Pituitary adenylate cyclase polypeptide (PACAP) stimulates cyclic AMP formation in pituitary fibroblasts and 3T3 tumor fibroblasts: lack of enhancement by protein kinase C activation

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Summary

A number of neuropeptides were shown to produce potent mitogenic effects on Swiss 3T3 fibroblasts by activating the phospholipase C pathway. Here we provide evidence for the activation by PACAP of the adenylate cyclase pathway in 3T3, as well as in non-tumoral pituitary fibroblasts, similarly to what was seen in pituitary endocrine cells. In these cells, PACAP triggered elevation of both intracellular and extracellular contents of cAMP and the effect was time- and dose-dependent, with half-maximal stimulations being induced with about 0.1 nM. Following activation of protein kinase C (PKC) by the phorbol ester phorbol 12-myristate 13-acetate (PMA), PACAP-induced cAMP production was amplified in pituitary endocrine cells, but was either unchanged or dampened in 3T3 and pituitary fibroblasts, respectively. Pretreatment of cells with pertussis toxin (PT) failed to change the effect of PMA on PACAP-stimulated adenylate cyclase activity, irrespective of the cell type being used. However, PT dramatically reduced the potentation by PMA of cAMP production enhanced by forskolin in 3T3 cells. These results provide new evidence pointing to the presence in fibroblasts of receptors for PACAP, coupled to cAMP production, which may play a role in the modulation of the mitogenic signal. They also indicate that, compared with pituitary endocrine cells, PKC activation in fibroblasts differentially affected PACAP-induced cAMP formation and that these effects were unaltered upon inhibition by PT of G_1-like proteins.

Introduction

Pituitary adenylate cyclase-activating polypeptides PACAP38 and PACAP27, with respectively 38 and 27 amino acid residues, have recently been isolated from and localized in ovine hypothalami (Miyata et al., 1989; Köves et al., 1990). These peptides, which share sequence homology with vasoactive intestinal peptide, were found to trigger cyclic AMP (cAMP) production in cultured rat anterior pituitary cells (Miyata et al., 1989), by binding to high affinity receptor sites (Shivers et al., 1991), most probably linked to G-proteins (Schäfer et al., 1991). The functional role of these peptides is not yet clearly established, as they failed to affect peptide output from pituitary cells incubated under static incuba-
tion conditions. In recent studies, however, we and others showed that PACAP exerted a stimulatory effect on growth hormone, prolactin and ACTH secretion from cultured tumor pituitary cell lines GH and AtT-20, as well as on α-melanocyte-stimulating hormone (α-MSH) secretion from cultured melanotrophs (Koch and Lutz-Bucher, 1992; Propato-Mussafari et al., 1992). Also, PACAP showed up to increase intracellular Ca2+ concentrations in pituitary gonadotropes and somatotropes (Canny et al., 1992).

Although PACAP has originally been defined after its ability to stimulate adenylyl cyclase activity in pituitary cell cultures (Miyata et al., 1989), functional binding sites for this family of peptides have recently also been localized in a number of other tissues, including the liver (Robberecht et al., 1991), the pancreas (Gourlet et al., 1991), adrenal PC12 cells (Watanabe et al., 1990), the testis and the brain (Arimura et al., 1991; Köves et al., 1991). Interestingly, PACAP receptors in the brain were shown to be present not only on neurons but also on astrocytes (Tatsumo et al., 1990), supporting the growing notion that glial cells may play important regulatory roles in brain function (for review see Martin, 1992). In this connection, we and others have also demonstrated the presence of guanylate cyclase-coupled receptors for atrial natriuretic peptide on glial cells in the brain and neurohypophysis (Luckman and Bicknell, 1991; Lutz-Bucher et al., 1991), as well as on rat tumoral fibroblasts and normal fibroblasts subcultured from pituitary tissue (Leitman et al., 1987; Lutz-Bucher et al., 1989). Among the tumoral cell lines, Swiss 3T3 fibroblasts provide a useful model system because they are responsive to a number of mitogenic neuropeptides, including bombesin, endothelin, bradykinin and vasopressin (Rozenburg et al., 1987; Zachary et al., 1991). Interestingly, there is also evidence for the existence in these cells of cross-talks between various signal transduction pathways, as protein kinase C (PKC) activation was found to enhance stimulated production of cAMP, most probably through a mechanism involving a pertussis toxin (PT)-sensitive substrate (Rozenburg et al., 1987).

Here we addressed the question as to whether PACAP receptors, coupled to cAMP formation, were similarly expressed in fibroblasts subcultured from pituitary cells and in Swiss 3T3 derived fibroblasts and examined the possible involvement of PKC, as well as of PT-sensitive G-like proteins, in cyclic nucleotide production. Our data show that PACAP stimulates cAMP formation not only in endocrine cells of the pituitary gland, but also in pituitary fibroblasts and in tumor fibroblasts. They also indicate that activation by phorbol ester of PKC induced differential effects on PACAP-induced formation of cAMP in fibroblasts compared to pituitary endocrine cells, while inhibition of guanine regulatory G-like proteins produced no significant change.

Materials and methods

Cells and reagents

Pituitary glands, obtained from male Wistar rats (300–350 g), were separated between anterior and neurointermediate lobes and the former were used in cell culture. 3T3 fibroblasts (SV3T3B; derived from Swiss fibroblasts) were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). PACAP38 and corticotropin-releasing factor (CRF) were obtained from Peninsula Laboratories (Merseyside, UK), culture medium (Dulbecco’s modified Eagle’s medium (DMEM without phenol red), forskolin, 3-isobutyl-1-methylxanthine (IBMX) and phorbol 12-myristate 13-acetate (PMA) from Sigma Chimie (La Verpillière, France). Pertussis toxin was purchased from List Biological Laboratories (Campbell, USA). Forskolin and PMA were added to incubation media as a 2000-fold concentrated stock solution in ethanol and dimethyl sulfoxide, respectively. The same amount of vehicle (final concentration of 0.05%) was included in media of control wells. Antiserum against CAMP was generously provided by G. Pelletier (Québec, Canada).

Cell cultures

Anterior pituitary tissue was enzymatically dispersed and dissociated cells were further purified as already reported (Koch and Lutz-Bucher, 1991). Cells were plated at a density of 2–3 × 10⁵ cells per well in 24-well cluster plates (Costar). They were cultured for 1–2 days in DMEM sup-
plemented with 7.5% horse serum, 2.5% fetal calf serum and antibiotics in a humidified atmosphere of 5% CO₂-95% air.

Fibroblasts were obtained by subculturing pituitary cells for 4–5 weeks in DMEM containing 10% fetal calf serum, with 4–5 cycles of dissociation-replating with 0.015% trypsin in phosphate-buffered saline containing 1 mM EDTA. This resulted in the generation of a highly enriched population of proliferating non-endocrine cells, which exhibited the morphological features of fibroblasts with the typical display of cytoskeletal vimentin filaments, as shown previously by immunocytochemical studies (Lutz-Bucher et al., 1989).

Tumor 3T3 fibroblasts were grown in DMEM enriched with 10% fetal serum and were used at near confluency.

**Cell productions of cAMP**

Cells were washed and equilibrated for 1 h, at 37°C, in Krebs-Hepes buffer composed of (in mM): 127 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 20 Hepes, 0.1 ascorbic acid, supplemented with 0.2% glucose, 0.02% glutamine, 0.1% bovine serum albumin (w/v) and 0.5% (v/v) amino acid mixture (50 ×), at pH 7.4. In some cases, cells were pretreated with PT (100 ng/ml) for 16 h and/or with PMA (10⁻⁷ M) for 10 min prior to and during exposure to test substances.

For measurement of cAMP production, cells were preincubated for 15 min with 0.5 mM IBMX in incubation medium and then incubated in the same medium with various concentrations of PACAP, CRF or forskolin. At the time periods indicated, the media were collected and to the cells was added 1 ml ice-cold 0.1 M HCl, followed by sonication. After centrifugation of media and cell extracts, the supernatants were saved at –25°C for measurement of cAMP by means of radioimmunoassay (Koch and Lutz-Bucher, 1991).

**Results**

**Time-course of the effect of PACAP on cAMP formation**

The time-courses of the effect of PACAP on cAMP production of pituitary fibroblasts and 3T3 fibroblasts are shown in Fig. 1 and Fig. 2, respectively. Upon exposure of both cell types to 5 nM PACAP there was a rapid elevation in intracellular cAMP concentration (2.5 to 5 times the basal level; p < 0.01) by 4–8 min. This was followed by a gradual drop of cellular cAMP, while there was at least 2–3 times, with representative results being reported.

**Statistics**

Statistical evaluation of the data was performed with analysis of variance, followed by the Bonferroni analysis for multiple comparisons (Instat-Graphpad software). Data displayed in figures are the means ± SE of 3–6 observations done in triplicate. Each experiment was repeated
a steadily rise in extracellular cAMP from 8 min to 90 min, at which time it reached about 13 times \( (p < 0.001) \) and 2 times \( (p < 0.01) \) the control value (in the absence of PACAP, at the same time point of 90 min) for pituitary fibroblasts and 3T3 cells, respectively.

**Dose dependency of the effect of PACAP**

Incubation of pituitary fibroblasts (Fig. 3A) and 3T3 fibroblasts (Fig. 3B) in the presence of increasing concentrations of PACAP produced a dose-related increment in intracellular cAMP contents. Maximal stimulations were achieved with 1–10 nM PACAP in both cell cultures, while half-maximal stimulations were obtained with \( (0.9-1.2) \times 10^{-10} \text{ M} \) and \( (3-4) \times 10^{-10} \text{ M} \) \( (n = 2) \), respectively.

**Effect of PKC activation on cAMP production**

Because PKC is thought to play a regulatory role in adenylate cyclase activity in various cell types (for review see Houslay, 1991), we tested the effect of PMA on cAMP production stimulated by PACAP in fibroblasts, as well as in endocrine cells of the pituitary gland. In 3T3 fibroblasts, activation by PMA of PKC activity was found to produce no significant change in cAMP accumulation stimulated by PACAP (Fig. 4B; \( p > 0.05 \)), while there was a dramatic potentiation upon cell exposure to forskolin (Fig. 4A; \( p < 0.001 \) compared with vehicle-treated cells). In sharp contrast, PMA treatment significantly dampened cAMP formation in pituitary fibroblasts exposed to PACAP (Fig. 5B; \( p < 0.001 \)), but failed to modulate the effect of \( 10^{-8} \) to \( 10^{-6} \).
Fig. 4. Effect of PMA on cAMP production of 3T3 fibroblasts incubated in the presence of increasing concentrations of forskolin (A) or PACAP (B). Points are means ± SE of 4–6 observations done in triplicate. In panel A: PMA treatment (100 nM) vs. vehicle was highly significant, with \( p < 0.001 \).

M forskolin (Fig. 5A; \( p > 0.05 \)). When similar experiments were carried out using pituitary endocrine cell cultures, we found that PKC activation resulted in enhancement of cAMP production stimulated by both PACAP (Fig. 6A; \( p < 0.008 \) in the concentration range of 1–10 nM) and CRF (Fig. 6A; \( p < 0.001 \) in the range of 0.1–10 nM). However, this sensitization of adenylate cyclase activity was consistently less pronounced in cells exposed to PACAP than in those exposed to CRF: respectively 1.5 times and 3–6 times the cAMP cell contents accumulated in the absence of PMA.

Effect of pretreatment of cells with pertussis toxin

We next tested whether inhibition by PT of \( G \)-like proteins would play a role in the effect of PACAP on cAMP formation in cells treated, or not, with PMA. Data displayed in Table 1 clearly show that PT pretreatment of cells (100 ng/ml for 16 h) failed to affect the ability of PACAP to activate adenylate cyclase in both 3T3 and pituitary fibroblasts, as well as in pituitary endocrine cells. Nor did it reverse either the inhibitory or the stimulatory effect of PMA on cAMP formation in non-tumoral fibroblasts and pituitary cells, respectively. However, under the same experimental conditions, PT consistently reversed PMA-amplified formation of cAMP triggered by \( 10^{-16} \) M forskolin in 3T3 fibroblasts: cellular cAMP content, which reached 30.1 ± 0.6 pmol/well in cells exposed to forskolin + 100 nM PMA (compared to 14.2 ± 0.4 with forskolin alone), was reduced to 5.1 ± 0.2 pmol/well after PT pretreatment. Furthermore, we also showed that long-term exposure of pituitary cells to PT (100
Fig. 6. Effect of PMA treatment on cAMP formation in pituitary endocrine cells exposed to increasing concentrations of CRF (A) or PACAP (B). The effects of treatment were highly significant, with \( p < 0.001 \) and \( p < 0.01 \) for, respectively, values in panel A (0.1–10 nM CRF) and those in panel B (1–10 nM PACAP).

ng/ml for 16 h) amplified cAMP formation elicited by \( 10^{-8} \) M CRF from 5.7 ± 0.2 to 7.8 ± 0.3 pmol/well for control and pretreated cells, respectively (two-tailed \( p \) value was 0.0008).

Discussion

Accumulating evidence suggests that a number of neuropeptides, including vasopressin, bombesin and endothelin, act as cellular growth factors in Swiss 3T3 fibroblasts (Rozengurt et al., 1987). These peptides interact with membrane receptors linked to GTP-binding proteins and trigger the breakdown of phosphatidylinositol phosphate and, at least for the effect of vasopressin on tumor fibroblasts, that of phosphatidylcholine as well (Huang and Cabot, 1990). This was followed by formation of diacylglycerol and subsequent activation of PKC and inositol trisphosphate-induced mobilization of intracellular Ca\(^{2+}\) stores. They also appear to signal through an additional pathway by stimulating tyrosine phosphorylation of specific substrates (Huang and Cabot, 1990; Zachary et al., 1991). Finally, bombesin was also shown to induce a membrane current in these cells by increasing Ca\(^{2+}\)-activated K\(^+\) conductance (Kusano and Gainer, 1991).

In the present investigation, we provide evidence which clearly indicates that in 3T3 fibroblasts, as well as in non-tumoral fibroblasts, the cAMP signaling pathway could likewise be stimulated by a neuropeptide, namely PACAP. This conclusion is supported by the findings that incubation of these cells in the presence of PACAP produced dose- and time-dependent increases in both intracellular and extracellular cAMP concentrations. This occurred with half-maximal stimulatory doses which are in good agreement with the \( K_d \) values of 0.2–0.5 nM reported for PACAP receptors in tissues such as the pituitary, the liver and the brain (Cauvin et al., 1991; Robberecht et al., 1991; Shivers et al., 1991). The

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3T3 fibroblasts</th>
<th>Pituitary fibroblasts</th>
<th>Pituitary endocrine cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PACAP</td>
<td>5.8 ± 0.2</td>
<td>9.1 ± 0.6</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>PACAP + PT</td>
<td>6.3 ± 0.1</td>
<td>9.8 ± 0.8</td>
<td>8.8 ± 0.5</td>
</tr>
<tr>
<td>PACAP + PMA</td>
<td>6.5 ± 0.3</td>
<td>5.7 ± 0.5 *</td>
<td>15.4 ± 0.9 *</td>
</tr>
<tr>
<td>PACAP + PT + PMA</td>
<td>6.0 ± 0.2</td>
<td>4.7 ± 0.7 *</td>
<td>14.4 ± 0.9 *</td>
</tr>
</tbody>
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* \( p < 0.05 \), compared with cells exposed to PACAP alone.
apparent biphasic pattern of the dose–response
curves depicted in Fig. 3 could be due to the
presence of more than one type of cells or, most
probably, to the existence of different popula-
tions of PACAP receptors, which actually com-
prise at least four distinct subtypes (for review
see Arimura, 1992). A study is currently under
way in order to characterize the type(s) of PACAP
receptors present on fibroblasts.

Thus PACAP, like other neuropeptides and
growth factors, may play a role as a mitogenic
factor and/or as a modulator of the mitogenic
signal and may thus be involved in such impor-
tant biological processes as development, tissue
regeneration and carcinogenesis. Although the
potential effect(s) of PACAP could involve
cAMP-dependent protein kinase phosphorylation
of multiple substrates, a recent study points to a
possible regulatory process in the expression of
some specific genes. Indeed, because it has been
demonstrated that stimulation of cAMP for-
mation in human fibroblasts induced the messenger
RNAs coding for interleukin-6 (IL-6) and the
proto-oncogenes c-fos and c-myc (Zhang et al.,
1988), it seems likely that PACAP-stimulated en-
hancement of the cAMP-dependent pathway may
participate in the regulation of these genes as
well. In this connection, it is of interest to note
that PACAP has actually been linked to IL-6
production by cultured pituitary cells (Tatsumo et
al., 1991). Finally, although it seems unlikely that
PACAP enhances tyrosine phosphorylation in a
way similar to that of bombesin and vasopressin
(Zachary et al., 1991), that possibility deserves to
be examined.

The cellular cAMP content is under control of
multiple regulatory processes, including both syn-
thesis and degradation by several isoforms of
phosphodiesterases of the cyclic nucleotide, as
well as cross-regulation with other signaling path-
ways. Among the latter, PKCs appear to play a
major role (for review see Houslay, 1991). These
kinases are able to either increase or decrease
cAMP formation by phosphorylating specific sub-
strates including various components of the G-
protein-linked receptor complex, subunits of
adenylate cyclase and phosphodiesterases. Fur-
thermore, PKC could additionally elicit the for-
mation of phosphatidylcholine and phosphatidic
acid, finally yielding diacylglycerol and arachi-
donic acid. In the present study we show that,
depending on the cell type, activation by PMA of
PKC activity produced differential effects on
PACAP-induced stimulation of cAMP accumula-
tion, that seem to reflect the functioning of these
multiple regulatory pathways. Exposure of pitu-
itary fibroblasts to PMA dampened cAMP forma-
tion elicited by PACAP, while it produced no
change in 3T3 fibroblasts. In the latter cells,
however, PKC activation resulted in a dramatic
amplification of adenylate cyclase activity stimu-
lated by forskolin, as previously shown (Rozen-
gurt et al., 1987). Interestingly, PMA treatment of
pituitary endocrine cells, which has been shown
to enhance the ability of CRF to stimulate accu-
mulation of cAMP (Cronin et al., 1986; Abou-
Samra et al., 1987; Lutz-Bucher et al., 1990),
likewise synergized with the effect of PACAP. In
that case, however, the potentiating effect was
less pronounced than with CRF, suggesting the
existence of specific differences in the mechanism
of action of the peptides, due to the selective
expression of some isoforms of adenylate cyclases
and/or in that of some PKC isozymes.

PT not only inactivates G-proteins coupled to
adenylyl cyclase activity, but also acts on various
other G-like proteins which may be related to
regulation of Ca\(^{2+}\) and K\(^+\) channels, as well as to
phospholipase C and A\(_2\) activities (for review see
Johnson and Dhanasekaran, 1989). In this con-
nection, it has been suggested that PT-sensitive
G-like proteins may be involved, in part, in the
sensitization effect of phorbol ester on cAMP
production triggered by forskolin in 3T3 fibro-
blasts (Rozengurt et al., 1987) and by CRF in
pituitary cells (Abou-Samra et al., 1987). The
present study, in addition to confirming the latter
findings, shows that, in contrast, pretreatment
with PT of 3T3 and pituitary fibroblasts, as well
as of pituitary endocrine cells, not only failed to
reverse the modulatory effects of PMA on
PACAP-stimulated production of cAMP, but also
proved ineffective in significantly amplifying the
ability of PACAP to elicit cAMP accumulation in
these cells. It thus seems that the functioning of
the PACAP receptor, as expressed at least in
terms of cellular cAMP production, may be unre-
related to PT-sensitive G-proteins.
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References