Potentiation of receptor-mediated cAMP production: role in the cross-talk between vasopressin V_{1a} and V_2 receptor transduction pathways

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Cross-talk between the phospholipase C and adenylyl cyclase signalling pathways was investigated in Chinese hamster ovary (CHO) cells transfected with the V_{1a} and V_2 vasopressin receptors. Cell lines expressing V_{1a}, V_2, or both V_{1a} and V_2 receptors, were established and characterized. Stimulation of V_2 receptors by vasopressin induced a dose-dependent increase in cAMP accumulation, whereas stimulation of V_{1a} receptor resulted in an increase in intracellular calcium without any change in basal cAMP. The simultaneous stimulation of V_2 and V_{1a} receptors by vasopressin elicited an intracellular cAMP accumulation which was twice that induced by stimulation of V_2 receptor alone with deamino-[D-Arg^8]vasopressin. This potentiation between V_{1a} and V_2 receptors was mimicked by activation of protein kinase C (PKC) with PMA, and was suppressed when PKC activity was inhibited by bisindolylmaleimide. The potentiation was observed in the presence or absence of 1 mM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, implying that an alteration in cAMP hydrolysis was not involved. Vasopressin, as well as PMA, had no effect on the forskolin-induced cAMP accumulation, suggesting that PKC did not directly stimulate the cyclase activity. On the other hand, vasopressin, like PMA, potentiated the cAMP accumulation induced by cholera toxin, an activator of $G\alpha_s$ protein. These results suggest that, in CHO cells, vasopressin V_{1a} receptor potentiates the cAMP accumulation induced by the V_2 receptor through a PKC-dependent increase in the coupling between Gs protein and adenylyl cyclase.

INTRODUCTION

The diversity of the signal transduction pathways linked to cell surface receptors raises the question of how these systems might relate to each other. With regard to the G-protein-coupled receptors, transmission of information is related to the interactions between ligands, receptors, G-proteins and enzymes that generate the second messengers. Different models for cross-talk at those different steps have been previously reviewed [1,2]. For example, a single receptor may activate alternative signalling pathways through different G proteins [3,4]; different receptors may share the same G protein pool [5]; second messengers may modulate their production of each other [6].

As regards the latter possibility, intracellular cAMP levels may be modified by calcium through regulation of the activity of some adenylyl cyclase isozymes. Calcium stimulates types 1, 3 and 8 adenylyl cyclase isoforms, inhibits types 5 and 6, and has no effect on types 2, 4 and 7 [7]. Such regulation may occur through either extracellular calcium entry or intracellular calcium mobilization with or without association with calmodulin (CAM). Regulation of the adenylyl cyclase activity by protein kinase C (PKC) has also been described. Phosphorylation of type 2 adenylyl cyclase by PKC has been observed in different cell lines, where phorbol esters, known activators of PKC, potentiate both basal and forskolin (FSK)-stimulated cAMP production [8–10]. Similar results have been reported for type 3 and type 5 adenylyl cyclase isoforms [9,11,12], whereas studies concerning the type 1 isoform have led to conflicting reports [9,10]. Intracellular messengers may also regulate the cAMP at the level of the cAMP hydrolysis. The phosphodiesterase (PDE) type 1 isoform is activated by the calcium–calmodulin complex, whereas type 2 and type 3 isoforms are respectively activated and inhibited by cGMP. The regulation of the two other cAMP-hydrolysing phosphodiesterases, type 4 and type 7 isoforms, is still unknown [13].

Such cross-talk between phospholipase C (PLC) and adenylyl cyclase transduction pathways was investigated in CHO cells transfected with both the V_{1a} and the V_2 vasopressin receptors. These two receptors have been identified as members of the G-protein-coupled receptor family [14,15]. In CHO cells, their respective signal transduction pathway is similar to the one described in native tissues. The stimulation of the V_{1a} receptor results in the activation of phospholipase C_β, and consequently in diacylglycerol (DAG) formation, inositol triphosphate generation and intracellular calcium mobilization. The V_2 receptor is coupled to a G_s protein and activates the adenylyl cyclase, which results in cAMP production [16].

We found that the V_2-induced cAMP accumulation was potentiated by the stimulation of the V_{1a} receptor transduction pathway. This potentiation occurs essentially via the PKC, which would possibly increase the coupling of the Gs protein with the adenylyl cyclase. Such a mechanism offers an additional regulatory step to the most currently described potentiating mechanisms, which imply the intrinsic adenylyl cyclase enzymatic activity or the phosphodiesterase activity.

EXPERIMENTAL

Materials

Ham's F-12 medium, fetal bovine serum (FBS) and penicillin–streptomycin were obtained from Gibco-BRL; hygromycin B

Abbreviations used: AVP, arginine vasopressin; αPDD, 4α-phorbol 12,13-didecanoate; BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, tetra (acetoxymethyl) ester; CaM, calmodulin; CHO, Chinese hamster ovary; DAG, diacyglycerol; DOG, 1,2-dioctanoyl-*sn*-glycerol; dDAVP, deamino-[D-Arg^8]AVP; FBS, foetal bovine serum; FSK, forskolin; IBMX, 3-isobutyl 1-methylxanthine; PDE, phosphodiesterase; PKC, protein kinase C; PLC, phospholipase C.

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was from Boehringer-Mannheim. Vasopressin and deamino-[D-Arg[8]]vasopressin (dDAVP) were from Neosystem, [^{3}H]vasopressin was from DuPont–NEN, bestatin, 3-isobutyl 1-methylxanthine (IBMX), BSA, PMA, 4α-phorbol 12,13-didecanoate (αPDD) were from Sigma, BAPTA-AM was from Calbiochem, cholera toxin and pertussis toxin were from Biomol, fura-2 was from Molecular Probes and cAMP enzymatic immunoassay was from Cayman Chemical. SR 49059 was a gift from Sanofi.

Cell culture

CHO cells were maintained at 37 °C in a humidified 5 % CO_2 atmosphere in Ham's F-12 medium (with glutamine) supplemented with 10 % FBS, 10 units/ml penicillin-streptomycin, and with 350 μg/ml hygromycin for transfected cells. Media were changed every 2 days and subculture was performed by trypsinization. After transfection, cells were used between passages 4 and 40.

Construction of CHO cell lines stably expressing rat V_{1a} and/or V_2 receptor

pCD SP6/T7 vector containing the V_{1a} cDNA [11], and pECE vector containing the V_2 cDNA, were either independently or simultaneously transfected into CHO cells by the calcium phosphate precipitation method, in the presence of a hygromycin resistance vector. Hygromycin-resistant clones were tested for V_{1a} and/or V_2 mRNA expression by RT-PCR using the primers described by Firsov et al. [17]. The positive clones were grown and tested for cAMP production. The expression of the vasopressin receptors on cell lines was also tested by binding studies, and pure clones were obtained by the limiting dilution technique.

Radioligand binding assays

Radioligand binding saturation assays were performed on confluent cells in 24-well plates. Cells were washed twice with ice-cold binding buffer [Tris/HCl 50 mM, 1 mM EGTA, 1 mM $MgSO_4$, 0.1 % BSA (w/v), 0.1 % bacitracin (w/v), pH 7.4] and incubated for 1 h at room temperature (20 °C) with [^{3}H]arginine vasopressin ([^{3}H]AVP) at different concentrations. Cells were then washed once rapidly with ice-cold buffer and lysed by 0.1 M NaOH. The radioactivity of the lysate was measured in a liquid scintillation counter. Specific binding was defined as the radioactivity displaceable by the addition of 1 μM unlabelled AVP. Specific V_{1a} or V_2 binding sites of CHO V_1V_2 cells were determined with [^{3}H]vasopressin and with unlabelled dDAVP (10^{-7} M) or V_1 antagonist SR 49059 (10^{-7} M) [18] as competitors, respectively. Each determination was carried out in duplicate.

cAMP measurement

Experiments were performed in 24-well plates. Confluent cells were rinsed twice with PBS, and preincubated for 10 min at 37 °C in PBS in the presence of 1 mM $MgCl_2$, 1 mM $CaCl_2$ (except in Ca^{2+}-free experiments), 1 mM Hepes, 0.1 % BSA (w/v), 0.1 % bacitracin (w/v), 10^{-6} M bestatin and 5×10^{-6} M indomethacin, pH 7.4. When used, IBMX and EGTA were at a concentration of 1 mM. Preincubation medium was removed and cells were stimulated for 4 min at 37 °C with similar medium containing the studied effectors. Each set of conditions was performed in duplicate. Medium was removed at the end of the stimulating period, and cells were lysed by addition of 5 % ethanol/formic acid (v/v) which was thereafter collected and evaporated overnight. The cAMP content of each sample was determined by enzymatic immunoassay according to Pradelles et al. [19].

Intracellular calcium measurement

Cells were grown to confluence in T_{25} flasks, trypsinized, centrifuged and incubated for 40 min with 2 μM fura-2 AM in culture medium supplemented with FBS. Cells were then centrifuged and resuspended at a concentration of 2 million cells per ml in PBS containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Hepes, 0.1 % BSA, 0.1 % bacitracin, 10^{-6} M bestatin and 5×10^{-6} M indomethacin, pH 7.4. When used, EGTA was at a concentration of 1 mM. Calcium measurements were performed with 1 ml of the cell suspension using an Amincon Bowman SLM 2 spectrofluorimeter. Cells were alternatively excited at either 340 nm or 380 nm, and the fluorescence emission was recorded at 510 nm. The ratio, R, between 340/380 nm was calculated each 0.5 s. At the end of the experiment, cells were permeabilized with 30 μM digitonin and R_{min} and R_{max} were determined by addition of 12 mM EGTA and saturating calcium concentration. The intracellular calcium concentration was calculated according to the Grynkiewicz equation [20].

Data are expressed as mean $\pm$ S.E.M. The significance of the results was tested by ANOVA (Dunnett's test, $P < 0.05$).

RESULTS

Expression of the vasopressin receptors in CHO cells

Clones obtained from cells transfected with the V_{1a} cDNA, the V_2 cDNA or both were named CHO V_1, CHO V_2 and CHO V_1V_2. Binding ability of transfected CHO cells was tested in saturation experiments by using [^{3}H]AVP as a ligand, from 0.125 nM to 5 nM. In CHO V_1, B_{max} was 82000 receptors per cell and the K_d for AVP was 0.8 nM. In CHO V_2, B_{max} was 81000 receptors/cell and K_d was 1.4 nM. In CHO V_1V_2 cells, experiments were performed in the absence or presence of the V_2 agonist dDAVP at 10^{-7} M and of the V_1 antagonist SR 49059 at 10^{-7} M. The number of V_1 and V_2 receptors per cell, calculated after Scatchard transformation, was 125600 ± 8400 and 94800 ± 9500. K_d values for V_1 and V_2 receptors were 1.00 ± 0.13 and 1.07 ± 0.04 nM ($n = 4$). The mean number of cells per well was 8×10^5.

Cellular response to dDAVP and AVP stimulation

Cells were tested for their capacity to produce calcium or cAMP in response to hormonal stimulation. Addition of AVP induced an increase of intracellular calcium concentration in CHO V_1 and CHO V_1V_2 cells (Figure 1). As expected, there was no cAMP accumulation in CHO V_1 cells after AVP stimulation (basal: 3.56 ± 0.41 pmol cAMP/well per 4 min; AVP 10^{-8} M: 3.89 ± 0.83 pmol cAMP/well per 4 min). In CHO V_2 cells, AVP and the specific V_2 receptor agonist dDAVP produced similar concentration-dependent cAMP accumulation with a maximum for 10^{-8} M AVP or dDAVP (data not shown). In CHO V_1V_2 cells, AVP was much more efficient than dDAVP in increasing cAMP level (Figure 2). The potentiating effect of AVP in CHO V_1V_2 was furthermore demonstrated by stimulating the cells with a fixed maximal dDAVP concentration (10^{-8} M) and various concentrations of AVP. The potentiation was concentration-dependent and peaked at 10^{-8} M AVP (Figure 3). This potentiation was not observed in CHO V_2 cells (Figure 3). Incubation of the CHO V_1V_2 cells with SR 49059, a specific V_{1a} antagonist, abolished the described potentiation without any influence on

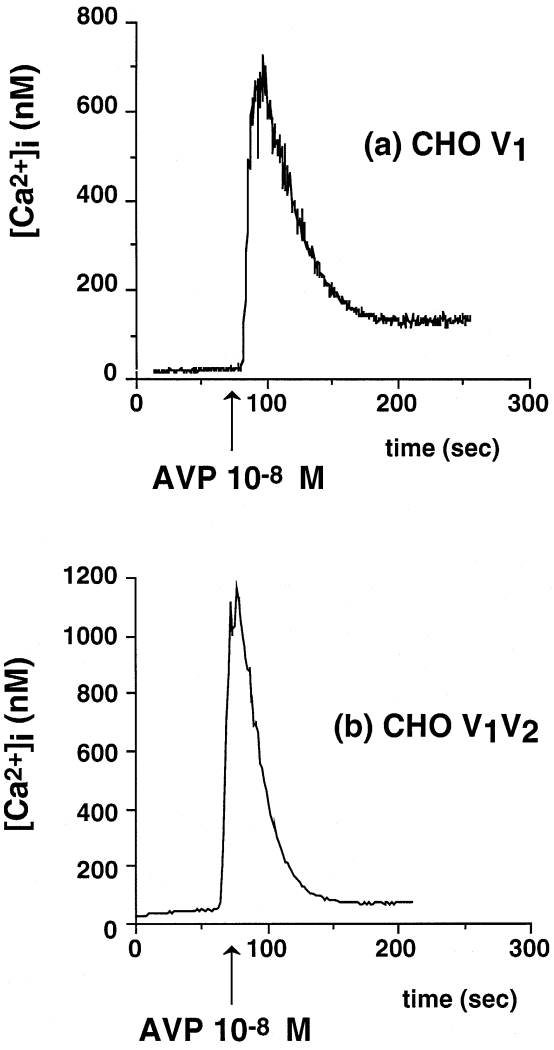

Figure 1 Effect of 10^{-8} M vasopressin on intracellular calcium concentration in CHO cells transfected with vasopressin V_{1a} and V_2 receptors

CHO cells were transfected with (**a**) either the V_{1a} vasopressin receptor (CHO V_1) or (**b**) with both the V_{1a} and the V_2 vasopressin receptors (CHO V_1V_2). Calcium increase in response to 10^{-8} M AVP was measured with 2 million cells loaded with fura 2-AM.

dDAVP-induced cAMP accumulation or on the basal cAMP level (Table 1).

Involvement of calcium, DAG and protein kinase C in the potentiation by AVP of dDAVP-induced cAMP accumulation

The role of calcium in the potentiation was tested by incubating CHO V_1V_2 cells in calcium-free medium supplemented with 1 mM EGTA and by addition of the intracellular calcium chelator BAPTA-AM at a concentration of 60 μM. The absence of extracellular Ca^{2+} did not modify the dDAVP-induced cAMP accumulation and its potentiation by AVP (Table 2). When intracellular calcium was chelated by 60 μM BAPTA-AM, the dDAVP-induced cAMP level was diminished and the potentiation was abolished (Table 2). BAPTA-AM also decreased the cAMP accumulation induced by 5×10^{-5} M of the cyclase activator forskolin from 44.8 ± 8.1 to 15.6 ± 3.2 pmol/well. The relative implication of intracellular calcium and DAG was assessed by stimulating the cells with dDAVP and either thapsi-

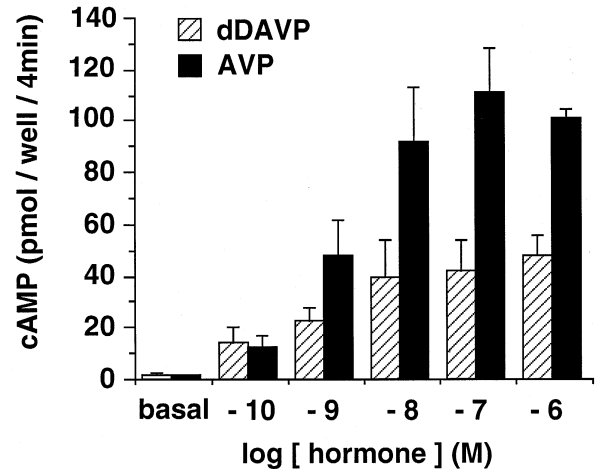

Figure 2 cAMP accumulation in response to AVP and dDAVP in CHO V_1V_2 cells

CHO cells transfected with both the V_{1a} and the V_2 vasopressin receptors (CHO V_1V_2) were stimulated for 4 minutes with various concentrations of AVP or of the V_2 receptor agonist dDAVP. Data (pmol cAMP/well per 4 min) represent the means $\pm$ S.E.M. of 3 experiments performed in duplicate.

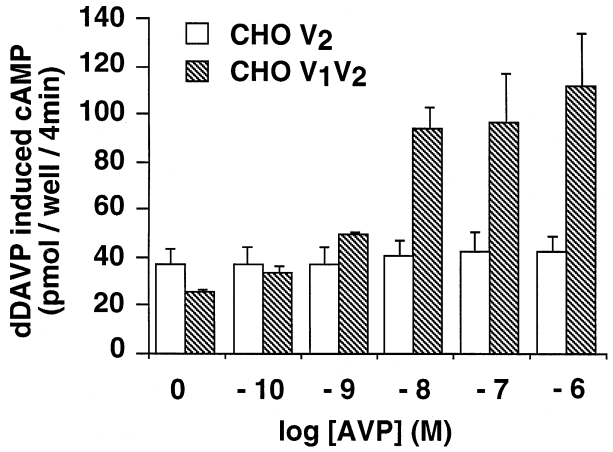

Figure 3 Effect of AVP stimulation on dDAVP-induced cAMP accumulation in CHO V_2 and CHO V_1V_2 cells

CHO V_2 and CHO V_1V_2 cells were stimulated for 4 min with 10^{-8} M dDAVP and various concentrations of AVP. The AVP-related potentiation of dDAVP-induced cAMP accumulation was observed in CHO V_1V_2 but not in the CHO V_2 cells. Data (pmol cAMP/well per 4 min) represent the means $\pm$ S.E.M. of three experiments performed in duplicate.

Table 1 Effect of the nonpeptide V_{1a} receptor antagonist SR49059 on hormone-induced cAMP accumulation in CHO V_1V_2 cells

Cells were pre-incubated for 10 min in the presence of 10^{-7} M SR49059 or vehicle alone (control), and were then stimulated for 4 min by either 10^{-8} M dDAVP or both 10^{-8} M dDAVP and AVP. Data (pmol cAMP/well per 4 min) represent the means $\pm$ S.E.M. of four experiments performed in duplicate. * Significantly different from dDAVP alone ($P < 0.05$).

	No stimulation	dDAVP	dDAVP + AVP
Control	1.7 ± 0.3	37.9 ± 2.8	87.1 ± 4.6*
SR49059	1.6 ± 0.2	38.8 ± 2.7	40.1 ± 2.6

Table 2 Role of extracellular and intracellular calcium in the V_{1a}-related potentiation of the V_2-induced cAMP accumulation

CHO cells expressing the V_{1a} and the V_2 vasopressin receptors were pre-incubated for 10 min in medium containing 1 mM calcium or in medium without calcium and supplemented with either 1 mM EGTA, or 1 mM EGTA and 60 μM of the intracellular calcium chelator BAPTA-AM. Cells were then stimulated for 4 min by either 10^{-8} M dDAVP or both 10^{-8} M dDAVP and AVP. Data (pmol cAMP/well per 4 min) represent the means ± S.E.M. of five experiments performed in duplicate. * Significantly different from dDAVP under calcium conditions ($P < 0.05$).

	dDAVP	dDAVP + AVP
Calcium	40.0 ± 2.7	92.2 ± 4.6*
Free calcium + EGTA	42.7 ± 3.1	82.8 ± 6.1*
Free calcium + EGTA + BAPTA-AM	20.4 ± 1.3*	24.1 ± 2.7*

Table 3 Effect of thapsigargin and diacylglycerol analogue DOG on dDAVP-induced cAMP accumulation

CHO cells expressing the V_{1a} and the V_2 receptors were pre-incubated for 10 min in medium without calcium and supplemented with EGTA 1 mM. Cells were then stimulated for 4 min by either 10^{-8} M dDAVP alone (control) or 10^{-8} M dDAVP and either 10^{-8} M AVP, 10^{-6} M thapsigargin, 5×10^{-5} M DOG, or both thapsigargin and DOG. Data (pmol cAMP/well per 4 min) represent the means ± S.E.M. of four experiments performed in duplicate. * Significantly different from dDAVP alone ($P < 0.05$).

	cAMP accumulation
dDAVP 10^{-8} M	40.2 ± 4.4
dDAVP 10^{-8} M + AVP	125.4 ± 10.7*
dDAVP 10^{-8} M + thapsigargin	50.1 ± 4.6
dDAVP 10^{-8} M + DOG	67.7 ± 5.9*
dDAVP 10^{-8} M + thapsigargin + DOG	89.2 ± 8.7*

Table 4 Comparison of AVP and PMA effect on dDAVP-induced cAMP accumulation in CHO V_1V_2 cells

CHO cells expressing the V_{1a} and the V_2 vasopressin receptors were pre-incubated for 10 min without or with 1 mM of the phosphodiesterase inhibitor IBMX. They were then stimulated for 4 minutes with 10^{-8} M dDAVP alone, or 10^{-8} M dDAVP and either 10^{-8} M AVP, 10^{-6} M of the inactive phorbol ester αPDD or 10^{-6} M of the PKC-activator PMA. Experiments were performed in the presence or in the absence of 1 mM IBMX. Data (pmol cAMP/well per 4 min) represent the means ± S.E.M. of four experiments performed in duplicate. * Significantly different from dDAVP alone ($P < 0.05$).

	dDAVP	dDAVP + AVP	dDAVP + αPDD	dDAVP + PMA
Without IBMX	23.9 ± 2.1	79.6 ± 9.9*	29.2 ± 4.0	80.5 ± 7.9*
With IBMX	52.5 ± 4.1	99.8 ± 9.8*	51.4 ± 6.7	99.8 ± 8.2*

gargin, diacylglycerol analogue DOG, or both. DOG partially mimicked the effect of AVP on dDAVP-induced cAMP accumulation. Thapsigargin alone, which increases intracellular calcium [34], did not potentiate cAMP accumulation, but enhanced the effect of DOG (Table 3). This suggests that DAG is the second messenger directly involved in the potentiation between V_{1a} and V_2 receptors.

Protein kinase C is the main cellular target of DAG. Its role in the potentiation between V_{1a} and V_2 receptors was investigated by using the PKC-activator PMA and its inactive analogue αPDD as a control. As in previous experiments we found that PMA dose-dependently potentiated the V_2-related cAMP accumulation from 10^{-10} to 10^{-6} M [34], the following experiments were performed at the maximal concentration of 10^{-6} M PMA. Like AVP, PMA, but not αPDD, significantly enhanced the dDAVP-induced cAMP accumulation in CHO V_1V_2 cells (Table 4). The effects of AVP and PMA were not additive (dDAVP + AVP + αPDD: 87.3 ± 13.3 pmol/well; dDAVP + AVP + PMA: 98.8 ± 15.4 pmol/well). The potentiating effect of AVP was abolished by preincubation of the cells with the selective PKC-inhibitor bisindolylmaleimide (10^{-5} M), or by desensitization of PKC by prolonged pre-incubation with 10^{-6} M PMA (Table 5). This indicates that the potentiating effect of the V_{1a} vasopressin receptor on the V_2 receptor-induced cAMP accumulation is mediated by a PMA-sensitive, i.e. PKC-dependent, mechanism.

Table 5 Effect of PKC inhibition or PKC desensitization on the potentiation by AVP of dDAVP-induced cAMP accumulation

(**a**) CHO cells expressing the V_{1a} and the V_2 vasopressin receptors were pre-incubated for 5 h with either vehicle alone (DMSO) or the PKC-inhibitor bisindolylmaleimide 10^{-5} M. (**b**) CHO cells expressing the V_{1a} and the V_2 vasopressin receptors were pre-incubated for 5 h with either vehicle alone (DMSO) or 10^{-6} M phorbol esters PMA or 10^{-6} M αPDD. Cells were then stimulated for 4 min by 10^{-8} M dDAVP alone or 10^{-8} M dDAVP and AVP. Data (pmol cAMP/well per 4 min) represent the means ± S.E.M. of three experiments (**a**) or five experiments (**b**) performed in duplicate. * Significantly different from dDAVP alone ($P < 0.05$).

Prolonged pre-incubation with	dDAVP	dDAVP + AVP
(**a**) Vehicle	30.5 ± 4.9	79.1 ± 10.5*
Bisindolylmaleimide	27.1 ± 3.6	40.5 ± 4.1
(**b**) Vehicle	24.7 ± 2.0	90.5 ± 6.7*
PMA	23.4 ± 2.2	41.7 ± 4.4
αPDD	27.1 ± 2.4	90.3 ± 7.9*

Cellular target of the potentiation

Phosphodiesterase involvement in the potentiation was assessed by incubating CHO V_1V_2 cells with 1 mM of the phosphodiesterase inhibitor IBMX prior to and during stimulation with hormones or PMA. As expected, IBMX increased the cAMP accumulation resulting from V_2 receptor stimulation by dDAVP. Neither its presence nor its absence made a difference to the AVP- or PMA-mediated potentiation (Table 4).

Cyclase involvement was investigated by using the diterpene forskolin, which directly activates the cyclase. In CHO V_1V_2 cells, FSK-induced cAMP accumulation was unaffected by the addition of PMA, in both the absence and presence of IBMX (Table 6). Similar results were observed in CHO V_1 cells following

Table 6 Effect of PKC stimulation by PMA on FSK-induced cAMP accumulation

CHO cells expressing the V_{1a} and the V_2 vasopressin receptors were pre-incubated for 10 min without or with 1 mM IBMX, and were then stimulated for 4 min with 5×10^{-5} M forskolin (FSK) alone or 5×10^{-5} M FSK and either 10^{-6} M PMA or 10^{-6} M αPDD. Data (pmol cAMP/well per 4 min) represent the mean ± S.E.M. of four experiments (without IBMX) or three experiments (with IBMX) performed in duplicate.

	Without IBMX	With IBMX
Unstimulated	1.8 ± 0.3	5.7 ± 1.0
FSK	19.0 ± 2.5	32.0 ± 1.5
FSK + PMA	25.9 ± 3.7	46.5 ± 3.0
FSK + αPDD	22.8 ± 3.1	38.3 ± 1.8

Table 7 Compared effect of FSK and AVP on dDAVP-induced cAMP accumulation

CHO cells expressing the V_{1a} and the V_2 vasopressin receptors were pre-incubated for 10 min with 1 mM IBMX, and were then stimulated for 4 min with 10^{-8} M dDAVP alone or 10^{-8} M dDAVP and either 10^{-8} M AVP, 5×10^{-5} M FSK or both. Data (pmol cAMP/well per 4 min) represent the means ± S.E.M. of three experiments performed in duplicate. * Significantly different from dDAVP or FSK alone ($P < 0.05$).

	cAMP accumulation
dDAVP	40.0 ± 2.2
dDAVP + AVP	80.1 ± 6.4*
FSK	35.1 ± 4.5
dDAVP + FSK	185.6 ± 3.9*
dDAVP + AVP + FSK	189.6 ± 15.7*

Table 8 Effect of AVP and PMA on cholera toxin-induced cAMP accumulation

CHO cells expressing the V_{1a} vasopressin receptors were pre-incubated for 3 h in culture medium with or without cholera toxin (CTX) 500 ng/ml. Both groups were then stimulated for 4 min with either vehicle alone, 10^{-8} M AVP, 10^{-6} M PMA or 10^{-6} M αPDD, in the presence of 1 mM IBMX. Data (pmol cAMP/well per 4 min) represent the means ± S.E.M. of four experiments performed in duplicate. * Significantly different from CTX alone (control) ($P < 0.05$).

	Control	AVP	αPDD	PMA
Without CTX	3.6 ± 0.5	3.9 ± 0.8	3.9 ± 1.5	4.1 ± 1.3
With CTX	58.4 ± 5.6	83.5 ± 9.6*	59.4 ± 4.2	83.8 ± 9.5*

their stimulation by forskolin and AVP (data not shown). On the other hand, forskolin not only increased the basal cAMP level in CHO V_1V_2 cells, but also potentiated the dDAVP-induced cAMP accumulation. Forskolin- and AVP-potentiating effects were not additive (Table 7).

G-protein involvement in the potentiation was assessed by using bacterial toxins which covalently modify either $G\alpha_s$ or $G\alpha_i$. Cholera toxin ADP-ribosylates the α_s subunit, resulting in the inhibition of the intrinsic GTPase activity, stabilization of the activated conformation and sustained cAMP production, while pertussis toxin inactivates the $G\alpha_i$ subunit.

The effect of AVP or PMA on the cholera toxin-induced cAMP accumulation was investigated in CHO V_1 cells. Cells were pre-incubated with or without cholera toxin at 500 ng/ml for 3 h and were then stimulated with either 10^{-8} M AVP or 10^{-6} M PMA. Neither AVP nor PMA modified the cAMP level in unstimulated cells. Both of them enhanced the cholera toxin-induced cAMP accumulation (Table 8).

The involvement of $G\alpha_i$ was assessed in CHO V_1V_2 cells. Cells were pre-incubated with pertussis toxin at different concentrations (0.01, 0.1 and 1 μg/ml) for 4 h and then stimulated with dDAVP (10^{-8} M), or dDAVP and AVP (10^{-8} M). Pertussis toxin neither enhanced the dDAVP-induced cAMP accumulation, nor modified the AVP-related potentiation (data not shown). This suggests that $G\alpha_s$ protein, but not $G\alpha_i$, is involved in the potentiation by PKC of the V_2-related cAMP production.

DISCUSSION

A complex range of regulatory mechanisms is available for the modulation of cellular cAMP level, and different signal transduction systems may interfere with the cyclase pathway [21–23]. In CHO cells transfected with the V_{1a} and the V_2 vasopressin receptors cDNA, we showed that the V_2 receptor-induced cAMP accumulation was potentiated by stimulation of the V_{1a} receptor. Since the V_{1a} receptor did not generate any cAMP increase by itself, we presumed that potentiation was related to the effect of V_{1a} receptor second messengers, i.e. calcium and DAG, on the V_2 receptor and its associated transduction pathway.

As regards the influence of calcium, the absence of extracellular calcium neither affected the dDAVP-induced cAMP accumulation nor its potentiation by AVP. In contrast, chelation of intracellular calcium decreased the cAMP accumulation linked to the stimulation of V_2 receptor, and abolished the V_{1a}-related potentiation. Calcium chelation also reduced the cAMP accumulation resulting from direct stimulation of the cyclase by forskolin. This indicates that calcium is necessary for the cyclase activity, which could explain the absence of potentiation under calcium chelation conditions. An increase in intracellular calcium induced by thapsigargin did not potentiate cAMP accumulation. On the other hand, DOG, a cell-permeable analogue of diacylglycerol, partially mimicked the potentiating effect of AVP, and thapsigargin somewhat enhanced this effect. Taken together, these data support the hypothesis that production of DAG, subsequent to the V_{1a} receptor stimulation, is directly involved in the potentiation, and that calcium has only a contributory effect. This suggests that protein kinase C is involved in the mechanism of potentiation.

Different results support this hypothesis. Short stimulation with the PKC-activator PMA mimicked the effect of AVP on the dDAVP-induced cAMP accumulation, and the effect of AVP was abolished in the presence of the PKC-inhibitor bisindolylmaleimide, or by long-term PMA pretreatment.

Three PKC isozymes, PKC α, ε and ζ, were identified in CHO cells by Liu et al. [24]. They described a rapid PMA-induced translocation of PKC α and ε from cytosol to membrane, and a drastic down-regulation of PKC α, but not PKC ε, after long-term PMA pretreatment. Thus PKC α could be the PKC isoform involved in the mechanism of potentiation.

A specific interaction between PKC and cAMP signal transduction has been observed in several cell types [25–27], and different mechanisms have been described. They include the direct phosphorylation of adenylyl cyclase [10,11,28,29], the phosphorylation of $G\alpha_i$, which decreases its ability to inhibit adenylyl cyclase [30,31] or the regulation of the phosphodiesterases [32], even if these latter enzymes seem to be preferentially regulated by PKA or CaM-kinases [33].

The phosphodiesterase pattern in CHO cells has been previously determined [34]. Among the four cAMP-hydrolysing isoforms found in these cells, the major ones are the PDE 4 isoform, which represents 50% of the total cAMP hydrolysing capacity, and an IBMX-insensitive (possibly PDE 7) isoform, which represents 30% of total activity. Given the high ratio of these isoforms, inhibition of one of them by calcium or PKC could result in potentiation. It has been previously shown that both fractions are insensitive to calcium [34]. The ability of PKC to inhibit some cAMP phosphodiesterase isoforms has been suggested before [35,36]. However, such a mechanism is unlikely to be involved in our experimental conditions, for the following reasons. On the one hand, the potentiating effect of AVP or PMA and the effect of the phosphodiesterase inhibitor IBMX were additive, and on the other hand, neither AVP nor PMA potentiated the FSK-induced cAMP accumulation.

Alternatively, our data may correspond to an activation of the cAMP synthesis system, which may be ascribed either to a modification of the cyclase catalytic site or to an enhancement of

the coupling between the Gs-protein and the cyclase. As described above, cAMP accumulation in response to the direct cyclase activator forskolin was not enhanced by AVP or PMA. It indicates that the potentiation is not mediated through a mechanism operating on the catalytic activity of the cyclase. This absence of PMA effect on basal and forskolin-stimulated cAMP accumulation is similar to that observed in HT4 cells or in kidney 293 cells transfected with either type I or type VI adenylyl cyclase [10].

The most specific feature of the potentiation between V_{1a} and V_2 receptors in CHO cells was that the enhancement of cAMP production by phorbol esters required the coupling between G protein and cyclase. The involvement of $G\alpha_s$ was demonstrated in CHO V_1 cells, where AVP as well as PMA synergistically increased the cholera toxin-induced cAMP accumulation. The possible implication of $G\alpha_i$ was also investigated because phorbol ester-mediated activation of PKC appeared to abolish the tonic inhibitory effect mediated by $G\alpha_i$ upon adenylyl cyclase in a number of cells [30,37]. Such a mechanism may be ruled out here, since pertussis toxin did not change the cAMP accumulation induced by V_2 receptor as its potentiation by V_{1a} receptor.

One possible mechanism for the PKC-dependent increase in cAMP production through Gs protein may involve the phosphorylation of the α_s subunit and/or the adenylyl cyclase, leading to enhanced reciprocal interaction and sustained activation of the cyclase. This hypothesis has been evoked to explain the synergistic effect of phorbol esters on cAMP production induced by stimulation of the adrenaline receptors in HT4 cells [38]. Another possible mechanism is that enhancement of the coupling would stabilize the catalytic site of the cyclase, resulting in increased cAMP synthesis. Such a stabilization could be similar to the one proposed to explain the potentiation by forskolin of receptor-mediated cAMP accumulation in different cell types [39–41]. The lack of additivity between AVP- and FSK-potentiating effects on dDAVP-induced cAMP accumulation in CHO V_1V_2 cells would support this hypothesis.

In conclusion, stimulation of the PLC pathway via the V_{1a} vasopressin receptor potentiates the cAMP accumulation related to activation of the V_2 receptor, when these receptors are both expressed in CHO cells. This potentiation results from the activation of PKC, which appears to enhance the coupling between the Gs protein and the adenylyl cyclase. This type of cross-talk, which requires co-activation of the receptors, might contribute to the accuracy of the cellular response at a step upstream of adenylyl cyclases and phosphodiesterases.

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