Eur. J. Clin. Chem. Clin. Biochem.
Vol. 31, 1993, pp. 273–276

Expression of Vasopressin Receptors (V_2-Subtype) on LLC-PK1 Cells During Cell Culture

By *T. Ehnis*[1], *B. Hocher*[1], *H. Abou-Rebyeh*[2], *W. Oelkers*[1] and *J. Hensen*[3]

[1] *Abteilung für Innere Medizin, Klinikum Steglitz, Freie Universität Berlin*
[2] *Institut für Molekularbiologie und Biochemie, Freie Universität Berlin*
[3] *Medizinische Klinik 1, Universitätsklinikum Erlangen, Germany*

(Received October 28, 1992/February 9, 1993)

Summary: Vasopressin receptor expression on LLC-PK_1-cells (a porcine renal tubular cell line) during cell culture is still not fully understood. We studied receptor expression using a novel vasopressin analogue with high specific radioactivity ([^{125}I][8-*p*-hydroxy-phenylpropionyl]-lys^8-vasopressin, 74EBq/mol (2000 Ci/mmol)). LLC-PK_1 cells were grown in monolayers for 1 to 6 days. *Scatchard* analysis performed with membranes of LLC-PK_1 cells revealed a single binding site with a binding constant (K_d) of 0.46 ± 0.04 nmol/l. During cell culture, the binding constant (K_d) was not altered, but receptor density increased significantly (21 115 ± 645 receptors per cell, day 2; 42 315 ± 1512 receptors per cell, day 6). A receptor occupancy of about 30% was found to be associated with a cAMP stimulation of 50%. The receptor reserve might be even higher because, by using a highly specific oxytocin antagonist, we found that 20% of the occupied [^{125}I][8-*p*-hydroxy-phenylpropionyl]-lys^8-vasopressin-binding sites are oxytocin receptors. For lys^8-vasopressin receptor studies, great care has to be taken to examine cells in identical culture phases.

Introduction

LLC-PK_1 cells, an established porcine renal cell line (1) with polar epithelial cell characteristics maintained in culture, has been shown to express some hormone receptors, e. g. for atrial natriuretic peptide and lys^8-vasopressin. LLC-PK_1 cells have been used as a model to study peptide hormone/hormone-receptor interactions (e. g. receptor downregulation) in vitro. Vasopressin, like other peptide hormones, performs its physiological function by binding to cell-surface receptors in association with a specific guanine-nucleotide-binding protein. These actions in the kidney are mediated by the adenylate-cyclase-coupled V_2-receptor subtype (2, 3). However, little is known about 'normal' vasopressin receptor expression and vasopressin-induced cAMP generation during cell culture. Therefore, we used the vasopressin analogue [^{125}I][8-*p*-hydroxy-phenylpropionyl]-lys^8-vasopressin prepared recently in our laboratory (4, 5) to analyse vasopressin receptor expression during cell culture. Furthermore, we analysed the relationship between lys^8-vasopressin binding to its receptor on LLC-PK_1 cells and the lys^8-vasopressin-induced cAMP generation.

Materials and Methods

Materials

We used LLC-PK_1 cells (a porcine renal tubular cell line, ATCC CRL 1392). [^{125}I][8-*p*-hydroxy-phenylpropionyl]-lys^8-vasopressin, 74EBq/mol (2000 Ci/mmol) was prepared in our laboratory (1); the [^{125}I]cAMP assay was from Amersham Int. (Buckinghamshire, England). Lys^8-vasopressin was obtained from Peninsula Laboratories, Inc. (Belmont, USA). Unless otherwise stated, all reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany) and Sigma (Munich, Germany). The oxytocin antagonist, [^{125}I]d(CH_2)$_5$(Tyr(Me)^2,Tyr^4, Tyr-NH_2^9)-oxytocin, first described by *Elands* et al. (14), was iodinated by the Iodogen® method and purified as described (14).

Cell culture

LLC-PK$_1$ cells were grown in monolayers for 1 to 8 days in 75 cm^2 culture flasks containing *Ham*'s F12 medium (Seromed, Germany), fetal calf serum (volume fraction 0.06) (Seromed, Germany), 2 mmol/l *L*-glutamine and antibiotics (100 mg/l streptomycin and 100 · 10^3 U/l penicillin). The cells were cultured at 37 °C in a humidified CO_2 incubator (95% air and 5% CO_2). 20 000 cells were sown per cm^2 on the first day of cultivation. The medium was changed every 48 hours. Viability was estimated by the trypan blue method. Cell counts performed with the *Neubauer*-counting system were used to establish the growth pattern.

Preparation of crude plasma membrane fractions

Partially purified membranes from LLC-PK$_1$ cells were prepared as previously described (4). Briefly, cells were detached by incubation for 30 min at 37 °C in 50 mmol/l Tris, 0.15 mol/l NaCl, 1 g/l Na_2EDTA, pH 7.4. Afterwards cells were lysed using a hypotonic buffer (5 mmol/l Tris-HCl, 1 mmol/l EDTA and 3 mmol/l $MgCl_2$, pH 7.4), and finally the lysate was homogenized. The homogenate was centrifuged at 2000 *g* for 10 min and the pellet thus obtained was washed twice in assay buffer and immediately processed.

Binding studies

Plasma membranes of LLC-PK$_1$ cells (150 µg/tube) were incubated with [^{125}I][8-*p*-hydroxy-phenylpropionyl]lys^8-vasopressin (5000–2 000 000 counts per min per tube, which is equivalent to 0.012 nmol/l–4 nmol/l; incubation volume was 150 µl) for "hot saturation" studies. For "cold saturation" studies, we used 30 000 counts per min tracer and increasing amounts of lys^8-vasopressin. Unless otherwise stated, binding studies were performed at 22 °C for 90 min as described previously (4, 5). Assay buffer for binding studies contained 1 g/l bacitracin, 100 mmol/l Tris, 5 mmol/l $MgCl_2$, and 1 g/l bovine serum albumin, pH 7.8. Non-specific binding was determined by addition of 3.3 mmol/l lys^8-vasopressin. Two millilitres of cold binding buffer were added. After separation of free and bound radioactivity by centrifugation at 3000 *g* for 10 min at 4 °C, the membranes were washed twice in cold binding buffer.

[^{125}I]cAMP assay

cAMP production in LLC-PK$_1$-cell monolayers was determined in 96-well culture plates. The culture medium was aspirated and washed with assay buffer consisting of 150 mmol/l NaCl, 5 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l $CsCl_2$, 1 mmol/l 3-*iso*-butyl-1-methylxanthine, 20 mmol/l Tris-HCl, 1 g/l bovine serum albumin, and 1 g/l bacitracin, pH 7.8. The buffer was than aspirated and replaced with 150 µl of assay buffer containing lys^8-vasopressin at 22 °C; the incubation volume was 150 µl. After 120 min incubation, 0.25 ml cold ethanol (100%) was added to lyse the cells and stop cAMP production. After centrifugation (41 000 *g*, 4 °C), the medium was further diluted in radioimmunoassay buffer for the messurement of cAMP using a radioimmunoassay ([^{125}I]cAMP assay from Amersham Int., Buckinghamshire, England).

Results

[^{125}I][8-*p*-hydroxy-phenylpropionyl]-lys^8-vasopressin binding studies

The confluence was 39% on day 2, 79% on day 3, 95% on day 4, 97% on day 5 and 100% on day 6. The binding constant (K_d) did not change and was 0.46 ± 0.04 nmol/l between day 2 and day 6 (fig. 1). The receptor density per cell (fig. 2) increased markedly until day 6 (achievement of confluence) and it decreased markedly when hyperconfluence was reached (data not shown). Cell size increased only slightly from day 2 after seeding to day 6 after seeding (about 9%) and did not correlate with the number of receptors per cell (data not shown). However, the picture changes when the number of receptors is related to the protein content. Figure 3 demonstrates the changes in receptor density during cell cultivation expressed as receptors per mg protein. We also compared the binding of [^{125}I]d(CH$_2$)$_5$(Tyr(Me)^2,Tyr^4,Tyr-NH$_2^9$)-oxytocin, an oxytocin antagonist (14), with the binding of [^{125}I][8-*p*-hydroxy-phenylpropionyl]-lys^8-vasopressin to LLC-PK$_1$ cell monolayers on day 6 after seeding. The receptor density of [^{125}]d(CH$_2$)$_5$(Tyr(Me)^2,Tyr^4,Tyr-NH$_2^9$)-oxytocin was about 20% compared with the receptor density of [^{125}I][8-*p*-hydroxy-phenylpropionyl]-lys^8-vasopressin (fig. 4). About 20% of the [^{125}I][8-*p*-hydroxy-phenylpropionyl-lys^8-vasopressin binding sites could be replaced by [^{125}I]d(CH$_2$)$_5$(tyr(Me)^2,Tyr^4,Tyr-NH$_2^9$)-oxytocin (data not shown).

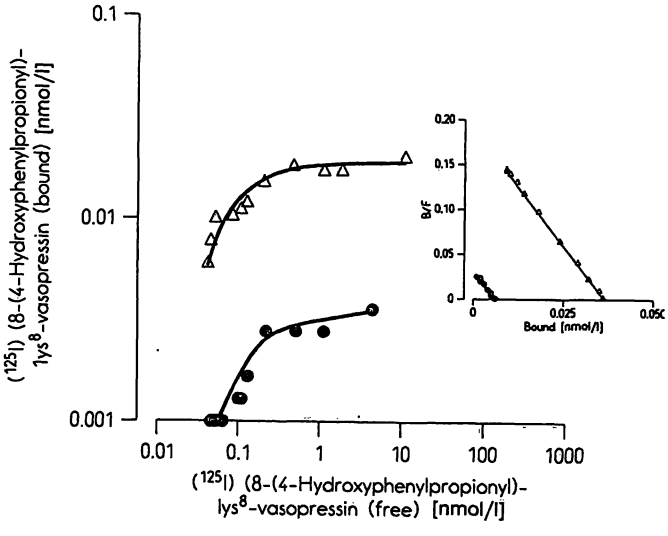

Fig. 1. Binding of [^{125}I][8-*p*-hydroxy-phenylpropionyl]-lys^8-vasopressin to LLC-PK$_1$ cell monolayers on day 2 (filled circles) and day 6 (open triangles) after seeding. The data were analysed according to *Scatchard* (insert). Binding assays (cold saturation) were carried out as described under Materials and Methods. Values are means from 2 individual determinations.

cAMP production in response to vasopressin

Addition of (8-lysine)-vasopressin to LLC-PK$_1$ cell monolayers on day 6 (100% confluence) was followed by a dose-dependent increase of intracellular cAMP accumulation (fig. 5). Lys^8-vasopressin (10 mmol/l)

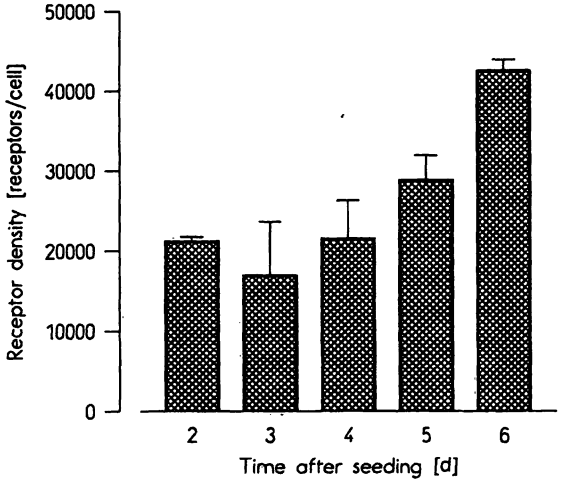

Fig. 2. Receptor density during cell cultivation expressed as receptors per cell. Values are means from 5 individual determinations ± S. D.

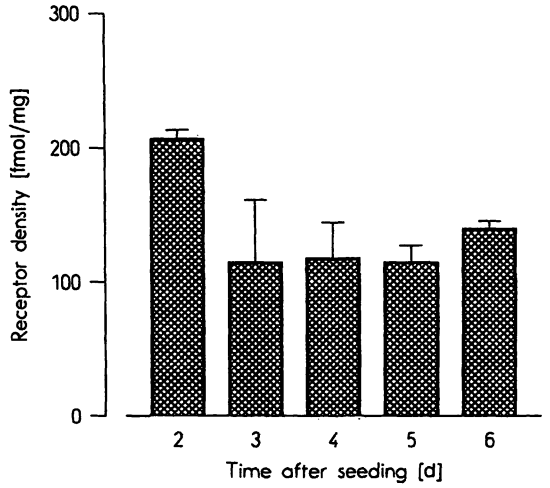

Fig. 3. Receptor density during cell cultivation expressed as receptors per mg of protein. Values are means from 5 individual determinations ± S. D.

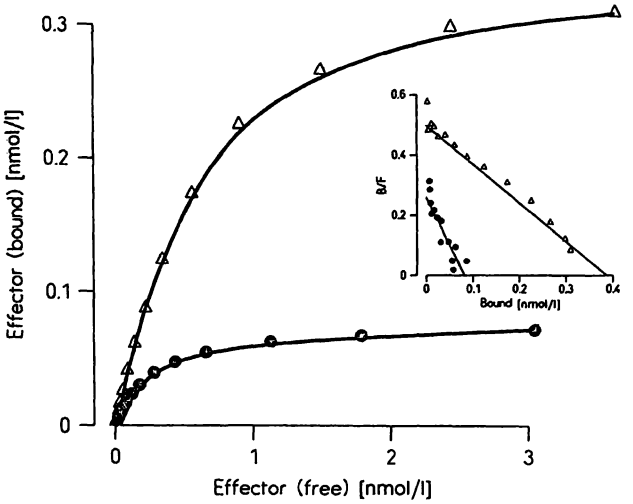

Fig. 4. Binding of [^{125}I][8-*p*-hydroxy-phenylpropionyl]-lys^8-vasopressin (open triangles) and [^{125}I]d(CH$_2$)$_5$(Tyr(Me)^2,Tyr^4,Tyr-NH$_2^9$)-oxytocin (filled circles) to LLC-PK$_1$ cells monolayers on day 6 after seeding. The data were analysed according to *Scatchard* (insert). Binding assays (hot saturation) were carried out as described under Materials and Methods. Values are means from 3 individual determinations.

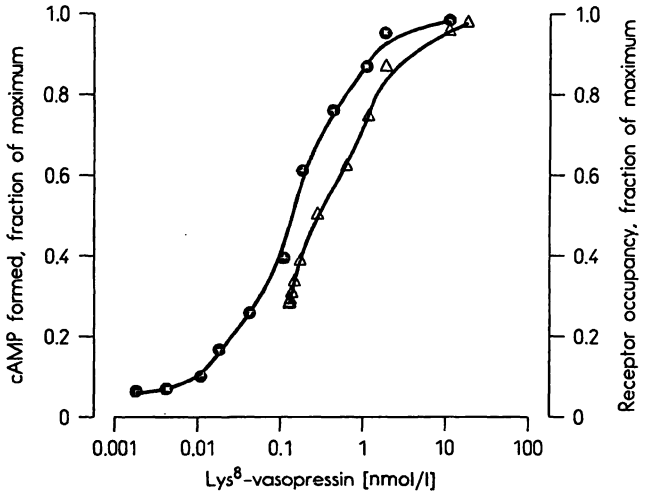

Fig. 5. Accumulation of cAMP in lys^8-vasopressin-treated LLC-PK$_1$ cells (filled circles) and lys^8-vasopressin binding to LLC-PK$_1$ cells (open triangles). The cAMP assay and the binding studies (cold saturation) were carried out as described under Materials and Methods. Values are means from 2 individual determinations.

increased intracellular cAMP to concentrations 16 times higher than the baseline cAMP production rate. The lowest lys^8-vasopressin concentration leading to a significant intracellular cAMP increase was 0.07 nmol/l. The binding constant (K_d), analysed in the same experiment was 0.48 nmol/l.

Discussion

The vasopressin V_2 receptor has been cloned previously and is a 370-amino-acid protein linked to adenylate cyclase with seven transmembrane domains and the characteristics of a G-protein-coupled receptor (6, 7). However, little is known about vasopressin-receptor expression during cell culture. Cell culture does not influence the binding constant of the vasopressin receptor (fig. 1), so that a modification of this receptor (altered phosphorylation or glycosylation) during cultivation is unlikely. On the other hand, the number of binding sites per cell is clearly related to confluence (fig. 2). Known regulators for the number of vasopressin binding sites per cell are vasopressin (homologous downregulation) (9) and other hormones or drugs (heterologous sensitation or desensitation) (6–8, 10). Growth behaviour in cell culture has not previously been reported to influence the expression of vasopressin receptors. The cell-to-cell interaction mechanisms (secreted autoregulatory factors or direct

cell-to-cell interactions) regulating the number of vasopressin receptors are unknown. The ratio of the number of receptors to the protein content is not a suitable quantity for investigation, because marked changes occur in the total cellular protein content during cell culture (fig. 3). Strictly identical culture phases and culture age are therefore important when studying the regulation of vasopressin receptors. An examination of the dose-effect relation between vasopressin – or [^{125}I][8-*p*-hydroxy-phenylpropionyl]-lys^8-vasopressin (fig. 5) – binding and receptor-mediated cAMP stimulation showed that a receptor occupancy of 30% is associated with a cAMP stimulation of 50%. But we should keep in mind that the relationship between vasopressin binding and cAMP formation is influenced by many other events, e. g.

(i) lateral mobility of the hormone-receptor complex in the plasma membrane,

(ii) receptor internalization,

(iii) G-protein-receptor interaction, and

(iv) G-protein-adenylate cyclase interaction (11, 12).

However, receptor reserve (under the conditions we used) might be even higher, since LLC-PK$_1$ cells express oxytocin receptors (13) and we found that approximately 20% of the occupied receptors are oxytocin receptors (fig. 4). [^{125}I][8-*p*-hydroxy-phenylpropionyl]-lys^8-vasopressin is also an oxytocin receptor ligand (data not shown). The oxytocin receptor does not stimulate cAMP formation and mainly interacts with the G-protein-mediated phosphatidylinositol pathway (13–15). Therefore, further studies should be performed to examine whether the relationship between vasopressin binding and receptor-mediated cAMP stimulation is also associated with the confluence of LLC-PK$_1$ cells in cell culture.

Acknowledgement

We wish to thank Prof. Dr. *F. Fahrenholz*, Frankfurt/Main, Germany for providing d(CH$_2$)$_5$(Tyr(Me)^3,Tyr^4,Tyr-NH$_2^9$)-oxytocin, and for valuable discussions. This study was supported by grants from the Deutsche Forschungsgemeinschaft (DFG He 1472/3-1).

References

1. Roy, C., Hall, D., Karish, M. & Ausiello, D. A. (1981) Relationship of (8-lysine)vasopressin receptor transition to receptor function properties in a pig kidney cell line (LLC-PK$_1$-cells). J. Biol. Chem. *256*, 3423–3427.
2. Bockaert, J., Roy, C., Rajerison, R. & Jard, S. (1973) Binding of [^{3}H]lysine-vasopressin to pig kidney plasma membranes. Relationship of receptor occupancy to adenylate cyclase acitivition. J. Biol. Chem. *248*, 5922–5931.
3. Orloff, J. & Handler, J. S. (1967) The role of adenosine 3′,5′-phosphate in the action of antidiuretic hormone. Am. J. Med. *42*, 757–768.
4. Dürr, J. A., Hensen, J. & Schrier, R. W. (1992) High specific activity ^{125}I- and ^{35}S-labeled vasopressin analogues with high affinity for the V$_1$ and V$_2$ vasopressin isoreceptors. J. Biol. Chem., in press.
5. Hocher, B., Merker, H.-J., Dürr, J. A., Schiller, S., Gross, P. & Hensen, J. (1992) Internalization of V$_2$-vasopressin receptors in LLC-PK$_1$-cells; Evidence for receptor-mediated endocytosis. Biochem. Biophys. Res. Commun. *186*, 1376–1383.
6. Birnbaumer, M., Antaramian, A., Themmen, A. P. N. & Gilbert, S. (1992) Desensitization of the human V$_2$ vasopressin receptor. J. Biol. Chem. *267*, 11783–11788.
7. Lolait, S. J., O'Carell, A.-M., McBride, O. W., Konig, M., Morel, A. & Brownstein, M. (1992) Cloning and characterization of vasopressin V$_2$ receptor and possible link to nephrogenic diabetes insipidus. Nature *357*, 336–339.
8. Lutz, W., Sanders, M., Salibury, J. & Kumar, R. (1990) Internalization of vasopressin analogues in kidney and smooth muscle cells: Evidence for receptor mediated endocytosis in cells with V$_2$ or V$_1$ receptors. Proc. Natl. Acad. Sci. USA *87*, 6507–6511.
9. Fishman, B. J., Dickey, B. F., Bucher, N. L. & Fine, R. E. (1985) Internalization, recycling, and redistribution of vasopressin receptors in rat hepatocytes. J. Biol. Chem. *260*, 12641–12646.
10. Hensen, J., Ehnis, T., Klein, C., Broser, M., Oelkers, W. & Dürr, J. A. (1992) Does chlorpropamide exert its antidiuretic action by receptor events? Acta Endocrinol. *126* (Suppl. 4); 141 (abstract).
11. Jans, D., Peters, R., Jans, P. & Fahrenholz, F. (1991) Vasopressin V$_2$-receptor mobile fraction and ligand-dependent adenylate cyclase activity are directly correlated in LLC-PK$_1$ renal epithelial cells. J. Cell Biol. *114*, 53–60.
12. Jans, D., Peters, R. & Fahrenholz, F. (1990) Lateral mobility of the phospholipase C-activating vasopressin V$_1$-type receptor in A7r5 smooth muscle cells: A comparison with the adenylate cyclase-coupled V$_2$-receptor. EMBO J. *9*, 2693–2699.
13. Canteau, B., Barjon, J. N., Chicot, D., Baskevitch, P. P. & Jard, S. (1990) Oxytocin receptors from LLC-PK$_1$-cells: Expression in Xenopus oocytes. Am. J. Physiol. *258*, F963–F972.
14. Elands, J., Barberis, C., Jard, S., Tribollet, E., Dreifuss, J.-J., Bankowski, K., Manning, M. & Sawyer, W. H. (1989) ^{125}I-Labeled d(CH$_2$)$_5$(Tyr(Me)^2,Tyr^4,Tyr-NH$_2^9$)OVP: A selective oxytocin receptor ligand. Eur. J. Pharmacol. *147*, 197–207.
15. Kimura, T., Tanizawa, O., Mori, K., Brownstein, M. J. & Okayama, H. (1992) Structure and expression of a human oxytocin receptor. Nature *356*, 526–529.

Dr. Berthold Hocher
Klinikum Steglitz der FU Berlin
Abteilung für Innere Medizin
Hindenburgdamm 30
W-1000 Berlin 45
Bundesrepublik Deutschland