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Oxytocin induced cAMP-dependent protein kinase activation and urokinase-type plasminogen activator production in LLC-PK_1 renal epithelial cells is mediated by the vasopressin V_2-receptor

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Using a variety of peptide analogues of oxytocin (OT) and Arg^8-vasopressin (AVP), OT-mediated induction of urokinase-type plasminogen activator (uPA) was examined in LLC-PK_1 renal epithelial cells, which possess distinct high-affinity receptors of both the OT- and vasopressin renal (V_2-) types. OT or OT-receptor specific agonists induced concentration-dependent cAMP synthesis, activation of the cAMP-dependent protein kinase (cAMP-PK) and uPA production consistent with their respective binding affinities for the V_2- and not the OT-receptor. OT-mediated uPA induction could be inhibited in a concentration-dependent fashion by coincubation with a V_2/V_1-receptor specific antagonist, but not by an OT-receptor specific antagonist. Results implied that stimulation of cAMP- and uPA responses in LLC-PK_1 cells by OT was V_2-receptor-mediated.

Neurohypophyseal nonapeptide hormone; Specific antagonist; Vasopressin V_2-receptor; Urokinase-type plasminogen activator

1. INTRODUCTION

The specific binding of the neurohypophyseal nonapeptide oxytocin (OT) to high-affinity receptors in myometrium [1–3] leads to inositol-trisphosphate turnover and Ca^{2+} influx [4,5], responses which are similar to those induced by the binding of the other neurohypophyseal nonapeptide, vasopressin, to the hepatic vasopressin V_1-receptor [6,7]. Interestingly, OT can have either diuretic or antidiuretic osmoregulatory effects depending on the presence or absence of vasopressin [8–10], which may be explained by the ability of OT to bind to the adenylate cyclase (AC)-stimulating vasopressin renal V_2-type receptor present on distal tubules and collecting ducts [9,10]. OT has also been reported to enhance glomerular filtration rate [8] and to have a natriuretic effect [8,9], both of which are OT-specific [8]. Stimulation of glomerular filtration is probably mediated by OT-receptors in the macula densa [11].

The LLC-PK_1 renal epithelial cell line has been reported to possess low density high-affinity OT-receptors [12,13], in addition to V_2-receptors (about 45,000/cell) [14], which couple to AC stimulation [14,15], activation of the cAMP-dependent protein kinase (cAMP-PK) [14–16], and production of the extracellularly secreted protease urokinase-type plasminogen activator (uPA) [14,15,17]. In addition to agents elevating intracellular cAMP, phorbol esters stimulate LLC-PK_1 cells to produce uPA by a cAMP-independent Ca^{2+}/phospholipid-dependent protein kinase (PK-C)-mediated pathway [14,18–21].

We were interested in the second messenger pathway of OT-mediated uPA induction in LLC-PK_1 cells. We show here that OT is capable of stimulating cAMP synthesis, cAMP-PK activation and uPA induction in LLC-PK_1 cells. All of these responses, however, appear to be mediated by OT-binding to the V_2-receptor.

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Abbreviations: cAMP-PK, cAMP-dependent protein kinase or ATP:protein phosphotransferase (EC 2.7.1.37); AC, adenylate cyclase or ATP pyrophosphatelyase (cyclicizing, EC 4.6.1.1); AVP, Arg^8-vasopressin; OT, oxytocin; uPA, urokinase-type plasminogen activator (EC 3.4.21.31); IBMX, 1-isobutyl-3-methylxanthine.

2. MATERIALS AND METHODS

2.1. *Materials*

5′-γ-[^{32}P]ATP, [^{3}H]Arg^8-vasopressin (AVP) and [^{3}H]OT were from Amersham, and phosphocellulose paper (P-81) from Whatman. The OT and AVP analogues used, listed in Table I, were synthesized as described [3,22–25]. Other materials were from previously described sources [14,26].

2.2. *Cell culture*

Cells of the LLC-PK_1 pig kidney epithelial cell line [27] and the V_2-receptor deficient mutant M18 [14] were cultured as described previously [14,26].

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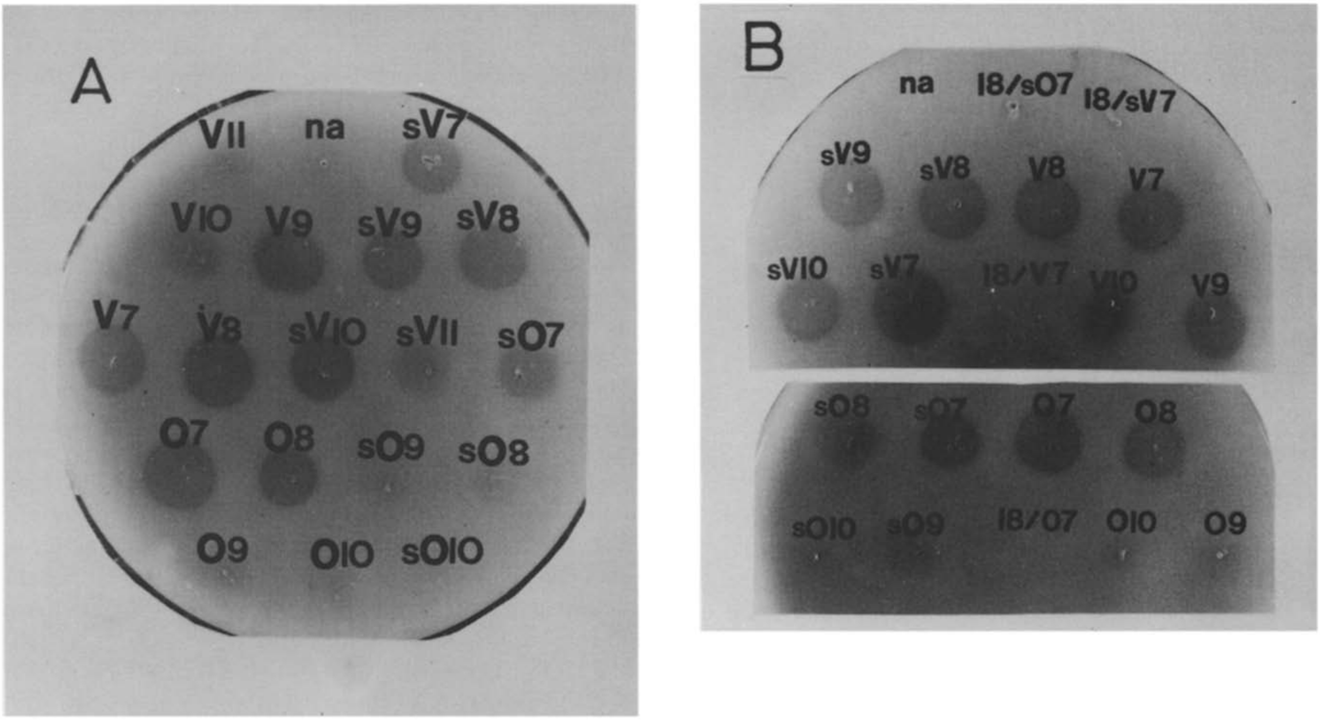

Fig. 1. (A) Concentration dependence of uPA production by LLC-PK$_1$ cells in response to OT and AVP analogues. Cell monolayers were washed extensively and then incubated for 8 h with agonists in serum-free DMEM as indicated: O (oxytocin), sO (dSOT), V (AVP), sV (SAVP), na (no addition), and the numbers represent the molar concentrations in negative log form. Medium (5 μl) was spotted onto the casein-plasminogen agar, and, after incubation at 37°C for 2–3 h, uPA activity became visible as zones of lysis in the agar. (B) uPA production by cells of the LLC-PK$_1$ and V$_2$-receptor deficient mutant M18 cell lines in response to OT and AVP analogues. Cell monolayers of the LLC-PK$_1$ and M18 (18) cell lines were treated as in Fig. 1A.

2.3. *Enzyme assays*

Extracts for the assay of cAMP-PK catalytic activity were prepared and assayed as previously, using Kemptide (L-R-R-A-S-A-G) as substrate [14,26]. 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) [14,26]. The ratio estimates the extent of cAMP-PK activation induced by different agents elevating intracellular cAMP levels [26,28,29]. uPA activity was detected using the previously described spot test [30,31], with activity indicated by zones of proteolytic clearing in the casein, due to plasmin activation. No proteolytic activity was observed in the absence of plasminogen. Protein was estimated using the dye binding assay of Bradford [32] with BSA (fatty-acid-free) as standard.

2.4. *cAMP determination*

Cells were treated with hormones and other agonists for 8 h as previously described [33]. Medium samples were then treated as described [25,26], and cAMP determined using the competitive protein binding assay of Tovey et al. [34].

2.5. *Receptor binding*

Vasopressin and oxytocin binding by EDTA-suspended cells was measured at 30°C as described [14,35]. Dissociation constants (K_D), the concentration of hormone corresponding to 50% maximal binding, were determined from competition binding experiments [3,14,23].

3. RESULTS

Maximal specific [^{3}H]AVP and [^{3}H]OT binding (10^{-8} M ligand) by LLC-PK$_1$ cells was 223 ± 24 (n = 4) and

Table I

Dissociation constants of the neurohypophyseal structural analogues used in this study for [^{3}H]AVP and [^{3}H]OT in LLC-PK$_1$ cells

Peptide/description [reference]	K_D for binding sites$^+$ (nM) for:	
	[^{3}H]AVP	[^{3}H]OT
[Arg^8]vasopressin (AVP)	1.4 ± 0.6	4.6
Oxytocin (OT)	776	2.8 ± 0.4
[Mpa^1,Sar^7]OT (dSOT)/OT-agonist [3,22]	820	4.5 ± 0.4
[Thr^4,Sar^7]OT (TSOT)/OT-agonist [3]	>103	ND*
[Sar^7]AVP (SAVP)/V$_2$-agonist [23]	3.6 ± 0.9	78
[Mpa^1,Val^4,Sar^7]AVP (dVSAVP)/ V$_2$-agonist [22]	ND*	ND*
[Mca^1,MeAla^7]AVP (MMAVP)/ V$_1$/OT-antagonist [23]	101	ND*
[Mca^1,D-Phe^2,Sar^7]AVP (MFSAVP)/ V$_1$/OT-antagonist [23,24]	52 ± 8	4.4 ± 1.0
[Mca^1,D-Phe^2,Ile^4,Lys^9]AVP (MFIKAVP)/ V$_2$/V$_1$-antagonist [25]	ND*	ND*
[Mca^1,Sar^7,Orn^8]OT (MSOOT)/OT-antagonist^a	6,510	40

$^+$$K_D$ was determined from competition binding studies with [^{3}H]AVP and [^{3}H]OT as described [23–25], using affinity constants (Scatchard analysis) for [^{3}H]AVP and [^{3}H]OT in LLC-PK$_1$ cells of 3.5 [14] and 1.9 [12], respectively. Results represent the mean from at least two separate experiments (n = 3 where S.D. is indicated).

*Not determined.

^{a} Inhibitory activity in rat uterus in vitro: pA$_2$ = 7.34 (Mg^{2+}-free medium); a detailed description of the synthesis and pharmacological properties of this OT-antagonist will be published elsewhere.

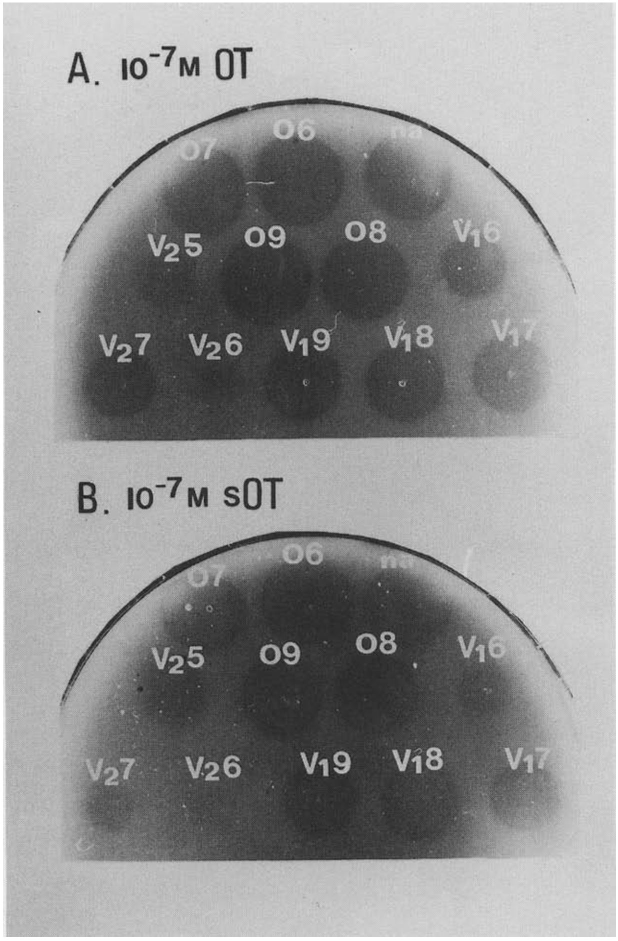

Fig. 2. uPA production by LLC-PK$_1$ cells stimulated by OT or dSOT in the absence (na) or presence of OT- (0), V_2/V_1- (V2) or V_1/OT- (V1) specific antagonists. Cell monolayers were treated as in the legend to Fig. 1A,B, in the presence of differing concentrations (designated as per Fig. 1A,B) of the antagonists MAAVP (V1), MFIKAVP (V2) or MSOOT (O).

34 ± 8.5 (n = 4) fmol/mg respectively. Binding was further characterized by competition with the various AVP and OT analogues listed in Table I. The dissociation constants (K_D) calculated from the displacement binding curves (not shown) are also listed. Reduced competition of [^{3}H]AVP binding was exhibited by OT, OT-receptor and V_1-specific ligands (15- to 15,000-fold difference in K_D), compared to the V_2-specific agonists SAVP and dVSAVP (Table I, and not shown). This implied that [^{3}H]AVP binds predominantly to the V_2-receptor of LLC-PK$_1$ cells.

[^{3}H]OT binding could be competed quite well by OT- and V_1-specific ligands compared to the V_2-agonist SAVP (about a 20-fold difference in K_D, Table I). AVP, however, also competed reasonably well for [^{3}H]OT-binding sites (K_D of 4.6 nM compared to 2.8 nM for OT itself), implying that the OT-receptor of LLC-PK$_1$ cells can bind AVP, consistent with similar observations for OT-receptors from other tissues [3,36,37]. In conclusion, the V_2-receptor accounted for about 95% of the specific [^{3}H]AVP binding at 10^{-8} M ligand (less than 5% of the binding likely to be due to binding to the OT-receptor); whilst the OT-receptor accounted for about 99% of the specific [^{3}H]OT binding at 10^{-8} M OT (Table I, and not shown).

3.1. *cAMP production and cAMP-PK activation in LLC-PK$_1$ cells in response to OT*

We tested the analogues from Table I for the stimulation of cAMP production and cAMP-PK activation in LLC-PK$_1$ cells (Table IIA,B). Results for the non-receptor-mediated AC-activator forskolin are shown for comparison. Whilst even high concentrations of the OT-agonists dSOT and TSOT only very weakly stimulated cAMP synthesis (Table IIA, and not shown) and cAMP-PK activation (Table IIB, and not shown), high concentrations of OT induced marked responses. Whereas half-maximal responses were elicited at about 10^{-7} M OT (Table IIA,B, and not shown) [12], half-maximal cAMP production and cAMP-PK activation in response to AVP are at about 10^{-9} M (Table IIA,B) [12,37,38]. The V_2-specific agonists dVSAVP and SAVP showed similar K_as (Table IIA,B, and not shown) [25]. Thus, although OT and to a minor extent OT-specific agonists were capable of inducing cAMP-mediated responses, the concentration-dependence of the responses was consistent with their respective affinities for the V_2-receptor of LLC-PK$_1$ cells. In contrast, half-maximal response in terms of Ca^{2+} influx occurs at about 5 × 10^{-9} M OT in LLC-PK$_1$ cells [12].

3.2. *uPA induction in LLC-PK$_1$ cells by OT and OT-specific agonists*

The above results were elaborated by testing the various analogues for the stimulation of uPA-production in LLC-PK$_1$ cells. uPA activity was detected by spotting 8h conditioned media onto a casein plasminogen agar, and incubating at 37°C [30,31], activity being indicated by zones of plasminogen-dependent proteolytic clearing in the agar (Fig. 1). Results (not shown) could be confirmed using a colorimetric assay with the synthetic peptide substrate S-2251 [14,19,20,26]. OT and dSOT stimulated uPA production (Fig. 1A) but only at concentrations about 100 times higher than those for maximal induction by AVP and SAVP, consistent with the affinity of the respective ligands for the V_2-receptor of LLC-PK$_1$ cells.

Treatment of the V_2-receptor deficient mutant M18 [14] resulted in no uPA production (Fig. 1B), supporting the conclusion that OT-stimulated induction of LLC-PK$_1$ cells was V_2-receptor mediated. Further evidence was provided by coincubating 10^{-9} M AVP or SAVP (not shown) or 10^{-7} M OT or dSOT (Fig. 2) with various concentrations of OT-, V_1/OT-, or V_2/V_1-specific antagonists. Whilst the OT-antagonist MSOOT failed to block OT- or dSOT-mediated uPA induction, the V_2/V_1-specific antagonist MFIKAVP inhibited uPA induction in a concentration-dependent manner (Fig. 2). These results were consistent with OT- and OT-specific agonist uPA induction in LLC-PK_1 cells being V_2-mediated.

4. DISCUSSION

The results here show that OT as well as OT-specific agonists can stimulate cAMP synthesis, cAMP-PK activation and uPA induction in LLC-PK_1 cells. The concentration dependence of the responses, the lack of OT-response of the V_2-deficient LLC-PK_1 mutant M18, and the inhibition of OT-mediated uPA induction by an V_2/V_1-receptor specific, but not OT-receptor specific antagonist, strongly indicate that these responses are all mediated by binding of the respective ligands to the V_2-, and not to the OT-receptor of LLC-PK_1 cells. Although activation of the Ca^{2+}-phospholipid dependent kinase (PK-C) by phorbol esters has been shown to stimulate uPA production in LLC-PK_1 cells independently of cAMP and cAMP-PK [14,18–21], binding of OT to the OT-receptor does not appear to lead to significant induction of uPA synthesis, at least within 8 h, as shown here. PK-C activation would accordingly not seem to couple to stimulation of the OT-receptor in LLC-PK_1 cells. Further investigations using specific OT-agonists and antagonists should assist in elaborating the precise events of signal transduction following OT-stimulation.

Table IIA

Extracellular cAMP production induced by V_2- and OT-specific agonists and antagonists in LLC-PK_1 and M18 cells

Treatment*	Extracellular cAMP production (pmol/10^6 cells)$^+$			
	LLC-PK_1		M18	
	−IBMX	+IBMX	−IBMX	+IBMX
No addition	21	116	15	59
50 μM forskolin	210	4,560	236	3,740
10^{-8} M AVP	380	8,830	14	141
10^{-8} M dVSAVP	282	6,010	14	112
10^{-7} M OT	38	555	11	52
10^{-7} M dSOT	23	112	13	50

*Cell monolayers were treated for 8 h in serum-free DMEM in the absence or presence of 500 μM IBMX as indicated.
$^+$Medium cAMP was determined as described in section 2, and is expressed per 10^6 cells. Data represent the mean from a single typical experiment where the S.D. was less than 22% the value of the mean.

Table IIB

cAMP-PK activation in LLC-PK_1 cells induced by forskolin, V_2- and OT-specific agonists and V_1/OT- and OT-specific antagonists

Treatment*	cAMP-PK activity ratio	
	−IBMX	+IBMX
No addition	0.02 ± 0.01	0.14 ± 0.04 (6)
50 μM forskolin	0.20 ± 0.04	0.63 ± 0.02
10^{-7} M AVP	0.18 ± 0.05	0.82 ± 0.07 (3)
10^{-8} M AVP	0.11 ± 0.01	0.58 ± 0.05 (3)
10^{-9} M AVP	0.07 ± 0.01	0.36 ± 0.06 (3)
10^{-6} M OT	0.07 ± 0.02	0.51 ± 0.05 (3)
10^{-7} M OT	0.05 ± 0.02	0.29 ± 0.12 (3)
10^{-8} M OT	0.03 ± 0.01	0.05 ± 0.01 (3)
10^{-6} M dSOT	0.03 ± 0.01	0.18 ± 0.02
10^{-7} M dSOT	0.03 ± 0.01	0.05 ± 0.01
10^{-6} M TSOT	0.06 ± 0.01	0.16 ± 0.02
10^{-7} M TSOT	0.02 ± 0.01	0.07 ± 0.01
10^{-8} M SAVP	0.08 ± 0.01	0.64 ± 0.06
10^{-6} M MAAVP	0.04 ± 0.01	0.08 ± 0.01

*Cell monolayers were treated for 30 min in serum-free DMEM in the absence or presence of 500 μM IBMX as indicated, prior to washing and preparation of cell extracts. Extracts were then assayed in the presence or absence of exogenously added cAMP (10 μM). Data represent the mean ± S.D. (n in parentheses where n > 2). Total cAMP-PK activity (assayed in the presence of 10 μM cAMP) was 2.8 ± 0.2 U/mg.

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REFERENCES

[1] Soloff, M.S. and Swatz, T.L. (1973) J. Biol. Chem. 248, 6471–6478.
[2] Soloff, M.S. and Grzonka, Z. (1986) Endocrinology 119, 1564–1569.
[3] Fahrenholz, F., Hackenberg, M. and Müller, M. (1988) Eur. J. Biochem. 174, 81–85.
[4] Schrey, M.P., Read, A.M. and Steer, P.J. (1986) Biosci. Rep. 6, 613–619.
[5] Batras, J.L. (1986) Eur. J. Pharmacol. 120, 57–61.
[6] Bojanic, D. and Fain, J.N. (1986) Biochem. J. 240, 361–365.
[7] Fitzgerald, T.J., Uhling, R.J. and Exton, J.H. (1986) J. Biol. Chem. 261, 16871–16877.
[8] Conrad, K.P., Gellai, M., North, W.G. and Valtin, H. (1986) Am. J. Physiol. 251, F290–296.
[9] Forsling, M.L. and Brimble, M.J. (1985) in: Oxytocin: Clinical and Laboratory Studies (J.A. Amico and A.G. Robinson, Eds.) Elsevier, Amsterdam, pp. 167–175.
[10] Lyness, J., Robinson, A.G., Sheridan, M.N. and Gash, D.M. (1985) Experientia 41, 1444–1446.
[11] Stoeckel, S. and Freund-Mercier, M.J. (1989) Am. J. Physiol. 257, F310–F314.
[12] Stassen, F.L., Heckman, G., Schmidt, D., Papadopoulis, M.T., Nambi, P., Sarau, H., Aiyar, N., Gellai, M. and Kinter, L. (1988) Mol. Pharmacol. 33, 218–224.
[13] Cantau, B., Barjon, J.N., Chicot, D., Baskevitch, P.P. and Jard, S. (1990) Am. J. Physiol. 258, F963–972.
[14] Jans, D.A., Resink, T.J., Wilson, E.R., Reich, E. and Hemmings, B.A. (1986) Eur. J. Biochem. 160, 407–412.
[15] 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.
[16] Hemmings, B.A. (1985) Curr. Topics in Cell Regulation 27, 117–132.
[17] Nagamine, Y., Sudol, M. and Reich, E. (1983) Cell 32, 1181–1190.
[18] Degen, J.D., Estensen, R.D., Nagamine, Y. and Reich, E. (1985) J. Biol. Chem. 260, 12426–12433.
[19] Jans, D.A. and Hemmings, B.A. (1986) FEBS Lett. 205, 127–131.
[20] Jans, D.A., Dierks-Ventling, C. and Hemmings, B.A. (1987) Exp. Cell Res. 172, 76–83.

[21] Pearson, D., Nigg, E.A., Nagamine, Y., Jans, D.A. and Hemmings, B.A. (1991) Exp. Cell Res. 192, 315–318.
[22] Grzonka, Z., Kasprzykowski, F., Kojro, E., Darlak, K., Melin, P., Fahrenholz, F., Crause, P. and Boer, R. (1983) J. Med. Chem. 29, 96–99.
[23] Fahrenholz, F., Boer, R., Crause, P., Fritzsch, G. and Grzonka, Z. (1984) Eur. J. Pharmacol. 100, 47–58.
[24] Grzonka, Z., Lammek, B., Kasprzykowski, F., Gazis, D. and Schwartz, I.L. (1983) J. Med. Chem. 26, 555–559.
[25] Jans, D.A., van Oost, B.A., Ropers, H.H. and Fahrenholz, F. (1990) J. Biol. Chem. 265, 15379–15382.
[26] Jans, D.A., Resink, T.J. and Hemmings, B.A. (1987) Biochem. J. 243, 413–418.
[27] Hull, R.N., Cherry, W.R. and Weaver, G.W. (1976) In Vitro 12, 670–677.
[28] Corbin, J.D. (1983) Methods Enzymol. 99, 227–232.
[29] Soderling, T.R., Corbin, J.D. and Park, C.R. (1974) Methods Enzymol. 38, 358–367.
[30] Luzius, H., Jans, D.A. and Fahrenholz, F. (1990) J. Receptor Res. 10, 61–80.
[31] Jans, D.A., Peters, R., Jans, P. and Fahrenholz, F. (1990) Exp. Cell Res. 191, 121–128.
[32] Bradford, W.M. (1976) Anal. Biochem. 72, 248–255.
[33] Steinberg, R.A., Steinberg, M.G. and van Daalen Wetters, T. (1979) J. Cell Physiol. 100, 579–588.
[34] Tovey, K.C., Oldham, K.G. and Whelan, J.A.M. (1974) Clin. Chem. Acta 56, 221–234.
[35] Jans, D.A. and Hemmings, B.A. (1991) FEBS Lett. 281, 267–271.
[36] Müller, M., Soloff, M.S. and Fahrenholz, F. (1989) FEBS Lett. 242, 333–336.
[37] Audigier, S. and Barberis, C. (1985) 4, 1407–1412.
[38] Jans, D.A., Zsigo, J., Peters, R. and Fahrenholz, F. (1989) EMBO J. 8, 2431–2438.
[39] Jans, D.A., Peters, R., Jans, P. and Fahrenholz, F. (1991) J. Cell Biol. 114, 53–60.