between 0 and 1, and a value of 1 corresponds to the highest selectivity for the damage. How well NER recognizes the damage compared to the non-damaged substrate can be expressed by the ratio of the dissociation constants, the versatility

\[
\omega = \frac{\sum^n_{i=1} k_{-i}/k_i^*}{\sum^n_{i=1} k_{-i}^*/k_i^*}. \tag{8.13}
\]

Where \(k_{-i}^*\) and \(k_i^*\) denote the dissociation and binding rate constants of the repair proteins to the non-damaged sites, respectively. The versatility varies between 0 and 1, \(\omega\) is equal 1 when all helix distortions are repaired with the same efficiency.

In Fig. 8.4 I plotted these two measures against each other. Clearly, the higher the affinity of the factors to the damaged site, low \(\omega\), the higher the selectivity. Because for each additional repair factor the substrate is verified anew, the selectivity increases with the number of factors involved in repair. Furthermore, for a multiprotein process the selectivity remains high even if the affinity for the lesions decreases, higher \(\omega\), whereas a single protein mediated repair \((n = 1)\) loses its selective power. As postulated above, to recognize a multitude of damage types it is desirable to have a moderate affinity for each type of lesion. Therefore, a multiprotein process allows to recognize and selectively repair a larger range of helix-distorting lesions compared to a process relying on a single protein.

The selectivity of NER towards the damaged substrate reminds kinetic proofreading [12, 68]. In my model the selectivity relies on the fact that the system is not in thermodynamic equilibrium. This is because the excision step is irreversible and the excised ssDNA containing the damage is quickly degraded. Strictly speaking, a kinetic proofreading mechanism also includes \(i\) irreversible, energy consuming, steps before the last catalytic one, and \(ii\) the possibility for the enzyme complex to fall off its substrate after such irreversible reactions. These latter mechanisms have been shown, using
Figure 8.4: Selectivity versus versatility for increasing number of repair factors (labels on curves). Increase in versatility is obtained by increasing the dissociation rate constant for the damaged substrate $k_-$. The curves are computed using Eq. (8.6) for low amounts of initial substrate. Parameters: $T = 0.44 \, \mu\text{M}$, $k = k^* = 0.2/\mu\text{M/s}$, $k^*_+ = 0.2/s$, $k_R = 0.007/s$.

Mathematical models, to dramatically increase the selectivity of a system towards the right substrate (see e.g. [103]). In NER, the opening of the DNA by the TFIIH helicases is known to require ATP [3]. It would be interesting to test, whether the introduction of such a step in the model and allowing for the factors to fall off the DNA at once before completion of the PC, could further increase the selectivity of repair.
8.3 Model variants

In the experimental literature, several alternative binding and dissociation mechanisms have been suggested. These will be discussed below and their efficiency compared to the sequential assembly mechanism.

8.3.1 Non-sequential binding of protein factors

The order of binding of the repair factors and whether it is strictly sequential or not has been a matter of controversy in the last years. It has been proposed that other factors, in addition to XPC-HR23B, are involved in the recognition of the damaged DNA [130, 132]. Furthermore, the results of Volker and coworkers indicate that ERCC1-XPF and XPG, the last two factors in our model, can bind randomly after XPA has been recruited to the DNA [180]. In this section, I will analyze the repair efficiency of alternative non-sequential assembly mechanisms.

In Fig. 8.5, I show the results for four alternative pathways to assemble the pre-incision complex (PC). They allow random binding of two factors to damaged DNA or to intermediary complexes (Fig. 8.5A). In assembly mechanism I, the first and second factor can recognize the damaged DNA, resulting in two different routes to complex S₀F₁F₂. Subsequent assembly of the other factors is assumed to proceed sequentially. For the assembly mechanisms II to IV, the constraint of strict sequential binding of factors is relieved more downstream. For a low (non-saturating) concentration of lesions Dₜ, one observes similar repair rates Vₐ for both the strict sequential assembly and mechanism I through IV (Fig. 8.5B). At a high DNA damage, mechanisms II and III are slightly more efficient than strict sequential assembly, which itself is slightly better than mechanism IV (inset Fig. 8.5B). Interestingly, the assembly mechanism I, which allows independent binding of two different factors to recognize damaged DNA, dramatically alters repair efficiency. The repair rate Vₐ reaches a maximum beyond which it rapidly declines with increasing damage concentration. This can be understood from the fact that, in the presence of a large number of lesions, many complexes will be formed containing only one of the two factors, hence lowering the probability for the other factor to be recruited, which in turn interrupts the assembly chain towards establishing a complete PC.

Therefore, albeit Vₐ for mechanism I hardly differs from that of a sequential assembly mechanism (Fig. 8.5B), non-sequential factor binding can readily become unfavorable at high concentrations of DNA damage. Consideration of more complex random binding mechanisms led to similar conclusions (data not shown). In particular, if more
Figure 8.5: Random binding of repair factors. (A) Five possible assembly mechanisms for the formation of the PC. Mechanism I to IV describe the random reversible binding of in each case two factors at a different step of the repair process. As in the previous model, after removal of the damage all factors are released. For clarity, the intermediary complexes are not shown. It is assumed that all factors bind equally well (same association/dissociation rate constants) to their respective binding sites and have equal total concentrations. This avoids bias, when comparing the alternative mechanisms. (B) Steady state repair rate versus total damage concentration. The colors and Roman numbers refer to the assembly mechanisms shown in (A). Inset is a magnification. For mechanism I repair is inhibited at high damage (red line). Parameters: each total factor concentration is $T = 0.2 \, \mu M$; binding and dissociation rate constants are $k = 1 \, (\mu M \, s)^{-1}$ and $k_0 = 0.005/s$, respectively; repair rate constant is $k_R = 0.007/s$.

than two factors can bind to the free damage the repair rate will decrease for high amount of lesions (similar curves as for mechanism I, Fig. 8.5B).

### 8.3.2 Non-simultaneous release of factors

Several studies have suggested that XPC-HR23B is released before the PC is established [136, 182, 200]. In addition, recent *in vivo* data showed that XPC-HR23B resides at the DNA damage significantly shorter than TFIIH, XPA, and ERCC1-XPF [66]. I, therefore, explored five mechanistic variants that consider early release of XPC-HR23B
Figure 8.6: Early release of the first factor. (A) Dissociation of the first factor $F_1$ at different steps of the assembly process. At step six all factors are released simultaneously (as hitherto). (B) Maximal repair rate as a function of the step where the first factor is released (shown in (A), dark encircled numbers). When the first factor is released before the other factors (step 5 to 1) the maximal repair rate increases. Parameter set 1 of Tab. 7.2 is used, except $T_1 = 0.2 \mu$M. (C) Residence times are calculated for saturating conditions (Appendix D.2.4). Only when the first factor is released before the other factors does its residence time fit with the experimental data.

(Fig. 8.6A) and compared their repair efficiencies and the time that NER proteins reside in the complexes. Figure 8.6B shows that repair efficiency significantly improves if XPC-HR23B is released prior to the other factors (the maximal repair rate $V_{max}$ increases). The rationale for this is that, at any time point, the first factor will now be sequestered in fewer complexes and can therefore initiate more repair events per unit time. This leads to a higher degree of immobilization of the other factors, so that more PCs are formed, and, thus, the maximal repair rate increases.

For distinguishing between the different alternatives, I calculated the mean time the factors reside in the repair complex (cf. Section 7.2.2 and Appendix D.2.4). Figure 8.6C demonstrates how the residence times of each factor depend on the step in which the first factor is released. As pointed in Section 7.3.3, for simultaneous release (step 6), the residence time of the first factor is higher than measured experimentally, whereas the
residence times of TFIIH (F₂), XPA (F₄) and ERCC1-XPF (F₅) are in agreement with the data [67, 71, 127]. Early release of the first factor (step 2–5) results in a lowering of its residence time²: One obtains a residence time of \( \sim 70 \) s, which has the same order of magnitude as what has been measured experimentally (\( \sim 44 \) s, [66]).

### 8.3.3 Preassembled repairosome

It has been a point of controversy whether NER proteins are recruited to lesions as individual factors [1, 67, 127, 180], larger protein complexes [50, 131, 183], or as a fully pre-assembled repairosome [160]. Although the current consensus is that the PC is assembled at sites of DNA damage, this does not imply that such on-the-spot assembly strategy is the most favorable per se. I already showed that repair mediated by the binding of a single protein, in this case the holocomplex, shows less selective power in distinguishing damaged and non-damaged sites than repair mediated by factors assembled at the DNA (Section 8.2). Here, I will analyze how both mechanisms differ with respect to repair efficiency.

Consider \( n \) factors that can interact with each other. For \( n = 3 \), all possible interactions to form a holocomplex are shown in Fig. 8.7A. Since repair by a pre-established repairosome complex involves only two steps, the repair time under non-saturating conditions, in analogy to Eq. (8.6), reads

\[
\tau^h_R = \frac{1}{kH} + \frac{1}{kR} + \frac{k_-}{kH kR},
\]

with \( H, k, k_- \), and \( kR \) being the concentration of the holocomplex, the binding and the dissociation rate constants at the damaged site, and the repair rate constant, respectively. In steady state, the concentration of the holocomplex \( H \) is given by the implicit expression (Appendix C.3)

\[
\left( \sqrt[1]{\frac{K_h}{T}} + n \sqrt[1]{\frac{H}{T}} \right)^{n-1} \sqrt[1]{\frac{H}{T}} = 1,
\]

where \( T \) is the total concentration of each factor, and \( K_h \) is the dissociation constant for factor interactions. Repair efficiency of the holocomplex is compared to the corresponding sequential pathway for which the repair time is, Eq. (8.6),

\[
\tau^s_R = \sum_{i=0}^{n} \left( \frac{k_-}{kT} \right)^i \left( \frac{n-i}{kT} + \frac{1}{kR} \right).
\]

²When XPC-HR23B can initiate additional repair complexes the free concentrations of downstream factors decrease. The consequence is that each binding step proceeds more slowly. This effect explains why, when XPC-HR23B is released prior step 5, its residence time slightly increases.
Figure 8.7: Preassembly of repair factors. (A) Repair factors are allowed to reversibly associate in solution to form intermediary complexes and the holocomplex in a damage-independent fashion (shown for the case of three protein factors). The holocomplex (H) contains all factors and is able to repair DNA damage through a single binding step and a single repair step. (B) Repair time of the holocomplex (\(\tau^h_R\), solid lines), and of the sequential assembly mechanism (\(\tau^s_R\), dashed line) as a function of the number of factors involved (\(n\)) for non-saturating damage. For the holocomplex, both weak and strong factor interactions were examined (\(K_h = 200\) nM and \(K_h = 10\) nM, respectively). Parameters: factors have equal concentrations \(T = 0.4\) \(\mu\)M, binding rate constant to the damaged site \(k = 0.5/\mu\)M/s, dissociation rate constant \(k_- = 0.1/s\), repair rate constant \(k_R = 0.007/s\).

In both models the repair time increases with the number of NER factors involved (Fig. 8.7B), but do so for different reasons: for the sequential pathway, the approximately linear increase in repair time results from the mere fact that each extra step requires extra time (Fig. 8.7, dashed curve). Repair by the pre-assembled holocomplex shows a strong non-linear increase in repair time (Fig. 8.7B, solid lines). Noticeably, pre-assembly of the repair complex is less favorable if the binding between factors is weak (Fig. 8.7B, \(K_h = 200\) nM). For strong affinities, repair by the holocomplex may be more efficient if only a few different factors are involved (in Fig. 8.7B if \(n < 3\)), yet repair readily becomes inefficient if holocomplex formation involves more proteins (Fig. 8.7B, \(K_h = 10\) nM). These results can be understood from the fact that holocomplex concentration decreases with increasing \(n\), as the individual factors become spread over more and more intermediary complexes.

Because most DNA-associated complexes that are involved in processes such as transcription, replication, and various repair mechanisms, involve a considerable number of macromolecules, fully pre-assembled complexes are probably less favorable than stepwise complex formation at the genomic site of action.
Chapter 9

Discussion part II: A model for nucleotide excision repair

Many nuclear processes are carried out by multi-component systems that have properties in common with nucleotide excision repair (NER). Sequential recruitment of protein components to their sites of action on chromatin has been suggested for a number of nuclear processes, including DNA double-strand break repair [193], base excision repair [115], transcription initiation by both RNA polymerase I [32] and RNA polymerase II [87], and DNA replication [17]. Therefore, the presented model, which describes the kinetic properties of NER in the nucleus of mammalian cells, may serve as a paradigm for the kinetic architecture of a broad variety of nuclear processes.

Since no mathematical framework for NER had been available to date, I was aimed to reduce the analysis to its essentials. The core model is based on sequential assembly of five repair factors and their simultaneous release after a successful repair event (Fig. 7.1). Clearly, the NER process is more complex than the six-step repair pathway that has been investigated here. Additional steps include, for instance, recruitment of RPA, opening of the DNA double helix by TFIH, and modulation of chromatin structure. Yet this does not prevent one to unveil general characteristics of the process as a whole, since these characteristics remain valid if the chain of steps is extended or truncated. The assumptions that entered the model led to several important predictions, which will be discussed below.
Chapter 9. Discussion part II: A model for nucleotide excision repair

No single rate-limiting step

In contrast to the often-held view that a multi-step process should be governed by a single rate-limiting step, I showed here that all reaction steps contribute to the efficiency of the sequential binding system. One may ask which step has the largest effect on repair efficiency? XPC-GFP accumulated remarkably slowly at damaged DNA in vivo. As the number of damages is assumed to be in large excess, and the rate XPC-GFP binding is proportional to the amount of damages, the binding to the DNA will be fast as soon as XPC-GFP is in the local damaged area. Therefore, to explain the slow accumulation of XPC-GFP, I proposed a diffusion-limited recruitment to the local damaged area, a process that may constrain the rate of formation of the pre-incision complex. However, the effective diffusion coefficient of XPC-GFP that was estimated computationally appeared ten times lower than those measured in living cells [66]. This may indicate that either other nuclear processes limit the recruitment of XPC-HR23B to sites of DNA damage (e.g. chromatin remodeling, recruitment of a repair factor prior to XPC-HR23B), or that the number of damage sites is not in such a high excess as assumed (see next Chapter, Section 10.1). Our analysis also revealed that the DNA repair step (i.e. last step in Fig. 7.1) plays a dominant role (Tables 8.1 and 8.2, and Fig. 8.2). The prediction of the model is that, for high damage, NER is limited by the rate of the repair step, which includes dual incision, removal of the damaged patch, and DNA resynthesis, and not by the time required for assembling the PC. Increasing the concentrations of all protein factors simultaneously and measuring the changes in maximal repair rate can experimentally verify this prediction. Depending on which process has the largest effect on repair efficiency, a first or second order dependency of the maximal repair rate on the factor concentrations is expected (see Fig. 8.2C).

On-site assembly results in more efficient repair

Many other chromatin-associated processes have been proposed to utilize a sequential assembly strategy [89, 146]. Since several nuclear proteins have multiple functions (e.g. TFIIH functions in both transcription and NER), on-site assembly seems advantageous as it allows flexible usage of proteins in different cellular contexts. However, this may not be the only reason why cells prefer a stepwise assembly strategy.

I showed that a strict sequential assembly mechanism can be remarkably efficient compared to alternative assembly schemes. For NER, XPC-HR23B, XPA, and RPA were recently proposed to cooperate in damage recognition through a random binding
mechanism [132]. Although such situation is conceivable, my theoretical analysis indicates that it might have a dramatic negative effect on repair efficiency, particularly at high DNA damage loads. Measurements of the amounts of immobilized NER factors in vivo have not indicated a decrease towards higher UV dose. Also, I could find no evidence in literature that repair capacity diminishes at high lesion concentrations. Therefore, damage recognition through different NER factors simultaneously (e.g. assembly mechanism I in Fig. 8.5A) seems unlikely. Random binding of factors further downstream in the chain cannot be excluded, but this is predicted to have no significant effect on repair efficiency.

Using the modeling approach, I demonstrated that a pre-assembling of NER proteins can dramatically reduce repair efficiency and the selectivity towards damaged sites. In agreement with this, it has been shown experimentally that in absence of DNA, NER factors only weakly interact in vitro [5]. Moreover, the in vivo diffusion properties of NER proteins strongly support the notion that, in the absence of UV damage, the factors are present as individual proteins, rather than being part of large complexes [67, 71, 127]. Thus, the model-based analysis indicates that a sequential repair mechanism is preferable over other assembly strategies.

In terms of efficiency, there are mechanistic features from which sequential systems benefit. For instance, I predicted that an early release of XPC-HR23B may significantly improve repair efficiency. In general, the shorter the time that a protein needs to reside in the protein complex, the more efficient that pathway will be. Recent experimental studies provide evidence that XPC-HR23B might indeed function as a "molecular matchmaker", leaving the NER complex before it is complete [136, 183]. It is likely that the NER pathway harbors more such differential release steps. To date, however, especially steps that follow the PC formation have remained ambiguous. Insight into the dynamic properties of these later events of the NER process will be essential for its comprehensive understanding (see also next Chapter, Section 10.3).

Usage of NER protein factors and their kinetics

The model shows that a direct consequence of a sequential assembly and simultaneous release mechanism is that the more upstream in the chain a factor is recruited, the higher the amount of that protein is used in the repair process (Fig. 7.4D–E, Fig. 7.5D–E, and Eq. (7.7)). The first factor will even become fully involved if there is an excess of lesions, and so represents a limiting diffusible recognition factor. Interestingly, in contrast to this prediction, experimental observations show that only about 40% of XPC-HR23B
is recruited to DNA damage in global damage experiments. Several explanations are possible. For instance, XPC-HR23B may exist in different states of which only one allows participation in the NER process. Alternatively, XPC-HR23B recruitment might not be the first step in NER. UV-damaged DNA binding protein (UV-DDB), for instance, has also been implicated to play a crucial role early in NER, especially for certain types of lesions [108, 162]. Its precise function in damage removal is still largely unclear, but UV-DDB was recently found to modulate XPC-HR23B recruitment by modifying its ubiquitylation state [159].

Although, the above propositions may explain why XPC-HR23B is not fully immobilized at saturating conditions, they cannot account for the differences in immobilized fractions in local and global damage experiments. Recent experiments show that for XPC-HR23B and all repair factors studied so far the fraction immobilized is about four times higher in global (∼40%) than in local damage (∼10%) experiments (cf. Section 6.2). Provided that the estimated immobile fractions in local and global damage experiments are not due to differences in the method used for estimation, and can therefore be compared, this is a surprising result. According to the assumed dependency of the number of damages on the UV light intensity (Eq. (7.10)), the number of lesions are about the same in local and global damage experiments. Indeed, model simulations show faster factor accumulation kinetics in global damage experiments, as the diffusional time becomes negligible, compared to local damage experiments, but eventually the attained immobilized fractions are the same (see next chapter, Fig. 10.2).

The initial model has been developed using the in vivo kinetic data of XPC-HR23B, TFIIH and ERCC1-XPF assembly, which it could reproduce satisfactorily. Recently, the assembly kinetics of XPG and XPA, two other essential repair factors, has also been analyzed. For XPA an extremely slow recruitment is measured, with a characteristic accumulation time of ∼810 s (M. Luijsterburg, personal communication). Initial modeling results show that, apparently, there is no parameter set for which the sequential assembly model with simultaneous release could reproduce all the five data sets. In particular, the fast accumulation of ERCC1-XPF and the very slow recruitment of XPA seem incompatible. This may indicate that either ERCC1-XPF binds before XPA, something quite improbable according to [180], or that the release does not occur simultaneously. In the next chapter, I will present first ideas and model extensions on how to accommodate these latest experimental facts (Section 10.3).
Chapter 10

Future directions for modeling NER

The considerations made in the discussion show that, for a further development of the NER model, one needs a better understanding on how damages are generated and recognized. Furthermore, it would be necessary to consider alternative release mechanisms in order to accommodate the latest results. In the next sections, I will discuss a few hypothesis to address the problems of NER saturation and assembly kinetics.

10.1 Damage generation

The probability $p(UV)$ to induce, for a given UV dose, a damage in a pair of adjacent bases can be derived from the experimental data. However, the reported dependency on UV dose vary strongly. Whereas Ye et al. (1999) and van Hoffen et al. (1999) claim linear dependencies [175, 198], Perdiz et al. (2000) show a saturation at higher doses [120]. The probabilities that a pair of adjacent nucleotides is a CPD or a 6-4PP, $p_{CPD}(UV)$ and $p_{PP}(UV)$, respectively, are shown in Fig. 10.1A-B. The total probability to have a damage is consequently $p(UV) = p_{CPD}(UV) + p_{PP}(UV)$. Unfortunately, the experiments have been performed at UV light intensities far below the doses used in local damage experiments (0–40 J/m$^2$ compared to 100–200 J/m$^2$). Thus, the interpolation to high UV doses may bear some errors. Depending on which $p(UV)$ curve is used the model predictions will be rather different (see section 10.2).

Future developments of the NER model should consider repair of both type of damages created by UV light. What is known from experiments is that 6-4PP are repaired about ten times faster than CPD [108]. Fig. 10.1C show typical data of damage removal. An improved mathematical model will help in elucidating the origins of the differences in repair efficiency between 6-4PP and CPD, and the possible contribution of CPD to
Chapter 10. Future directions for modeling NER

Figure 10.1: Damage generation and removal. The probability to form a CPD (A) or a 6-4PP (B) increases linearly, according to [175, 198], or saturates according to [120] (data points). Compared to 6-4PP, CPD are formed with a 2–4 times higher probability. (C) The removal of CPD is ten times slower than of 6-4PP (data from [108]). One estimates a first order damage removal rate constant of $0.68 \pm 0.143$/hour for 6-4PP and of $0.071 \pm 0.05$/hour for CPD.

the observed recruitment kinetics. To account for CPD repair, it will be necessary to include the protein UV-DDB, which is known to be crucial for repairing CPD and which facilitates 6-4PP repair [159].

10.2 How and why NER saturates: a case study

NER is saturated when an increase in UV dose does neither causes further involvement of factors in repair, nor does it produce faster accumulation of repair factors at the local damage (cf. Section 6.2). A plausible explanation was that, at high damage loads, nearly the totality of the recognition factor is bound to damages, so that no further repair complexes can be initiated. As pointed in the discussion, some problems follow the assumption of such a limiting recognition factors. Resolving these problems can give a better understanding of NER.

I will discuss two alternative hypothesis. In the first case, not all damages can be recognized/processed simultaneously, I denote this hypothesis lateral inhibition. In the second case, the number of induced damages per UV light saturates (see above). These two alternatives are tested against three different experiments: (i) the immobilized fraction at different UV doses in local damage experiments (LDE) and global damage experiments (GDE), (ii) the recruitment kinetics to multiple local damages, and (iii) the long time behavior at the local damage. For comparison, I recapitulate below the results
obtained with the previous model, which accounted for a limiting diffusible recognition factor.

10.2. How and why NER saturates: a case study

10.2.1 Limiting recognition factor

When the number of damages is an increasing function of the UV dose (Fig. 10.2A) and the recognition factor is limiting, the maximal amounts of immobilized factors in GDE and LDE become identical for high UV doses (dashed and solid lines in Fig. 10.2B, respectively). The differences in immobile fractions between GDE and LDE at their respective typical doses of 10 J/m², solid square, and 100 J/m², opaque square, are minimal. This is due to the dissipation of the spatial gradients after the initial transient, so that it is irrelevant if the same number of damages are concentrated locally or evenly distributed throughout the nucleus. The recognition factor is limiting at high UV doses and will be completely bound to the DNA (red lines in Fig. 10.2B). The dose-response curves can be used to read out the effect of augmenting the damaged area. One predict only a small increase of the immobile fraction along the arrows shown in Fig. 10.2B, the changes become negligible for factors binding more downstream in NER (green lines).

If more than one local damage is applied to a nucleus, the accumulation kinetics at each of these local damages is faster and the immobile fraction lower compared to a nucleus with one single local damage (blue and black line respectively, Fig. 10.2C). Characteristic for the presence of a limiting diffusible recognition factor is that, at saturating UV, the sum of the immobile fraction in the three local damages approaches the value obtained with an unique local damage (dotted line, Fig. 10.2C). This is due to the fact that the recognition factor is fully immobilized in both cases.

A further prediction of the model is that an increase in UV dose is reflected in a longer immobilization time (Fig. 10.2D). As shown in Chapter 7, the changes in immobilization/repair time are proportional to changes in the initial damage. This type of kinetics is the proof that, indeed, more damages have been induced by increasing the UV dose.

10.2.2 Limited number of detectable damages: lateral inhibition

The central idea, is that only part of the damages can be recognized/processed at a time, and that the number of detectable damages saturates with increasing number of lesions. This may be due to the limited capacity of chromatin remodeling, thought to
Figure 10.2: Limiting recognition factor. (A) Linear increase in damages with UV intensity, Eq. (7.10). (B) Immobilized fraction of the recognition factor (red lines) and the last factor ERCC1-XPF (green lines) in global and local damage experiments (dashed and solid lines, respectively). The arrows indicate the changes obtained by increasing the size of the local damage. The squares show the typical doses used in GDE and LDE. (C) Kinetics of ERCC1-XPF accumulation in the damaged area, when the nucleus has one (black line) or three different local damages (blue line). After the initial transient, the immobilized fraction in all three local damages (dotted line) is similar to that for one single local damage (solid black line). UV dose is 100 J/m$^2$. (E) Immobile fraction of ERCC1-XPF after a dose of 100 and 200 J/m$^2$. Parameters Tab. 7.2, set 1.

be necessary to make the DNA accessible for the protein factors [23, 96]. One can think at an initial event, which mediator is not diffusible, acting locally in the damaged area. Alternatively, physical hindrances may exist. For example, in a volume around a pre-incision complex no other repair complexes can be initiated. Both possibilities could be implemented in the model and I expect similar results. Here, I will elaborate on the situation where physical hindrances exist.

The pre-incision complex is composed of about 10 proteins each with a radius of 4-7 nm, which yields a total radius of 10-23 nm. As 10 bp have a length of about 3.4 nm, the pre-incision complex occupies at least 60–140 bp [3]. This value may be higher if
one consider that the DNA is packed. For example, around a nucleosome unit one finds 146 bp and a radius of just 6 nm [3]. In the following, I will derive a mathematical expression that account for the lateral inhibition around a PC.

Let assume that all damages on a DNA string of \( N \) nucleotides are recognized as one damage. The generation of damages is assumed to be a random process, so that the probability of having \( j \) damages in a string of \( N \) nucleotides follow a binomial distribution

\[
\binom{N/2}{j} p(UV)^j [1 - p(UV)]^{N/2 - j}
\]

where \( N/2 \) is the maximal number of damages physically possible in the string (\( N \) even). Thus the probability of having one or more damages in \( N \) nucleotides is

\[
w(UV) = 1 - [1 - p(UV)]^{N/2}.
\]

The average number of initial damages \( d \) and detectable damages \( \hat{d} \) follows

\[
d = \frac{V_d N_T/2}{V_n} p(UV)
\]

\[
\hat{d} = \frac{V_d N_T/2}{V_n N/2} w(UV) = \frac{V_d N_T}{V_n N} [1 - (1 - p(UV))^{N/2}]
\]

Where \( N_T = 3.2 \times 10^9 \) bp is the total size of dsDNA, \( V_d \) and \( V_n \) the volumes of the damaged area and the nucleus, respectively. Converting these expressions in concentrations yields a relation, valid for all times, between detectable damage concentration \( \hat{S}_0 \) and total free damage \( S_0 \)

\[
\hat{S}_0 = \frac{D}{N/2} \left[ 1 - (1 - S_0/D)^{N/2} \right], \tag{10.1}
\]

where the concentration of DNA pairs is \( D \approx 8.7 \) mM.

The function given by Eq. (10.1) is plotted in Fig. 10.3A. Whereas the concentration of detectable damages saturates (dot-dashed line), the concentrations of damages continue to rise linearly with increasing UV dose (solid line). Because the recognition factor binds only to detectable damages, the time evolution of the free damage concentration and the first intermediary complex changes to

\[
\frac{dS_0}{dt} = - k_1 \hat{S}_0 F_1 + k_{-1} S_1 \tag{10.2}
\]

\[
\frac{dS_1}{dt} = k_1 \hat{S}_0 F_1 - (k_{-1} + k_2 F_2) S_1 + k_{-2} S_2 \tag{10.3}
\]

The equations for the other repair factors remain unchanged.
Figure 10.3: Lateral inhibition. (A) Concentration of detectable damages saturates at high UV light intensities (dot-dashed line, Eq. (10.1) $N = 1000$), whereas the concentration of damages increases (solid line). (B) Fraction immobilized of the first factor in global and local damage experiments (dashed and solid lines, respectively). (C) ERCC1-XPF accumulation in nuclei with one or three different local damages (black and blue line, respectively). The total amount of proteins immobilized in all three local damages (dotted line) is higher than for a single local damage. (D) ERCC1-XPF accumulation after a dose of 100 and 200 J/m$^2$. Parameters are determined by fitting the model, with Eqs. (10.2)–(10.2) and factor diffusion, to the accumulation kinetics. Differently from the original model, the immobile fractions as obtained from local damage experiments are used (Tab. 6.2). Parameters: $T_1 = 0.4$, $T_2 = 0.4$, $T_3-T_5 = 0.2$ µM; $k_1 = 0.0014$, $k_2 = 0.9$, $k_3-k_5=5$ /µM/s; $k_{-1} = 0.022$, $k_{-2} = 2.54$, $k_{-3}-k_{-5} = 0.273$/s; $k_R = 0.007$/s; $D_1 = 1$ µm$^2$/s.

Model predictions are shown in Fig. 10.3B–D. As observed experimentally, the model predicts that in GDE the maximal fraction of immobilized factors is higher than in LDE (dashed and solid line in Fig. 10.3B, respectively). The reason are the additional detectable damages in globally irradiated nuclei. An aggrandizement of the local damaged area will cause a significant increase in the immobile fraction (arrow). Since saturation is due to a limited number of recognizable damages, at high UV dose the recognition factor will not be fully immobilized (Fig. 10.3B). Furthermore, different from the previously
10.2. How and why NER saturates: a case study

described model, the recruitment of XPC-HR23B is not solely dependent on its diffusion to the local damage. With a value for the diffusion coefficient of XPC-HR23B similar to what has been found in experiments, $1 \, \mu m^2/s$ compared to $0.16 \, \mu m^2/s$ with a limiting recognition factor, the model well reproduces the experimental data (not shown). In the presence of multiple local damages one finds a faster accumulation kinetics and a lower immobile fraction (Fig. 10.3C, blue line). Here, the main difference to the previous case is that the total amount of immobile factors in a nucleus with several local damages is higher compared to a nucleus with a single local damage (dotted and black solid line, respectively, Fig. 10.3C, compare to Fig. 10.2C). Since the number of damages increases with the dose, one observes a longer immobilization/repair time when the UV intensity is increased (Fig. 10.3D).

10.2.3 Limited number of damages

The model predictions, when the number of damages, as suggested by [120], saturates with increasing UV light intensities, are shown in Fig. 10.4. As for the case of lateral inhibition, one finds, in agreement with the data, that the maximal immobile fraction in GDE is higher than in LDE (dashed and solid lines, Fig. 10.4B) and that the first factor does not become fully immobilized at high UV light intensities. Furthermore, the total immobile fraction in a nucleus which has three local damages is higher than in a nucleus with one local damage (Fig. 10.4C). The main feature, characterizing a limited number of damages, is that the long-time immobilization kinetics at the local damage are very similar for increasing UV light intensities (100 and 200 J/m$^2$ in Fig. 10.4D). This behavior, which is rather different from the two previously discussed cases, characterizes the fact that for increasing UV dose the number of lesions only minimally increases.
Figure 10.4: Limiting number of damages. (A) Saturating generation of damages as function of UV light intensity. (B) Fraction immobilized of the first factor in global and local damage experiments (dashed and solid lines, respectively). An increase in the size of the local damage causes large changes in the immobile fraction (arrow). (C) ERCC1-XPF accumulation, with one (black line) or three different local damages (blue line). The total amount of proteins immobilized in all three local damages is higher than for a single local damage (dotted line). Dose is 100 J/m$^2$. (D) Increasing the dose from 100 to 200 J/m$^2$ does not change the long-time kinetics of ERCC1-XPF. Parameters estimated as in Fig. 10.3: $T_1 = 0.4$, $T_2 = 0.4$, $T_3-T_5 = 0.2$ µM; $k_1 = 0.0263$, $k_2 = 0.3$, $k_3-k_5=3.88$ /µM/s; $k_{-1} = 3.6$, $k_{-2} = 0.096$, $k_{-3-k-5} = 0.2$/s; $k_R = 0.007$/s; $D_1 = 1 \mu m^2$/s.

10.3 Understanding the last steps of NER

In the last years more and more cell lines have been developed with a fluorescent version of one of the repair factors, and it will soon be possible to measure the recruitment kinetics and residence times of all known repair factors. The recent data indicate that the recruitment kinetics are not closely correlated to the assumed binding position in the NER pathway. For example XPA accumulates about 10 times slower than ERCC1-XPF although ERCC1-XPF binds only in the presence of XPA (M. Luijsterburg, personal communication). As pointed out in the discussion, this large variance in the accumulation kinetics cannot be accommodated in a model where the factors are released.
simultaneously. The assumption of simultaneous release was a simplification and did not take into account two differential steps at the end of repair: the incision of the damaged DNA and the resynthesis of the new strand (cf. Section 6.1). First theoretical models indicate that the resolution of the enzymatic steps at the end of repair, which already exerted strong control on the repair efficiency in our initial model (Fig. 8.2), can provide the required answers.

I illustrate this idea with a minimal model containing two repair factors, $F_1$ and $F_2$ (Fig. 10.5A). Both $F_1$ and $F_2$ can bind to the damage, however, due to its high affinity for the lesion ($D^*D$), factor $F_1$ will be recruited first. After incision, there is a net dissociation of factors from the single stranded DNA ($D$). The complex $(D)F_1F_2$ is unstable and decays to $(D)F_1$ by releasing $F_2$ first. The departure of $F_1$ allows for other proteins, responsible for resynthesis and ligation of the DNA, to dock at the DNA.

\footnote{In fact, when the differences in affinities of the factors to the damage are large, the depicted binding scheme is equivalent to a sequential binding scheme.}
As resynthesis and ligation are assumed to be slow processes, the complex (D)F\textsubscript{1} has a delayed accumulation. This is reflected in the simulated accumulation kinetics shown in Fig. 10.5B, where F\textsubscript{1} needs much more time to reach the plateau (solid line, \(t_{1/2} = 690\) s) compared to F\textsubscript{2} (dashed line, \(t_{1/2} = 96\) s). Although, the accumulation kinetics of the two repair factors are extremely different, their residence times are similar (Fig. 10.5C).

### 10.4 Concluding remarks

In this last chapter, I showed how the gained knowledge from the initial NER model was used to approach some of the open questions of DNA repair.

I proposed a series of experiments to improve the understanding of the initial events in repair. These experiments can readily be implemented as they are based on the available techniques. They consist in monitoring the factor kinetics on a long time scale at different UV doses, and vary the size and number of local damages. The obtained results can be used to distinguish between several hypothesis.

The theoretical analysis can be helpful in understanding the course of the final events of NER. I showed how the presence of two separate enzymatic steps, e.g. incision and resynthesis, can account qualitatively for the observed recruitment kinetics. After incision, the release of repair factors does not occur simultaneously, and some factors can reside longer on the single stranded DNA. Whether this prolonged permanence has a biochemical significance remains to be elucidated. For example RPA, which complexes with XPA, is thought to protect the single stranded DNA from misfolding and endonucleases [56]. For a more quantitative analysis, it is necessary to estimate the kinetic parameters of different binding and release mechanisms, and compare the model predictions with the numerous experimental data. This procedure could be largely simplified by deriving, for each alternative mechanism, analytical expressions characterizing the repair factor kinetics (cf. Appendix D.1).
Bibliography


[49] Holmquist. G.P. Endogeneous lesions, S-phase-independent spontaneous mutations, and evoluo-


Part III

Appendices
Appendix A

Ca$^{2+}$ feedbacks on IP$_3$ metabolism: alternative models

I will discuss exemplarily four mathematical models that have been proposed in the literature and how they respond to IP$_3$K and IP$_3$ buffer expression. The model proposed by Houart and coworkers includes Ca$^{2+}$ activation of IP$_3$K the model by Shen and Larter Ca$^{2+}$ activation of PLC [69, 147]. These two models use similar terms for the description of the IP$_3$R as the model in the main part. Differently the model by Sneyd and Dufour (1999), which includes covalent modifications of the IP$_3$R, e.g. phosphorylation [154]. The last model has been proposed by Cuthbertson and Chay [25]. It is a so-called pure IP$_3$ oscillator where the oscillations in IP$_3$ are absolutely necessary for the Ca$^{2+}$ oscillations. I show simulations using the parameter set given in the original articles and to highlight some of the conclusions with slightly modified parameters.

The results shown here indicate that my theoretical conclusions are not dependent on the exact choice of kinetic expressions for the different processes considered. The results obtained with the four alternative models are in agreement with the results obtained by using the model presented in the main text.

A.1 Model with IP$_3$3K

The model proposed by Houart and coworkers [69] consider three variables: calcium in the cytosol (Z), Ca$^{2+}$ in the internal stores (Y), and IP$_3$ (A). The two major feedbacks are the Ca$^{2+}$ and IP$_3$ activation of the IP$_3$R and the Ca$^{2+}$ dependent degradation of IP$_3$ by the IP$_3$3K.

To simulate the overexpression of IP$_3$3K and the presence of the IP$_3$ buffer, with con-
The equation for the IP$_3$ concentration in the original article is modified to
\[
\frac{dA}{dt} = \frac{V_{M5}}{1 + B_T K_A/(K_A + A)^2} \left( \beta V_4^4 - \frac{A^2}{K_0^4 + A^2 K^4_d + Z^4} - \frac{\varepsilon}{V_{M5}} A \right)
\]  
(A.1)

The maximal IP$_3$ production rate $V_4$ is rescaled with the maximal degradation flux by the IP$_3$3K $V_{M5}$. These modifications avoid the depletion of IP$_3$ after overexpression of the IP$_3$3K. For $V_{M5} = 5 \mu$M/min one has the original parameter set. The parameter $\beta \in [0, 1]$ accounts for the stimulus dependent activation of the PLC. For further details see the original work [69].

The result of the simulations are shown in Fig. A.1. Using the original parameter set overexpression of IP$_3$3K cannot abolish the oscillations. Nevertheless, one observes a decrease in the maximal period of the oscillations with increasing level of IP$_3$3K (Fig. A.1A). On the other hand, introduction of the IP$_3$ buffer increases the period range (Fig. A.1B). These simulations are in agreement with the results shown in the main text: The role of IP$_3$3K in generating long period oscillations and the requirement of slow IP$_3$ dynamics. After modification of two parameters in the original set (the sensitivity of the IP$_3$R to IP$_3$ and the sensitivity of the IP$_3$3K to Ca$^{2+}$) one obtains, at high level of IP$_3$3K, a decrease in the period range (Fig. A.1C) and amplitude (not shown), and eventually abolishment of the oscillations. This effect, as in my model, is not due to depletion of IP$_3$ (Fig. A.1D).

### A.2 Model with Ca$^{2+}$ activation of PLC, I

The model proposed by Shen and Larter [147] has three dynamic variables: cytoplasmic Ca$^{2+}$ $x$, internal store concentration $y$, and IP$_3$ concentration $z$. In this model PLC is Ca$^{2+}$ dependent. The IP$_3$R is activated by IP$_3$ and shows a bell-shaped dependence on cytoplasmic Ca$^{2+}$ (activated at low and inactivated at high Ca$^{2+}$ concentrations). The equation for IP$_3$ in the original article is modified to account for the IP$_3$ buffer with concentration $B_T$

\[
\frac{dz}{dt} = \frac{1}{1 + B_T K_1/(K_1 + x)^2} \left( A R \frac{x}{K_3 + x} - Bz \right).
\]  
(A.2)

$R \in [0, 1]$ quantifies the stimulus dependent activation of the PLC, which has a maximal activity $A$. For further details on the model see [147].

The effects of introducing an IP$_3$ buffer are summarized in Fig. A.2. In the presence of high levels of IP$_3$ buffer the oscillations have a reduced amplitude (Fig. A.2A) and a decreased period (Fig. A.2B). By comparing these oscillations with the oscillations...
Figure A.1: Effect of IP$_3$3K overexpression and IP$_3$ buffer expression in the model for the Ca$^{2+}$ and IP$_3$ dynamics proposed by Houart and coworkers [69]. (A) Overexpression of IP$_3$3K causes a decrease in the period range. (B) The slowing of the IP$_3$ dynamics by the IP$_3$ buffer causes an increase in the period range. In particular longer period oscillations arise. (C) In the same system overexpression of IP$_3$3K can abolish the oscillations, when e.g. the sensitivity of the IP$_3$R to IP$_3$ and the sensitivity of the IP$_3$3K to Ca$^{2+}$ are modified. (D) The impairment of Ca$^{2+}$ oscillations by IP$_3$3K is not due to depletion of IP$_3$. Ca$^{2+}$ oscillates in the absence of IP$_3$3K (left panel), whereas at high level of IP$_3$3K, although the IP$_3$ concentration is the same, one observes only a transient increase in [Ca$^{2+}$]$_c$ (right panel). Parameters: (A) and (B) See [69]. (C) and (D) as in [69] except (C) $K_A = 0.5$ µM and $K_d = 0.3$ µM. (D) left panel $K_A = 0.5$ µM, $K_d = 0$, $V_4 = 3.07$ µM/min, $\beta = 0.75$; right panel $K_A = 0.5$ µM, $K_d = 0.3$ µM, $V_{M5} = 30$ µM/min, $V_4 = 2$ µM, $\beta = 0.75$. The parameters are chosen so that at $\beta = 0.75$ the steady states are identical with and without IP$_3$3K.
obtained in the absence of Ca\(^{2+}\) feedback on PLC (by setting \(K_3 = 0\)), one observes that both have similar amplitude and period range. This shows that, on the one hand positive feedback on PLC increases the period range, on the other hand, an IP\(_3\) buffer erases this effect. In Fig. A.2C I show the location of the two Hopf bifurcations as function of the buffer concentration \(B_T\) and stimulus strength \(R\). The picture is qualitatively similar to Fig. 3.3. A slight modification of the parameters of the core oscillator strengthens the effect of the IP\(_3\) buffer (dashed line in Fig. A.2C). Introducing an IP\(_3\) buffer extinguishes the oscillations in a large stimulatory domain. For the parameter used, at high IP\(_3\) buffer concentrations oscillations can persist, but with reduced amplitude and high frequency. In Fig. A.2D typical oscillations in the absence of the IP\(_3\) buffer (left panel) and a slow unique Ca\(^{2+}\) spike in the presence of IP\(_3\) buffer (right panel) are shown.

### A.3 Model with Ca\(^{2+}\) activation of PLC, II

The model proposed by Sneyd and Dufour [151] for the IP\(_3\)R dynamics considers recent experimental facts on the inactivation mechanisms of the IP\(_3\)R type 2. The kinetic description of the IP\(_3\)R differs strongly from the previous two models and the model I used. I was interested whether the theoretical results depend on the particular IP\(_3\)R model. The model used to simulate the Ca\(^{2+}\) and IP\(_3\)R dynamics is given in [154]. I implemented it with an extra equation for the IP\(_3\) concentration (in the original model the IP\(_3\) concentration is a parameter), which accounts for Ca\(^{2+}\) activation of PLC and expression of the IP\(_3\) buffer

\[
\frac{dp}{dt} = \frac{1}{1 + B_T/K_B} \left( V_{PLC} \frac{c^2}{K_{PLC}^2 + c^2} - k_{3PP} \right). \tag{A.3}
\]

I also slightly modified the term describing the Ca\(^{2+}\) influx from the extracellular medium

\[J_{influx} = \alpha_1 + \alpha_2 V_{PLC}. \tag{A.4}\]

This modification conserves the property of a stimulation dependent influx, which does not oscillate together with Ca\(^{2+}\).

The effects of slowing the IP\(_3\) dynamics with the IP\(_3\) buffer are illustrated in Fig. A.3. The results are similar to the results shown in Fig. 3.3. High concentrations of IP\(_3\) buffer completely abolish the oscillations. Instead, one observes a slow transient increase in [Ca\(^{2+}\)]\(_c\).
Figure A.2: Effect of an IP$_3$ buffer in the model proposed by [147], which includes Ca$^{2+}$ activation of PLC. (A) Slowing of the IP$_3$ dynamics with an IP$_3$ buffer reduces the oscillatory domain and amplitude and (B) considerably decreases the period range. In particular, long period oscillations disappear (circle and square denote the longest ans shortest period, respectively, obtained when varying the stimulus). (C) Locus of the two Hopf-bifurcations as function of stimulus $R$ and IP$_3$ buffer concentration for the original parameter set (solid line). With a slight parameter change, the effect of the IP$_3$ buffer becomes more pronounced (dashed line). (D) Example of the Ca$^{2+}$ (solid) and IP$_3$ (dashed) dynamics prior (left panel) and after (right panel) addition of IP$_3$ buffer. The oscillations disappear, instead a broad and slow Ca$^{2+}$ spike is observed. Parameters: (A), (B) as given in Shen and Larter (1995) the IP$_3$ buffer concentration is 100 $\mu$M. (C) solid line: as given in [147], dashed line: Ca$^{2+}$ activation constant of the IP$_3$R is $K_5 = 0.4 \, \mu$M (instead of 0.69 $\mu$M) and of PLC $K_3 = 0.2 \, \mu$M (instead of 1 $\mu$M.). (D) $K_3 = 0.2 \, \mu$M, $K_5 = 0.4 \, \mu$M. At $t = 0$ s $R$ is increased from 0.15 to 0.25. Left panel: $B_T = 0$. Right panel: $B_T = 15 \, \mu$M.
Appendix A. Ca\(^{2+}\) feedbacks on IP\(_3\) metabolism: alternative models

A.4 Pure IP\(_3\)/DAG oscillator

Cuthbertson and Chay [25] analyzed different oscillator models. The common point between all these models is that the oscillation mechanism is upstream the Ca\(^{2+}\) release, Ca\(^{2+}\) barely follows the oscillations in IP\(_3\). In all models the positive action of Ca\(^{2+}\) on the IP\(_3\)R is not considered. The principal positive feedback mechanism is the activation of PLC by diacylglycerol (DAG), the other product of the PLC mediated hydrolysis of PIP\(_2\). Although the activation of PLC by DAG has not been clearly demonstrated experimentally [62], these models allow to analyze the effect of an IP\(_3\) buffer on a so called pure IP\(_3\) oscillator.

Here, I show the simulation results for one of the models developed in [25] (called example 1). This model accounts for the activation of PLC by DAG and the inactivation of the G\(_\alpha\) by protein kinase C (PKC). PKC depends on both DAG and Ca\(^{2+}\). In the original article the model variables are Ca\(^{2+}\) in the cytoplasm, the concentration of G\(_\alpha\) bound to GTP, and DAG. IP\(_3\) was assumed to be equal to DAG, to analyze the effect of the IP\(_3\) buffer I add an equation for the IP\(_3\) concentration, which reads:

\[
\frac{dp}{dt} = \frac{1}{1 + BTK_s/(K_s + p)}(k_d R_{PLC} - h_dp + l_d).
\]  (A.5)
A.4. Pure IP$_3$/DAG oscillator

Figure A.4: Model proposed by Cuthbertson and Chay ([25]) implemented with the IP$_3$ dynamics, Eq. A.5. Typical oscillations observed for four different IP$_3$ buffer concentrations. DAG appears to be only minimally affected by the buffer. The oscillations in IP$_3$ and Ca$^{2+}$ have a strong reduced amplitude but the period is unaffected as it is dictated by the oscillations in DAG. Parameters as in [25].

The other equations are described in [25]. As expected for a pure IP$_3$/DAG oscillator mechanism, the presence of an IP$_3$ buffer strongly damps the IP$_3$ oscillations and so the Ca$^{2+}$ oscillations (Fig. A.4). Strictly speaking, although the Ca$^{2+}$ oscillations are very small in amplitude they never cease. This is because the DAG and G$_\alpha$ oscillations remain practically unaffected by the IP$_3$ buffer. In fact, the oscillations in DAG persist even after clamping Ca$^{2+}$ and IP$_3$ (not shown). This is because, in the model by Cuthbertson and Chay, PKC is mainly controlled by DAG and is not very sensitive to Ca$^{2+}$ variations.
Appendix A. Ca$^{2+}$ feedbacks on IP$_3$ metabolism: alternative models
Appendix B

Phosphoinositide metabolism

B.1 $IP_3$ signal duration and amplitude

To calculate the signal duration and amplitude (Eqs. (4.18)–(4.19)) one must first derive the integrals. Writing the linear ordinary differential equation system in matrix form yields

$$\frac{dX(t)}{dt} = MX(t),$$

where $X(t) = (X_1(t), \cdots, Y_1(t))^T$ is the vector of concentrations and $M$ the matrix characteristic for the pathway. Integrating from $t = 0$ to $\infty$ gives a vector containing the 0-moments in time of the concentration distributions

$$\int_0^\infty \Delta X(t) dt = -M^{-1}\Delta X(0), \quad (B.1)$$

with $\Delta X(t) = X(t) - X(\infty)$. Integration by part gives following relation between the vector of the $n-1$-moments and the vector of the $n$-moments

$$\int_0^\infty t^{n-1}\Delta X(t) dt = \frac{t^n}{n}\Delta X(t)\bigg|_{t=0}^\infty - \int_0^\infty \frac{t^n}{n} \frac{dX(t)}{dt} dt = -M \int_0^\infty \frac{t^n}{n} \Delta X(t) dt. \quad (B.2)$$

Combining Eq. (B.1) and (B.2) leads to the general expression

$$\int_0^\infty t^n \Delta X(t) dt = n!(-1)^{n+1}M^{-(n+1)}\Delta X(0). \quad (B.3)$$

Matrix inversion and multiplication gives the integrals required for calculating signal duration and amplitude. For the case without phosphatases and PI 3/5 kinase these two measures are given in Eq. (4.20) and (4.21).
The phosphatases are introduced by conserving the reference unstimulated steady state concentrations. This is guaranteed when the corresponding kinase rate constant is increased according to Eq. (4.11). In the absence of PIP$_2$ phosphatase the formulas for the signal duration and amplitude are not modified when PIP phosphatase is active ($k_{-1} \neq 0, k_{-2} = 0$). The result is different, when instead of PIP phosphatase, PIP$_2$ phosphatase is active ($k_{-1} = 0, k_{-2} \neq 0$). The signal duration and amplitude are given by

\[ \theta = 2 \sqrt{\frac{1}{k_3^2} + \frac{1}{k_4^2} + \frac{(k_3^2 + k_{-2})^2 + 2k_3k_{-2}}{k_2^2k_3^2} - (k_2 + k_{-2})^{-2}} \]  

and

\[ \alpha = \frac{\Upsilon_1 \left( \frac{1}{k_3} - \frac{1}{k_5} \right) k_2 + k_{-2}}{k_2}, \]

respectively. In both equations the PIP 5-kinase rate is determined by Eq. (4.12).

The expression for the signal duration and amplitude when both phosphatases are present, but the PI 3/5 kinase is inactive ($k_{-1}, k_{-2} \neq 0, \kappa = 0$), is very lengthy and I forbear from listing it here. However, one can show, using symbolic computation as provided by Mathematica, that for this situation the signal duration further increases.

### B.2 Agonist dependent kinases

There are indications that some of the enzymes involved in the phosphoinositide pathway are activated by agonist dependent G-proteins (see reviews [94, 188]). This putative
B.3. Linear chain with substrate inhibition

An increase in enzymatic rates does not consequently imply that the enzymes must be activated by the stimulus. If some of the enzymes are inhibited by their substrate the activation of PLC could lead to an overall increase in flux, and so to a prolonged increase in \([\text{IP}_3]\). Indeed, for PI 4-kinase and PIP 5-kinase substrate inhibition has been reported [93, 117]. To assess the potential role of substrate inhibition for sustained \([\text{IP}_3]\) signals, the rate of phosphorylation by PI 4-kinase and PIP 5-kinase are described by

\[
v_1 = V_{1}^{\text{max}} \frac{A_1 X_1}{1 + A_1 X_1 + A_2 X_2} \quad \text{and} \quad v_2 = V_{2}^{\text{max}} \frac{B_2 X_2}{1 + B_2 X_2 + B_3 X_3},
\]

respectively. Where \(A_1\) \((B_2)\) is the affinity of PI 4-kinase \((\text{PIP} 5\)-kinase\) for its substrate PI \((\text{PIP})\) and \(A_2\) \((B_3)\) the affinity to its product PIP \((\text{PIP}_2)\). The two enzymes catalyze phosphorylation of their substrate with consumption of ATP. The rather improbable reverse reaction, dephosphorylation of the phosphoinositide and transformation of ADP into ATP is neglected. The PLC reaction and \([\text{IP}_3]\) degradation are described as shown before.
Figure B.2: Stimulating the kinases produces a steady increase in the [IP3]. (A) Agonist activates all forward reactions (PI synthase, PI 4-kinase, PIP 5-kinase and PLC). (B) [IP3] remains elevated during stimulation as observed experimentally (data points). (C) The densities of phosphoinositides decrease during stimulation. Data points for PI, PIP and PIP_2 from [189]. Rate constants during stimulation are determined from the steady state in stimulated cells (see Tab. 4.3) $k_1 = 0.0079/s$, $k_2 = 0.074/s$, $k_3 = 0.066/s$. A better agreement with data is obtained with partial receptor desensitization (insets in B and C). This is modeled similar to Fig. 4.5 by $k_2 = 0.074(1 + 0.3e^{-t/100})/s$ and $k_3 = 0.066(1 + 0.7e^{-t/100})/s$.

The increase in IP_3 production flux mediated by substrate inhibition works as follows: the activated PLC causes a decrease in PIP_2 density ($X_3$). This relieves product inhibition of PIP 5-kinase and $v_2$ increases. The ensuing fall in PIP density ($X_2$) leads to an increase in $v_1$. Given the concentrations of metabolites before and during stimulation, one can evaluate the increase in phosphorylation flux brought about by this mechanism. The ratio $\gamma_1$ between the steady state PI phosphorylation rate in stimulated and unstimulated state is

$$\gamma_1 = \frac{v_1^*}{v_1} = \frac{X_1^*/X_1(1 + A_1X_1 + A_2X_2)}{1 + A_1X_1 + A_2X_2}.$$  \hspace{1cm} (B.7)

Where * indicates the lipid densities and phosphorylation rate during stimulation. From
the previous equation one can express the affinity of PI 4-kinase for its product PIP as

\[
A_2 = \frac{A_1 X_1^* (\gamma_1 - 1) + \gamma_1 - X_1^* / X_1}{X_2 X_1^*/X_1 - \gamma_1 X_2^*}.
\]  (B.8)

The nominator of this equation is, according to data, positive (\(\gamma_1 > 1\) and \(X_1^*/X_1 \geq 1\), see Tab. 4.3), therefore, for a positive affinity \(A_2\) the denominator has to be positive. This means that there is an upperbound \(\gamma_1^{\text{max}}\), corresponding to a singularity in Eq. (B.8), for the increase in PI phosphorylation rate during stimulation. This upper bound is a function of the concentration ratio in stimulated and unstimulated state, that is

\[
\gamma_1 < \gamma_1^{\text{max}} = \frac{X_2 X_1^*}{X_2^* X_1}.
\]  (B.9)

According to the experimental data in Tab. 4.3 \(\gamma_1^{\text{max}} = 4 - 5\).

Similarly, one derives an equation for \(B_3\), the affinity of the PIP 5-kinase for its product PIP\(_2\), which yields when \(B_3 \to \infty\) a maximal fold increase in PIP phosphorylation during stimulation

\[
\gamma_2^{\text{max}} = \frac{X_3 X_2^*}{X_3^* X_2}.
\]  (B.10)

As PIP and PIP\(_2\) densities decrease of about the same amount \(\gamma_2^{\text{max}} \approx 1\).

In steady state the phosphorylation of PI and PIP must have equal rates (\(v_1 = v_2\)). Therefore, the fold increase in IP\(_3\) production flux, \(\gamma^{\text{max}}\), obtained after activating PLC is limited by the slowest enzyme, that is \(\gamma^{\text{max}} = \min(\gamma_1^{\text{max}}, \gamma_2^{\text{max}})\). As \(\gamma_2^{\text{max}} \approx 1\), product inhibition alone does not explain the observed sustained increase in [IP\(_3\)] during stimulation.
Appendix C

Steady state, holocomplex, and unspecific binding in NER

C.1 Binding of repair proteins to undamaged sites

Factor $F_i$ reversibly associates to an undamaged site $u_i$ and forms a complex $U_i$ according to the reaction $u_i + F_i \leftrightarrow U_i$. The balance equations for the concentrations of $U_i$ and the free form of the factors are

$$\frac{dU_i}{dt} = k_{+u_i}(T_{u_i} - U_i)F_i - k_{-u_i}U_i$$ \hspace{1cm} (C.1)

and

$$\frac{dF_i}{dt} = k_{RS_i} - \tilde{k_i}F_iS_{i-1} + k_{-i}S_i - \frac{dU_i}{dt},$$ \hspace{1cm} (C.2)

respectively. $k_{+u_i}$ and $k_{-u_i}$ are the binding and dissociation rate constants of $F_i$ to the undamaged site $u_i$, respectively, and $T_{u_i} = U_i + u_i$ is the total concentration of undamaged sites. The conservation relation (7.6) changes to

$$T_i = F_i + I_i + U_i.$$ \hspace{1cm} (C.3)

If the binding of factors to undamaged sites is fast compared to other processes, one can apply a quasi-steady state approximation ($dU_i/dt \approx 0$) to obtain $U_i = T_{u_i}F_i/(K_{u_i} + F_i)$ for the concentration of the complexes, where $K_{u_i} = k_{-u_i}/k_{+u_i}$. For low affinity binding sites ($K_{u_i} \gg F_i$) one has

$$U_i \approx \frac{T_{u_i}}{K_{u_i}}F_i.$$ \hspace{1cm} (C.4)

With this formula and after addition of Eq. (C.1) and (C.2) one yields

$$\frac{dF_i}{dt} = (k_{RS_i} - \tilde{k_i}F_iS_{i-1} + k_{-i}S_i)/\beta_i,$$
where $\beta_i = 1 + \frac{T_u}{K_u}$ is referred to as the buffering coefficient.

Equations (C.3) and (C.4) give

$$F_i = \frac{(T_i - I_i)}{\beta_i},$$  \hspace{1cm} (C.5)

indicating that in the absence of damage ($I_i = 0$) the fraction of repair factor transiently immobilized to non-damaged sites is $1/\beta_i$. With the latter formula, the model which considers non-damaged binding sites reads

$$\frac{dS_0}{dt} = V_{\text{dam}} - k_1(T_1 - I_1)S_0 + k_{-1}S_1$$  \hspace{1cm} (C.6)

$$\frac{dS_i}{dt} = k_i(T_i - I_i)S_{i-1} - k_{-i}S_i - k_{i+1}(T_{i+1} - I_{i+1})S_i + k_{-(i+1)}S_{i+1}$$  \hspace{1cm} (C.7)

$$\frac{dS_n}{dt} = k_n(T_n - I_n)S_{n-1} - k_{-n}S_n - k_RS_n,$$  \hspace{1cm} (C.8)

$i = 1, \cdots, n - 1$. The buffering coefficients are included in the effective binding rate constants $k_i = \bar{k}_i/\beta_i$. Equation (C.7) corresponds to Eq. (7.9).

### C.2 Quasi steady state repair rate

A stationary state in the intermediary complex concentrations is reached, when the DNA is damaged constantly at a rate $V_{\text{dam}}$ lower than the maximal repair rate $V_{\text{max}}$. One derives from Eqs. (C.6)–(C.8)

$$S_i = \frac{V_{\text{dam}} + k_{-(i+1)}S_{i+1}}{k_{i+1}(T_{i+1} - I_{i+1})} \text{ and } S_n = \frac{V_{\text{dam}}}{k_R},$$  \hspace{1cm} (C.9)

where the concentration of the immobilized $i$th factor is defined by $I_i = \sum_{j=i}^n S_j$. With Eq. (C.9) one calculates recursively, starting from $i = n$, the steady state concentrations as a function of the damage-induction rate $V_{\text{dam}}$.

Plotting the steady state concentration of DNA lesions $D_T$ against $V_{\text{dam}}$ results in a hyperbolic curve (Fig. C.1A). Obviously, a steady state can only be established if $V_{\text{dam}}$ is lower than the maximal repair rate $V_{\text{max}}$. When $V_{\text{dam}}$ approaches $V_{\text{max}}$ the concentration of damaged sites dramatically increases. For $V_{\text{dam}} > V_{\text{max}}$ the system can no longer cope, resulting in a continuous accumulation of lesions. At steady state the damage induction rate equals the repair rate, one can therefore plot the steady state repair rate $V_R$ as function of the total damage concentration (Fig. C.1B, black solid line). Although in most experimental NER studies all DNA damage is introduced during a short time interval only, rather than continuously, the time dependent repair rate does
C.2. Quasi steady state repair rate

Figure C.1: (A) Total damage steady state concentration $D_T$ as a function of the damage induction rate $V_{dam}$. At steady state the damaging rate equals the repair rate $V_{dam} = V_R$. $V_{max}$ and $K_d$ are the maximal repair rate and half-saturation constant (total damage for which $V_{dam} = V_{max}/2$) of the repair process, respectively. Both quantities characterize repair efficiency. (B) Time dependent repair rate $V_R$ as a function of the damage concentration $D_T$. Initial damage concentrations to the whole nucleus: 0.5 µM, 1 µM, and 5 µM (blue lines 1, 2 and 3, respectively). Arrows indicate progression in time. Within a large time interval the repair rate is well represented by its steady state value (black line). Differences are observed during the comparatively fast binding phase, which is characterized by a nearly constant $D_T$ and a strong increase in repair rate $V_R$. Inset: total damage as a function of time. (C) Exact steady state repair rate and approximation Eq. (C.10) (solid and dashed line, respectively). Parameter set 1 of Tab. 7.2 has been used.

not strongly deviates from its steady state value (Fig. C.1B, blue solid lines). This is true during both the repair phase, where the repair rate is nearly constant and the number of lesions decreases linearly, and during the release phase, in which the repair rate drops sharply. Thus, the steady state relationship can be employed as a measure for repair efficiency.

An approximation for the steady-state repair rate curve is

$$V_R \approx V_{max} \frac{D_T}{D_T + K_d}$$  \hspace{1cm} (C.10)

where $K_d$ is the damage concentration for which $V_R = V_{max}/2$. This approximated curve shows good agreement with the exact solution (Fig. C.1C, solid and dashed line, respectively).
Appendix C. Steady state, holocomplex, and unspecific binding

C.3 Holocomplex concentration

The repair factors interact with each other to form a holocomplex. A simple expression for the steady state is obtained when equal total concentrations \( T_i = T \), and equal dissociation constants \( K_h \) between factors are assumed. Then the concentrations of free diffusing complexes that contain the same number of factors are identical. One derives following conservation relation

\[
T = I + \sum_{i=0}^{n-1} \binom{n}{i} f_{i+1}.
\]  
(C.11)

The concentration of a free diffusing complex containing \( i \) factors is denoted by \( f_i \). Thus the holocomplex concentration is \( H = f_n \). \( I \) is the concentration of the holocomplex bound to the damaged site and \( \binom{n}{i} \) is the binomial coefficient. In the absence of damage \( (I = 0) \) and in steady state, one derives

\[
f_i = \frac{f_1}{K_h^{i-1}}.
\]  
(C.12)

Using the above formula Eq. (C.11) simplifies to

\[
T = f_1 \left( 1 + \frac{f_1}{K_h} \right)^{n-1}.
\]  
(C.13)

From Eq. (C.12) one has \( f_1 = \sqrt[n-1]{f_n/K_h^{n-1}} \) which together with Eq. (C.13) gives an implicit expression for the holocomplex concentration \( H = f_n \)

\[
\left( \sqrt[n-1]{K_h} + \sqrt[n-1]{H} \right)^{n-1} \sqrt[n-1]{H} = 1
\]  
(C.14)

This formula has been used to calculate the holocomplex concentration and hence the repair time by the holocomplex.

C.4 Maximal repair rate

C.4.1 Condition for maximal repair rate

Theorem C.4.1 The repair system defined by the set of differential equations (C.6)–(C.8), reaches its maximal repair rate when the first factor is completely bound to the damage

\[
V_R = V_{max} \text{ for } T_1 = I_1.
\]  
(C.15)
Proof Theorem C.4.1 is analogous to enzymatic reactions where the maximal reaction rate is reached when all enzyme molecules are bound to substrate. By taking the derivative of \( S_i \) (Eq. (C.9)) with respect to \( V_{\text{dam}} \) one easily shows that the immobilized concentrations \( I_i \) of the factors increase monotonously with \( V_{\text{dam}} \). However, for any factor an upper limit is given by

\[
I_i \leq T_i. \tag{C.16}
\]

For calculating the maximal repair rate one needs to identify the value of \( V_{\text{dam}} \) at which one of the above conditions is violated. From Eq. (C.9) one obtains

\[
I_i = \frac{V_{\text{dam}}}{k_R} + \sum_{j=i+1}^{n} \frac{V_{\text{dam}} + k_{-j}S_j}{k_j(T_j - I_j)}. \tag{C.17}
\]

Clearly, for any \( j > i \) one obtains \( I_i \to \infty \) for \( I_j \to T_j \), contradicting condition (C.16). Therefore, when increasing \( V_{\text{dam}} \), the condition for the first factor, \( I_1 \leq T_1 \), is violated first. The maximal repair rate is reached accordingly when condition (C.15) is fulfilled. One yields a polynomial of order \( 2^{n-1} \) for \( V_{\text{max}} \). Only the smallest positive real root is of interest, since all other solutions would violate condition (C.16). By searching the roots of the polynomial numerically one obtains the maximal repair rate \( V_{\text{max}} \).

C.4.2 Formula for the maximal repair rate

I was able to derive a formula for the maximal repair rate in the case of equal total concentrations and when the unbinding reactions are slow (\( k_{-i} \approx 0 \)). First some algebraic transformations of the steady state equations need to be done. From Eq. (C.9) one obtains \( T - I_n = T - V/k_R = V/(k_nS_{n-1}) \) which gives a relation between \( S_{n-2} \) and \( S_{n-1} \)

\[
S_{n-2} = S_{n-1} \frac{k_n}{k_{n-1}} \frac{V}{V - k_nS_{n-1}}.
\]

For clarity sake, here and in all subsequent formulas, the damaging rate \( V_{\text{dam}} \) is denoted by \( V \). Similarly, from Eq. (C.9) one obtains \( T - I_{i+2} = V/(k_{i+2}S_{i+1}) \) which gives

\[
S_i = S_{i+1} \frac{k_{i+2}}{k_{i+1}} \frac{V}{V - k_{i+2}S_{i+1}} \text{ for } i \leq n - 2 \tag{C.17}
\]

Lemma C.4.2 The concentration of the intermediary complexes and the free damage can be expressed by \( i \leq n - 2 \)

\[
S_i = S_{i+1} \frac{k_{i+2}}{k_{i+1}} \frac{C_i}{C_i + V_{i+1}}, \tag{C.18}
\]
where the coefficients $C_i$ and $V_i$ are obtained recursively from

$$C_i = k_{i+1}(C_{i+1} - V_{i+1})^2 \text{ and } V_i = k_{i+2}V_{i+1}C_{i+1},$$

(C.19)

with $C_{n-1} = k_n(T - V/k_R)^2$ and $V_{n-1} = V$. These coefficients satisfy

$$V - k_{i+2}S_{i+1}^2 = V \frac{C_{i+1} - V_{i+1}}{C_{i+1}}$$

(C.20)

**Proof** Lemma C.4.2 is proven using complete induction. For $i = n - 2$ it is easy to see that

$$V - k_{n-1}S_{n-1}^2 = V \frac{V_n - k_n(T - V/k_R)^2 - V}{k_n(T - V/k_R)^2}$$

(C.21)

which yields

$$S_{n-2} = S_{n-1} - \frac{k_n}{k_n - 1} \frac{C_{n-1}}{C_{n-1} - V_{n-1}}$$

This relation together with Eq. (C.21) gives the next denominator of Eq. (C.17) for $i = n - 3$

$$V - k_{n-2}S_{n-2}^2 = V - \frac{k_n V_{n-1} C_{n-1}}{k_{n-1}(C_{n-1} - V_{n-1})^2}$$

$$= V \frac{k_{n-1}(C_{n-1} - V_{n-1})^2 - k_n V_{n-1} C_{n-1}}{k_{n-1}(C_{n-1} - V_{n-1})^2}$$

$$= V \frac{C_{n-2} - V_{n-2}}{C_{n-2}}$$

with $C_{n-2}$ and $V_{n-2}$ given by Eq. (C.19). Using Eq. C.17 one derives that, for $i = n - 3$, Eqs. (C.18)–(C.20) hold

$$S_{n-3} = S_{n-2} - \frac{k_{n-1}}{k_{n-2} - 1} \frac{C_{n-2}}{C_{n-2} - V_{n-2}}.$$

Assuming that the lemma is valid for $i \geq j$, with $j < n - 2$, it is sufficient to prove that its validity for $i = j - 1$. From (C.20) one has $S_{j+1} = \sqrt{VV_{j+1}/(k_{j+2}C_{j+1})}$ so that

$$V - k_{j+1}S_j^2 = V - \frac{k_{j+2}V_{j+1}C_{j+1}}{k_{j+1}(C_{j+1} - V_{j+1})^2}$$

$$= V \frac{k_{j+2}(C_{j+1} - V_{j+1})^2 - k_{j+2}V_{j+1}C_{j+1}}{k_{j+1}(C_{j+1} - V_{j+1})^2}$$

$$= V \frac{C_j - V_j}{C_j}.$$

This gives

$$S_{j-1} = S_j - \frac{k_{j+1}}{k_j} \frac{C_j}{C_j - V_j}.$$

Therefore, Lemma C.4.2 is true for $i \leq j - 1$ and so for $i = 0, \ldots, n - 2.$
Theorem C.4.3 For a system defined by Eq. (C.9), and assuming equal total factor concentrations \( T_i = T, \forall i \) and negligible dissociation rate constants \( (k_{-i} \approx 0) \), the maximal repair rate where \( I_1 = T \) is given by

\[
V_{\text{max}} = \frac{k_R}{2\lambda_{n-1}k_n} \left( 2\lambda_{n-1}k_nT + k_R - \sqrt{4\lambda_{n-1}k_Rk_nT + k_R^2} \right) \tag{C.22}
\]

where the coefficient \( \lambda_{n-1} \) is obtained recursively from

\[
\lambda_i = \frac{2\lambda_{i-1}k_i + k_{i+1} - \sqrt{4\lambda_{i-1}k_i k_{i+1} + k_{i+1}^2}}{2\lambda_{i-1}k_i} \tag{C.23}
\]

for \( i = 2, \cdots, n-1 \) with \( \lambda_1 = 1 \).

Proof According to Lemma C.4.2 (Eq. (C.18)), the free damage is given by

\[
S_0 = S_1 \frac{k_2}{k_1} \frac{C_1}{C_1 - V_1}. \tag{C.24}
\]

The condition for the maximal repair rate is when \( I_1 = T \) which corresponds to a singularity in the formula for \( S_0 \) (Eq. (C.9)). Using the new formulation (C.24), the singularity \( T = I_1 \) is equivalent to \( C_1 = V_1 \). With \( V_1 = k_3V_2C_2 \) and \( C_1 = k_2(C_2 - V_2)^2 \) (Eq. (C.19)). The condition \( C_1 = V_1 \) gives a quadratic equation in \( V_2 \), which roots are

\[
V_2 = C_2 \frac{k_3 + 2k_2 \pm \sqrt{4k_2k_3 + k_3^2}}{2k_2}.
\]

\( C_2 \) and \( V_2 \) can be replaced by \( V_3 \) and \( C_3 \), which yields again a quadratic equation in \( V_3 \). One finds following recursive relation

\[
V_i = C_iL_i, \text{ for } i = 1, \cdots, n-1
\]

with the coefficient \( L_i \) given by

\[
L_i = \frac{2L_{i-1}k_i + k_{i+1} \pm \sqrt{4L_{i-1}k_i k_{i+1} + k_{i+1}^2}}{2L_{i-1}k_i}. \tag{C.25}
\]

The last equation for \( i = n-1 \) gives a quadratic expression in \( V \), which roots are

\[
V = \frac{k_R}{2L_n-1k_n} \left( 2L_{n-1}k_nT + k_R \pm \sqrt{4L_{n-1}k_Rk_nT + k_R^2} \right)
\]

This equation, together with (C.25), gives all the \( 2^{n-1} \) roots of \( T = I_1 \) (as we have \( 2^{n-2} \) different \( L_{n-1} \)). By taking the smallest of these roots, one obtains \( V_{\text{max}} \) as given by Eq. (C.22). \( \blacksquare \)
C.4.3 Consequences of the theorem

The dependency of $V_{\text{max}}$ on $T$ given by Eq. (C.22) can be checked experimentally by measuring $V_{\text{max}}$ at different factor concentrations (see Fig. 8.2C). In particular, these experiments allow to distinguish which processes are limiting for repair.

I) If repair is limited by the binding of the factors, the maximal repair rate has a second order dependency on the total factor concentrations

$$V_{\text{max}} \approx \lambda_{n-1} k_n T^2. \tag{C.26}$$

The above formula is valid, when the total concentrations of the factors are small and/or the binding rate constants are low compared to the repair rate constant ($k_i \ll k_R$). Then $4k_n T \lambda_{n-1}/k_R \ll 1$ and the root of Eq. (C.22) is given by a second-order Taylor series approximation

$$\sqrt{4k_R k_n T \lambda_{n-1} + k_R^2} \approx k_R + 2k_n T \lambda_{n-1} - 2\frac{k_n^2 T^2 \lambda_{n-1}^2}{k_R}.$$

Using this Equation one obtains (C.26).

II) Conversely, if repair is limited by the repair step (i.e $k_i \gg k_R$ and/or high factor concentrations) one can approximate the numerator of Eq. (C.22) with $2\lambda_{n-1} k_R k_n T$ yielding

$$V_{\text{max}} \approx k_R T, \tag{C.27}$$

which is first order in $T$. 

Appendix D

Metrics of NER

In the introduction, I described how local damage experiments with fluorescent-protein tagged repair factors are performed and data collected (Chapter 6, Section 6.2). In this appendix I will first define, for comparison of experimental and simulated data, their counterparts in the model. Then, I will present measures for the time needed to recruit the repair factor to the damaged sites, the accumulation time, and the time spent in a repair complex, the residence time. Together with the time needed to remove the damage, the repair time, one has a “metric” system to characterize kinetics and efficiency of NER. If the system is simplified in the case of low and saturating damage, these three characteristic times can be calculated analytically. The obtained formulas are used to deduce general properties of NER.

D.1 Mapping of experimental data

D.1.1 Fluorescence

Fluorescence emitted by a repair factor is directly proportional to its concentration. In experiment, fluorescence is monitored in the local damaged area $F_{1,i}$ and in a control region $F_{2,i}$. The corresponding model definitions are given in Fig. D.1.

The accumulation curve of the $i$th factor in the local damaged area, i.e. the fraction of factors involved in repair as obtained from experiments, is defined by

$$ Acc_i(t) = \frac{[F_{1,i}(t)/V_d - F_{2,i}(t)/V_c]V_d}{T_i V_{nuc}}. \quad (D.1) $$

Where $V_d$, $V_c$ and $V_{nuc}$ are the volumes of the damaged region, the control region, and the nucleus, respectively (Fig. D.1). The life time of a protein in the repair complex
Appendix D. Metrics of NER

Figure D.1: Fluorescence is monitored in the local damaged area ($Fl_{1,i}(t)$) and in a control region ($Fl_{2,i}(t)$). In the model fluorescence is the sum of factors bound to damaged and undamaged DNA, and freely diffusing factors. During FLIP experiments fluorescence is bleached in region 3. $V_d, V_c$: volume of damaged and control area, respectively; $\vec{x} = (x, y, z)$: spatial coordinate.

is measured using the FLIP technique (Chapter 6, section 6.2). In such an experiment fluorescence is continuously bleached in a region opposite to the local damage (region 3 in Fig. D.1). In the model the residence curve corresponds to

$$R_i(t) = \frac{Fl_{1,i}(t)}{Fl_{1,i}(0)}. \tag{D.2}$$

The accumulation and residence curves can be computed for a particular choice of geometry after solving the corresponding system of partial differential equations. This rather time consuming procedure has been used to fit the model parameters to the experimental data (Appendix E).

D.1.2 Accumulation and residence time

For comparing the accumulation and residence curves of different repair factors, it is useful to summarize their course using characteristic times. I define the mean accumulation time of the $i$th factor

$$\alpha_i = \frac{\int_0^{t_p} [Acc_i(t_p) - Acc_i(t)] dt}{Acc_i(t_p) - Acc_i(0)}, \tag{D.3}$$

and its residence time

$$\rho_i = \frac{\int_0^\infty R_i(t) dt}{R_i(0)}. \tag{D.4}$$
Both quantities can be estimated from data. The time $t_p$ is when maximal accumulation, i.e. the plateau, is reached.

Further simplification is obtained if the factors are homogeneously distributed so that $F_i(\vec{x}, t) = F_i(t)$ and $I_i(\vec{x}, t) = I_i(t)$. This is justified by the fact that diffusion is fast compared to the time needed for a repair event. Measured residence and accumulation times are 2–5 minutes, the diffusion coefficients are around $5 \, \mu m^2/s$ (see Chapter 6). Thus, it takes about 10 sec to diffuse through a typical nucleus of 10 $\mu m$ diameter. In FLIP experiments, one can assume that fluorescence of the free factors will be completely bleached at the beginning of the experiment. Therefore, during FLIP experiments, solely the unbinding of the protein from the repair complex is monitored, its binding to the DNA cannot be observed as the free factors do not fluoresce. This corresponds to $F_i(t > 0) = 0$. Under these two assumptions, the accumulation and residence time curves read

$$Acc_i(t) = \frac{I_i(t)V_d}{T_iV_{nuc}} \quad (D.5)$$
$$R_i(t) = \frac{I_i(t)}{I_i(0)} \quad (D.6)$$

The time evolution of $I_i(t)$ is described by a system of ODE’s instead of PDE’s. The solution is quickly obtained by numerical integration. Application of formula (D.5) and (D.6) gives the accumulation and residence time of each factor. In the following sections, I will derive analytical expressions for the characteristic times when the initial damage is low or saturating.

## D.2 Non-saturating damage

### D.2.1 Definitions

The central idea is that the free factor concentrations do not change significantly during the measurements ($F_i \approx const$). This is indeed the case for residence time measurements, which are performed after a plateau in the immobilized fraction has been reached. Additionally, for most of the factors studied with local damage experiments, the fraction immobilized is around 10% of the total concentration so that one can assume $F_i \approx T_i$. Together with the assumed homogeneous distribution (see above), the system is described by a set of linear ordinary differential equations

$$\frac{dS(t)}{dt} = MS(t). \quad (D.7)$$
The vector $S(t)$ contains the free damage and intermediary complex concentrations. The matrix $M$ characterizes the stoichiometry and kinetics of NER. I introduce the matrix $W$, which computes, from the concentrations of the intermediary complexes, the concentration of the immobile factors

$$(I_1, \cdots, I_n)^T = WS_0.$$ 

The superscript $T$ stands for the transposed. $S_0$ is a subvector of $S$ without the free damage.

For a pathway with sequential binding and simultaneous release of the factors one has

$$S = (S_0, \cdots, S_n)^T \text{ and } S_0 = (S_1, \cdots, S_n)^T.$$ 

The $(n-1) \times (n-1)$ matrix $W$ reads

$$W = \begin{pmatrix}
1 & 0 & \cdots & 0 \\
1 & 1 & \ddots & \\
\vdots & \vdots & \ddots & 0 \\
1 & \cdots & 1 & 1
\end{pmatrix},$$

the $n \times n$ system matrix $M$ is

$$M = \begin{pmatrix}
-k_1 & k_{-1} & 0 & \cdots & 0 \\
k_1 & -k_2 - k_{-1} & k_{-2} & \cdots & 0 \\
0 & k_2 & -k_3 - k_{-2} & \cdots & 0 \\
\vdots & \vdots & \vdots & \ddots & \vdots \\
0 & \cdots & k_n & -k_{n+1} - k_{-n}
\end{pmatrix} \quad (D.8)$$

For clarity sake, I introduced $\kappa_i = k_i T_i$ for $i \leq n$ and $\kappa_{n+1} = k_R$.

### D.2.2 Repair time

**General definition**

The repair time is defined by

$$\tau_R = \frac{\int_0^{\infty} D_T(t) \, dt}{D_T(0)} = \frac{\sum_{i=0}^{n} \int_0^{\infty} S_i(t) \, dt}{\sum_{i=0}^{n} S_i(0)}.$$ 

The integrals over time are given by

$$\int_0^{\infty} S(t) \, dt = -M^{-1}S(0).$$
Where $M^{-1}$ is the inverse of $M$. The initial condition is $S(0) = (D_T(0), 0, \cdots, 0)^T$. This leads to a definition of the repair time under non-saturating conditions

$$\tau_R = \frac{-[M^{-1}S(0)]^T 1}{S(0)^T 1}.$$  \hspace{1cm} (D.9)

Where $1 = (1, \cdots, 1)^T$ is the unit vector.

This formula for the repair time can be applied to any pathway structure (different binding schemes, and kinetics) characterized by different system matrices $M$ and $W$. Therefore, this approach is suited to fast compute the efficiency of pathway alternatives. The expressions derived for the sequential pathway are given in the main text (Section 8.1).

### D.2.3 Accumulation time

**General definition**

If the time for accumulation of the factors is short compared to the time needed to remove the damage, so that $S_0(t) \approx$ const, the kinetics of accumulation are described by

$$\frac{dS_0(t)}{dt} = M_0 S_0(t) + b_0.$$  \hspace{1cm} (D.11)

Where $M_0$ is the $(n-1) \times (n-1)$ submatrix of $M$ without the first column and row, $S_0$ contains the concentration of the intermediary complexes only. The inhomogeneity vector $b_0$ of size $n-1$ gives the flux of the intermediary complexes resulting from the binding of free factors to the damage.

As the free damage is assumed constant, the integration to calculate the mean accumulation times (Eq. (D.5)) can be carried till $t_b = \infty$

$$\alpha_i = \int_0^\infty \left[ I_i(\infty) - I_i(t) \right] dt / I_i(\infty) - I_i(0).$$

The steady state is $S_0(\infty) = -M_0^{-1}b_0$ and $(I_1(\infty), \cdots, I_n(\infty))^T = -WM_0^{-1}b_0$. The integrals of the intermediary complexes are

$$\int_0^\infty [S_0(\infty) - S_0(t)] dt = M_0^{-2}b_0,$$

where $M_0^{-2}$ is the product of the inverse $M_0^{-1}$. The accumulation times read in a compact notation

$$(\alpha_1, \cdots, \alpha_n)^T = D^{-1}WM_0^{-2}b_0.$$  \hspace{1cm} (D.10)
**D** is the diagonal matrix containing the steady state immobilized factor concentrations

\[
D = \begin{pmatrix}
I_1(\infty) & 0 & \cdots & 0 \\
0 & I_2(\infty) & \ddots & \vdots \\
\vdots & \vdots & \ddots & 0 \\
0 & \cdots & 0 & I_n(\infty)
\end{pmatrix}.
\]

Using this general definition, the accumulation times obtained from pathways alternatives can easily be computed.

**Application to the sequential pathway**

For a sequential binding and simultaneous release of repair factor one has \(b_0 = (k_1S_0, 0, \cdots, 0)\) and \(S_0 = (S_1, \cdots, S_n)^T\). When binding is irreversible \((k_{-i} \approx 0)\) the accumulation time reads

\[
\alpha_i = \sum_{j=i+1}^{n+1} \frac{1}{\kappa_j} \left( \sum_{l=2}^{j} \frac{1}{\kappa_l} \right).
\]  

(D.11)

The difference in accumulation time between two successive factors (the \(i\)th and \((i+1)\)th) is always positive for any \(i\)

\[
\alpha_{i+1} - \alpha_i = \frac{\sum_{j=i+2}^{n+1} \frac{1}{\kappa_j} \left( \sum_{l=i+2}^{j} \frac{1}{\kappa_l} \right)}{\kappa_{i+1} \left( \frac{1}{\kappa_{i+1}} + \sum_{j=i+2}^{n+1} \frac{1}{\kappa_j} \right)} > 0.
\]

This shows that early binding factors accumulate faster than late binding factors. The same is true when binding of repair factors to the damaged DNA is reversible \((k_{-i} > 0)\). In this case the expressions become very lengthy and I was not able to derive a compact notation as for irreversible binding. However, the hierarchy in accumulation times appears to be conserved. Using symbolic computation as provided by programs like *Mathematica* for solving Eq. D.10, one can prove that

\[
\alpha_j > \alpha_i \text{ for } j > i.
\]

(D.12)

This appears to be true at least up to 100 repair factors \((n = 100)\).
D.2.4 Residence time

General definition

During FLIP experiments with a GFP-tagged $i$th factor, $F_i(t > 0) \approx 0$. The loss in fluorescence in the local damage is described by

$$\frac{dS_i}{dt} = M_i S_i,$$  \hspace{1cm} (D.13)

$M_i$ is a submatrix of $M$ which describes the reactions of the intermediary complexes that contain the $i$th factor, but not the binding reactions of the $i$th factor. The vector $S_i$ is a subvector of $S$, where only complexes which contain the $i$th factor are included. At $t = 0$ bleaching is initiated, the initial condition $S_i(0)$ is set by the intermediary complex concentrations in the plateau phase after damage induction. Using Eq. (D.13) one obtains the general expression

$$\rho_i = - \frac{[M_i^{-1}S_i(0)]^T 1}{S_i(0)^T 1}.$$ \hspace{1cm} (D.14)

The 1 is again the unit vector. This definition, like the former ones, can be applied to any putative NER pathway structure.

Application to the sequential pathway

For sequential binding and simultaneous release of the repair factors $S_i = (S_i, \cdots, S_n)^T$, the matrix $M_i$ is a $(n - i) \times (n - i)$ submatrix of $M$ where the rows and columns from 1 to $i$ are omitted, for example

$$M_2 = \begin{pmatrix}
-\kappa_3 - k_{-2} & \cdots & 0 \\
\vdots & \ddots & \vdots \\
\cdots & & -\kappa_{n+1} - k_{-n}
\end{pmatrix}.$$ \hspace{1cm} (D.15)

The initial condition is set by the steady state for $S_0(t) = \text{const}$. That is $S_1(0) = -M_0^{-1}b_0$ with $b_0 = (k_1 S_0, \cdots, 0)$. The residence time reads

$$\rho_i = \frac{\sum_{j=i+1}^{n+1} \frac{1}{\kappa_j} \left( \sum_{l=i+1}^{j} \frac{1}{\kappa_l} \right)}{\sum_{j=i+1}^{n+1} \frac{1}{\kappa_j}}.$$ \hspace{1cm} (D.16)

From this formula, one can prove that early binding factors reside longer in the repair complex than late binding factors

$$\rho_j < \rho_i \text{ for } j > i.$$ \hspace{1cm} (D.17)
Furthermore, one recognizes that the residence time of the first factor equals its accumulation time (Eq. (D.11)).

When the unbinding reactions are included \((k_{-i} > 0)\), I was not able to derive a closed expression for the residence times. Nevertheless, a few general conclusions can be formulated. According to Eq. (D.10) and (D.14) the residence time of the first factor equals its accumulation time, \(\alpha_1 = \rho_1\), when \(S_1(0) = -M_0 b_0\), that is the plateau concentration reached at the end of the accumulation phase equals the initial concentration for the FLIP experiment. When factors bind equally well at their respective substrate \((\kappa_i = \kappa\) and \(k_{-i} = k_-, i \leq n)\), one can show, using symbolic computation, that early binding factors reside longer in the repair complex than late binding factors, that is Eq. (D.17) holds (formula (D.14) has been computed with Mathematica till \(n = 100\)). The hierarchy in residence times as given by Eq. (D.17) can break when the binding properties of the factors to the damaged sites differ. This is already observed for the last two factors where the repair times read

\[
\rho_n = \frac{1}{\kappa_n + k_{-n}} \\
\rho_{n-1} = \frac{\kappa_n^2 + [k_+ + \kappa_n + 1]^2 + \kappa_n[\kappa_{n+1} + 2k_- + k_{-(n-1)}]}{[\kappa_n + \kappa_{n+1} + k_-][\kappa_n \kappa_{n+1} + k_{-(n-1)}(\kappa_n + \kappa_{n+1})]}.
\]

For example when the first intermediary complex of the \(n - 1\) factor is unstable \((k_{n-1}\) is large) then \(\rho_{n-1} \approx \rho_n \kappa_n / (\kappa_n + \kappa_{n+1} + k_-) < \rho_n\).

**D.3 Saturating damage**

**D.3.1 Repair time**

As shown in Appendix C.2, the time dependent repair rate is well characterized by its steady state value. Using Eq. (C.10) the approximation for the time dependent changes of the total damage reads

\[
\frac{dD_T}{dt} \approx -V_{\text{max}} \frac{D_T}{D_T + K_d}
\]

Separation of variables

\[
D_T dt = -\frac{D_T + K_d}{V_{\text{max}}} dD_T,
\]

and integration leads to an expression for the repair time under saturating conditions

\[
\tau_R = \frac{1}{D_T(0)} \int_0^\infty D_T(t) dt \approx \frac{1}{2} \frac{D_T(0)}{V_{\text{max}}} + \frac{K_d}{V_{\text{max}}} \tag{D.18}
\]
D.3. Saturating damage

Figure D.2: (A) Solid line: exact repair time calculated numerically by solving the system of differential equations (Eqs. (C.6)–(C.8)) and Eq. (8.1) for different initial damage concentrations. Dashed line: approximation of the repair time obtained from Eq. (D.18). (B) Ratio of the numerically calculated repair time and the approximated value as a function of initial damage. For large initial damages this ratio approaches one. Parameters from set 1, Tab. 7.2.

As shown in Fig. D.2A, this formula well approximates the exact repair time (dashed and solid line, respectively). The two values become practically identical for saturating initial damages (Fig. D.2B).

D.3.2 Accumulation time

At very high damage concentrations, the first factor is nearly completely immobilized to the damage for a long time period (see (C.4)). If its diffusion is fast compared to the binding of the subsequent factors, the initial condition can be approximated by 

\[(S_1(0), \cdots, S_n(0))^T \approx (T_1, \cdots, 0)^T.\]

Over time, the first factor redistributes between the intermediary complexes. In the sequential pathway following conservation relation holds

\[S_1(t) = T_1 - \sum_{i=2}^{n} S_i(t). \quad \text{(D.19)}\]

Assuming constant free factor concentrations the kinetics of the intermediary complexes \(S_2 = (S_2, \cdots, S_n)^T\) are described by a set of linear ODE’s

\[
\frac{dS_2}{dt} = M_2 S_2 + \left( \begin{array}{c}
\kappa_2(T_1 - \sum_{i=2}^{n} S_i) \\
\vdots \\
0
\end{array} \right)
\quad \text{(D.20)}
\]
Where $M_2$ has been defined in (D.15). The concentration of the first intermediary complex is obtained from (D.19).

Due to the high concentration of damaged sites the first factor binds to the DNA as soon as it enters the damaged area. Therefore, its accumulation time is solely determined by the time it takes to diffuse from the undamaged to the damaged area (a process which I do not further consider in this section). To calculate the accumulation time of the other repair factors we can apply the same method as before (section D.2.3). For the sequential accumulation and simultaneous release of the factors the formulas are cumbersome and I forbear of listing them here. Using symbolic computation, one can prove that the repair factors accumulate faster for saturating damage than for low damage, and that the position of binding is positively correlated with a longer accumulation time (i.e. formula (D.12) holds).

### D.3.3 Residence time

When the repair system is saturated, the amount of immobilized and free factors does not change during a long time (as the binding and release of the factors is balanced). Thus, one can use the same method as for non-saturating conditions to calculate the residence times (Eq. (D.14)). The initial intermediary complex concentrations $S_i(0)$ and the free factor concentrations $F_j$ are set by the condition $T_1 = I_1$. For a sequential irreversible binding ($k_{-i} \approx 0$) the residence times read

$$\rho_i = \frac{1}{k_{Ri}} + \sum_{j=i+1}^{n} \frac{1}{k_j F_j} \left(1 - \frac{I_j(0)}{I_i(0)}\right)$$

and

$$\rho_n = \frac{1}{k_{Rn}}. \tag{D.21}$$

One has

$$\frac{1}{k_j F_j} \left(1 - \frac{I_j}{I_k}\right) < \frac{1}{k_j F_j} \left(1 - \frac{I_j}{I_i}\right) \tag{D.22}$$

when $I_i > I_k$, which occurs in the sequential pathway when $k > i$ (see Eq. 7.8). Using the above relation, one shows that

$$\rho_j < \rho_i \text{ for } j > i \tag{D.23}$$

holds. This means that early binding factors reside longer in the repair complex than late binding factors also for saturating conditions. For reversible binding, numerical calculations indicate that if the binding and dissociation rate constants are the same for all factors this hierarchy in the residence times (Eq. (D.17)) is conserved (data not shown).

To calculate the residence times in the alternative pathways where the first factor is released prior to the other factors (page 118), one proceeds in a similar way.
Appendix E

Data fitting

E.1 NER model including diffusion

The NER model is extended to include diffusion of the free factors and is used to simulate the data of the local UV-damage experiments and fit the model parameters to the accumulation curves of XPC-HR23B, TFIH, and ERCC1-XPF. The model accounts for the spatial gradients that arise after application of a high UV dose to a local area of the nucleus.

The nuclear geometry is approximated by a cylinder with radius $R_n$. As shown in Fig. E.1, I assume that UV irradiation induces damages in a cylindrical volume of radius $R_d$, thus the system has axial symmetry and is described in cylinder coordinates. The concentration of the diffusible free factor at a distance $r$ from the center is determined by

$$
\frac{\partial F_i(r, t)}{\partial t} = k_R S_n + k_{-i} S_i - \bar{k}_i F_i S_{i-1} + \frac{1}{r} \frac{\partial}{\partial r} \left( \bar{D}_i r \frac{\partial F_i}{\partial r} \right),
$$

where $\bar{D}_i$ is the diffusion coefficient of the free $i$-th repair factor. For the intermediary complexes

$$
\frac{\partial S_i(r, t)}{\partial t} = \bar{k}_i F_i S_{i-1} - k_{-i} S_i - \bar{k}_{i+1} F_{i+1} S_i + k_{-(i+1)} S_{i+1}
$$

holds. Similar expressions for the free damage $S_0$ and the PC concentration ($S_n$) are used. At $t = 0$, DNA damage is induced instantaneously. The initial conditions are

$$
S_0(r, 0) = \begin{cases} D_T(0) & \text{for } 0 \leq r \leq R_d, \\ 0 & \text{for } R_d < r \leq R_n \end{cases}
$$

$$
S_i(r, 0) = 0 
$$

$$
F_i(r, 0) = T_i / \beta_i.
$$
Note that the presence of unspecific binding sites, $\beta_i > 1$, reduces the initial concentration of free factors available for repair (Eq. (E.4)).

In the presence of undamaged binding sites, the equation for the free factors is

$$
\frac{\partial F_i(r, t)}{\partial t} = \frac{k R}{\beta_i} S_n + \frac{k_{i-1}}{\beta_i} S_i - \frac{\tilde{k}_i}{\beta_i} F_i S_{i-1} + \frac{1}{r} \frac{\partial}{\partial r} \left( \frac{\tilde{D}_i}{\beta_i} \frac{\partial F_i}{\partial r} \right)
$$

(E.5)

where a quasi-steady state approximation is used as in section C.1. This indicates a reduction of the diffusibility of $F_i$, which diffuses with an effective diffusion coefficient

$$
D_i = \frac{\tilde{D}_i}{\beta_i}.
$$

When DNA damage is uniform throughout the nucleus ($R_d = R_n$), as is the case in global damage experiments, the spatial gradients vanish ($\frac{\partial F_i}{\partial r} = 0$) and everywhere in the nucleus the conservation relation $T_i = F_i \beta_i + I_i$ holds. In this case the model with diffusion reduces to the basic model (Eqs. (C.6)–(C.8)).

### E.2 Immobile fraction

The parameters and confidence intervals were estimated using the initial accumulation curves of XPC-HR23B, TFIH, and ERCC1-XPF. In experiments it is not possible to distinguish between freely diffusing factors and immobile factors (see Appendix D.1),
E.2. Immobile fraction

so that the amount of immobilized factors involved in repair is approximated by taking the difference between total fluorescence in the damaged area and total fluorescence in an area of equal size outside the damaged region. In the model, this is accounted for by taking the difference \( M_i \) between the average concentration of factors in damaged and undamaged volume, that is (see Fig. E.1)

\[
M_i = \frac{\int_0^{R_d} (F_i \beta_i + I_i) r dr}{R_d^2} - \frac{\int_0^{R_n} (F_i \beta_i) r dr}{R_n^2}.
\]

Accordingly, the observable fraction of immobilized factors is given by

\[
F r_i = \frac{M_i R_d^2}{T_i R_n^2}. \tag{E.6}
\]

The binding rate constants were estimated by minimizing the sum of squares differences between simulations and data [125]

\[
\chi^2 = \sum_i \sum_{j=1}^{N_i} \left( \frac{T_i(t_{j,i}) - F r_i(t_{j,i})}{\sigma_i(t_{j,i})} \right)^2 \tag{E.7}
\]

where \( i = 1 \) (XPC-HR23B), 2 (TFIIH), 5 (ERCC1-XPF). In this formula, \( T_i(t_{j,i}) \) and \( \sigma_i(t_{j,i}) \) are the observed averages and standard deviations of the immobilized fraction of the \( i \)-th factor at time points \( t_{j,i} \), respectively. To get the value of \( F r_i \) at the desired time points, Eqs. (E.1)–(E.5) are solved numerically on a regular spaced grid (see Appendix F). For minimization of \( \chi^2 \), the non-linear curve fitting procedure \texttt{lsqnonlin} included in the package \texttt{matlab} was used (MathWorks Inc., Natick, MA).

For the distance between two grid points I used \( \Delta r = 0.5 \mu m \), and for the time step \( \Delta t = 2.77 \) ms. Higher spatial or temporal resolutions did not increase the accuracy of the calculations. The radius of the nucleus is \( R_n = 10 \mu m \). The ratio of damaged volume to total nuclear volume is \( \gamma = V_d / V_n = R_d^3 / R_n^3 \). Thus, the radius of the damaged area is given by \( R_d = R_n \sqrt{\gamma} = 3.77 \mu M \) (in the experiments \( \gamma \approx 1/7 \)).
Appendix F

Simulation of reaction-diffusion equations

F.1 Type of problems

A substance $c$ diffuses in a medium with diffusion constant $D$. In one dimension or with cylindrical, or spherical symmetry, the equation describing the changes in concentration $c$ is given by

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} + D \frac{\alpha}{x} \frac{\partial c}{\partial x} + f(c) \tag{F.1}$$

with $\alpha = 0$, 1 or 2 for linear, cylindrical, or spherical coordinates, respectively. The term $f(c)$ incorporates all reactions in which $c$ is involved. The boundary conditions at $x = l$ and $x = L$ are

$$-D \frac{\partial c}{\partial x} \bigg|_{x=l} = J_l \tag{F.2}$$
$$-D \frac{\partial c}{\partial x} \bigg|_{x=L} = J_L \tag{F.3}$$

Note that due to symmetry in cylindrical and spherical coordinates ($\alpha = 1, 2$) the flux is zero at the boundary $l = 0$. To solve this type of diffusion problems the space is subdivided in $N$ equally spaced points (Fig. F.1). The distance between each grid points is $\Delta x$ so that $N\Delta x = L$. The concentration at the grid point $j$ situated at $x = \Delta x(j + 1/2)$ is denoted by $c_j$.  

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Figure F.1: Scheme of the lattice used for the numerical solution of Eq. (F.1). The first grid point, $c_j$, is at $\Delta x(j_l + 1/2) = l + \Delta x/2$, the last point $c_{N-1}$ at $\Delta x(N - 1/2) = L - \Delta x/2$, the $j$-th point at $\Delta x(j_l + 1/2)$. The index varies between $j = j_l$ and $N - 1$.

**F.2 Difference scheme**

**F.2.1 Away from the boundaries**

A Taylor expansion of the concentrations $c_{j+1}$ and $c_j$ ($N - 1 > j > j_l$), yields

$$
c_{j+1} = c_j + \Delta x \left. \frac{\partial c}{\partial x} \right|_{\Delta x(j+1/2)} + \frac{(\Delta x)^2}{2} \left. \frac{\partial^2 c}{\partial x^2} \right|_{\Delta x(j+1/2)} + \frac{(\Delta x)^3}{6} \cdots + O((\Delta x)^4) \tag{F.4}
$$

$$
c_{j-1} = c_j - \Delta x \left. \frac{\partial c}{\partial x} \right|_{\Delta x(j+1/2)} + \frac{(\Delta x)^2}{2} \left. \frac{\partial^2 c}{\partial x^2} \right|_{\Delta x(j+1/2)} - \frac{(\Delta x)^3}{6} \cdots + O((\Delta x)^4) \tag{F.5}
$$

The difference between the previous equations gives an approximation for the first derivative

$$
\left. \frac{\partial c}{\partial x} \right|_{\Delta x(j+1/2)} = \frac{c_{j+1} - c_{j-1}}{2\Delta x} + O((\Delta x)^2) \tag{F.6}
$$

The sum of Eq. (F.4) and (F.5) yields an approximation for the second derivative

$$
\left. \frac{\partial^2 c}{\partial x^2} \right|_{\Delta x(j+1/2)} = \frac{c_{j+1} - 2c_j + c_{j-1}}{(\Delta x)^2} + O((\Delta x)^2) \tag{F.7}
$$

**F.2.2 At the boundaries**

At the left-hand side boundary a Taylor expansion of the first space derivative at $x = l$ and $l + \Delta x$ yields

$$
\left. \frac{\partial c}{\partial x} \right|_{l+\Delta x} = \left. \frac{\partial c}{\partial x} \right|_{l+\Delta x/2} + \frac{\Delta x}{2} \left. \frac{\partial^2 c}{\partial x^2} \right|_{l+\Delta x/2} + \frac{(\Delta x)^2}{8} \cdots + O((\Delta x)^3) \tag{F.8}
$$

$$
\left. \frac{\partial c}{\partial x} \right|_{l} = \left. \frac{\partial c}{\partial x} \right|_{l+\Delta x/2} - \frac{\Delta x}{2} \left. \frac{\partial^2 c}{\partial x^2} \right|_{l+\Delta x/2} + \frac{(\Delta x)^2}{8} \cdots - O((\Delta x)^3). \tag{F.9}
$$
By summing the two equations one obtains
\[
\frac{\partial c}{\partial x} \bigg|_{l+\Delta x/2} = \frac{1}{2} \left( \frac{\partial c}{\partial x} \bigg|_{l+\Delta x} + \frac{\partial c}{\partial x} \bigg|_{l} \right) + O((\Delta x)^2). \tag{F.10}
\]

With Eq. (F.2) and (F.6) the first derivative in space at the left boundary reads
\[
\frac{\partial c}{\partial x} \bigg|_{l+\Delta x/2} = \frac{c_{j+1} - c_{j}}{2\Delta x} - \frac{J_l}{2D\Delta x} + O((\Delta x)^2). \tag{F.11}
\]

Taking the difference of Eq. (F.8) and (F.9) yields
\[
\frac{\partial^2 c}{\partial x^2} \bigg|_{l+\Delta x/2} = \frac{c_{j+1} - c_{j}}{(\Delta x)^2} + \frac{J_l}{D\Delta x} + O((\Delta x)^2). \tag{F.12}
\]

Similarly, at the right boundary one derives for the first derivative
\[
\frac{\partial c}{\partial x} \bigg|_{L-\Delta x/2} = \frac{c_{N-1} - c_{N-2}}{2\Delta x} - \frac{J_N}{2D\Delta x} \tag{F.13}
\]
and for the second derivative
\[
\frac{\partial^2 c}{\partial x^2} \bigg|_{L-\Delta x/2} = \frac{c_{N-2} - c_{N-1}}{(\Delta x)^2} - \frac{J_N}{D\Delta x}. \tag{F.14}
\]

### F.2.3 An explicit scheme

By taking a forward difference for the time derivative
\[
\frac{\partial c}{\partial t} = \frac{c(t + \Delta t) - c(t)}{\Delta t} = \frac{c^* - c}{\Delta t}
\]

one obtains at the left boundary
\[
c^*_j = \left( 1 - \nu \frac{2j_l + 1 + \alpha}{2j_l + 1} \right) c_j + \nu \frac{2j_l + 1 + \alpha}{2j_l + 1} c_{j+1} + \frac{\Delta t}{\Delta x} \frac{2j_l + 1 - \alpha}{2j_l + 1} J_l \tag{F.15}
\]

where \( \nu = D\Delta t/((\Delta x)^2) \), away from the boundaries
\[
c^*_j = \nu \frac{2j + 1 - \alpha}{2j + 1} c_{j-1} + (1 - 2\nu) c_j + \nu \frac{2j + 1 + \alpha}{2j + 1} c_{j+1} \tag{F.16}
\]

and at the right boundary
\[
c^*_{N-1} = \nu \frac{2N - 1 - \alpha}{2N - 1} c_{N-2} + \left( 1 - \nu \frac{2N - 1 - \alpha}{2N - 1} \right) c_{N-1} - \frac{\Delta t}{\Delta x} \frac{2N - 1 + \alpha}{2N - 1} J_L \tag{F.17}
\]
F.2.4 A mixed implicit-explicit scheme

The average of an implicit and explicit difference scheme gives a better numerical stability. One obtains

\[-\alpha_j c^*_j - \beta_j c^*_j - \gamma_j c^*_{j+1} = \delta_j\]  (F.18)

with

\[
\begin{align*}
\alpha_j &= 0 \\
\beta_j &= 1 + \theta \nu \frac{2j + 1 + \alpha}{2j + 1} \\
\gamma_j &= \theta \nu \frac{2j + 1 + \alpha}{2j + 1} \\
\delta_j &= (1 - (1 - \theta) \nu \frac{2j + 1 + \alpha}{2j + 1}) c_j + (1 - \theta) \nu \frac{2j + 1 + \alpha}{2j + 1} c_{j+1} \\
&+ \frac{\Delta t}{\Delta x} \frac{2j + 1 - \alpha}{2j + 1} J_l
\end{align*}
\]

The averaging coefficient is \(\theta \in [0, 1]\), with \(\theta = 0\) for the explicit scheme and \(\theta = 1\) for a pure implicit scheme. Away from the boundaries \((N - 1 > j > j_i)\)

\[
\begin{align*}
\alpha_j &= \theta \nu \frac{2j + 1 + \alpha}{2j + 1} \\
\beta_j &= 1 + \theta 2\nu \\
\gamma_j &= \theta \nu \frac{2j + 1 + \alpha}{2j + 1} \\
\delta_j &= (1 - \theta) \nu \frac{2j + 1 - \alpha}{2j + 1} c_{j-1} + (1 - (1 - \theta) 2\nu) c_j \\
&+ (1 - \theta) \nu \frac{2j + 1 + \alpha}{2j + 1} c_{j+1}
\end{align*}
\]

and

\[
\begin{align*}
\alpha_{N-1} &= \theta \nu \frac{2N - 1 + \alpha}{2N - 1} \\
\beta_{N-1} &= 1 + \theta \nu \frac{2N - 1 + \alpha}{2N - 1} \\
\gamma_{N-1} &= 0 \\
\delta_j &= (1 - \theta) \nu \frac{2N - 1 + \alpha}{2N - 1} c_{N-2} + (1 - (1 - \theta) \nu \frac{2N - 1 + \alpha}{2N - 1}) c_{N-1} \\
&+ \frac{\Delta t}{\Delta x} \frac{2N - 1 + \alpha}{2N - 1} J_L
\end{align*}
\]
F.2.5 Accuracy and stability

The Crank-Nicolson scheme is when $\theta = 0.5$ in this case the numerical scheme is $(\Delta t)^2$ and $(\Delta x)^2$ accurate. Following stability conditions have to be fulfilled (derived for $\alpha = 0$)

when $0 \leq \theta < 1/2$, stable if and only if $\nu \leq \frac{1}{1 - 2\theta}$
when $1/2 \leq \theta \leq 1$, stable for all $\nu$

Moreover, in order for the solution to be positive at all times (see [85])

$$\nu \leq \frac{1}{4} \frac{2 - \theta}{(1 - \theta)^2}. \tag{F.19}$$

This is a more severe condition than for stability. In particular, it should be applied if the initial conditions are not smooth. Depending on the problem higher values for $\nu$ can be chosen.

The previous stability conditions apply in the absence of reactions. In the presence of reactions the choice of $\Delta t$ is also determined by their kinetics. In the absence of diffusion, the numerical scheme is a simple forward Euler. Take, for example, following degradation reaction

$$\frac{du}{dt} = -cu, \ c > 0.$$

Applying forward Euler one obtains

$$u^* = (1 - c\Delta t)u$$

This map is stable if $1 > 1 - c\Delta t > -1$, that is $\Delta t < 2/c$ and $c > 0$. Thus, when reactions are fast, $\nu$ needs possibly to be chosen lower than given in Eq. (F.19).
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>inositol 1,4,5-trisphosphate receptor</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;P</td>
<td>inositol 1,4,5-trisphosphate 5-phosphatase</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;K</td>
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<td>PLC</td>
<td>phospholipase C</td>
</tr>
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<td>PI</td>
<td>phosphatidylinositol</td>
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<tr>
<td>PIP</td>
<td>phosphatidylinositol-4-phosphate</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>1,2-diacylglycerol</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco endoplasmic reticulum Ca&lt;sup&gt;2+&lt;/sup&gt; ATPase</td>
</tr>
<tr>
<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sub&gt;c&lt;/sub&gt;</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; concentration in the cytoplasm</td>
</tr>
<tr>
<td>PMCA</td>
<td>plasma membrane Ca&lt;sup&gt;2+&lt;/sup&gt; ATPase</td>
</tr>
<tr>
<td>CICR</td>
<td>calcium induced calcium release</td>
</tr>
<tr>
<td>XP</td>
<td>Xeroderma Pigmentosum</td>
</tr>
<tr>
<td>PC</td>
<td>pre-incision complex</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
</tbody>
</table>
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Publications


In preparation


Selbständigkeitserklärung

Ich versichere hiermit, die vorliegende Arbeit selbständig und ausschließlich unter Verwendung der angegebenen Mittel und ohne unerlaubte Hilfen angefertigt zu haben.

Antonio Politi