

## SHORT COMMUNICATION

### PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP)-EVOKED INCREASE IN CYCLIC AMP PRODUCTION IN CHICK CEREBRAL CORTEX: LACK OF A ROLE OF THE PROTEIN KINASE C PATHWAY

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*Pituitary adenylate cyclase-activating polypeptide (PACAP)-evoked increase in cyclic AMP production in chick cerebral cortex: lack of a role of the protein kinase C pathway.* J.B. ZAWILSKA, P. NIEWIADOMSKI, J.Z. NOWAK. *Pol. J. Pharmacol.*, 2003, 55, 245–250.

Pituitary adenylate cyclase-activating polypeptide 38 (PACAP38) is a potent activator of cyclic AMP formation in the chick brain. The peptide also stimulates inositol phosphates accumulation and protein kinase C (PKC) activity in the chick cerebral cortex. In this work, we analyzed whether PACAP38-induced increase in cyclic AMP production in the chick cerebrum can be modified by a PKC pathway. 4 $\beta$ -Phorbol 12,13-dibutyrate (4 $\beta$ -PDB), a PKC activator, did not significantly affect the PACAP38-evoked increase in [<sup>3</sup>H]cyclic AMP production in [<sup>3</sup>H]adenine-prelabeled slices of the chick cerebral cortex. Of the tested PKC inhibitors, i.e. chelerythrine, H-7, NPC-15437 and staurosporine, only chelerythrine markedly decreased, in a concentration-dependent manner, the PACAP38-activated cyclic AMP accumulation in the chick cerebrum. These results suggest that (1) the process of cyclic AMP production stimulated by PACAP in the cerebral cortex of chick is not PKC-dependent, and that (2) chelerythrine, a widely used PKC inhibitor, influences the intracellular signaling pathway(s) associated with PACAP receptors in the chick brain in a way not involving PKC.

**Key words:** *pituitary adenylate cyclase-activating polypeptide, PACAP, chick, cerebral cortex, protein kinase C, adenylyl cyclase, PDB, chelerythrine, H-7, NPC-15437, staurosporine*

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## INTRODUCTION

Pituitary adenylyl cyclase-activating polypeptide (PACAP) was originally isolated from ovine hypothalamus based on its ability to stimulate adenylyl cyclase in the rat anterior pituitary cells. The peptide exists in two biologically active forms, i.e. PACAP38 and PACAP27, consisting of 38 and first 27 amino acids, respectively. The short form of PACAP shows 68% amino acid sequence homology with vasoactive intestinal peptide (VIP). The primary structure of PACAP has been remarkably well conserved in the course of evolution. It is identical in all mammals studied so far, and shows only 1–4 amino acid differences among other vertebrate species. PACAP displays a widespread distribution and appears to be biologically active in various tissues, including the central nervous system, pituitary, adrenal gland, liver, pancreas, as well as the nerve fibers in the lung and the gut. In addition to playing a role of a hormone, neurotransmitter/neuromodulator, and hypophysiotrophic factor, PACAP is also endowed with strong neuroprotective and neurotrophic activity [1, 20, 25].

The diverse biological actions of PACAP are mediated *via* specific membrane-bound receptors, which belong to the class B of a superfamily of G protein-coupled receptors. Three types of receptors for PACAP have been discovered so far: PAC1 (Type I; PACAP-specific) receptor shows selective affinity for both forms of PACAP, whereas VPAC1 and VPAC2 (Type II) receptors are equally sensitive to PACAP and VIP [5, 20]. The adenylyl cyclase/cyclic AMP is the main intracellular signal transduction pathway coupled with all mentioned types of receptors [5, 20]. A fraction of PACAP receptors can be also coupled to phospholipase C (PLC) [20], an enzyme responsible for the phosphoinositide turnover with concomitant production of two second messengers, i.e. inositol(1,4,5)trisphosphate (IP<sub>3</sub>) and 1,2-diacylglycerol (DAG).

Recently, we have shown that PACAP is a potent stimulator of cyclic AMP production in the brains of domestic fowls, including young cocks [13, 14, 16]. Using [<sup>125</sup>I]-PACAP27 and [<sup>125</sup>I]-VIP as specific radioligands, the presence of PAC1 and VPAC type receptors in the chick cerebral cortical membranes has been demonstrated, both being linked to the activation of adenylyl cyclase-related signal transduction pathway [16, 22, 23]. In addition to its action on the cyclic AMP-generating sys-

tem, PACAP concentration-dependently enhanced inositol phosphate accumulation and activated protein kinase C (PKC) in the cerebral cortex of chick [12, 15].

PKC is present in a vast range of different cells. Activation of PKC results in phosphorylation of numerous substrates, and such an action has been linked to a wide variety of biological responses [11]. There is growing evidence that stimulation of PKC can significantly influence (either potentiate or inhibit) the formation of cyclic AMP elicited by various transmitters and hormones (e.g. [4, 7, 10, 21, 23]). In view of all these facts, a hypothesis that the PACAP-induced cyclic AMP production in the chick brain is, at least in part, a PKC-dependent/sensitive event, seemed plausible. Thus, the aim of this study was twofold: (1) to examine whether the stimulatory effect of PACAP on cyclic AMP accumulation in the chick cerebral cortex involves PKC, and (2) to see whether the PACAP-related cyclic AMP response can be modulated by a concomitant activation of PKC.

## MATERIALS and METHODS

### Animals

White male leghorn chicks (*Gallus domesticus*; HyLine) were purchased locally on the day of hatching, and kept in temperature-controlled warmed brooders (28 ± 1°C during the first 5 days and 25 ± 1°C afterwards) with standard food and tap water available *ad libitum*, for a minimum of two weeks before use. The animals were maintained under a 12 h light : 12 h dark (LD) lighting schedule (lights on between 21.30 and 9.30). The lighting cycle was produced by overhead cool fluorescent lamps providing light intensity at the level of the animals' eyes of approximately 150 lx. The experiments were carried out in strict accordance with the Polish regulations concerning experiments on animals (Dz.U. 97.11.724) and rules followed at the Department of Biogenic Amines.

On the day of an experiment, the animals were killed by decapitation under standard laboratory illumination between 9.00 and 9.30, and their cerebral cortex was rapidly isolated. Cross-chopped slices (250 µm; prepared with McIlwain tissue chopper) of cerebral cortex were suspended in cold, O<sub>2</sub>/CO<sub>2</sub> (95:5)-gassed, glucose-containing modified Krebs-Henseleit medium (KHM; mmol/l): 118

NaCl, 5 KCl, 1.3 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 11.7 D-glucose; pH 7.4.

### Assay of cyclic AMP formation

The formation of [<sup>3</sup>H]cyclic AMP in [<sup>3</sup>H]adenine pre-labeled tissues was assayed according to Shimizu et al. [19]. In brief, slices were resuspended in 15 ml of O<sub>2</sub>/CO<sub>2</sub> (95:5)-gassed fresh KHM. Then, [<sup>3</sup>H]adenine was added to the incubation mixture. After a 45 min incubation at 37°C, the slices were washed, distributed into Eppendorf tubes containing freshly gassed O<sub>2</sub>/CO<sub>2</sub> KHM, and preincubated for 10 min (experiments with 4β-PDB and with PKC inhibitors without IBMX) or 15 min (experiments with IBMX, and with chelerythrine and IBMX; added 15 and 10 min, respectively, before exposition of slices to PACAP) at 37°C with the tested compounds. Subsequently PACAP38 was added, and the incubation continued for 15 min. The reaction was terminated by adding 0.55 ml of 10% trichloroacetic acid. The slices were homogenized and centrifuged, and the resulting supernatants (of which 50 μl aliquots were saved for the determination of total radioactivity) were transferred into test tubes. The [<sup>3</sup>H]cyclic AMP formed was isolated by a sequential co-chromatography using Dowex 50Wx4 and aluminum oxide columns, with a tracer [<sup>14</sup>C]cyclic AMP used for measurement of the recovery of each assay (the mean recovery was 40–48%) according to the method of Salomon et al. [18]. Data presented have been corrected for recovery and expressed as per cent conversion ([<sup>3</sup>H]cyclic AMP × 100/total [<sup>3</sup>H]).

### Chemicals

2,8-[<sup>3</sup>H]Adenine (sp. activity 28.8 Ci/mmol) and [<sup>14</sup>C]cyclic AMP (sp. activity 52.3 mCi/mmol) were purchased from DuPont New England Nuclear (Boston, MA, USA). Chelerythrine, 3-isobutyl-1-methylxanthine (IBMX), NPC-15437 (S-2,6-diamino-N[[1-(1-oxotridecyl)-2-piperidinyl]methyl]hexanamide), 4β-phorbol 12,13-dibutyrate (4β-PDB), PACAP38 and staurosporine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). H-7 ((±)-1-5-(isoquinolinesulfonyl)-2-methylpiperazine) was from Tocris Cookson Ltd., Bristol, UK. Other chemicals were of analytical purity and were obtained mainly from Sigma Chemical Co. (St. Louis, MO, USA).

Data were expressed as mean ± SEM values. To calculate statistical significance of differences be-

tween group means, a one-way analysis of variance followed by post-hoc Student-Newman-Keuls test was employed, using GraphPad software (GraphPad, San Diego, CA, USA).

## RESULTS and DISCUSSION

To investigate a possible participation of PKC in the stimulatory action of PACAP38 on cyclic AMP production in chick cerebral cortex, we have employed four inhibitors of PKC with different chemical formulas and potencies, i.e. chelerythrine, staurosporine, H-7 and NPC-15437. Prior treatment of the chick cortical slices with these compounds did not affect the basal cyclic AMP accumulation. Of the tested PKC inhibitors, only chelerythrine (1–50 μM) potently reduced, in a concentration-dependent manner, increases in cyclic AMP formation evoked by 30 nM (data not shown) and 100 nM PACAP38 (Fig. 1). Staurosporine (0.3–3 μM) and H-7 (100 and 300 μM) did not affect the stimulatory action of PACAP38 on cyclic AMP formation in the chick cerebrum. There was a tendency of NPC-15437 (3–300 μM) to decrease the PACAP-evoked cyclic AMP response, yet the obtained values did not reach the level of statistical significance

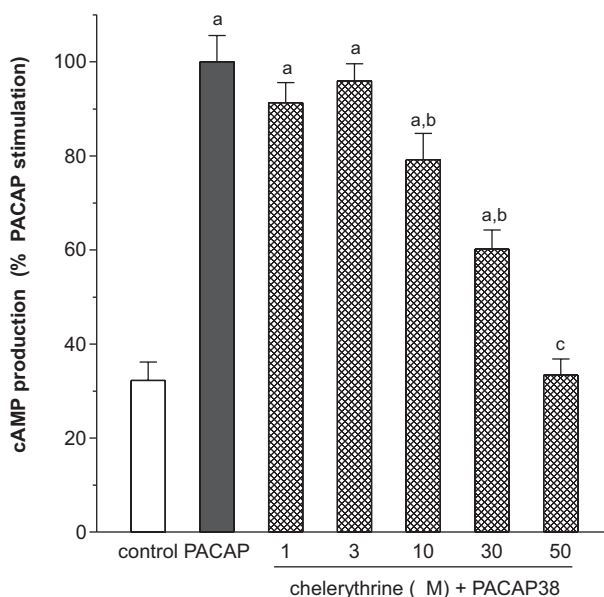


Fig. 1. Chelerythrine inhibits PACAP38 (100 nM)-stimulated increase in cyclic AMP production in the [<sup>3</sup>H]adenine pre-labeled slices of the chick cerebral cortex. Results are means ± SEM values from n = 9–20 measurements, expressed as per cent of PACAP38 response (7.69 ± 0.43 % conversion defined as 100%; n = 19). <sup>a</sup> p < 0.001 vs. control, <sup>b</sup> p < 0.01 vs. PACAP38, <sup>c</sup> p < 0.001 vs. PACAP38

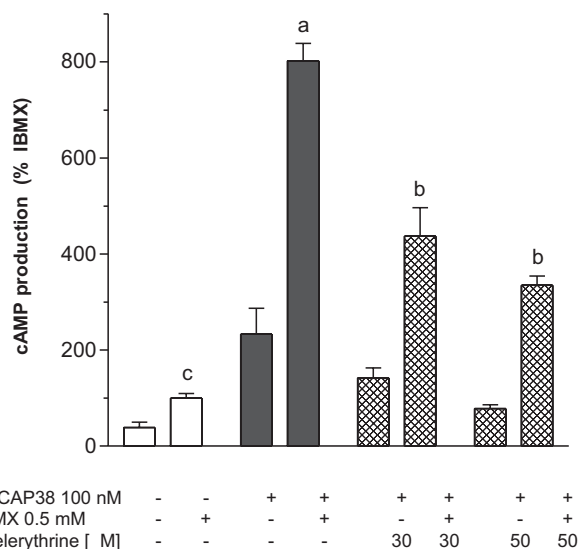


Fig. 2. Lack of effect of 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM) on the suppressive action of chelerythrine on PACAP38 (100 nM)-stimulated increase in cyclic AMP production in the [ $^3$ H]adenine prelabeled slices of the chick cerebral cortex. Results are means  $\pm$  SEM values from  $n = 4-17$  measurements, expressed as per cent of basal cyclic AMP production in the presence of 0.5 mM IBMX (2.80  $\pm$  0.26% conversion defined as 100%;  $n = 9$ ). <sup>a</sup>  $p < 0.001$  vs. IBMX, <sup>b</sup>  $p < 0.001$  vs. PACAP38 with IBMX, <sup>c</sup>  $p < 0.01$  vs. control (incubation in the absence of IBMX)

(Tab. 1). The effectiveness of chelerythrine in counteracting the PACAP-induced increase in cyclic AMP formation in the chick cerebrum, together with a non-significant activity of NPC-15437, and lack of effect of H-7 and staurosporine, suggests that the observed action of chelerythrine may be independent of PKC. It should be noted that although chelerythrine is widely used as a specific PKC inhibitor [6], some interactions of this compound with other effectors, such as, e.g. activation of cyclic nucleotide phosphodiesterase or inhibition of 5-phosphatase activity, have already been reported [3, 9]. In our experimental model, the action of chelerythrine was observed both in the presence and absence of 0.5 mM IBMX (Fig. 2), a phosphodiesterase inhibitor, implying that an alteration of cyclic AMP hydrolysis was not involved. Thus, the exact molecular mechanism(s) underlying the suppressive effect of chelerythrine on the PACAP-induced increase in cyclic AMP formation in the cerebral cortex of chick remains to be established.

An interaction between intracellular systems linked to adenylyl cyclase and PKC is a subject of increasing interest [17]. The tumor-promoting phor-

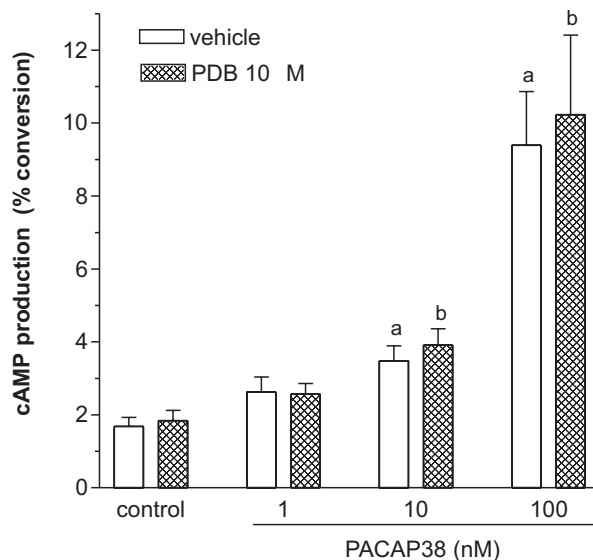


Fig. 3. Effects of 4 $\beta$ -phorbol 12,13-dibutyrate (4 $\beta$ -PDB; 10  $\mu$ M) on basal and PACAP38 (1–100 nM)-stimulated cAMP production in the [ $^3$ H]adenine prelabeled slices of the chick cerebral cortex. Results are means  $\pm$  SEM values from  $n = 8-17$  measurements, expressed as per cent conversion. <sup>a</sup>  $p < 0.001$  vs. control, <sup>b</sup>  $p < 0.001$  vs. PDB 10  $\mu$ M

bol esters, which directly activate PKC by substituting for the endogenous activator DAG [11], enhance cyclic AMP accumulation elicited by various neurotransmitters and hormones in a variety of tissues (e.g. [4, 7, 8, 17]). We have recently demonstrated that these compounds potentiate increases in cyclic AMP formation mediated by H<sub>2</sub>-like histamine receptors in the cerebral cortex and pineal gland of chick [21, 24]. Hence, it could be hypothesized that the PACAP-evoked stimulation of cyclic AMP formation in the chick cerebral cortex may also be a subject of modulation by PKC. To test this assumption, we examined effects of a biologically active 4 $\beta$ -phorbol ester, i.e. 4 $\beta$ -PDB, known to enter cells and activate PKC [2], on the action of PACAP. Pretreatment of the chick cerebral cortical slices with 10  $\mu$ M 4 $\beta$ -PDB did not significantly affect either the basal or PACAP38-stimulated cyclic AMP formation (Fig. 3). These data indicate that a concomitant stimulation of PKC does not modulate the studied action of PACAP. One likely explanation of this observation is that adenylyl cyclase isozyme linked to PAC1-type receptors in the chick cerebral cortex is not sensitive to phosphorylation by the PKC subtype coexpressed in the same cells, a situation being already described for other experimental models [7, 8].

Table 1. Lack of significant effect of H-7, staurosporine, and NPC-15437 on PACAP38 (100 nM)-stimulated increase in cyclic AMP production in [<sup>3</sup>H]adenine prelabeled slices of the chick cerebral cortex

	n	Antagonist concentration (μM)	Cyclic AMP production	
			% conversion	% PACAP38 response
<i>Experiment 1</i>				
PACAP38	15		8.51 ± 0.59	100.00 ± 6.93
PACAP38 + H-7	17	100		95.35 ± 3.44
	12	300		106.22 ± 3.40
<i>Experiment 2</i>				
PACAP38	23		7.65 ± 0.61	100.00 ± 7.960
PACAP38 + taurosporine	6	0.3		93.66 ± 13.52
	30	1		97.30 ± 3.61
	20	3		98.04 ± 2.83
<i>Experiment 3</i>				
PACAP38	13		5.59 ± 0.60	100.00 ± 12.34
PACAP38 + NPC-15437	15	3		92.78 ± 3.78
	15	10		84.50 ± 2.93
	14	30		84.42 ± 4.98
	19	100		76.93 ± 9.61
	5	300		75.98 ± 7.82

Results are means ± SEM values from n measurements

In summary, although PACAP, in addition to its ability to potently increase cyclic AMP formation, stimulates phosphoinositide turnover and activates PKC in the cerebral cortex of chick, we were unable to clearly demonstrate a cross-talk between these two signaling pathways, at least with respect to cyclic AMP generation.

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