Volume 281, number 1,2, 267–271 FEBS 09602 April 1991

ADONIS 001457939100311 5

cAMP-dependent protein kinase activation affects vasopressin V_2-receptor number and internalization in LLC-PK_1 renal epithelial cells

David A. Jans[1] and Brian A. Hemmings[2]

[1]*Max-Planck-Institut für Biophysik, Heinrich Hoffmannstr. 7, D-6000 Frankfurt am Main 71, Germany* and [2]*Friedrich Miescher Institut, Postfach 2543, CH-4002 Basel, Switzerland*

Received 11 February 1991

The relationship between activation of the cAMP-dependent protein kinase (cAMP-PK) and ligand binding and internalization by the vasopressin renal (V_2-type) receptor of LLC-PK_1 renal epithelial cells was examined. Upon cAMP-PK activation through 1 h treatment with the cAMP analogue 8-bromo-cAMP (BrcA), a marked reduction in V_2-receptor steady state number and internalization in LLC-PK_1 cells was effected. In cells treated for 17 h with BrcA and hence down-regulated for cAMP-PK, the V_2-receptor number was normal but internalization was markedly reduced. Cells of the LLC-PK_1 mutant FIB4, which possesses about 10% parental cAMP-PK catalytic subunit activity, exhibited lower V_2-receptor steady state number and internalization in comparison to untreated LLC-PK_1 cells. A negative correlation was thus evident between cAMP-PK activation and V_2-receptor number, and internalization. Phosphorylation by cAMP-PK may effect ligand-independent removal of receptor from the plasma membrane.

Down-regulation; Renal epithelial cell; Vasopressin V_2-receptor internalization; cAMP-dependent protein kinase

1. INTRODUCTION

Phosphorylation is a universal regulatory mechanism of cellular processes including growth, differentiation and transformation. In the adenylate cyclase (AC) system, the cAMP-dependent protein kinase (cAMP-PK) is activated by a concerted series of events initiated by the binding of hormone to receptor at the external surface of the plasma membrane [1,2]. cAMP production by AC leads to the elevation of intracellular cAMP levels, and dissociation of the cAMP-PK holoenzyme complex to release the active catalytic (C-) subunit [3,4]. A variety of cellular proteins serve as substrates for phosphorylation by the kinase, resulting in the modulation of a number of metabolic pathways and gene regulation [1,2]. Down-regulation or desensitization begins at the level of the receptor [5–7], but occurs at all stages of the above [2,5,8], including cAMP-PK itself [9,10]. The cAMP-PK [5–7,11,12] as well as the β-adrenergic receptor kinase [13,14] are capable of phosphorylating the β-adrenergic receptor, thereby regulating its transducing capacity and/or endocytosis.

This study examines the effect of in vivo activation of cAMP-PK on the vasopressin V_2-type receptor of LLC-PK_1 renal epithelial cells [15] which possess distinct receptors for vasopressin and calcitonin, both of which activate AC [16,17]. In addition to agents elevating intracellular cAMP, phorbol esters stimulate LLC-PK_1 cells to produce urokinase-type plasminogen activator by a cAMP-independent Ca^{2+}/phospholipid-dependent protein kinase (PK-C) -mediated pathway [18,19]. Here we examine cells of the LLC-PK_1 cell line, and those of the FIB4 mutant, which possesses normal amounts of cAMP-PK C-subunit, but only about 10% wild-type activity [18,20]. Treatments activating cAMP-PK or PK-C to differing extents were examined for their effects on V_2-receptor binding. We demonstrate a negative correlation between cAMP-PK activation and both V_2-receptor number and internalization.

Correspondence address: D.A. Jans, c/o Dr. F. Fahrenholz, Max Planck Institut für Biophysik, Kennedyallee 70, D-6000 Frankfurt am Main 70, Germany. Fax: (49) (69) 630 3423

Abbreviations: cAMP-PK, cAMP-dependent protein kinase or ATP:protein phosphotransferase (EC 2.7.1.37); cAMP, adenosine 3′,5′-monophosphate; BrcA, 8-bromo-cAMP; AC, adenylate cyclase or ATP pyrophosphatelyase (cyclicizing, EC 4.6.1.1); AVP, Arg^8-vasopressin; IBMX, 1-isobutyl-3-methylxanthine

2. MATERIALS AND METHODS

2.1. *Materials*

5′-[γ-^{32}P]ATP and [^{3}H]Arg^8-vasopressin (AVP) were from Amersham, and phosphocellulose paper (P-81) from Whatman. All other materials were from previously described sources [17,21].

2.2. *Cell culture*

The LLC-PK_1 pig kidney epithelial cell line [15] and the FIB4 mutant [18,20] were cultured as described previously [17].

2.3. *Enzyme assays*

Extracts for the assay of cAMP-dependent protein kinase (cAMP-PK) catalytic activity were prepared and assayed using Kemptide (L-R-R-A-S-A-G) as a substrate [17,21]. The cAMP-PK activity ratio expresses the C-subunit activity present in cell extracts (assayed in the absence of cAMP) relative to the total stimulatable activity (assayed

in the presence of cAMP) [17,21]. The ratio estimates the extent of cAMP-PK activation induced by different agents elevating intracellular cAMP levels [21–23]. Agonist-induced cAMP-PK activity could be completely inhibited by 100 nM protein kinase inhibitor peptide 5–24 [24]. Protein was estimated using the dye binding assay of Bradford [25] with BSA (fatty-acid-free) as a standard.

2.4. *Receptor binding*

Vasopressin binding by EDTA-suspended cells was measured using a filter assay [17]. Maximal binding capacity of cells at steady state was determined by measuring hormone binding after 60 min at 4°C. Internalized ligand was measured by incubating cells, subsequent to binding, with 200 mM Gly-HCl pH 3, 200 mM NaCl for 2 min at 4°C to remove externally bound ligand [26,27], prior to washing and filtration. Total specific binding at 30°C represents the summation of binding capacity, internalization and receptor recycling (see section 3).

3. RESULTS

We initially modified our binding assay for LLC-PK$_1$ cells in suspension [17] to quantitate the relative contribution of receptor internalization/recycling etc. to total maximal V_2-receptor binding activity (section 2). Half-maximal binding at 30°C and 4°C was achieved at 2.2 ± 0.1 min (Fig. 1, squares) and 18.6 ± 1.4 min (Fig. 1, closed circles) respectively (mean ± SEM for $n > 3$). Ligand internalization, estimated using a pH 3 treatment subsequent to binding, was half-maximal at 6.9 ± 1.6 min at 30°C (Fig. 1, triangles). No internalization was observed at 4°C (Fig. 1, empty circles). Maximal specific binding at 4°C (64 fmol/10^6 cells)

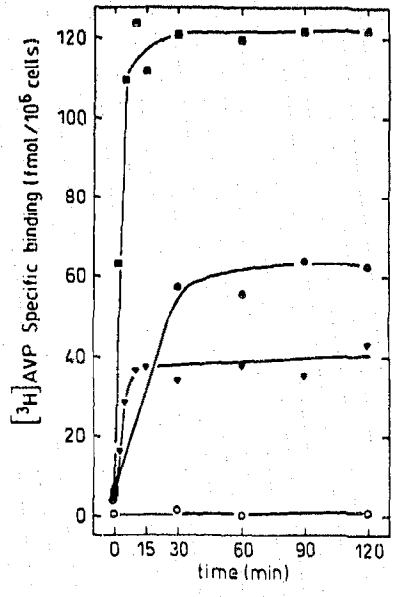

Fig. 1. Time course of [^{3}H]AVP specific binding and internalization in EDTA-suspended LLC-PK$_1$ cells. Total specific binding at 30°C (■—■) or 4°C (●—●), or internalization at 30°C (▼—▼) or 4°C (○—○) was determined as described in section 2. Results are the means from a single typical experiment for which the SEM was less than 11% the value of the mean. Maximal binding was achieved at 40.0 ± 3.4 and 10.8 ± 2.1 min (mean ± SEM, $n > 3$) at 4°C and 30°C, respectively; internalization was maximal at 15.7 ± 3.3 min.

Table I

[^{3}H]AVP specific binding and internalization in LLC-PK$_1$ cells

Treatment*		[^{3}H]AVP specifically bound (fmol/10^6 cells)*	
Before	After	4°C, 60 min	30°C, 30 min
None	None	63.9	120.4
None	pH 3	0.6	39.9
None	Trypsin	18.6	81.8
pH 3	None	77.5	123.2
pH 3	pH 3	0.6	41.0
Trypsin	None	42.7	52.4
NaN_3	None	60.2	97.7
NaN_3	pH 3	0.5	19.7

* Values represent the means from a single typical experiment from a series of similar experiments, where the SEM was less than 12% the value of the mean ($n = 4$).

† Treatments were identical before or after the binding test on suspended cells, and were at 4°C for 2 and 5 min for pH 3 (200 mM Gly-HCl, pH 3, 200 mM NaCl) and trypsin (0.25%). NaN_3 (10 mM) pretreatment was for 60 min at 37°C.

represents the number of binding sites at steady state (no contribution of internalization or receptor cycling/recycling); and accounted for about 53% of total maximal binding at 30°C (120 fmol/10^6 cells). Internalization at 30°C (40 fmol/10^6 cells) accounted for a further 33%, which is comparable to that observed in other systems (e.g. [28]).

Short trypsin treatment (0.25%, 5 min, 4°C) subsequent to binding was not as effective as pH 3 treatment in removing non-internalized ligand, since 29% of ligand bound at 4°C remained cell-associated (Table I). Cells pretreated at pH 3 prior to binding showed no reduction of either maximal specific [^{3}H]AVP binding nor internalization at 4°C or 30°C (Table I), indicating that pH 3 treatment did not irreversibly denature the V_2-receptor. In contrast, short trypsin pretreatment reduced maximal binding by 33% or 56% at 4°C or 30°C, respectively. NaN_3, which inhibits energy-dependent processes such as receptor endocytosis, did not affect the number of AVP-binding sites (4°C binding), but reduced internalization by 51% compared to untreated cells (Table I).

3.1. *[^{3}H]AVP binding and internalization in LLC-PK$_1$ cells treated with agents elevating intracellular cAMP levels*

LLC-PK$_1$ cells were pretreated with the cAMP analogue 8-bromo-cAMP (BrcA) for either 1 h or 17 h, and then cAMP-PK activation (Table II) and maximal specific [^{3}H]AVP binding and internalization were determined (Fig. 2). 1 h treatment (cAMP-PK activity ratio of 0.42) resulted in a marked reduction of both AVP internalization (54% reduced compared to untreated cells) and binding at 4°C (35% decreased). 17 h treatment (conditions of down-regulated cAMP-PK; activity ratio of 0.24) also induced a reduction in internalization (51% reduced compared to untreated con-

Table II

cAMP-PK activities in cell-free extracts from the LLC-PK$_1$ and FIB4 cell lines in response to various agonists

Treatment	cAMP-PK activity (U/mg)*		cAMP-PK activity
	− 10 μM cAMP (A)	+ 10 μM cAMP (B)	ratio (A/B)
I. LLC-PK$_1$ cell line			
No addition	0.10	2.95	0.03
10^{-3} M IBMX	0.31	2.84	0.11
10^{-7} M AVP	0.27	2.73	0.10
IBMX/AVP	2.09	2.75	0.76
10^{-3} M BrcA (1 h)	1.21	2.88	0.42
10^{-3} M BrcA (17 h)	0.20	0.85	0.24
3×10^{-8} M PMA (1 h)	0.10	2.72	0.04
3×10^{-8} M PMA (48 h)	0.10	2.66	0.04
II. FIB4 cell line			
No addition	0.04	0.43	0.10
10^{-3} M IBMX	0.07	0.42	0.17
10^{-7} M AVP	0.09	0.43	0.22
IBMX/AVP	0.34	0.41	0.82
10^{-3} M BrcA (1 h)	0.16	0.40	0.40
10^{-3} M BrcA (17 h)	0.02	0.08	0.28

* Cell monolayers were treated for 30 min or the times indicated in serum-free DMEM, prior to washing and preparation of cell extracts. Extracts were then assayed in the presence or absence of exogenously added cAMP. Data represent a single typical experiment performed in triplicate, for which the SEM was less than 11% the value of the mean.

trol). Reduced AVP internalization did not appear to result from an altered V_2-receptor affinity for ligand (not shown).

Results were compared to cells pretreated for 1 h or 48 h with 3×10^{-8} M phorbol-myristate acetate (PMA), treatments which induce activation and down-regulation

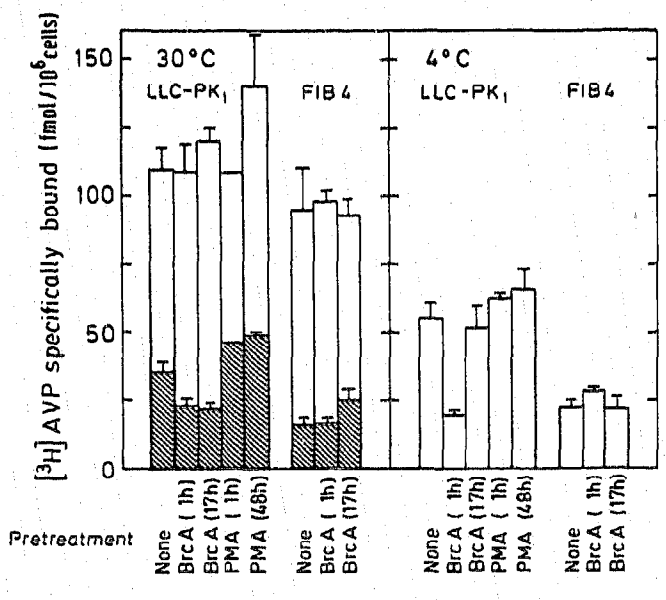

Fig. 2. Maximal specific [^{3}H]AVP binding and internalization in cells of the LLC-PK$_1$ and cAMP-PK mutant FIB4 cell lines. Total specific binding (□) and internalization (▨) were determined after 30 and 60 min at 30°C and 4°C, respectively, on cells pretreated as indicated. The results shown are the means with SEM shown for more than 3 experiments performed in duplicate.

respectively of PK-C [18,19,29]. Compared to untreated cells, PMA-treated cells exhibited slightly increased internalization at 30°C and binding at 4°C. Total binding at 30°C was essentially comparable for all of the variously treated and untreated cells, with the possible exception of PK-C-down-regulated cells, which showed 19% higher total binding.

The data for LLC-PK$_1$ cells under the above various conditions (from Table II and Fig. 2) revealed a negative correlation ($r = -0.92$, $x = 65.8$, $y = -96.7$, $n = 5$) between the cAMP-PK activity ratio and the number of V_2-receptors at steady state (binding at 4°C). A negative correlation ($r = -0.89$, $x = 47.1$, $y = -69.6$, $n = 5$) was also observed for cAMP-PK activity ratio and AVP internalization.

3.2. *[^{3}H]AVP binding and internalization in the cAMP-PK C-subunit mutant FIB4*

The FIB4 mutant [20] was similarly analyzed for AVP binding and internalization. FIB4 cells, with or without BrcA pretreatment, showed total binding at 30°C essentially comparable to that of LLC-PK$_1$ cells (Fig. 2). The extent of cAMP-PK activation (cAMP-PK activity ratio) upon BrcA treatment (1 h or 17 h) was also comparable for both cell lines (Table II), although the absolute cAMP-PK activities were largely different due to the C-subunit mutation of FIB4. However, maximal specific [^{3}H]AVP internalization was markedly lower (more than 40% reduced), as was steady state binding activity (4°C) (50% reduced) compared to untreated LLC-PK$_1$ cells. FIB4 cells thus largely resembled BrcA-treated (down-regulated) LLC-PK$_1$ cells in

their [^{3}H]AVP steady state binding and internalization (Fig. 2).

3.3. *The effect of other cAMP agonists on V_2-receptor function in LLC-PK$_1$ cells*

To determine whether other agents elevating intracellular cAMP levels have an effect on AVP-binding, LLC-PK$_1$ cells were pretreated with either forskolin (AC activator) or salmon calcitonin for 17 h and compared to BrcA-treated or untreated cells for [^{3}H]AVP binding (Fig. 3). All agonist pretreatments reduced [^{3}H]AVP internalization (Fig. 3), indicating that agents other than BrcA which bring about cAMP-PK down-regulation [10] also effect a reduction in V_2-receptor internalization.

4. DISCUSSION

The results here suggest a role for the cAMP-PK in regulating ligand binding and internalization by the V_2-receptor of LLC-PK$_1$ cells. Treatments resulting in the stimulation and subsequent down-regulation of cAMP-PK markedly reduced the steady state V_2-receptor number and internalization. Interestingly, the cAMP-PK C-subunit mutant FIB4 also showed a low steady state receptor number and internalization. Activation of the cAMP-PK by BrcA (and probably also by vasopressin itself) appears to effect endocytosis of plasma membrane receptors even though they are not occupied by ligand, and concomitant reduction in ligand-dependent internalization, presumably mediated by phosphorylation. That heterologous hormone (calcitonin)- or forskolin-mediated stimulation of LLC-PK$_1$ cells similarly affected V_2-receptor binding implies the physiological relevance of this effect. The V_2-receptor thus probably resembles the β-adrenergic and muscarinic G-protein-coupled receptors [5,11,12,30] in the regulatory role of specific kinases in receptor desensitization.

Other kinases may also have an influence on V_2-receptor function. PK-C plays a role in desensitization in the β-adrenergic system [5,31], but we observed no marked effect of PMA on V_2-internalization here, and an elevated rather than reduced steady state V_2-receptor number (see Fig. 2). In the case of the receptors for the tumour necrosis factor and EGF, PMA induces receptor down-regulation in the absence of ligand, due to a reduction in the number of plasma membrane receptors [32]. Interestingly, this parallels the effects of cAMP agonists on the V_2-receptor. Further examination of the various complex feedback mechanisms using in vivo systems, together with mutants affected in specific components of signal transduction, should assist in elucidating the processes regulating receptor function.

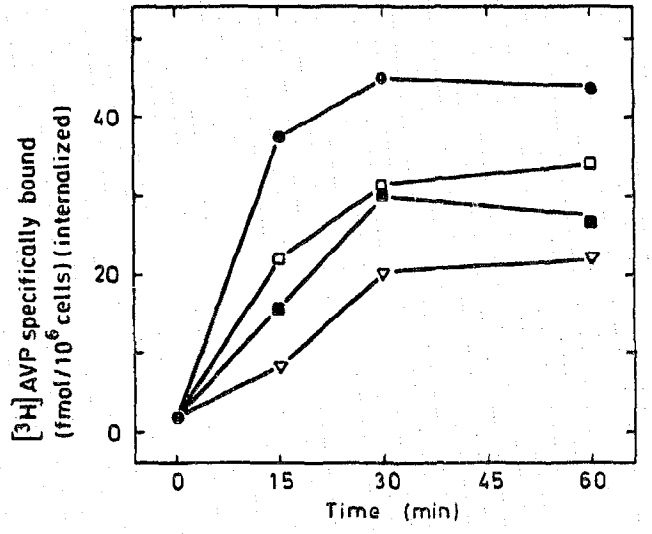

Fig. 3. Influence of agents elevating intracellular cAMP on [^{3}H]AVP internalization in LLC-PK$_1$ cells. The time course of internalization at 30°C was followed in LLC-PK$_1$ cells which had been pretreated for 17 h without (●—●) or with 1 mM BrcA (▽—▽), 10 μM forskolin (□—□), or 30 nM salmon calcitonin (■—■). Results are the means from a single typical experiment for which the SEM was less than 12% the value of the mean.

Acknowledgements: The authors gratefully acknowledge the cheerful technical assistance of Patricia Jans, T. Riemenschneider and A. Kraft for careful reading of the manuscript, and Dr. Falk Fahrenholz and the Deutsche Forschungs Gemeinschaft (SFB 169) for financial support.

REFERENCES

[1] Lohmann, S.M. and Walter, U. (1984) Adv. Cyclic Nucleot. Prot. Phosphorylation Res. 18, 63-117.
[2] Steinberg, R.A. (1983) Biochem. Actions Horm. 11, 25-65.
[3] Flockhart, D.A. and Corbin, J.D. (1982) CRC Crit. Rev. Biochem. 12, 133-186.
[4] Bechtel, P.J., Beavo, J.A. and Krebs, E.G. (1977) J. Biol. Chem. 252, 2691-2697.
[5] Benovic, J.L., Bouvier, M., Caron, M.G. and Lefkowitz, R.J. (1988) Annu. Rev. Cell Biol. 4, 405-428.
[6] Sibley, D.R. and Lefkowitz, R.J. (1987) Nature 317, 124-129.
[7] Sibley, D.R., Strasser, R.H., Caron, M.G. and Lefkowitz, R.J. (1986) Cell 48, 913-918.
[8] Jans, D.A. and Hemmings, B.A. (1988) Adv. Second Messenger Phosphoprotein Res. 21, 109-121.
[9] Alhanaty, E., Tauber-Finkelstein, M., Schmeeda, H. and Shaltiel, S. (1985) Curr. Top. Cell. Regulation 27, 267-278.
[10] Hemmings, B.A. (1986) FEBS Lett. 196, 196-200.
[11] Sibley, D.R., Peters, J.R., Nambi, P., Caron, M.G. and Lefkowitz, R.J. (1984) J. Biol. Chem. 259, 9742-9749.
[12] Benovic, J.L., Pike, L.J., Cerione, R.A., Staniszewski, C., Yoshimasa, T., Codina, J., Caron, M.G. and Lefkowitz, R.J. (1985) J. Biol. Chem. 260, 7094-7101.
[13] Benovic, J.L., Regan, J.W., Matsui, H., Mayor, F., Cotecchia, S., Leeb-Lundberg, L.M.F., Caron, M.G. and Lefkowitz, R.J. (1987) J. Biol. Chem. 262, 17251-17253.
[14] Benovic, J.L., Kuhn, H., Weyand, I., Codina, J., Caron, M.G. and Lefkowitz, R.J. (1987) Proc. Natl. Acad. Sci. USA 84, 8879-8882.
[15] Hull, R.N., Cherry, W.R. and Weaver, G.W. (1976) In vitro 12, 670-677.
[16] Dayer, J.-M., Vassalli, J.-A., Bobbit, J.L., Hull, R.N., Reich, E.R. and Krane, S.H. (1981) J. Cell Biol. 91, 195-200.
[17] Jans, D.A., Resink, T.J., Wilson, E.R., Reich, E. and Hemmings, B.A. (1986) Eur. J. Biochem. 160, 407-412.

[18] Jans, D.A. and Hemmings, B.A. (1986) FEBS Lett. 205, 127–131.
[19] Degen, J.D., Estensen, R.D., Nagamine, Y. and Reich, E. (1985) J. Biol. Chem. 260, 12426–12433.
[20] Botterell, S.H., Jans, D.A. and Hemmings, B.A. (1987) Eur. J. Biochem. 164, 39–44.
[21] Jans, D.A., Resink, T.J. and Hemmings, B.A. (1987) Biochem. J. 243, 413–418.
[22] Soderling, T.R., Corbin, J.D. and Park, C.R. (1974) Methods Enzymol. 38, 358–367.
[23] Corbin, J.D. (1983) Methods Enzymol. 99, 227–232.
[24] Scott, J.D., Glaccum, M.B., Fischer, E.H. and Krebs, E.G. (1986) Proc. Natl. Acad. Sci. USA 83, 1613–1616.
[25] Bradford, W.M. (1976) Anal. Biochem. 72, 248–255.
[26] Schneider, H.-G., Raue, F., Zink, A., Koppold, A. and Ziegler, R. (1988) Mol. Cell. Endocrinol. 58, 9–15.
[27] Jans, D.A., Peters, R., Zsigo, J. and Fahrenholz, F. (1989) EMBO J. 8, 2481–2488.
[28] Zidovetzki, R., Yarden, Y., Schlessinger, J. and Jovin, T.M. (1981) Proc. Natl. Acad. Sci. USA 78, 6981–6985.
[29] Jans, D.A., Dierks-Ventling, C. and Hemmings, B.A. (1987) Exp. Cell Res. 172, 76–83.
[30] Haga, K. and Haga, T. (1989) Biomed. Res. 10, 293–299.
[31] Sibley, D.R., Nambi, P., Peters, J.R. and Lefkowitz, R.J. (1984) Biochem. Biophys. Res. Commun. 21, 973–977.
[32] Aggarwal, B.B. and Eessalu, T.E. (1987) J. Biol. Chem. 262, 16450–16455.