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# Determination of the Kinetics of $Na^+/H^+$ Exchange in Platelets Using the Coulter S-Plus Cell Counter

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**Summary:** Cell swelling, as measured by electronic cell sizing, is a good indicator of the $Na^+/H^+$ exchange activation. In this study the kinetic properties of the $Na^+/H^+$ exchanger were determined with the aid of the Coulter S-Plus VI D haematological cell counter. Cell swelling was measured in platelets suspended in Na-propionate medium. The rapid entry and intracellular dissociation of propionic acid induces activation of the exchanger, and in turn the uptake of water by osmosis. The fractional volume increase measured by the Coulter S-Plus was dependent on the external Na-concentration, with $K_m$ = 86 mmol/l. Saturation was reached at a propionate concentration of 140 mmol/l. Inhibition by amiloride was dose-dependent with $K_i$ = 24 μmol/l. The activity of the exchanger was not modified by ouabain. These data are generally consistent with those published in previous reports, and indicate that automated haematological analysers are appropriate for the study of this aspect of platelet function.

## Introduction

The cell membrane $Na^+/H^+$ exchange system plays a fundamental role in the regulation of intracellular pH ($pH_i$) and in the control of volume, growth and proliferation of many cell types (1, 2). Some findings suggest that the $Na^+/H^+$ exchanger is very active in blood platelets and that it probably plays a critical role in the activation of platelets by several agonists (3–5), as well as in the regulation of cytoplasmic pH (6) and of platelet volume (7). The exchanger, which is virtually quiescent at physiological $pH_i$, can be activated by lowering $pH_i$ and by other stimuli such as growth factors, hormones, protein kinase C, etc. (1, 2, 8, 9).

The activation of the $Na^+/H^+$ antiport has been determined by the measurement of changes in cell volume using electronic cell sizing (10, 11). By this method the exchanger was characterized in platelets using a Coulter Counter ZF with a channelyzer (12).

In the present study, the kinetic properties of the $Na^+/H^+$ antiport were determined in platelets, using a Coulter mod. S-Plus VI D haematological analyser.

## Materials and Methods

### Reagents and solutions

The acid-citrate-dextrose (ACD) consisted of 65 mmol/l citric acid, 11 mmol/l glucose, 85 mmol/l trisodium citrate. The NaCl medium contained 140 mmol/l NaCl, 1 mmol/l KCl, 1 mmol/l $MgCl_2$, 10 mmol/l glucose, 20 mmol/l Hepes, pH 7.35. The Na-propionate and K-propionate media were of similar composition but NaCl was replaced by Na-propionate or by K-propionate, respectively. $CaCl_2$ (1 mmol/l) was added to the assay media before use; the pH of the assay media was 6.7 and osmolarity was adjusted to 290 mosmol/l with the major salt. Amiloride was kindly supplied by Merck Sharp & Dohme, Italy; all other reagents were purchased from Sigma.

### Preparation of platelet suspension

Venous blood from healthy volunteers was collected in plastic tubes containing ACD as anticoagulant at a 6 : 1 blood/anticoagu-

lant ratio. Platelet-rich plasma was obtained by centrifugation at 120 *g* for 10 min: it was kept at room temperature and used within four hours.

### Determination of $Na^+/H^+$ exchange by cell sizing

$Na^+/H^+$ exchange was estimated from changes in platelet volume measured by electronic cell sizing by means of the Coulter S-Plus VI D. The method measures the platelet swelling associated with the activation of the exchanger upon addition of a small volume of platelet-rich plasma to Na-propionate media. Free propionate anion is in equilibrium with propionic acid in solution: the latter penetrates the plasma membranes and dissociates intracellularly, thereby inducing cytoplasmic acidification and activation of the antiporter which then exchanges extracellular $Na^+$ for intracellular $H^+$. The sustained presence of the weak acid and continued activity of the $Na^+/H^+$ exchanger determines the accumulation of Na-propionate and thus a shift of intracellular water, leading to cell swelling. If platelet-rich plasma is added to Na-free K-propionate media, cell acidification occurs in the same way as described, but the activation of the exchanger is hampered by the lack of external $Na^+$; thus no, or minimal swelling occurs.

### Measurements

Volume measurements were carried out with the Coulter Counter S-Plus VI. Platelet suspensions were diluted to a final platelet content of $50 \times 10^9$/l. The electronic pulses related to the particle passage through a 70 µm diameter orifice were analysed. Mean platelet volume was calculated from size distribution curves displayed through a histogram on a X-Y recorder.

Measurements were taken in triplicate at room temperature at 10–30 s intervals upon addition of one of the following media:

a) NaCl for baseline volume measurement,
b) Na-propionate,
c) K-propionate
d) Na-propionate containing amiloride or ouabain.

Exchange rates were expressed as the increase in platelet volume from the baseline upon addition of platelet-rich plasma to Na-propionate media after subtraction of the (minimal) volume change occurring in K-propionate media (used as control).

## Results

Time-courses of platelet swelling in Na-propionate versus K-propionate media are shown in figure 1: a fractional volume increase of up to 0.48 was observed with Na-propionate, and this increase was complete after 4 min.

The dependence of $Na^+/H^+$ exchange on $[Na^+]_o$ concentration is shown in figure 2: the reaction follows *Michaelis-Menten*-type kinetics with a $K_m$ of 86 mmol/l for $[Na^+]_o$.

Volume increase is also dependent on propionate concentration in the media with no evidence of saturation (fig. 3).

The inhibitory effect of amiloride on cell swelling is apparent from figures 4 and 5: the inhibition was dose-dependent with a $K_i$ of 24 µmol/l (fig. 5).

Finally, prolonged treatment (60 min) with ouabain (fig. 6) did not affect $[Na^+]_o$-dependent platelet swelling at any of the two concentrations tested.

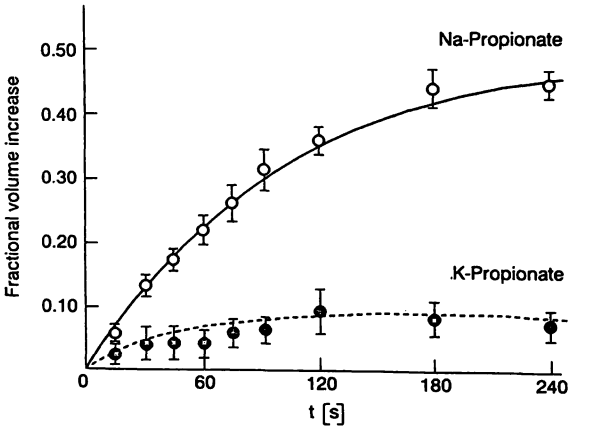


**Fig. 1** Fractional volume increase of platelets in Na-propionate or K-propionate media. Time course of changes in platelet mean volume was measured with the Coulter S-Plus Counter, after suspension of platelet rich plasma in isotonic media (pH 6.7) containing Na-propionate or K-propionate (140 mmol/l) (see Materials and Methods). Data are means ± SE from three similar experiments.

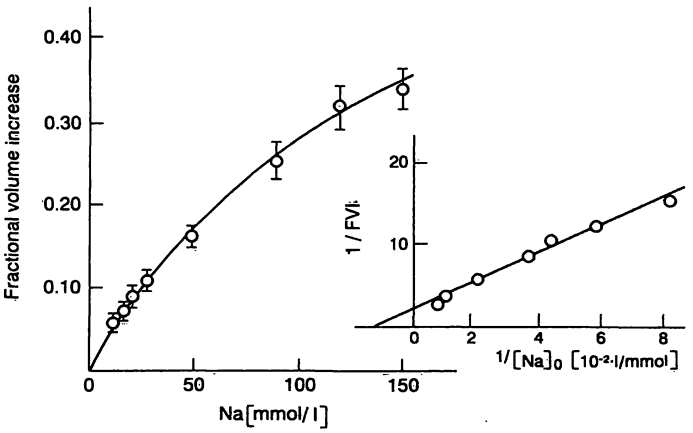


**Fig. 2** Dependence of propionate-induced platelet swelling on external sodium concentration. Experimental details as in figure 1; each data point represents the mean ± SE of 3 determinations. A double reciprocal plot of the fractional volume increase (1/FVI) as affected by $1/[Na]_0$ is represented. The kinetic values derived from the plot are $K_m$ = 86 mmol/l; r = 0.99; $v_{max}$ = 0.48 FVI.

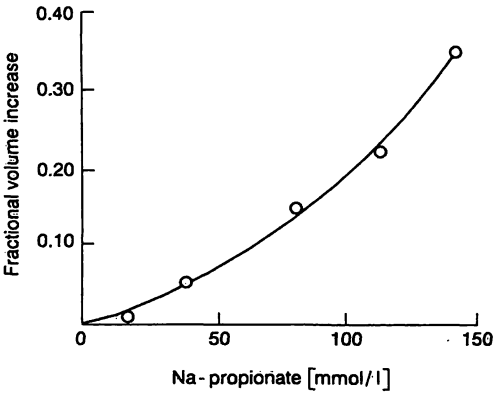


**Fig. 3** Dependence of platelet swelling on external propionate concentrations. Effect of propionate concentration in the suspending medium on the fractional volume increase.

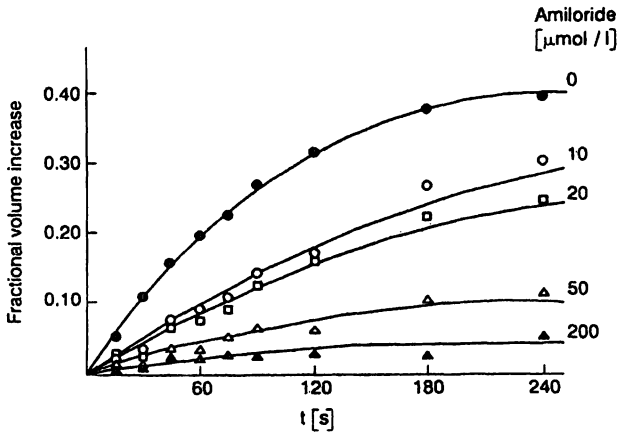


**Fig. 4** Amiloride inhibition of platelet swelling in Na-propionate. Time course of changes in mean platelet volume in Na-propionate media (140 mmol/l), in the absence (control) and presence of various amiloride concentrations (10–200 μmol/l).

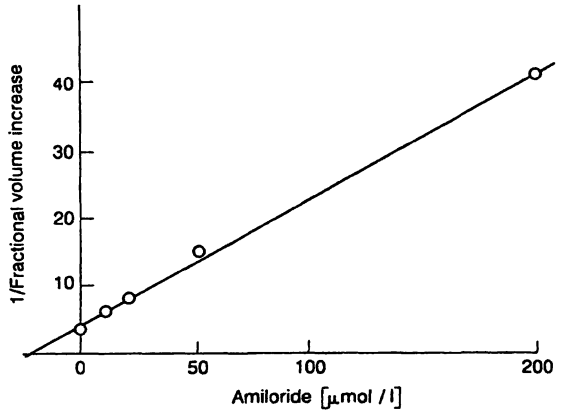


**Fig. 5** Amiloride inhibition of platelet swelling in Na-propionate. Plot of 1/FVI rate constant of platelet swelling vs amiloride concentration. Platelets were suspended in 140 mmol/l Na-propionate and osmotically balanced with K-propionate at varying concentrations of amiloride. Data are means ± SE of 3 determinations. $K_i$ = 24 μmol/l.

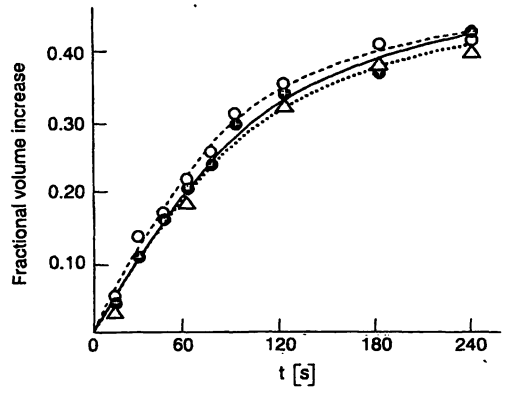


**Fig. 6** Effect of ouabain on platelet swelling in Na-propionate. Platelet rich plasma was treated with 0.5 mmol/l (○), or 0.8 mmol/l (△) ouabain or K propionate (● = control), then analysed for volume change in Na-propionate (140 mmol/l, pH 6.5).

## Discussion

The estimation of $Na^+/H^+$ exchange through the measurement of Na-propionate-induced platelet swelling with the Coulter S Plus VI provided data consistent with those published in previous reports and obtained with other instrumentation (10–12). In particular, the dependence of the rate of volume gain on external $Na^+$ concentration showed a saturation curve comparable to previous findings: the $K_m$ value for $[Na^+]_o$ of 86 mmol/l is consistent with previous data on platelets (12) and a few other cell types (8, 10). The increase in the rate of swelling with increased Na-propionate concentration is due to the progressive increase in cell acidification; this is also in agreement with previous findings (11), as are the kinetics of inhibition by amiloride.

However, prolonged treatment with ouabain, which leads to an increased intracellular $Na^+$ concentration (13), had no effect on the rate of increase of platelet volume.

The most important difference between our results and those obtained with the Coulter Counter ZF channelyzer (12), is a lower initial rate of platelet swelling. The reason for this discrepancy could be that *Grinstein* and coworkers (10–12) used a cell counter system in which platelet-rich plasma was diluted up to 120 × $10^6$ cells per litre and thus washed free of any contamination by $Na^+$. In cell counts performed with the Coulter S-Plus, the platelet-rich plasma is diluted 1 : 20 before addition to Na-propionate and K-propionate media to start the reaction. Thus a residual amount of $Na^+$ still present in the system probably accounts for the small difference observed in the initial rate of cell swelling. This, however, does not constitute a problem, because platelet swelling appears to be linear for up to at least 90 s; at this time the rate of fractional volume increase obtained with our method is not significantly different from that reported by other authors (11, 12).

In conclusion our study suggests that the kinetics of $Na^+/H^+$ exchange in platelets can be adequately evaluated in clinical laboratories equipped with automated haematological analysers.

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