Pituitary adenylate cyclase-activating polypeptide increases \([\text{Ca}^2+]_i\) in rat gonadotrophs through an inositol trisphosphate-dependent mechanism

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) increases cAMP production and stimulates hormone release from a variety of anterior pituitary cells. However, in anterior pituitary gonadotrophs PACAP stimulates oscillations in cytosolic free Ca^{2+} concentration \(([\text{Ca}^2+]_i)\) that appear to be independent of cAMP. To study the mechanisms involved in this response, we used the patch-clamp technique to microperfuse various agents into single rat gonadotrophs while monitoring \([\text{Ca}^2+]_i\) with microfluorometry. Extracellular application of PACAP to single gonadotrophs stimulated high amplitude (> 1 microM) oscillations in \([\text{Ca}^2+]_i\), which were blocked by intracellular application of GDP beta S (guanosine 5'-O-2-thiodiphosphate), indicating the involvement of a G-protein. To identify the intracellular messenger(s) involved, we microperfused gonadotrophs with cAMP, inositol 1,4,5-trisphosphate (Ins(1,4,5)P3), or heparin, an antagonist of the Ins(1,4,5)P3 receptor. A high concentration of cAMP (100 microM) had no significant effect on basal \([\text{Ca}^2+]_i\) and did not alter the PACAP-stimulated Ca^{2+} response. Heparin, but not its inactive [...]
Pituitary Adenylate Cyclase-activating Polypeptide Increases \([\text{Ca}^{2+}]_{\text{i}}\), in Rat Gonadotrophs through an Inositol Trisphosphate-dependent Mechanism*

(Received for publication, June 22, 1993, and in revised form, September 14, 1993)

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Pituitary adenylate cyclase-activating polypeptide (PACAP) increases cAMP production and stimulates hormone release from a variety of anterior pituitary cells. However, in anterior pituitary gonadotrophs PACAP stimulates oscillations in cytosolic free \([\text{Ca}^{2+}]_{\text{i}}\) that appear to be independent of cAMP. To study the mechanisms involved in this response, we used the patch-clamp technique to microperfuse various agents into single rat gonadotrophs while monitoring \([\text{Ca}^{2+}]_{\text{i}}\) with microfluorometry. Extracellular application of PACAP to single gonadotrophs stimulated high amplitude (>1 μm) oscillations in \([\text{Ca}^{2+}]_{\text{i}}\), which were blocked by intracellular application of GDPβS (guanosine 5’-O-2-thiodiphosphate), indicating the involvement of a G-protein. To identify the intracellular messenger(s) involved, we microperfused gonadotrophs with cAMP, inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃), or heparin, an antagonist of the Ins(1,4,5)P₃ receptor. A high concentration of cAMP (100 μM) had no significant effect on basal \([\text{Ca}^{2+}]_{\text{i}}\) and did not alter the PACAP-stimulated \([\text{Ca}^{2+}]_{\text{i}}\) increase in \([\text{Ca}^{2+}]_{\text{i}}\), while Ins(1,4,5)P₃ stimulated oscillations in \([\text{Ca}^{2+}]_{\text{i}}\), very similar to those observed in response to PACAP. These results strongly suggest that PACAP mobilizes \([\text{Ca}^{2+}]_{\text{i}}\) through an Ins(1,4,5)P₃-dependent mechanism. The fact that PACAP stimulates two signaling pathways in pituitary cells could substantially enhance the signaling potential of this hypothalamic peptide.

Pituitary adenylate cyclase-activating polypeptide (PACAP)¹ is the most recently discovered member of the secretin/glucagon/vasoactive intestinal polypeptide (VIP) family of peptides. It is a 38-amino acid, C terminus-aminated, peptide (PACAP38), which also exists in a 27-amino acid form (PACAP27) (Miyata et al., 1989, 1990). The first 28 amino acids of PACAP38 share 68% sequence homology with porcine VIP, although the remaining sequence is unique (Miyata et al., 1989). However, PACAP38 is approximately 1000 times more potent at stimulating CAMP production in anterior pituitary cells than is VIP (Miyata et al., 1989). PACAP-like immunoreactivity is found in the median eminence of the hypothalamus (Koves et al., 1990) and specific PACAP binding sites, which do not significantly bind VIP, are present on anterior pituitary cells (Gottschall et al., 1990). In addition, PACAP can modulate the in vitro and in vivo release of a variety of pituitary hormones including luteinizing hormone (LH) (Culler and Pascall, 1991; Hart et al., 1992; Osuga et al., 1992), and growth hormone (Goth et al., 1992; Hart et al., 1992; Jarry et al., 1992). Thus it has been proposed that PACAP may act as a hypothalamic regulator of anterior pituitary cell function.

The intracellular mechanisms regulated by PACAP have yet to be fully determined. In addition to its effect on CAMP levels, PACAP has been shown to increase \([\text{Ca}^{2+}]_{\text{i}}\), in a number of anterior pituitary cell types (Canny et al., 1992; Vigh et al., 1992; Gracia-Navarro et al., 1992). In somatotrophs, which secrete growth hormone, PACAP stimulates \([\text{Ca}^{2+}]_{\text{i}}\) influx through a CAMP-dependent mechanism (Canny et al., 1992; Rawlings et al., 1993). In contrast, in gonadotrophs PACAP stimulates \([\text{Ca}^{2+}]_{\text{i}}\) release from an intracellular store, an effect that is not blocked by an antagonist to CAMP-dependent protein kinase (Canny et al., 1992; Rawlings et al., 1993). It thus appears that PACAP can stimulate two different intracellular pathways in closely related cell types. This study addresses the mechanisms by which PACAP stimulates the mobilization of \([\text{Ca}^{2+}]_{\text{i}}\) in rat gonadotrophs.

A number of intracellular messengers are capable of stimulating the release of \([\text{Ca}^{2+}]_{\text{i}}\) from intracellular stores including inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) (Berridge and Irvine, 1989), cAMP (Cadiot et al., 1991), cADP ribose (Galiane, 1993) and \([\text{Ca}^{2+}]_{\text{i}}\) itself (Fabiato, 1983). The present study has investigated the mechanisms underlying the PACAP-stimulated \([\text{Ca}^{2+}]_{\text{i}}\) release in single identified gonadotrophs. The whole-cell patch clamp technique was used to apply various compounds intracellularly while \([\text{Ca}^{2+}]_{\text{i}}\) was monitored with microfluorometry. It was found that the action of this peptide in the gonadotroph is G-protein-mediated, and stimulates the release of \([\text{Ca}^{2+}]_{\text{i}}\) from an intracellular store through an Ins(1,4,5)P₃-dependent, CAMP-independent, mechanism.

**EXPERIMENTAL PROCEDURES**

*Anterior Pituitary Cell Dissection—Male Sprague-Dawley rats (200-300 g, IFPA Credo, l'Arbresle, France) were kept in a controlled environment before use (lights on, 0700-1900, 25 °C). For each experimental day, a single rat was decapitated, the anterior pituitary gland removed, finely diced, and the cells dissociated in Spinner's minimum essential medium (Life Technologies, Inc.) containing 0.1% trypsin...*
PACAP38 Regulation of [Ca\(^{2+}\)]\(_i\) in Rat Gonadotrophs

(Difo), 0.2% penicillin/streptomycin (Life Technologies, Inc.), and 0.1% bovine serum albumin at 37°C for 1 h. The cells were resuspended in RPMI medium (Life Technologies, Inc.), containing 0.1% bovine serum albumin and 0.2% penicillin/streptomycin, at 400,000 cells/ml, at 37°C for at least 1 h before use.

\([\text{Ca}^{2+}]\), Measurements—Anterior pituitary cells were loaded with Indo-1AM (2.5 μM) in RPMI for 30 min at room temperature, washed with RPMI to remove any remaining ester, and then kept at room temperature. A coverslip holding Indo-1-loaded cells was fixed onto a chamber on the stage of an inverted microscope. Nikon Diaphot) equipped for dual emission recordings (De Maurex et al., 19931, and changes in [Ca\(^{2+}\)]\(_i\) were monitored by recording the fluorescent emission of the Indo-1 in response to UV light. A high wavelength light was passed through a monochromator and was attenuated by neutral density filters (x 256), passed through a 355-nm interference filter, and reflected to the stage of the microscope by a dichroic mirror (critical wavelength \(\lambda_{\text{c}}\) 440 nm). The emitted fluorescent light was collected by an objective (Nikon Fluor x 40, oil immersion) and split into two by a second dichroic mirror (\(\lambda_{\text{c}}\) 455 nm), the high wavelength light then passing through a 480-nm interference filter to be detected by one photomultiplier, while the low wavelength light passed through a 405-nm interference filter to be detected by a second photomultiplier. Emitted Indo-1 fluorescence at the two wavelengths was recorded simultaneously at 50 Hz using a 12-bit analogue–digital converter system (Hamill et al., 1981) interfaced to an IBM personal computer. The output of the two photomultipliers was also recorded directly onto a digital tape recorder (DTR-1201 Biological, 38640 Clais, France) at 12 kHz to provide a backup of the recording.

Whole Cell Patch Clamp Electrophysiology—The whole-cell configuration of the patch clamp technique (Hamill et al., 1981) was used to voltage-clamp and micropuncture substances into the cells. Borosilicate glass pipettes were made using a BB-CH-PC puller (Mecanex, Nyon, Switzerland) and had resistances of between 6 and 12 megarohms when using the standard solutions described below. Pipette-cell membrane (resistance) resistances were between 20 and 50 megarohms, and access resistances upon establishing the whole cell configuration ranged from 15 to 40 megarohms. Recordings were made with an Axopatch 200A amplifier (Axon Instruments Inc., Foster City, CA), in the voltage-clamp mode. The recording of current and holding current were made by the same A/D interface used for the [Ca\(^{2+}\)]\(_i\) measurements, allowing for concurrent recordings of electrophysiological and [Ca\(^{2+}\)]\(_i\) data. The data were also backed up onto the digital tape recorder at a sampling rate of 24 kHz.

Whole-cell recordings were made with cells voltage-clamped to a potential close to the normal resting membrane potential. To determine this value, recordings of membrane potential at zero applied current were made for several seconds, with the membrane potential of identified gonadotrophs was found to range between −59 and −43 mV, with a mean value of −50 ± 2 mV (mean ± S.E., n = 10). Thus whole-cell voltage-clamp recordings were made at a holding potential of −50 mV.

Calculation of [Ca\(^{2+}\)]\(_i\), Values—Values for [Ca\(^{2+}\)]\(_i\) were calculated using the technique of Hamill et al. (1981) \(K_{\beta}=R_{\text{mem}}/R_{\text{pip}}\) (R inocula-

\[K_{\beta}=R_{\text{mem}}/R_{\text{pip}}\]

) was determined after perfusing cells with the standard pipette solution buffered to free [Ca\(^{2+}\)] < 10 nM with 10 mM EGTA. \(R_{\text{pip}}\) (1.61 ± 0.05; n = 10) was measured with a pipette containing 10 mM Ca\(^{2+}\); \(R_{\beta}\) (127 ± 30; n = 9) was calculated by solving the above equation using the \(R_{\beta}\) value measured following breaking in with a pipette containing 5.2 mM EGTA and 5.4 mM Ca\(^{2+}\), which produced a fixed Ca\(^{2+}\) concentration of 291 nM, as measured with a Ca\(^{2+}\)-sensitive electrode.

Solutions—Experiments were conducted at 22°C in experimental medium containing (in mM): 127 NaCl, 5 KCl, 2 MgCl\(_2\), 1.8 CaCl\(_2\), 5 NaHCO\(_3\), 10 HEPES-NaOH, and 10 glucose, pH 7.4. For the nominally Ca\(^{2+}\)-free medium, the CaCl\(_2\) was replaced with the Ca\(^{2+}\)-free buffer EGTA (1 mM). The standard pipette solution contained (in mM), 120 potassium aspartate, 20 KCl, 2 MgCl\(_2\), 20 HEPES-NaOH, 0.1 GTP, 2 ATP, 0.04 indol-1, pH 7.4. This pipette solution had a free Ca\(^{2+}\) concentration of between 0.5 and 85 nM, measured with a Ca\(^{2+}\)-sensitive electrode.

Micropuncture of Cells—The use of the whole-cell configuration of the patch clamp technique allowed compounds to be microperforated into the inside of single cells during recording. Where appropriate, heparin, de-N-sulfated heparin, Ins(1,4,5)P\(_3\), or CAMP were added to the standard pipette solution. For the experiments involving GDPβS, this nucleotide replaced GTP in the pipette solution. Achievement of the whole-cell configuration generally resulted in little change in [Ca\(^{2+}\)]. However, when Ins(1,4,5)P\(_3\) was included in the pipette solution, the cells were not perforated at all (data not shown). This indicates that the diffusion of low molecular weight compounds from the pipette into the cell is very rapid. A high access resistance between the pipette and the inside of the cell significantly reduces the ability of the pipette contents to diffuse into the cell (Marty and Neher, 1983), an effect that is particularly profound for large molecular weight compounds. When blockers were introduced into the cell, only recordings where the access resistance was less than 40 megarohms were considered. In general PACAP was applied to the cell 2 min after establishing the whole-cell configuration to ensure that even the large molecular weight compounds such as heparin had sufficient time to diffuse into the cell.

Cell Identification—Only 3–5% of anterior pituitary cells secrete LH in response to LHRH and thus can be identified as gonadotrophs (Rawlings et al., 1991). LHRH is a specific stimulator of gonadotrophs, and thus can be used as a tool to identify such cells (Naor, 1996). In this study lobed cells of 10–12 μm diameter were selected for recordings, since a high proportion of such cells have been shown to be LH-secreting gonadotrophs (Rawlings et al., 1991). In our hands, >95% of the cells selected on these morphological criteria showed a clear Ca\(^{2+}\) response to LHRH. Only cells exhibiting a Ca\(^{2+}\) response to LHRH (Leong and Thorner, 1981), added at the end of the experiment, were included in the statistical analysis. Cells that did not respond to IHRH were used for the identification of the potential blocking actions of intracellulary applied heparin and GDP(βS (Tse and Hille, 1992).

Materials—PACAP (1–38) was obtained from either Peninsula Laboratories Europe Ltd. (St. Helera, UK), or Calbiochem (Lauffelfingen, Switzerland). PACAP (1–38) was synthesized in the laboratories of Drs. Naor, Rawlings, and Tse. PACAP (1–38) was therefore synthesized inhouse. Materials used had an M\(_r\) of 6,000 (product no. H-2149; Sigma). All other peptides and reagents, unless otherwise stated, were obtained from Sigma.

RESULTS

This study is the first to employ whole-cell patch clamp recordings to study the action of PACAP at the single cell level. As such, it was necessary to test whether this technique significantly alters the responsiveness of gonadotrophs to PACAP. Thus the peptide was added to intact cells and cells in whole-cell recording configuration to compare the stimulated changes in [Ca\(^{2+}\)], under otherwise identical experimental conditions.

In intact gonadotrophs, a maximum concentration of PACAP (100 nm; Rawlings et al., 1993) stimulated an increase in [Ca\(^{2+}\)], (Fig. 1A). Of 18 gonadotrophs tested in this way, 15 cells (83%) showed an increase in [Ca\(^{2+}\)], and 12 of these 15 cells (80%) showed high amplitude (>1 μM) oscillations in [Ca\(^{2+}\)], as shown in Fig. 1A. The other three cells exhibited a low amplitude (0.2–0.4 μM), steplike increase in [Ca\(^{2+}\)], as previously described (Canny et al., 1992; Rawlings et al., 1993). In gonadotrophs subjected to whole-cell voltage-clamp recordings, PACAP (100 nm) stimulated an increase in [Ca\(^{2+}\)], (Fig. 1B, top). Of 23 cells tested in this way, 21 cells (91%) showed increases in [Ca\(^{2+}\)], and of these 21 cells, 19 (90%) showed the Ca\(^{2+}\) oscillations response (Fig. 1B). Hence, the responsiveness of the gonadotrophs to PACAP in the whole-cell recording configuration was not significantly altered compared to intact cells. Significantly, in both intact and patch clamp recording configuration, of PACAP in the medium did not preclude the Ca\(^{2+}\) response to a high concentration (10 nM) of LHRH (Fig. 1F). The whole-cell voltage-clamp technique also allowed us to monitor changes in cell membrane conductance. With cells voltage-clamped at −50 mV, close to the resting membrane potential of these cells (see “Experimental Procedures”), both PACAP and LHRH stimulated changes in membrane conductance, which appeared to closely follow their effects on changes in [Ca\(^{2+}\)], (Fig. 1B, bottom). Similar LHRH-stimulated currents have been previously reported (Kulkjyan et al., 1992; Tse and Hille, 1992) and have been used instead of indo-1 to monitor changes in [Ca\(^{2+}\)], (Tse and Hille, 1992). In the present study,
the measurements of membrane conductance were used simply as a monitor of the cell stability during microperfusion.

A maximal concentration of LHRH (10 nM) generally stimulates a characteristic "spike-plateau" Ca^{2+} response pattern in gonadotrophs, as most clearly observed in Fig. 1A (Shangold et al., 1988; Iida et al., 1991; Leong and Thorner, 1991). In contrast, lower concentrations of LHRH (around the picomolar range) stimulate repetitive Ca^{2+} oscillations, as shown in Fig. 1C (Shangold et al., 1988; Iida et al., 1991; Leong and Thorner, 1991). Similarities in the Ca^{2+} response patterns for PACAP and LHRH (Fig. 1C) suggest the possibility of a common mechanism of action. The present study has investigated the intracellular mechanisms regulating the PACAP-stimulated Ca^{2+} oscillation response in gonadotrophs. The frequently observed steplike Ca^{2+} response pattern (Rawlings et al., 1993) has not been specifically addressed in the present study.

**GTP-binding Proteins (G-proteins)**

The cell membrane receptor-mediated stimulation of the Ca^{2+} mobilization may occur through G-protein-dependent (Taylor et al., 1991) and G-protein-independent (Kim et al., 1991; Gusovsky et al., 1993) mechanisms. To test for the involvement of a G-protein in the PACAP stimulation of Ca^{2+} mobilization, the competitive inhibitor of GTP-binding to G-proteins GDPβS replaced GTP in the pipette solution. Microperfusion of GDPβS (intrapipette concentration = 2 mM) into the cell blocked the Ca^{2+} response to the subsequent addition of PACAP in all cells tested (Fig. 2, n = 6). This concentration of GDPβS also blocked the Ca^{2+} mobilizing action of low concentrations of LHRH (data not shown), which is known to involve a G-protein (Conn et al., 1987; Naor, 1990). However, as previously suggested (Tse and Hille, 1992), the use of a maximal concentration of LHRH (10 nM) was capable of overriding this block, thus allowing the identification of the gonadotrophs (Fig. 2). In conclusion, the effect of PACAP on Ca^{2+} mobilization in gonadotrophs appears to be mediated by the activation of a G-protein.

**Cyclic AMP**

A previous report using a competitive antagonist to cAMP-binding of cAMP-dependent protein kinase has demonstrated that the PACAP-stimulation of Ca^{2+} mobilization in rat gonadotrophs is not through the activation of cAMP-dependent protein kinase (Rawlings et al., 1993). However, cAMP is also known to have cAMP-dependent protein kinase-independent actions (Nakamura and Gold, 1987; DiFrancesco and Tortora, 1991). To test for the effect of cAMP on Ca^{2+}, and the subsequent response to PACAP, a high concentration of cAMP (100 μM) was microperused into identified gonadotrophs. This concentration of cAMP significantly stimulates Ca^{2+} mobilization in guinea pig hepatocytes (Capio et al., 1991). However, it had no effect on [Ca^{2+}], in four gonadotrophs, and produced a small decrease in basal [Ca^{2+}], in one cell (Fig. 3). Microperfusion of cAMP had no noticeable effect on the subsequent Ca^{2+} response to the addition of PACAP (Fig. 3, n = 5). These results strongly suggest that the PACAP stimulation of Ca^{2+} mobilization is not through a cAMP-dependent mechanism.

**Ins(1,4,5)P_{3}**

**Effect of the Ins(1,4,5)P_{3} Receptor Antagonist Heparin**—Previous reports have used the competitive Ins(1,4,5)P_{3} receptor blocker heparin (Ghosh et al., 1988) to distinguish between Ins(1,4,5)P_{3}-dependent and -independent stimulation of Ca^{2+} mobilization in a variety of cell types including gonadotrophs (Pape et al., 1988; Capiod et al., 1991; Tse and Hille, 1992; Takasawa et al., 1993). Heparin (300 μM) microperused into gonadotrophs completely blocked the PACAP-stimulated Ca^{2+} response in all cells tested (Fig. 4A, n = 7). In contrast, inclusion of 300 μM of the inactive form of heparin (de-N-sulfated heparin), had no effect on the PACAP-stimulated Ca^{2+} mobilization response (Fig. 4B, 6 of 7 cells). In agreement with an earlier study (Tse and Hille, 1992), we have found that the Ca^{2+} response to low concentrations of LHRH were blocked by this concentration of heparin (data not shown). However, addi-
allowed for the microperfusion of the cell with 100 p~

medicated through CAMP.

Reproduction of the maximal concentration of LHRH (10 nM) to identify the cell type was able to elicit a Ca²⁺ response. Such effects of high concentrations of LHRH to overcome the heparin block have been previously reported (Tse and Hille, 1992).

**Reproduction of the PACAP-stimulated Ca²⁺ Response by Ins(1,4,5)P₃**—If the effect of PACAP to mobilize Ca²⁺ was through the production and action of Ins(1,4,5)P₃, it would be expected that the microperfusion of Ins(1,4,5)P₃ into the cell should reproduce the Ca²⁺ response patterns observed for PACAP. Four concentrations of Ins(1,4,5)P₃ were tested. Microperfusion of 5 μM Ins(1,4,5)P₃ into gonadotrophs resulted in either no response (3 cells; Fig. 5A), or the production of a single Ca²⁺ spike after a delay of 40 s (1 cell). Microperfusion of 10 μM Ins(1,4,5)P₃ resulted in either no effect (4 cells), or repetitive Ca²⁺ oscillations (3 cells; Fig. 5B). Microperfusion of 20 μM Ins(1,4,5)P₃ stimulated repetitive oscillations in [Ca²⁺] in all of the cells tested (Fig 5C; n = 7). The top concentration of Ins(1,4,5)P₃ tested with this protocol (50 μM) produced a series of rapid Ca²⁺ oscillations (Fig 5D; n = 6). Comparison of the Ca²⁺ oscillation response pattern stimulated by PACAP (Fig. 1, 3, and 4B) and the response to the microperfusion of Ins(1,4,5)P₃ (Fig. 5) clearly demonstrate that physiological concentrations of Ins(1,4,5)P₃ can mimic the Ca²⁺ mobilizing action of PACAP.

**Block of the PACAP Response by a High Concentration of Intracellular Ins(1,4,5)P₃**—The intracellular messenger Ins(1,4,5)P₃ is known to address specific intracellular Ca²⁺ stores. If PACAP acts through an Ins(1,4,5)P₃-dependent mechanism, the emptying of these Ca²⁺ stores should preclude further responses to PACAP. To empty the Ins(1,4,5)P₃-sensitive stores we microperfused a pharmacologically high concentration of Ins(1,4,5)P₃ (200 μM) into gonadotrophs bathed in a nominally Ca²⁺-free medium containing 1 mM EGTA. In five of six cells tested in this way, Ins(1,4,5)P₃ stimulated a rapid, large amplitude rise in [Ca²⁺], which was followed by a number of high amplitude Ca²⁺ oscillations before the [Ca²⁺] returned to basal levels, generally within 3 min (Fig. 6A). In the other cell, Ins(1,4,5)P₃ microperfusion stimulated a single Ca²⁺ spike, which was not accompanied by any repetitive Ca²⁺ oscillations. Clamping the intracellular Ins(1,4,5)P₃ level in this way completely blocked the Ca²⁺ response to a subsequent addition of PACAP (n = 5) (Fig. 6A). In control cells (no Ins(1,4,5)P₃ microperfusion, in Ca²⁺-free medium), 4 of 6 cells tested exhibited a Ca²⁺ response to PACAP (Fig. 6B). The addition of a high concentration of LHRH at the end of the experiment was generally capable overcoming the block by Ins(1,4,5)P₃ and eliciting a small response (Fig. 6A). Only cells identified in this way were included in the results detailed above.

In conclusion, these data demonstrate that PACAP can stimulate the release of Ca²⁺ from intracellular Ca²⁺ stores through an Ins(1,4,5)P₃-dependent mechanism in the rat gonadotroph.

**DISCUSSION**

PACAP was first isolated, and in fact was named, by its action to stimulate the production of cAMP in anterior pituitary cells (Miyata et al., 1989). A number of its actions in the regulation of pituitary cell function appear to be through the cAMP system, including the stimulation of changes in Ca²⁺ influx in somatotrophs (Rawlings et al., 1993), the release of interleukin-6 from folliculo-stellate cells (Tatsuno et al., 1991), and the release of alpha-melanocyte-stimulating hormone from melanotrophs (Koch and Lutz-Bucher, 1992). The present study has directly demonstrated at the single cell level that PACAP can have a specific cellular action through the activation of the Ins(1,4,5)P₃ system.

**PACAP Stimulation of Ca²⁺ Mobilization**—The effect of PACAP to mobilize Ca²⁺ from intracellular Ca²⁺ stores was blocked by the Ins(1,4,5)P₃ receptor antagonist heparin. This result is argued against the involvement of such Ca²⁺ mobilizing agents as cADP ribose and Ca²⁺ itself, which act through a heparin-insensitive nystatin-sensitive receptor (Galione, 1993; Palade et al., 1989). The mechanism of cAMP-stimulated Ca²⁺ mobilization in hepatocytes is unclear, however, it is not blocked by heparin (Capiod et al., 1991). Furthermore, in the present study, 100 μM CAMP could not stimulate Ca²⁺ mobilization in the rat gonadotroph, nor could it inhibit the response to PACAP. Thus it appears that the PACAP-stimulated Ca²⁺ rise is mediated through the Ins(1,4,5)P₃ receptor on the intracellular Ca²⁺ store. The fact that microperfusion of physiological concentrations of Ins(1,4,5)P₃ can mimic, and that pharmacological concentrations of Ins(1,4,5)P₃ can block, this effect of...
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A. 5 μM Ins(1,4,5)P_3

5μM Ins(1,4,5)P_3

LHRH

[Ca^{2+}]_i (μM)

0 1 2 3 4 5 6

Time (min)

B. 10 μM Ins(1,4,5)P_3

10μM Ins(1,4,5)P_3

LHRH

[Ca^{2+}]_i (μM)

0 1 2 3 4 5 6

Time (min)

C. 20 μM Ins(1,4,5)P_3

20μM Ins(1,4,5)P_3

LHRH

[Ca^{2+}]_i (μM)

0 1 2 3 4 5 6

Time (min)

D. 50 μM Ins(1,4,5)P_3

50μM Ins(1,4,5)P_3

LHRH

[Ca^{2+}]_i (μM)

0 1 2 3 4 5 6

Time (min)

Fig. 5. Microperfusion of Ins(1,4,5)P_3 into gonadotrophs can mimic the PACAP-stimulated Ca^{2+} response. Measurement of [Ca^{2+}]_i during a whole-cell recording of gonadotrophs voltage-clamped at ~50 mV. The whole-cell configuration was established at the times indicated by the arrows and allowed for the microperfusion of the cells with Ins(1,4,5)P_3 (5–50 μM, as indicated in panels A–D). LHRH was applied as in Fig. 1, to identify the cells as gonadotrophs.

PACAP supports this conclusion.

PACAP Stimulation of Phospholipase C—But how is PACAP causing this effect? The most logical explanation is that the stimulation of the PACAP receptor leads to the activation of phospholipase C (PLC) and the subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate into the intracellular messengers Ins(1,4,5)P_3, and 1,2-diacylglycerol. A similar mechanism exists in gonadotrophs for the activation of the LHRH receptor (Naor et al., 1986; Morgan et al., 1987; Chang et al., 1988), and the endothelin receptor (Stojilkovic et al., 1992). In support of this idea, PACAP has been recently shown to stimulate the production of inositol phosphates in clonal adrenal medullary cells (Deutsch and Sun, 1992; Isobe et al., 1993). In addition, PACAP-stimulated LH release from the gonadotroph appears to be, at least in part, on the activation of protein kinase-C (Hart et al., 1992). Thus PACAP action in the gonadotroph may involve, in addition to Ins(1,4,5)P_3-mediated Ca^{2+} mobilization, the activation of protein kinase C by 1,2-diacylglycerol.

There are a number of possible mechanisms for the activation of phospholipase C isoforms. Activation of many growth factor receptors is through a direct (non-G-protein-mediated) tyrosine phosphorylation of the γ-isozyme of phospholipase C (phospholipase C-γ) (e.g. see Kim et al. (1991)). In a variation of this system, the muscarinic receptor-mediated tyrosine phosphorylation of phospholipase C-γ is also dependent upon Ca^{2+} influx (Gusovsky et al., 1993). The present study has demonstrated that the PACAP action is mediated by a G-protein consistent with a recent binding study demonstrating the existence of a G-protein-complexed PACAP receptor in rat brain (Schafer et al., 1991). In addition, the PACAP-stimulation of the Ca^{2+} oscillations response pattern is independent of extracellular Ca^{2+} (Fig. 6B) (Canny et al., 1992). Thus the PACAP stimulation of Ins(1,4,5)P_3 in the gonadotroph is most likely to be through the G-protein-mediated activation of the β-isozyme of phospholipase C (Taylor et al., 1991).

Two PACAP-stimulated Intracellular Pathways in Pituitary Cells—The present study has demonstrated that PACAP action in gonadotrophs can be mediated by Ins(1,4,5)P_3, whereas previous data has shown that in somatotrophs PACAP action is mediated by cAMP (Rawlings et al., 1993). Thus PACAP can apparently stimulate both the cAMP and the phosphoinositol signaling pathways in anterior pituitary cells. How could PACAP influence these two systems? Recent cloning data may provide an answer to this question. The PACAP type I receptor has been very recently cloned (Pisegna and Wank, 1993; Hashimoto et al., 1993; Spengler et al., 1993), and shown to exist in at least five splice variants (Spengler et al., 1993). One of the splice variants is linked solely to the stimulation of cAMP production, while the other four splice variants of the PACAP receptor can be linked to the activation of adenylate cyclase and phospholipase C (Spengler et al., 1993). Although PACAP38 and PACAP27 stimulate cAMP production with approximately equal potency for these four splice variants of the receptor, PACAP38 stimulates inositol phosphate production with a potency 2–3 logs greater than for PACAP27. These results are consistent with an earlier study showing a differential action of PACAP38 and PACAP27 on the stimulation of cAMP.
生产及inositol phospholipid turnover in the sympathetic adrenal PC12 cell line (Deutsch and Sun, 1992). Thus, if the action of PACAP in the rat gonadotroph is through the activation of one of these receptor subtypes we may expect to see a differential action of PACAP38 and PACAP27 on Ins(1,4,5)P3-stimulated Ca2+ oscillations in these cells.

In initial experiments we tested three concentrations of PACAP38 and PACAP27 (10 nM, 100 nM, and 1 μM) on their ability to stimulate Ca2+ oscillations in the rat gonadotroph. Remarkably, given the data described above, we found no striking difference in the potency of these two PACAP isomers. For both PACAP38 and PACAP27 the threshold concentration for the stimulation of Ca2+ oscillations was between 10 nM and 100 nM (n = 12). How can we reconcile such data? Perhaps the simplest explanation lies in the differences in the methods of detecting inositol phosphate production between our study and the studies described above (Deutsch and Sun, 1992; Spengler et al., 1993). Our study, by measuring the release of Ca2+ from Ins(1,4,5)P3-sensitive stores, detects the production of an effective concentration of Ins(1,4,5)P3 over a time period of seconds at the single cell level, although it can give only an approximate idea of the levels of Ins(1,4,5)P3 formed in contrast, the other studies measured the cumulative production of all inositol phosphates (InsP4-e) over a time period of 50 min (Deutsch and Sun, 1992) and 20 min (Spengler et al., 1993).

Whatever the reasons for the differences between these results, the new found complexity in the PACAP receptor subtype field and our initial results given above show that a simple comparison of the effects of PACAP38 and PACAP27 will not provide a clear answer on the PACAP receptor subtype(s) expressed in the rat gonadotroph. Further studies, probably involving molecular biology techniques, will be necessary to identify the PACAP receptor subtype(s) involved in Ins(1,4,5)P3 production, and hence Ca2+ mobilization, in this cell type.

Possible Physiological Role of PACAP—in the gonadotroph lHRR can produce similar oscillations in [Ca2+]i, those observed by PACAP stimulation in the present study (Fig. 1C) (Strangold et al., 1988; Iida et al., 1991; Leong and Thorner, 1991; Tse and Hille, 1992). Such Ca2+ oscillations are closely coupled with exocytosis (and thus probably LH release) (Tse et al., 1993) and have also been proposed to be a method of regulating lHRR receptor numbers and LH synthesis (Leong and Thorner, 1991; Leong, 1991). Thus the action of PACAP to stimulate Ca2+ oscillations may play an important role in the regulation of gonadotroph function.

The fact that PACAP can act through two different intracellular signaling systems provides the possibility of an enhanced signaling potential for this peptide’s effects on anterior pituitary cell function. On the one hand, the same peptide could selectively address different pituitary cell types for which one or the other pathway appears to be dominant (phospholipase C for gonadotrophs; cAMP for somatotrophs). Furthermore, if the two pathways coexist in the same cells, PACAP could produce a more complex signal, dependent upon the interactions or “cross-talk” (Houslay, 1991) between these two signaling pathways. There is some evidence that other hypothalamic factors may stimulate more than one signaling pathway, for example the action of thyrotropin-releasing hormone on clonal pituitary cells (Paulssen et al., 1992). However, this is the first clear demonstration that a hypothalamic factor can significantly stimulate both of these systems in normal identified pituitary cells, and, furthermore, it may address different systems in different cell types (see above). These results should thus encourage further studies on the action of PACAP on anterior pituitary cell physiology.

Acknowledgments—We thank Dr. Karl-Heinz Krause and Dr. Rosemary Murray-Whelan for helpful comments on an earlier draft of the manuscript. We also thank the group of Jóel Bockaert for providing a reprint of the paper on the cloning of the PACAP receptor (Spengler et al., 1993).

REFERENCES
Leong and Thorner, M. O. (1992) Endocrinology 130, 939–944
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