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ORIGINAL ARTICLE

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Differential calcium responses to the pituitary adenylate cyclase-activating polypeptide (PACAP) in the five main cell types of rat anterior pituitary

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Abstract We have compared the effects of pituitary adenylate cyclase-activating polypeptide (PACAP-27) on the five main cell types of rat anterior pituitary in primary culture by monitoring changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in single fura-2-loaded cells. Cells were typed by multiple sequential primary immunocytochemistry at the end of the Ca^{2+} measurements. PACAP-27 increased $[Ca^{2+}]_i$ by three different mechanisms, each one dominant in a given cell type. These involved Ca^{2+} entry or release from the stores and mediation through different second messenger pathways: (1) stimulation of Ca^{2+} entry mediated by cAMP was the main mechanism in somatotrophs; (2) Ca^{2+} release from the intracellular Ca^{2+} stores mediated by phospholipase C (PLC) was the dominant modality in gonadotrophs; (3) stimulation of Ca^{2+} entry not mediated by cAMP was the main mechanism in lactotrophs. A minor fraction of somatotrophs (11%) may also use mechanism 3. Corticotrophs and thyrotrophs exhibited weak responses to PACAP (<10% of the cells responded), which in all cases were mediated by mechanism 1. Mechanism 3 represents a novel effect of PACAP which cannot be explained by interaction with the conventional PACAP receptor families.

Key words Adenylate cyclase · Calcium influx · L-type calcium channels · Lactotrophs · Pituitary adenylate cyclase-activating polypeptide · Pituitary cells

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) was originally isolated from ovine hypothalami by its ability to stimulate cAMP production in rat anterior pituitary (AP) cells. PACAP shares significant sequence homology with vasoactive intestinal polypeptide (VIP). Alternative processing of mRNA produces two polypeptides of different lengths, PACAP-38 and PACAP-27, with similar biological activity [1, 7, 11]. The wide tissue distribution of PACAP suggests that it has a variety of physiological roles [1, 7, 9]. One of the main roles is to act as a hypothalamic factor regulating AP cell function [18]. The actions of PACAP are mediated through three receptor subtypes: PAC1 (type I, PVR1), VPAC1 (PVR2) and VPAC2 (PVR3), which can be distinguished by their relative specificity for PACAP versus VIP and by their coupling to different intracellular messenger pathways [11, 12, 16, 18, 21]. VPAC1 and VPAC2 exhibit no specificity for PACAP over VIP and are coupled to the activation of adenylate cyclase (AC). PAC1 strongly prefers PACAP over VIP and it has numerous splice variants. They are coupled to AC and some of the variants are coupled to phospholipase C (PLC) [4, 11, 12, 22]. A novel variant of the PAC1 receptor, named PACAPR TM4, of unknown physiological significance was recently cloned from rat cerebellum. PACAPR TM4 does not activate AC or PLC, but it does increase Ca^{2+} entry through L-type Ca^{2+} channels [5].

It has been reported that PACAP stimulates the synthesis and secretion of growth hormone (GH) [8, 24] and gonadotropins [6, 23]. Most of the studies investigating how PACAP works have been carried out on adenoma cells or inmortalized cell lines, sometimes with conflicting results [12, 18]. In somatotrophs the effects of PACAP seem to be mediated by the stimulation of AC through VPAC2, resulting in membrane depolarization and Ca^{2+} entry [3, 10, 15, 18, 19, 31]. In gonadotrophs the effects of PACAP seem to be mediated through PAC1, coupled to PLC and Ca^{2+} release from intracellular Ca^{2+} stores [2, 13, 15, 16, 17, 18, 19, 20, 23]. Expression of either PAC1 in somatotrophs [2, 25] or VPAC2 in gonadotrophs [18] has also been reported, but their contribution to the physiological responses is obscure. There is little information on the actions of PACAP on the other AP cell types [12, 18].

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Here we have carried out a systematic study of the effects of PACAP on $[Ca^{2+}]_i$ in AP cells in primary culture. $[Ca^{2+}]_i$ measurements were complemented by in situ typing of the cells at the end of the experiments. The on-line identification of AP cell type sometimes relies on their responses to the different hypothalamic releasing hormones, but up to one-third of the cells are able to respond to several hypothalamic releasing hormones [27, 28]. In one study, cell typing was performed by multiple sequential immunocytochemistry of the AP hormones using fluorescent secondary antibodies [10]. This requires relocating the cells on which the functional studies were done, after their reaction with each primary antibody, and cross-reactions by the secondary antibodies are unavoidable. In the present study these difficulties were circumvented by introducing multiple primary sequential immunocytochemistry (MSPI). MSPI allows the simultaneous identification of the different AP cell types present in the same microscope field, and the comparison of their responses to PACAP not to be biased by variability among experiments. Using this procedure, the mechanism of action of the PACAP-induced $[Ca^{2+}]_i$ increase has been studied for each cell type, especially with regard to the origin, i.e., intracellular or extracellular, of Ca^{2+} and coupling to AC.

Materials and methods

AP cells were prepared from 8- to 10-week-old male Wistar rats as described previously [26, 28, 29] and cultured in RPMI 1640 medium containing 10% fetal calf serum over glass coverslips coated with poly-L-lysine (0.01 mg/ml, 10 min). After 1–24 h in culture, cell-coated coverslips were loaded with fura-2 by incubation with 4 µM fura-2/AM at room temperature for 1 h. Measurements of $[Ca^{2+}]_i$ and time-resolved digital image analysis were performed as described elsewhere [26, 28, 29]. All the experiments were performed at 37°C. The standard incubation solution had the following composition (in mM): NaCl, 145; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; sodium-HEPES, 10; pH 7.4. Treatments were added by bath perfusion with the solutions containing drugs and hormones.

At the end of the $[Ca^{2+}]_i$ measurements, we typed the AP cells present in the microscope field by MSPI, using antibodies raised against pituitary hormones [26, 27]. The identification of several AP cell types by sequential immunocytochemistry using primary antibodies and fluorescent secondary antibodies had already been done [10], but it required relocating the cells of interest after staining with each single antibody. The use of primary antibodies allows faster processing, necessary for in situ analysis, and circumvents cross-reactions, which are unavoidable when using secondary antibodies with multiple primaries. In addition, as there is no need to move the preparation during the whole process, image processing techniques can be used with MSPI to improve analysis. Fluorescent antibodies were prepared from antisera provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and purified over a protein A-Sepharose [26, 27]. The reaction times were shortened as much as possible. Briefly, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.3% Triton X-100 in PBS for 3 min and washed with PBS for 5 min Then 10% goat serum in PBS was added. After 5 min the antibody against one of the AP hormones labelled with Oregon green 488 was added and the incubation continued for 15–30 min After washing, a fluorescence image was captured (excitation, 490 nm; emission, >510 nm) with the image processor. The process was repeated with a second and a third fluorescent antibody. Specific staining with each antibody was obtained by subtracting from each image the previous one. Finally, nuclei were stained with Hoechst 33258 (0.5 µg/ml, 10 min) and another fluorescence image was acquired (excitation, 340 nm; emission, >420 nm). The image from the fluorescence-stained nuclei allowed us to define the boundaries of cells that were physically close to one another. In routine experiments, antibodies against adrenocorticotrophic hormone (ACTH), GH and prolactin (PRL) were used in this sequence. In other experiments antibodies against thyrotropin (TSH) and follicle-stimulating hormone (FSH) were used sequentially in order to positively identify thyrotrophs and gonadotrophs.

For cAMP measurements AP cells were plated in culture trays at $5 \cdot 10^5$ cells/well (15 mm) and cultured for 18–24 h. Then the culture medium was replaced by standard incubation medium and incubated for 30 min at 37°C. At this time forskolin (5 µM) or PACAP (100 nM) was added and the incubation continued for 15 min. The incubation period was terminated by the addition of 2 vol. of absolute ethanol containing 5 mM EDTA. After 5 min at room temperature with shaking, the contents of the wells were transferred to an Eppendorf tube and centrifuged for 5 min at 4°C and 12,000 *g*. The supernatant was transferred to a clean tube and evaporated under vacuum. The cAMP contents were determined using the "Cyclic AMP [^{3}H] Assay System" kit from Amersham Ibérica, Madrid, Spain.

Antisera against rat PRL (rabbit, no. AFP425–10–91), β-TSH (rabbit, no. AFP1274789), GH (monkey, no. AFP4115), β-FSH (guinea pig, no. AFP85GP9691BFSHB) and anti-human ACTH (rabbit, no. AFP39013082) were generous gifts from the National Hormone and Pituitary Program, NIDDK, the National Institute of Child Health and Human Development, and the US Department of Agriculture, Rockville, Md., USA. Furnidipine was a generous gift from Laboratorios Alter, Madrid, Spain. PACAP-27 was obtained from RBI, Natick, Mass., USA. 8-Br-cAMP was from Biolog Life Sciences Institute, Bremen, Germany. Fura-2/AM and Oregon green 488-isothiocyanate were purchased from Molecular Probes, Eugene, Ore., USA. Other chemicals were obtained either from Sigma, Madrid, Spain or from E. Merck, Darmstadt, Germany.

Results

Stimulation of AP cells with either the AC activator forskolin or PACAP-27 resulted in a $[Ca^{2+}]_i$ increase in many cells. The mean response, obtained by averaging the data from all the cells present in the microscope field, was similar in amplitude for both stimuli. However, stimulation by PACAP increased cAMP by about tenfold whereas stimulation by forskolin produced a 100-fold increase (Fig. 1B). This outcome suggests that either the PACAP-induced increase of cAMP has a maximal effect on $[Ca^{2+}]_i$ or, alternatively, that the effects of PACAP on $[Ca^{2+}]_i$ are mediated not only by cAMP, but by other mechanisms not shared with forskolin.

Figure 2A illustrates identification of AP cell type by MSPI. Three somatotrophs, three lactotrophs and one corticotroph can be positively identified within the section of the microscope field shown. The fura-2 ratio image on the left was taken during stimulation by PACAP. Two of the somatotrophs and two of the lactotrophs responded to the peptide with a $[Ca^{2+}]_i$ increase in this particular example, whereas the corticotroph did not respond. This methodology allowed us to define the contours of all the positively identified cells for quantification of $[Ca^{2+}]_i$. In similar experiments gonadotrophs and

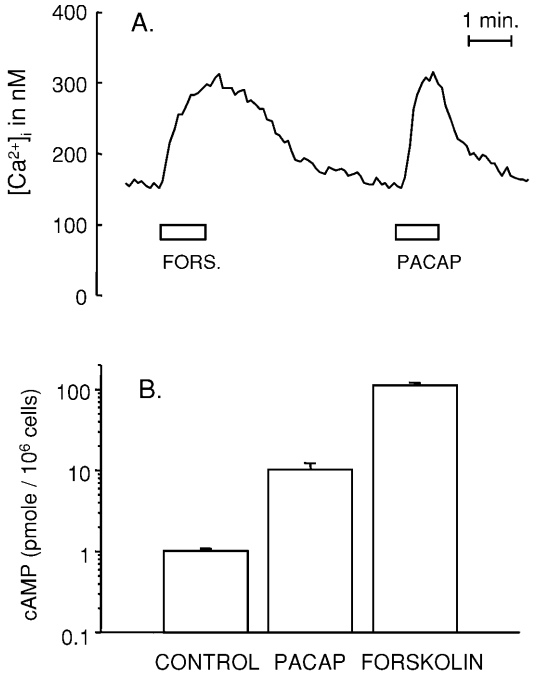
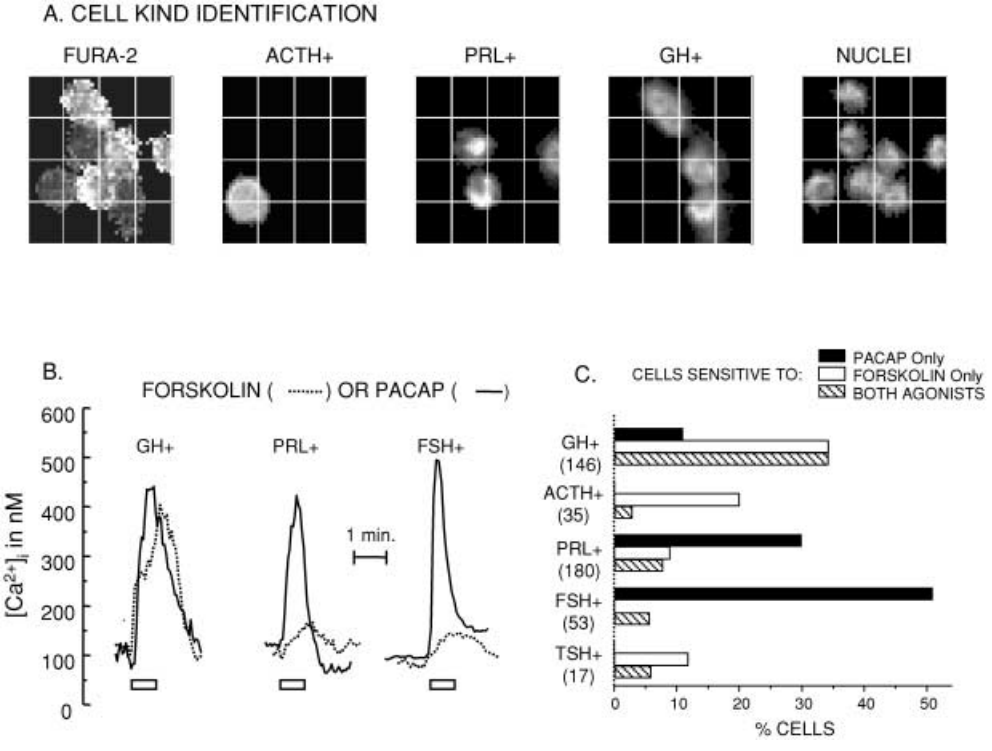


Fig. 1 Comparison of the effects of pituitary adenylate cyclase-activating polypeptide (*PACAP*, 100 nM) and forskolin (5 µM) on $[Ca^{2+}]_i$ (**A**) and on cAMP contents of rat anterior pituitary (*AP*) cells (**B**). **A** Average data of 47 cells present in the same microscope field. Data in **B** are averages of 18 determinations with 6 different cell batches. *Vertical bars* represent SEM

thyrotrophs were identified by sequential staining with antibodies against the corresponding AP hormones. Cells reacting with more than one antibody were rare. In some cases apparent double positives were caused by the spatial superposition of two cells, as suggested by the presence of two nuclei, and the percentage of true multihormonal cells amounted to only 1–3%. These cells were excluded from the analysis.

The responses of the different cell types to PACAP were not homogeneous. Corticotrophs and thyrotrophs responded very poorly (not shown, see below). In contrast, about 50% of somatotrophs, lactotrophs and gonadotrophs responded to the peptide with a clear (>100 nM) increase of $[Ca^{2+}]_i$. Figure 2B illustrates the typical responses to PACAP in these three cell types. The responses to forskolin in the same cells are also shown for comparison. Somatotrophs responded the most to both PACAP and forskolin, and typically responded similarly to both stimuli. The response to

Fig. 2A–C Comparison of the effects of PACAP and forskolin in the five main AP cell types. **A** Identification of three different AP cell types by multiple sequential primary immunocytochemistry (*MSPI*). The image on the *left* (*FURA-2*) is a ratio image taken during PACAP stimulation. It is coded in grey levels, the brighter ones corresponding to higher $[Ca^{2+}]_i$. The following three images correspond to the same field after staining with antibodies against adrenocorticotrophic hormone (*ACTH+*), growth hormone (*GH+*) or prolactin (*PRL+*). In each case, the previous image has been substracted (see Materials and methods). The *rightmost image* corresponds to staining of nuclei with Hoechst 33258. **B** Cells were stimulated first with forskolin (5 µM; *dotted traces*) for 1 min and 10 min later with PACAP (100 nM; *continuous traces*) for 1 min and then typed by MSPI. Traces for both stimuli are averaged for all the cells within each cell type and superimposed. The actual number of cells was: somatotrophs (*GH+*), 29; lactotrophs (*PRL+*), 19; gonadotrophs (*FSH+*), 11. Representative of three to six similar experiments. **C** Cells from nine similar experiments were classified into the three groups shown. Sensitive cells are defined as those responding to the stimuli with a $[Ca^{2+}]_i$ >100 nM. The figures in *parentheses* indicate the total number of cells studied for each cell type



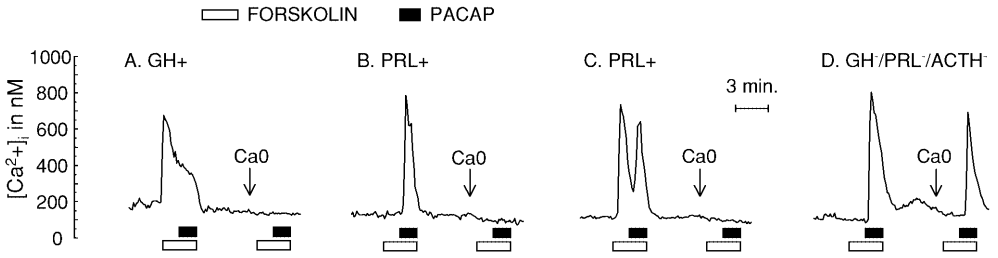


Fig. 3A–D Additivity and the Ca^{2+} dependence of the effects of forskolin and PACAP. Cells were stimulated for 90 s with forskolin and then with forskolin and PACAP together for an additional 90-s period. Stimulation was performed first in standard Ca^{2+}-containing, medium and then in Ca^{2+}-free medium (*Ca0*, containing 0.1 mM EGTA; switched at the time marked by the *arrow*) as shown. MSPI for ACTH, GH and PRL was performed after the $[Ca^{2+}]_i$ measurements. Single cells representative of the different effects observed are shown. For all of them, a previous stimulation with PACAP had produced a $[Ca^{2+}]_i$ response (not shown). Other details as in Figs. 1 and 2

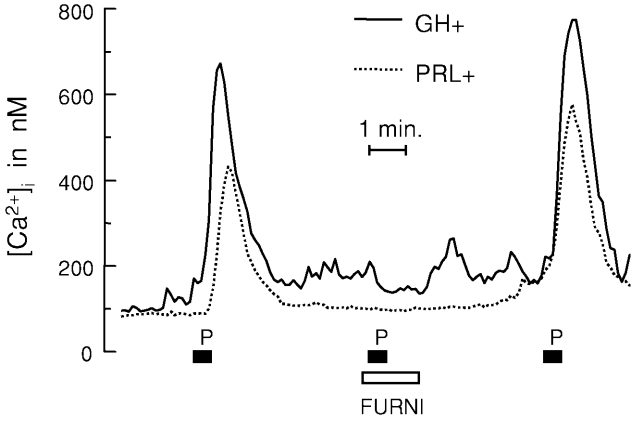


Fig. 4 Furnidipine (*FURNI*) blocks the effect of PACAP (*P*) in somatotrophs and lactotrophs. The concentrations of furnidipine and PACAP were 1 µM and 100 nM, respectively. The traces are the average of nine PACAP-responding cells of each type present in the same microscope field. Other details as in Fig. 2

PACAP was also somewhat longer in somatotrophs than in the other cell types. In lactotrophs and gonadotrophs there was a clear-cut dissociation of the effects of PACAP and forskolin, as the AC activator had a very small effect on $[Ca^{2+}]_i$. The average responses in all the PACAP-responding cells studied, measured as the mean area of the $[Ca^{2+}]_i$ peak during the 60-s stimulation period, were [in nM; mean ±SD (*n*)]: somatotrophs, 313±174 (65); lactotrophs, 173±62 (78); gonadotrophs, 208±70 (33).

Figure 2C summarizes the results of experiments in which every single cell was classified according to their responses to forskolin and PACAP. Cells giving a $\Delta[Ca^{2+}]_i$ larger than 100 nM were classified as responsive to the stimulus. Most of the tyrotrophs and corticotrophs (94–97%) were insensitive to PACAP. In contrast, a large fraction (40–60%) of somatotrophs, lactotrophs and gonadotrophs were responsive to the peptide. For somatotrophs, most of the PACAP-responding cells (45%) were also sensitive to forskolin (34%). There was a significant fraction of the cells (11%), however, which was sensitive to PACAP but not to forskolin. Other cells (34%) were sensitive to forskolin but not to PACAP. In gonadotrophs and lactotrophs the opposite pattern was found: the dominant subpopulation was composed of cells sensitive to PACAP but not to forskolin (51 and 30%, respectively) (Fig. 2C). As a matter of fact, these cell types, specially gonadotrophs, were barely sensitive to forskolin. In another series of experiments 8-Br-cAMP (500 µM) was used instead of forskolin to look for cAMP-mediated changes in $[Ca^{2+}]_i$. An increase of $[Ca^{2+}]_i$ was observed in some somatotrophs and corticotrophs but there was little effect on the other cell types (results not shown).

The above results suggest that the effects of PACAP on gonadotrophs and lactotrophs are not mediated via cAMP. In order to obtain additional evidence on this point the additive effects of PACAP and forskolin were investigated. Cells were stimulated with forskolin for 2 min and then with forskolin plus PACAP for a further minute. The results obtained in illustrative single somatotrophs and lactotrophs are shown in Fig. 3. The effects of PACAP were occluded by previous treatment with forskolin in somatotrophs (Fig. 3A). In lactotrophs, most of the cells did not respond to forskolin but were activated by PACAP (Fig. 3B). Forskolin-sensitive lactotrophs (which are a minor fraction of all the cells) were stimulated further by PACAP (Fig. 3C). Therefore, these experiments indicate that the $[Ca^{2+}]_i$-increasing ability of PACAP is most probably not mediated by cAMP in lactotrophs.

The $[Ca^{2+}]_i$-increasing ability of cAMP in AP cells has been attributed to a protein-kinase-A-dependent stimulation of Ca^{2+} entry through voltage-operated channels. In the experiments shown in Fig. 3 the effects of forskolin and PACAP in Ca^{2+}-free medium were also investigated (last half of the traces). In both somatotrophs and lactotrophs, the effects of both agonists, forskolin and PACAP, depended strictly on the presence of Ca^{2+} (Fig. 3A–C). In many of these cells thyrotropin-releasing hormone (TRH, 100 nM) was able to increase $[Ca^{2+}]_i$ in Ca^{2+}-free medium (not shown). This result demonstrates that the lack of effect of PACAP in Ca^{2+}-free medium cannot be attributed to the absence of a mobilizable Ca^{2+} pool in these cells. The blockade of voltage-dependent Ca^{2+} channels with the dihydropyridine antagonist furnidipine also prevented the $[Ca^{2+}]_i$ increase induced by PACAP in both somatotrophs and lactotrophs, and this

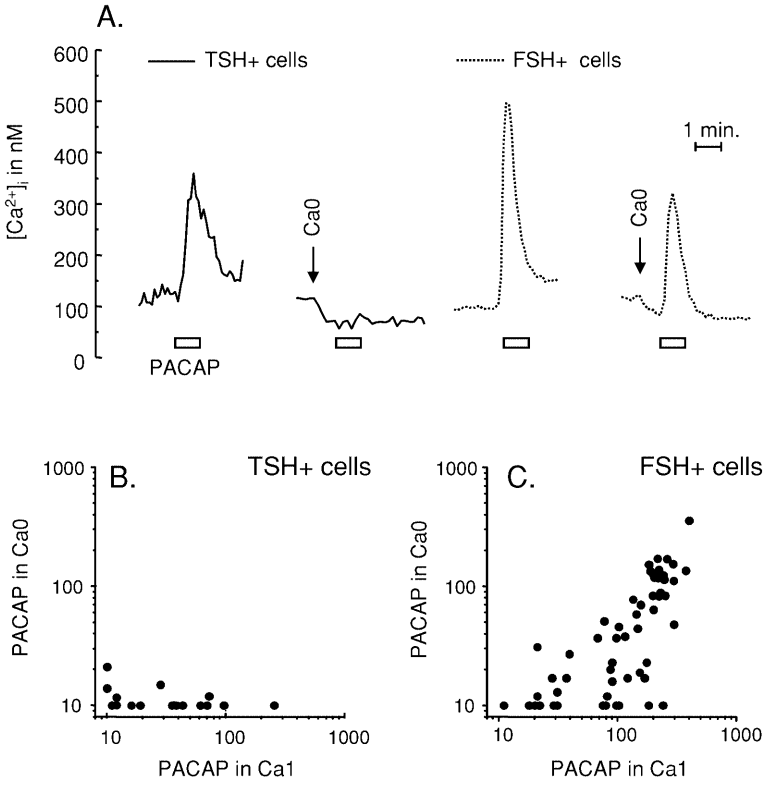


Fig. 5A–C Comparison of the responses of thyrotrophs and gonadotrophs to PACAP in Ca^{2+}-containing and in Ca^{2+}-free media. **A** Cells were stimulated with PACAP (100 nM) for 1 min as shown by the *horizontal bars*. Traces correspond (from *left* to *right*) to the averages of 6 TSH+ and to 21 FSH+ cells, respectively, which were present in the same microscope field. Other details as in Fig. 2 and 3. **B**, **C** The single-cell responses from three similar experiments, expressed as $\Delta[Ca^{2+}]_i$ (in nM), are shown. $\Delta[Ca^{2+}]_i$ was quantified as the mean height of the area under the $[Ca^{2+}]_i$ peak during the 1-min stimulation period. All the values below 10 nM have been equated to 10 nM for plotting. Notice the logarithmic scales

effect was reversed on washing the dihydropyridine antagonist out (Fig. 4).

In the experiments of Fig. 3, some of the non-identified cells, not stained with antibodies against GH, ACTH or PRL, were able to respond to PACAP both in Ca^{2+}-containing and in Ca^{2+}-free medium (Fig. 3D). In a new series of experiments, the Ca^{2+} dependence of the effect of PACAP was studied in gonadotrophs and thyrotrophs. A representative experiment is shown in Fig. 5A and the results of the single-cell analysis of several similar ones are summarized in Fig. 5B and C. In thyrotrophs the effects of PACAP were rather poor and strictly dependent on external Ca^{2+} (Fig. 5B). In contrast, within the gonadotroph cell group a large majority of cells was able to respond to PACAP in Ca^{2+}-free medium (Fig. 5C). This outcome indicates that the main effect of the peptide in gonadotrophs is to release Ca^{2+} from the intracellular Ca^{2+} stores.

Discussion

We find that PACAP-27 is able to increase $[Ca^{2+}]_i$ in somatotrophs, lactotrophs and gonadotrophs through three different mechanisms involving different transduction cascades: (1)PLC-mediated Ca^{2+} release from intracellular stores; (2) cAMP-mediated stimulation of Ca^{2+} entry; and (3) stimulation of Ca^{2+} entry not mediated through cAMP. Each one of the above mechanisms seems to predominate in a given AP cell type. In gonadotrophs the main effect of PACAP is to release Ca^{2+} from the intracellular stores (Fig. 5). This finding is consistent with previous proposals, as well as with the main type of PACAP receptor identified in gonadotrophs, PAC1, which can couple to PLC [2, 16, 17].

Ca^{2+} entry mediated by an increase of cAMP is the main mechanism in somatotrophs. This is consistent with previous reports and fits the type of PACAP receptor dominant in these cells, VPAC2, which is coupled to AC [12, 18, 19]. A small fraction of somatotrophs (11%) may respond to PACAP through a cAMP-independent mechanism similar to the dominant one in lactotrophs (see below). Even though PAC1 mRNA has been detected in a fraction of somatotrophs [2, 25] we did not find that PACAP induced the release of Ca^{2+} from the intracellular stores in these cells (Figs. 3, 4). It has been reported recently that PACAP-38, but not PACAP-27, is able to induce Ca^{2+} mobilization from the stores in porcine somatotrophs [14]. Therefore, the possibility remains that PAC1 may be stimulated selectively by PACAP-38 and should be investigated in future experiments. In thyrotrophs and corticotrophs the effects of PACAP were very poor, but, in the few responding cells, PACAP seems to act as in somatotrophs.

In lactotrophs the increase in $[Ca^{2+}]_i$ induced by PACAP was due to Ca^{2+} entry through L-type Ca^{2+} channels (Figs. 3, 4). However, at variance with the observations in somatrophs, the stimulation of Ca^{2+} entry was not mediated by AC as most of the PACAP-responding lactotrophs were not sensitive to forskolin and even in the lactotrophs that were responsive to forskolin the AC activator did not occlude the effect of PACAP (Fig. 4).

This mode of action of PACAP is, to our knowledge, entirely new. The type of PACAP receptor expressed in normal rat lactotrophs has not been identified yet. VPAC2 is expressed in lactotroph-like cell lines, such as GH_3 or GH_4C_1 [18], but this receptor, coupled to AC, would not explain the results found here. A novel variant of the PACAP receptor – PACAPR TM4, similar to the short splice variant of PAC1 but differing from it by discrete sequences located in transmembrane domains II and IV – has been cloned recently from rat cerebellum [5]. PACAPR TM4 does not activate either AC or PLC, but is able to activate Ca^{2+} influx though L-type Ca^{2+} channels in transfected Chinese hamster ovary (CHO) cells [5]. PACAPR TM4 has been detected by polymerase chain reaction in pancreatic β cells [5], where PACAP induces insulin secretion dependent on the presence of external Ca^{2+} [30]. The presence of this novel receptor in lactotrophs would fit the actions of PACAP on $[Ca^{2+}]_i$ documented here. About 11% of individual somatotrophs were insensitive to forskolin but responded to PACAP (Fig. 2), suggesting that, in a minor fraction of these cells, the PACAP-induced Ca^{2+} entry may not be mediated through AC either. The diversity in the modes of action of PACAP in the different AP cell types is reminiscent of the tissue-specific expression of PACAP receptors and may allow a very flexible control of hormone secretion.

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