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PAC_1 Receptor Activation by PACAP-38 Mediates Ca^{2+} Release from a cAMP-dependent Pool in Human Fetal Adrenal Gland Chromaffin Cells*

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Previous studies have shown that human fetal adrenal gland from 17- to 20-week-old fetuses expressed pituitary adenylate cyclase-activating polypeptide (PACAP) receptors, which were localized on chromaffin cells. The aim of the present study was to identify PACAP receptor isoforms and to determine whether PACAP can affect intracellular calcium concentration ($[Ca^{2+}]_i$) and catecholamine secretion. Using primary cultures and specific stimulation of chromaffin cells, we demonstrate that PACAP-38 induced an increase in $[Ca^{2+}]_i$ that was blocked by PACAP (6–38), was independent of external Ca^{2+}, and originated from thapsigargin-insensitive internal stores. The PACAP-triggered Ca^{2+} increase was not affected by inhibition of PLCβ (preincubation with U-73122) or by pretreatment of cells with Xestospongin C, indicating that the inositol 1,4,5-triphosphate-sensitive stores were not mobilized. However, forskolin (FSK), which raises cytosolic cAMP, induced an increase in Ca^{2+} similar to that recorded with PACAP-38. Blockage of PKA by H-89 or (R_p)-cAMPS suppressed both PACAP-38 and FSK calcium responses. The effect of PACAP-38 was also abolished by emptying the caffeine/ryanodine-sensitive Ca^{2+} stores. Furthermore, treatment of cells with orthovanadate (100 μM) impaired Ca^{2+} reloading of PACAP-sensitive stores indicating that PACAP-38 can mobilize Ca^{2+} from secretory vesicles. Moreover, PACAP induced catecholamine secretion by chromaffin cells. It is concluded that PACAP-38, through the PAC_1 receptor, acts as a neurotransmitter in human fetal chromaffin cells inducing catecholamine secretion, through nonclassical, recently described, ryanodine/caffeine-sensitive pools, involving a cAMP- and PKA-dependent phosphorylation mechanism.

Pituitary adenylate cyclase-activating polypeptide is a 38-residue α-amidated neuropeptide (PACAP-38)[1] originally isolated from the ovine hypothalamus for its ability to stimulate cAMP formation in rat anterior pituitary cells. Processing of PACAP-38 can generate a 27-amino acid amidated peptide (PACAP-27) that exhibits 68% sequence identity with vasoactive intestinal polypeptide (VIP), thus identifying PACAP as a member of the VIP/secretin/glucagon superfamily of regulatory peptides (1, 2).

The effects of PACAP are mediated through interaction with two types of high affinity receptors: type I receptors are selectively activated by PACAP, whereas type II receptors bind PACAP and VIP with similar affinity (3). Three isoforms of PACAP receptors have now been cloned and designated as PACAP-specific receptor I (PAC_1-R) (4, 5) and VIP/PACAP mutual receptors 1 and 2 ($VPAC_1$-R and $VPAC_2$-R) (6, 7). Both PAC_1-R (type 1 receptors) and $VPAC_1$-R/$VPAC_2$-R (type 2 receptors) belong to the seven-transmembrane domain, G-protein-coupled receptor family, and are all positively coupled to adenylyl cyclase (2). Eight isoforms of PAC_1-R, resulting from alternative splicing, have been characterized to date. These variants display differential signal transduction properties with regard to adenylyl cyclase and phospholipase C (PLC) stimulation (1, 2). In addition to these classical signaling pathways, PACAP has been found to stimulate a Ca^{2+}-calmodulin nitric oxide synthase (8) and mitogen-activated protein kinase activity (9). These various transduction mechanisms are involved in the neurotrophic activities exerted by PACAP (*i.e.* inhibition of apoptosis and stimulation of neurite outgrowth) during development (9–11).

PACAP and its receptors are actively expressed in the adrenal medulla (12–14). In particular, we have previously demonstrated the occurrence of PACAP-38 (15) and PACAP binding sites (16) in chromaffin cells from 16- to 20-week-old fetal human adrenal glands. Activation of these receptors by PACAP-38 causes stimulation of cAMP production and induces a modest increase in inositol 1,4,5-triphosphate (IP_3) formation (16), suggesting a role for the neuropeptide in the developing

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[1] The abbreviations used are: PACAP-38, pituitary adenylate cyclase-activating polypeptide of 38 residues; VIP, vasoactive intestinal polypeptide; PAC_1-R, PACAP-specific receptor I; VPAC, VIP/PACAP mutual receptor; R, receptor; PLC, phospholipase C; RT, reverse transcriptase; cDNA, complementary DNA; IP_3, inositol 1,4,5-triphosphate; TG, thapsigargin; XeC, Xestospongin C; FSK, forskolin; PKA, protein kinase A; MEM, minimal essential medium; (R_p)-cAMPS, R_p-adenosine 3′,5′-cyclic monophosphorothioate.

adrenal gland. During the process of adrenal gland development, pheochromoblasts originating from the neural crest migrate throughout the fetal cortex, acquiring progressive differentiation through contact with the steroidogenic cells (17, 18) (for review see Ref. 19). However, the neuroendocrine regulation of catecholamine release by chromaffin cells in the human fetus has not been investigated.

Although PACAP is known to be a potent activator of catecholamine secretion from rat and porcine adrenochromaffin cells (20, 21), the differential coupling of PACAP receptor variants to the various Ca^{2+} sources and to the adenylyl cyclase and PLC signaling pathways is still poorly understood. In particular, the effect of PACAP on fetal chromaffin cells has never been investigated. The aim of the present study was therefore to identify the PACAP receptor isoforms expressed in the human fetal adrenal gland and to analyze the signaling pathways responsible for PACAP-evoked $[Ca^{2+}]_i$ increase and catecholamine secretion.

MATERIALS AND METHODS

Chemicals—The chemicals used in the present study were obtained from the following sources: RNAqueous™-4PCR purchased from Ambion (Austin, TX); dithiothreitol, $p(dT)_{12-18}$, rRNasin Ribonuclease Inhibitor, Moloney murine leukemia virus reverse transcriptase (RT) and DNA ladder from Promega (Madison, WI); deoxy-NTPs and *Taq* DNA polymerase from Amersham Pharmacia Biotech (Piscataway, NJ); Xestospongin C, U73122, forskolin, thapsigargin, and H-89 from Calbiochem-Novabiochem Corp. (San Diego, CA); methacholine, sodium orthovanadate, nicotine, and DNase from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada); collagenase, MEM Eagle's medium and OPTI-MEM from Invitrogen (Burlington, Ontario, Canada); (R_p)-cAMPS from Biomol (Plymouth Meeting, PA); Fluo-4 from Molecular Probes (Eugene, OR); PACAP-38 was synthesized by the solid phase methodology as previously described (22); and PACAP (6–38) was from American Peptide Co. (Sunnyvale, CA). All other chemicals were of A grade purity.

Retrieval and Preparation of Glands—Fetal adrenal glands were obtained from fetuses aged 14–20 weeks (post fertilization) at the time of therapeutic abortion. Fetal ages were estimated by foot length and time after menstruation, according to Steeter *et al.* (23). The project was approved by the human subject review committee of our institution. After retrieval, glands were cleansed of fat and processed immediately for cellular or RNA preparation.

RT-PCR—RNA isolation and complementary DNA (cDNA) synthesis: total RNA was isolated from whole human fetal adrenal glands (14, 17, and 20 weeks of gestation), and fetal human brain (18 weeks) using RNAqueous™-4PCR, according to the manufacturer's recommendations. RNA content and quality were determined photometrically. 5 μg of total RNA was denatured (70 °C, 10 min) and reverse-transcribed in the presence of 200 μM $p(dT)_{12-18}$ at 42 °C for 50 min in 20 μl of 1× RT buffer (25 mM Tris-HCl, pH 8.3, 37.5 mM KCl, 1.5 mM $MgCl_2$) containing: 15 mM dithiothreitol, 200 μM deoxy-NTPs, 25 units of rRNasin ribonuclease inhibitor, and 200 units of Moloney murine leukemia virus RT. Inactivation of the enzyme (70 °C, 10 min) was followed by glyceraldehyde-3-phosphate dehydrogenase PCR to assess the quality of the cDNA template (24).

PCR Amplifications—cDNA samples (2 μl) were used for subsequent PCR amplifications in 50 μl of 1× PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) containing 200 μM deoxy-NTPs, 10 pmol of each of the sense and antisense primers, and 2.5 units of *Taq* DNA polymerase. Primers used for the amplification of PAC_1-R splice variant cDNAs were sense, 5′-CTTGTGCAGAAACTTCAGTCTCCAGACATG, and antisense, 5′-TCGGTGCTTGAAGTCCACAGCGAAGTAACGGTTCACCTT (25), corresponding to base sequences 1235–1264 and 1499–1537 of human PAC-R (4). These primers flank the insertion site of 84-bp cassettes (5) and were designed to give a 303-bp amplicon for the normal (null) PACAP receptor, a 387-bp amplicon for the SV-1 or SV-2 splice variants (insertion of one 84-bp cassette), and a 471-bp amplicon for the insertion of two cassettes. PCR was carried out in a PerkinElmer Life Sciences (geneAmp PCR System 2400) thermocycler at 94 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 61 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. Primers used for the amplification of $VPAC_1$-R cDNA were the sense, 5′-ATGTGCAGATGATCGAGGTG, and antisense, 5′-TGTAGCCGGTCTTCACAGAA (26), corresponding, respectively, to base sequences 127–146 and 431–450 of human $VPAC_1$-R (6). PCR was carried out at 94 °C for 2 min followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min, giving a 324-bp amplicon. Primers used for the amplification of $VPAC_2$-R cDNA were the sense, 5′-TCAAACAGAAAAACACAAAGC, and antisense, 5′-ACCTGTTCCTGTCCTTCATCC (7), corresponding, respectively, to base sequences 294–314 and 653–673 of human $VPAC_2$-R (7). PCR was carried out at 95 °C for 2 min, followed by 35 cycles at 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min, giving a 380-bp amplicon.

In every PCR experiment, amplification in the absence of cDNA and in the presence of 2 μg of RNA was performed as a control (not shown). Negative controls using cDNA templates from NCI-H295R and Chinese hamster ovary cells were performed (not shown). PCR products (10 μl) were analyzed on 2% (w/v) agarose gel and visualized by ethidium bromide staining. Length of PCR products was estimated using a 100-bp DNA ladder. Identification of the PCR products was confirmed by enzymatic digestion and electrophoresis on 3% (w/v) agarose with a 25-bp DNA ladder (not shown). Experiments were performed with RNA isolated from three different adrenal glands from 14-, 17-, and 20-week-old human fetuses. Three experiments were performed for each age.

Cell Culture—Glands were processed as described previously (16). Whole tissues from one or two glands were used for each cell preparation, without separation of fetal zone, neocortex, or chromaffin cells. Briefly, small portions of glands (1–2 mm^3) were dissociated with collagenase (2 mg/ml) and DNase (25 μg/ml) in Eagle's minimal essential medium containing 2% antibiotics. After three 20-min incubations, cells were dissociated, filtered, and centrifuged for 10 min at 100 × *g*. The cell pellet was suspended in OPTI-MEM medium containing 2% fetal calf serum, antibiotics, and antimycotics. Cells were plated at a density of ~2×10^5 on plastic coverslips (25 mm). Cells were grown for 3 days in a humidified atmosphere of 95% air/5% CO_2, at 37 °C.

Calcium Measurement—For dye loading, cells were incubated for 30 min at 37 °C in the physiological medium OPTI-MEM containing 4 μM of the fluorescent calcium indicator Fluo-4/AM. Hydrolysis was performed for 30 min at 37 °C in a medium containing: 140 mM NaCl; 5.4 mM KCl; 2 mM $CaCl_2$; 1 mM $MgCl_2$; 10 mM HEPES, pH 7.4; and 1 g/liter glucose. The coverslips were then mounted on the stage of an inverted microscope (Nikon Diaphot, Mississauga, Ontario, Canada). The light source was generated by a 100-watt mercury lamp. Band-pass filters (450–490 nm) and (520–560 nm) were used for excitation and emission, respectively. The emitted light was recorded by a photon-counting unit. Calcium calibration was performed as described previously (27). However, because calcium dye properties may be different in cytoplasmic and nucleoplasmic compartments (28), $[Ca^{2+}]_i$ should be considered only as semiquantitative data (29).

Catecholamine Secretion—An amperometric technique was used to record secretion from chromaffin cells as described previously (30). Briefly, a 5-μm carbon fiber electrode (Ala Scientific Instrument Inc., Westbury, NY) connected to a Patch Clamp PC-501A amplifier (Warner Instrument Corp., Hamden, CT) modified for voltametry was positioned in close proximity of a cell. The potential of the electrode was fixed to 800 mV, and current traces were filtered at 4 kHz and recorded on a DAS-75 digital recorder (Dagan Corp., Minneapolis, MN).

Data Analysis—Curves were fitted with SigmaPlot (version 7.0, Chicago, IL). The data are presented as means ± S.E. from the number of experiments indicated in the legends or in the text. Statistical analyses of the data were performed using the one-way analysis of variance test. Homogeneity of variance was assessed by Bartlett's test, and *p* values were obtained from Dunnett's tables.

RESULTS

Molecular Identification of PACAP Receptors—Using autoradiography, we have previously shown that PACAP receptors in the human fetal adrenal gland were localized only on chromaffin cells (16). An RT-PCR approach was used to identify the PACAP receptor subtypes expressed in adrenal glands from 14- to 20-week-old fetuses. As indicated under "Materials and Methods," PAC_1-R splice variants were discriminated using primers flanking the insertion site of the 84-bp cassettes. As shown in Fig. 1*A*, two bands were detected: a 303-bp band corresponding to the short form of the receptor and a 387-bp band corresponding to an isoform containing a single insertion cassette. Amplicons of 324 and 380 bp corresponding, respectively, to $VPAC_1$-R and $VPAC_2$-R sequences were also detected

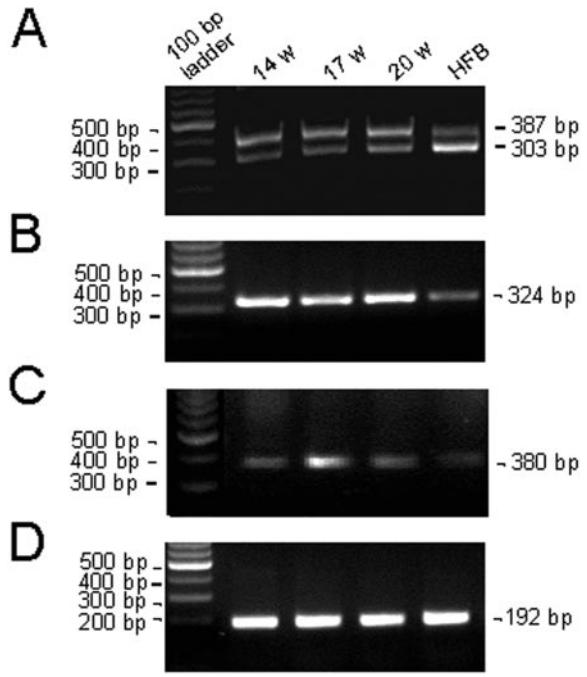

FIG. 1. **Expression of PACAP receptor isoforms in the human fetal adrenal gland.** cDNA from adrenal glands 14-, 17-, and 20-week-old fetuses and from 18-week-old human fetal brain (*HFB*) were amplified using oligonucleotide primers encoding: *A*, nucleotide sequences 1235–1264 and 1499–1537 of the PAC_1 receptor, designed to give three possible amplicons of 303, 387, and 471 bp; *B*, nucleotide sequences 127–146 and 431–450 of the human $VPAC_1$ receptor; and *C*, nucleotide sequences 294–314 and 653–673 of the human $VPAC_2$ receptor. *D*, amplification of glyceraldehyde-3-phosphate dehydrogenase (192 bp) was used to ensure RNA quality and amounts.

in all fetal adrenal glands. However, based on our previous results, these receptors have a more diffuse distribution throughout the adrenal gland, whereas PAC_1-Rs are only detected in chromaffin cells (16). Expression of the PAC_1-R, $VPAC_1$-R, and $VPAC_2$-R RNAs was also observed in 18-week-old fetal brains. Amplification of glyceraldehyde-3-phosphate dehydrogenase (Fig. 1*D*) confirmed that cDNA was present in each reaction.

Effect of PACAP on Cytosolic Ca^{2+} Concentration—Chromaffin cells, which gather into small clusters with numerous processes, could be easily distinguished from the large individual fetal steroidogenic cells (Fig. 2, *white arrows*). Measurements of $[Ca^{2+}]_i$ were performed on peripheral cells of the clusters, selected by a pinhole placed in the optical path (Fig. 2, *asterisk*). Mean $[Ca^{2+}]_i$ in chromaffin cells incubated in medium containing 2 mM Ca^{2+} was 77 nM ± 7 (n = 6) in resting conditions. Application of 1×10^{-9} M PACAP-38 induced a transient elevation of $[Ca^{2+}]_i$ (Fig. 3*A*). This $[Ca^{2+}]_i$ increase was characterized by a rapid upstroke followed by a slower decrease to basal levels. The time course of the decrease could be fitted by a monoexponential function. At a concentration of 1×10^{-9} M PACAP-38, the time constant τ was 57.3 ± 7.4 s (n = 9). In Ca^{2+}-free medium (0 Ca^{2+} plus 1 mM EGTA), application of 1×10^{-9} M PACAP-38 provoked an increase in $[Ca^{2+}]_i$ similar to that recorded in 2 mM Ca^{2+}-containing medium (Fig. 3*B*) with a time constant (49.7 ± 3.5 s; n = 11) that was not significantly different (p = 0.34), suggesting that PACAP-38 causes mobilization of intracellular Ca^{2+} stores. Indeed, application of Ni^{2+}, a known Ca^{2+} channel blocker, during the decreasing phase of the Ca^{2+} response, had no effect on the kinetics of the Ca^{2+} spike induced by application of PACAP-38 (1×10^{-9} M) in 2 mM Ca^{2+}-containing medium, whereas a second application of PACAP-38 in the presence of

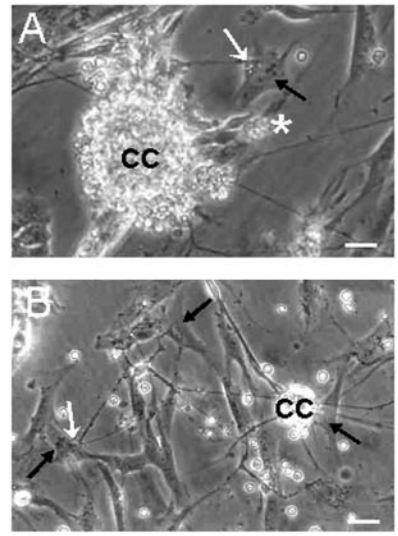

FIG. 2. **Phase-contrast morphology of a human fetal adrenal gland cell culture.** *A* and *B*, representative illustrations from two different cell cultures. After 24 h in culture, chromaffin cells (*CC*) were gathered in small clusters that exhibited several long processes. Cells from the fetal zone (*white arrows*) were easily identified by their size and their polygonal morphology. *Black arrows* indicate contact between a chromaffin cell extension and a fetal cell. The *asterisk* indicates a cell selected by a pinhole placed in the optical path. *Scale bars* represent 10 μm.

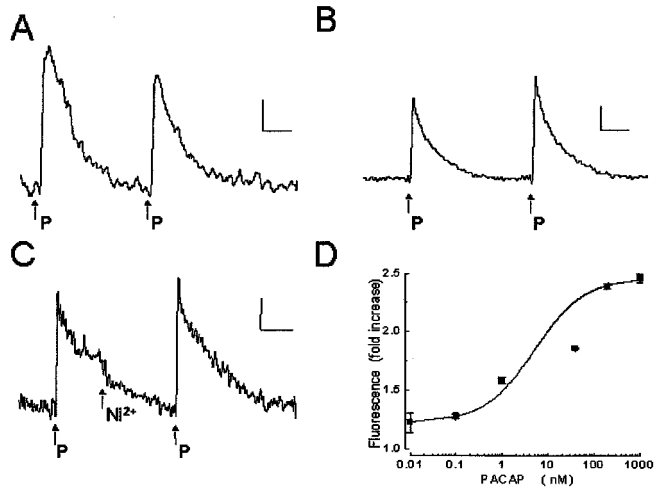

FIG. 3. **Effects of PACAP-38 on $[Ca^{2+}]_i$ in human fetal chromaffin cells.** *A*, PACAP-38 (*P*) 1×10^{-9} M was applied in a medium containing 2 mM of Ca^{2+} (representative of 9 cells from 9 different cell cultures). Scale: *vertical*, 25 nM; *horizontal*, 50 s. *B*, PACAP-38 (*P*) 1×10^{-9} M was applied in a Ca^{2+}-free medium containing 1 mM EGTA (representative of 11 cells from 9 different cell cultures). Scale: *vertical*, 20 nM; *horizontal*, 50 s. *C*, application of Ni^{2+} (500 μM) during the falling phase of the Ca^{2+} response induced by PACAP-38 (*P*) 1×10^{-9} M had no effect on the time course of the response nor on the subsequent response induced by a new application of PACAP-38 (representative of 5 cells from 5 different cell cultures). Scale: *vertical*, 11 nM; *horizontal*, 100 s. *D*, relationship between the concentration of PACAP-38 and the Ca^{2+} increase (n = 3, 5, 15, 3, 3, and 3 for 0.01, 0.1, 1, 40, 200, and 1000 nM of PACAP-38, respectively). The experimental points were fitted by the logistic function with an ED_{50} of 5 nM.

Ni^{2+} provoked a Ca^{2+} response similar to that obtained in the absence of the blocker (Fig. 3*C*). Application of increasing concentrations of PACAP-38 resulted in a dose-dependent rise in the amplitude of the $[Ca^{2+}]_i$ response with an ED_{50} value of 5 nM (Fig. 3*D*), a value similar to that obtained (2.6 nM) by measuring the $[Ca^{2+}]_i$ increase on hippocampal neurons (31). Administration of repeated pulses of PACAP-38 (1×10^{-9} M) at various time intervals induced a reproducible $[Ca^{2+}]_i$ increase,

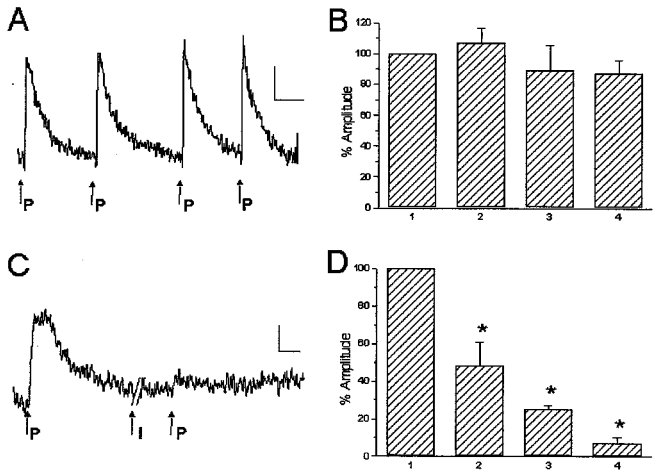

FIG. 4. **Frequency dependence of the PACAP-induced Ca^{2+} increase in the human fetal adrenal gland.** *A*, four identical concentrations of PACAP-38 (*P*) 1×10^{-9} M were applied on the same cell. Note that the four Ca^{2+} increases are identical with no apparent desensitization. Scale: *vertical*, 20 nM; *horizontal*, 100 s. *B*, compilation of data obtained in three different cells from three different glands where PACAP-38 was applied four successive times indicated by *1*, *2*, *3*, and *4* corresponding to the first, second, third, and fourth PACAP-38 applications. Data are normalized to the first response. *C*, effect of PACAP (6–38) on the PACAP-induced Ca^{2+} increase in the human fetal adrenal gland. A first application of PACAP-38 (*P*) (1×10^{-7} M), which elicited a Ca^{2+} increase, was followed by application of 10 μM of the PAC_1-R antagonist PACAP (6–38) (*I*). After 10 min, PACAP-38 (*P*) (1×10^{-7} M) was further applied. Scale: *vertical*, 13 nM; *horizontal*, 20 s. *D*, inhibition of the PACAP-38 (1×10^{-7} M) Ca^{2+} responses by PACAP (6–38) applied during 10 min; *1*, normalized Ca^{2+} increase in control condition; *2*, blockage of the Ca^{2+} response by 0.3 μM PACAP (6–38) obtained in four different cells from three different glands; *3*, blockage of the Ca^{2+} response by 1 μM of PACAP (6–38) obtained in two different cells from two different glands; *4*, blockage of the Ca^{2+} response by 10 μM PACAP (6–38) obtained in four different cells from three different glands. *, significantly different at the $p = 0.05$ level.

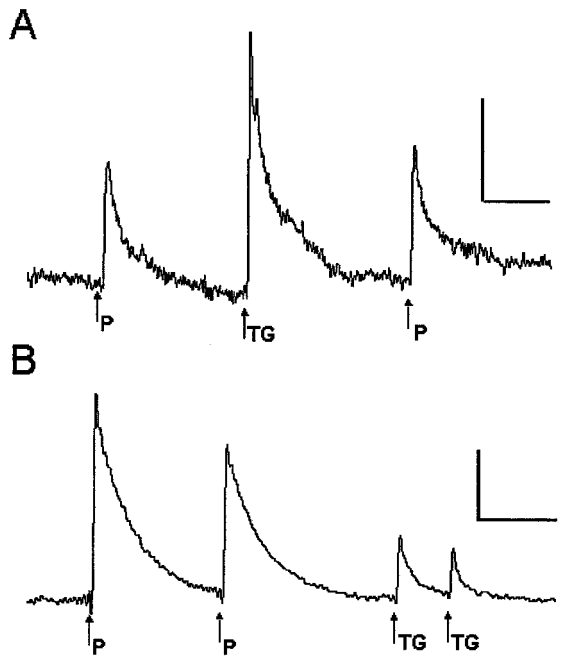

FIG. 5. **Lack of sensitivity of the PACAP-induced Ca^{2+} increase to thapsigargin in the human fetal adrenal gland.** *A*, a first application of PACAP-38 (*P*) 1×10^{-9} M, which elicited a Ca^{2+} increase, was followed by application of 4×10^{-6} M of thapsigargin (*TG*) to deplete the TG-sensitive Ca^{2+} pools. A subsequent application of PACAP-38 at the same concentration triggered a Ca^{2+} response, which was not affected by TG (representative of three cells, three different cells cultures); experiments conducted in a Ca^{2+}-free medium. Scale: *vertical*, 14 nM; *horizontal*, 100 s. *B*, the cells were preincubated in a medium containing 8×10^{-6} M of TG during 30 min. Application of PACAP-38 (*P*) 2.5×10^{-9} M elicited a Ca^{2+} increase similar to the response obtained in control conditions, whereas TG (4×10^{-6} M) elicited a slight increase indicating that the TG-sensitive Ca^{2+} stores are depleted (representative of four cells, three different cells cultures). Scale: *vertical*, 38 nM; *horizontal*, 100 s.

even when applied at very short intervals (Fig. 4*A*). The amplitudes of the responses to any of the four pulses applied were not significantly different (Fig. 4*B*). To further confirm that the PACAP-induced $[Ca^{2+}]_i$ increase was due to the activation of the PAC_1-R type receptor, we used PACAP (6–38), a PAC_1 receptor-specific antagonist shown to inhibit the activation of adenylyl cyclase with a K_i of 7 nM (32). A first application of PACAP-38 (1×10^{-7} M) triggered a Ca^{2+} response as previously described. The antagonist PACAP (6–38) was then applied (10 μM) for a 10-min period and followed by a second application of PACAP-38, at the same concentration, which induced a lower Ca^{2+} increase (Fig. 4*C*). For concentrations of PACAP (6–38) of 0.3, 1, and 10 μM, the Ca^{2+} response to PACAP-38 (1×10^{-7} M) was reduced to 48.5 ± 12% ($n = 4$), 25 ± 2% ($n = 2$), and 6.9 ± 3.4% ($n = 4$) of the control, respectively (Fig. 4*D*).

Nature of the Intracellular Ca^{2+} Pool—We have previously shown that PACAP-38 induces a 3.4-fold increase in cAMP production and a modest increase in IP_3 formation in fetal human chromaffin cells (16). In the present study, we first investigated whether or not the PACAP-sensitive Ca^{2+} pool was responsive to thapsigargin (TG), a known inhibitor of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase pumps but without effect on plasma membrane Ca^{2+}-ATPase activity (33). In one series of experiments, the cells were bathed in Ca^{2+}-free medium. Application of PACAP-38 (1×10^{-9} M) produced an increase in Ca^{2+} as described previously (Fig. 5*A*). The subsequent application of TG (4 μM) triggered an additional increase in $[Ca^{2+}]_i$ resulting from blockage of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase pumps. When PACAP-38 was applied further, the amplitude and kinetics of the Ca^{2+} increase were similar to those obtained prior to TG application, indicating that TG and PACAP-38 did not mobilize the same Ca^{2+} pool(s). In a second series of experiments, cells were preincubated for 30 min in a calcium-free medium containing 8 μM TG. Under these conditions, the Ca^{2+} response to TG (4 μM) was greatly reduced, whereas the response to PACAP-38 (2.5×10^{-9} M) was not affected (Fig. 5*B*).

In some other cell types, it has been shown that PACAP receptors are coupled to PLC through a $G_{q/11}$ protein to produce diacylglycerol and IP_3 (34). In the specific case of human fetal chromaffin cells, we previously found that IP_3 production is relatively low (16). Hence, experiments were performed to assess the putative role of IP_3-sensitive calcium pools in the PACAP-induced increase in $[Ca^{2+}]_i$. Cells were treated with U-73122 compound, a specific PLCβ inhibitor (35), at a concentration of 1 μM for 18 h. PACAP-38 (1×10^{-9} M) was then added in the presence or absence of Ca^{2+} in the external medium as described above. In five different cells, the amplitude as well as the kinetics of the Ca^{2+} response to PACAP-38 were similar to those obtained in control cells (Fig. 6*A*), indicating that the Ca^{2+} increase was not dependent on IP_3-sensitive pools. Further confirmation of these results was provided from experiments using Xestospongin C (XeC), a potent specific blocker of the inositol 1,4,5-triphosphate (IP_3) receptors (36). In this experimental design, PACAP-38 (1×10^{-9} M) was first applied to induce a $[Ca^{2+}]_i$ increase. XeC (20 μM) was then applied for 10 min prior to a second application of PACAP-38. Fig. 6*B* shows that the amplitude of the Ca^{2+} increase was not affected by XeC, thus indicating that IP_3-sensitive Ca^{2+} pools are not

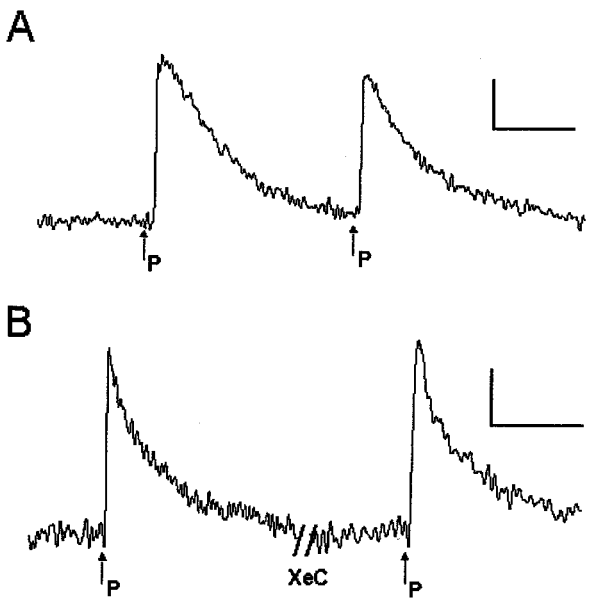

FIG. 6. **PACAP-38 does not mobilize IP_3-sensitive Ca^{2+} stores in the human fetal adrenal glands.** *A*, cells were preincubated for 18 h in the presence of the PLC inhibitor U73122 (1×10^{-6} M). Experiments were conducted in a Ca^{2+}-free medium. This treatment did not modify the PACAP-38 (*P*) 1×10^{-9} M-induced Ca^{2+} increase (representative of five cells, three different cell cultures). Scale: *vertical*, 20 nM; *horizontal*, 50 s. *B*, Xestospongin C (*XeC*) 20×10^{-6} M was applied after a first response elicited by PACAP-38 (*P*) 1×10^{-9} M. The second application of PACAP-38 (*P*) triggered a similar Ca^{2+} increase (representative of five cells, five different cells cultures). Scale: *vertical*, 14 nM; *horizontal*, 100 s.

involved. Similar results were obtained from five different cells.

Involvement of a cAMP-sensitive calcium pool in the PACAP response was tested by directly activating cAMP production with forskolin (FSK). Fig. 7*A* demonstrates that FSK (1×10^{-5} M) and PACAP-38 (1×10^{-9} M) triggered identical calcium increases. Similar results were obtained in the absence of Ca^{2+} in the bathing medium (data not shown, $n = 3$). The involvement of a PKA-dependent phosphorylation step in the increase in Ca^{2+} triggered by PACAP was assessed by using H-89, an inhibitor of PKA. When cells were pretreated with H-89 (10 μM) for 15 min, subsequent stimulation with PACAP-38 failed to trigger a Ca^{2+} increase (Fig. 7*B*, $n = 7$). The FSK-triggered Ca^{2+} increase was also abolished (Fig. 7*B*, $n = 4$). When (R_p)-cAMPS (1 mM), a more specific membrane-permeant inhibitor of PKA was used, the response to PACAP-38 (2×10^{-7} M), but not to TG, was abolished (Fig. 7*C*, $n = 4$).

Caffeine is known to activate ryanodine channels and to induce $[Ca^{2+}]_i$ increase in numerous cell types (37). Application of caffeine (20 mM) to chromaffin cells induced an increase in $[Ca^{2+}]_i$ (Fig. 8*A*). More importantly, the Ca^{2+} response to PACAP-38 (1×10^{-9} M) was significantly decreased following caffeine application to chromaffin cells ($-51 \pm 6.7\%$, $n = 5$). Similar results were obtained with ryanodine (20 μM, $n = 4$, data not shown).

A recent study reported that neuroendocrine cells contained dense core secretory vesicles that could constitute a dynamic Ca^{2+} store, whereby the P-type Ca^{2+} pump was responsible for Ca^{2+} uptake in these secretory vesicles (38). To assess such putative participation, we used orthovanadate at 100 μM, a concentration known to inhibit the ATP-dependent P-type Ca^{2+} pump. In Ca^{2+}-free medium, cells were first challenged with PACAP-38 (1×10^{-9} M) followed by application of Na_3VO_4 (100 μM) for 10 min. Thereafter, the first addition of PACAP-38 induced a Ca^{2+} response not significantly different in amplitude ($9.2 \pm 9\%$ decrease, $n = 6$) from that obtained prior to

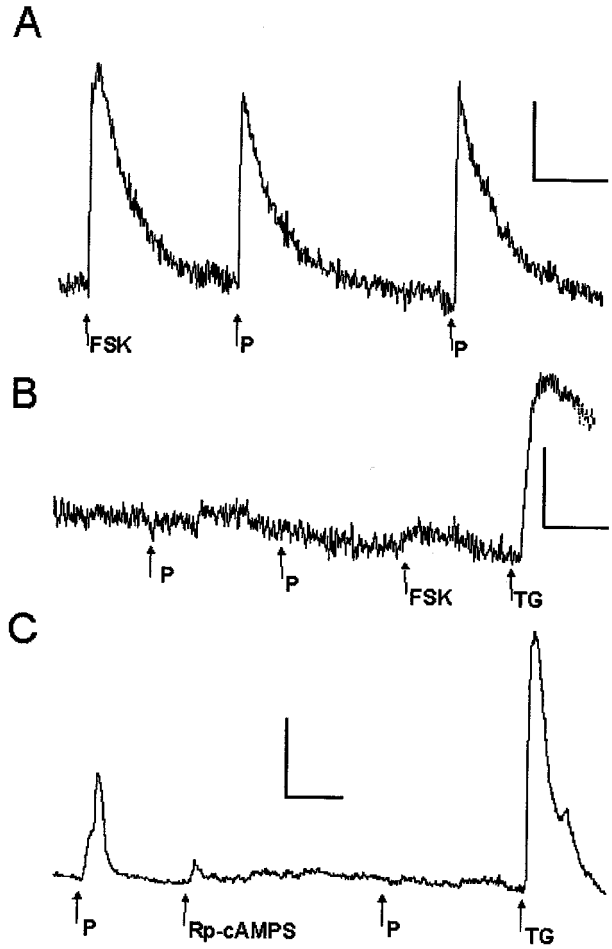

FIG. 7. **PACAP-38 uses the cAMP/PKA pathway to trigger Ca^{2+} increase in the human fetal adrenal gland.** *A*, forskolin (*FSK*) 1×10^{-5} M triggered a Ca^{2+} increase similar to that evoked by PACAP-38 (*P*) 1×10^{-9} M (representative of three cells, three different cell cultures). Scale: *vertical*, 38 nM; *horizontal*, 100 s. *B*, cells were treated with H-89 (10×10^{-6} M) for 15 min. Application of PACAP-38 (*P*) 2×10^{-7} M as well as FSK (1×10^{-6} M) were not able to trigger Ca^{2+} increase (representative of seven cells, five different cell cultures). Scale: *vertical*, 20 nM; *horizontal*, 50 s. *C*, a first application of PACAP-38 (*P*) 2×10^{-7} M elicited a Ca^{2+} response. The second application, after (R_p)-cAMPS (1×10^{-3} M) did not induce any response. In all panels, thapsigargin (*TG*) is always able to elicit a large increase in intracellular Ca^{2+} (representative of four cells, four different cell cultures). Scale: *vertical*, 100 nM; *horizontal*, 200 s.

adding Na_3VO_4. However, subsequent applications of PACAP-38 gave rise to a lower amplitude of Ca^{2+} increase (-63% and -86% for the second and third PACAP-38 applications, respectively) (Fig. 8*B*). Data obtained from three different cell cultures are summarized in Fig. 8*C*.

Functional Properties of Chromaffin Cells—Activation of cholinergic receptors in chromaffin cells is the main stimulus for mobilizing Ca^{2+} to induce catecholamine secretion (39). Hence, experiments were undertaken to determine if chromaffin cells from 17- to 20-week-old human fetuses express functional cholinergic receptors. Release of Ca^{2+} from IP_3-sensitive Ca^{2+} pools was tested using methacholine, a muscarinic receptor agonist. Fig. 9*A* shows that application of methacholine (10 μM) to chromaffin cells induced a large, transient increase of $[Ca^{2+}]_i$ followed by a plateau indicating an influx of Ca^{2+}as previously shown for guinea pig adrenal chromaffin cells (40). Moreover, voltage-dependent Ca^{2+} channels are functional in 17- to 20-week-old human fetal adrenal chromaffin cells. Indeed, membrane depolarization caused by activation of nicotinic receptors with nicotine (10 μM) induced a $[Ca^{2+}]_i$ increase

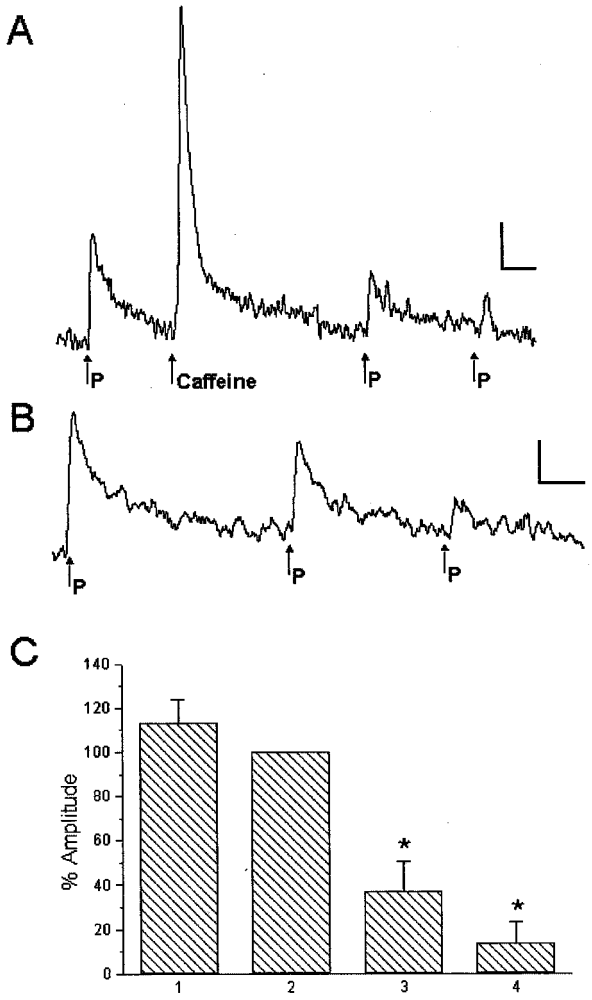

FIG. 8. **PACAP-38 mobilizes Ca^{2+} from a ryanodine/caffeine-sensitive stores in the human fetal adrenal gland.** *A*, addition of caffeine (20 mM) triggered a Ca^{2+} increase, revealing the presence of a ryanodine/caffeine store. Emptying this store blunted the Ca^{2+} response to PACAP-38 (*P*) 1×10^{-9} M (representative of five cells, three different cell cultures). Scale: *vertical*, 11 nM; *horizontal*, 100 s. *B*, cells were treated for 10–15 min with Na_3VO_4 (100 μM) and challenged several times by PACAP-38 (*P*) 1×10^{-9} M. The amplitude of the Ca^{2+} response decreased as a function of the number of trials (representative of six cells from four different cell cultures). Scale: *vertical*, 10 nM; *horizontal*, 50 s. *C*, plot of the amplitude of the Ca^{2+} responses for subsequent trials. *1*, response in control conditions; *2*, first response after treatment with Na_3VO_4; *3*, second response after Na_3VO_4; *4*, third response after Na_3VO_4; data are normalized to the amplitude of the first response after Na_3VO_4 (*bar number 2*). Compilation of data from *B*. *, significantly different at the $p = 0.05$ level.

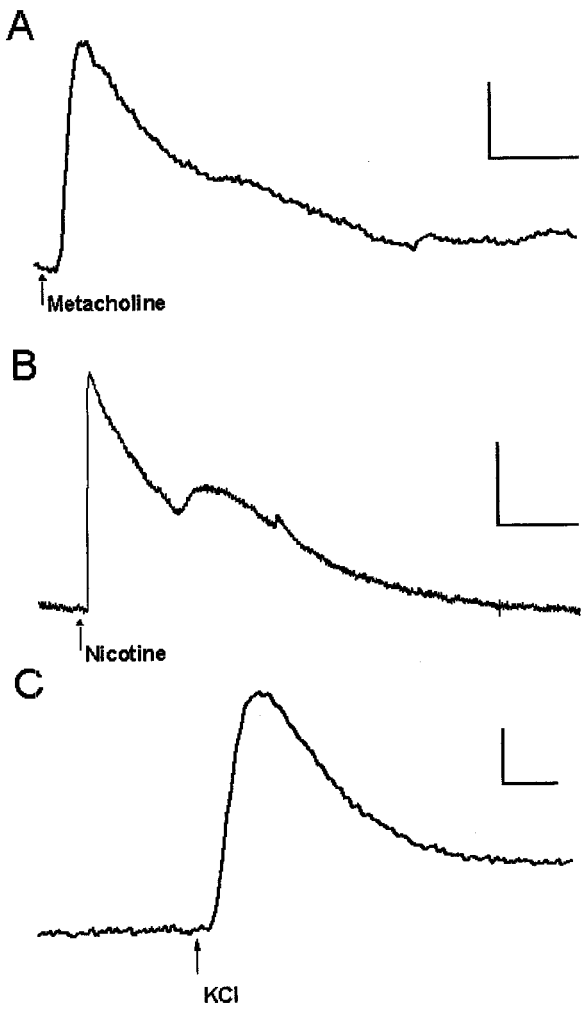

FIG. 9. **Cholinergic agonists and KCl increase $[Ca^{2+}]_i$ in fetal human chromaffin cells in the human fetal adrenal gland.** *A*, methacholine (10×10^{-6} M), a muscarinic agonist, induced a Ca^{2+} increase when applied in the bath (representative of three cells, three different cell cultures). Scale: *vertical*, 50 nM; *horizontal*, 200 s. *B*, Ca^{2+} increase upon addition of nicotine (10×10^{-6} M) (representative of three cells, three different cell cultures). Scale: *vertical*, 38 nM; *horizontal*, 200 s. *C*, depolarization by 30 mM KCl induced a Ca^{2+} increase (representative of three cells, three different cell cultures). Scale: *vertical*, 14 nM; *horizontal*, 50 s.

(Fig. 9*B*) similar to that obtained after depolarization using KCl (30 mM) (Fig. 9*C*).

Because PACAP-38 increased $[Ca^{2+}]_i$ in human chromaffin cells, the question arises as to whether these cells are capable of catecholamine secretion. A carbon fiber (5 μm) for amperometric detection (41) was used to monitor catecholamine secretion from cell clusters. Stimulation with PACAP-38 (5×10^{-7} M) in a 2 mM Ca^{2+} medium, triggered the quantal release of catecholamine by chromaffin cells as demonstrated by the occurrence of spikes under the carbon electrode (Fig. 10*A*, $n = 5$). If the cells are preincubated for 20 min with the PAC_1-R antagonist PACAP (6–38) (10 μM), application of PACAP-38 (5×10^{-7} M) failed to stimulate catecholamine release (Fig. 10*B*, $n = 4$). Similar results were obtained if the cells are preincubated with (R_p)-cAMPS (1 mM, 10 min) prior application of PACAP-38 (data not shown, $n = 2$).

DISCUSSION

Our study demonstrates that the fetal human adrenal gland expresses both type I and type II PACAP receptors. Activation of chromaffin PACAP receptors with PACAP-38 induced a transient increase in $[Ca^{2+}]_i$ originating exclusively from intracellular calcium pools and did not involve Ca^{2+} influx from the external medium. Moreover, the PACAP-sensitive Ca^{2+} pool was not mobilized by IP_3 or TG. More importantly, PACAP-38 activated a ryanodine/caffeine-sensitive pool, which involved cAMP and a phosphorylation step by PKA. In addition, we were able to demonstrate that activation of the PAC_1 receptor induced secretion of catecholamine by the chromaffin cells.

The results presented herein indicate that, among the various isoforms of the PACAP receptors, the short fragment and one isoform A of an hip-hop insertion cassette of the PAC_1 receptors are present in the human fetal adrenal gland at the second trimester of gestation. By using PACAP (6–38), a PAC_1 receptor-specific antagonist (32), we demonstrate that the Ca^{2+} increase induced by PACAP-38 is mediated by the activation of PAC_1-R coupled to adenylyl cyclase (2).

One important finding of this study is the observation that

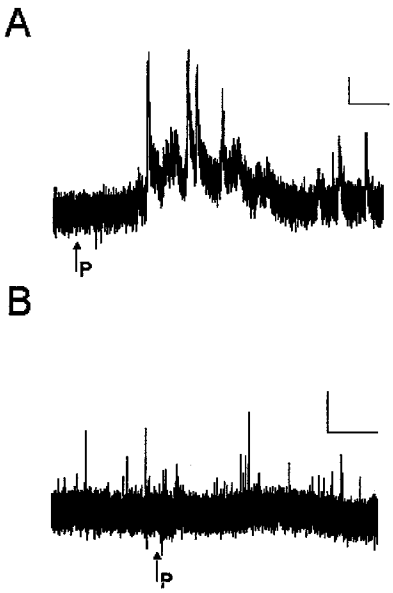

FIG. 10. **Amperometric recordings of catecholamine secretion by fetal human chromaffin cells.** Current spikes were recorded by a carbon electrode positioned near the cell. *A*, secretion induced by application of PACAP-38 (*P*) 5×10^{-7} M. Scale: *vertical*, 0.8 pA; *horizontal*, 625 s. (representative of five cells, three different cells cultures). *B*, cells were preincubated for 20 min in the presence of PACAP (6–38) 1×10^{-5} M and challenged with PACAP-38 (*P*) (5×10^{-7} M). Scale: *vertical*, 0.8 pA; *horizontal*, 800 s (representative of four cells, two different cells cultures).

the PACAP-induced Ca^{2+} rise was not affected by the absence of Ca^{2+} in the external medium. This clearly indicated that PACAP-38 does not trigger a Ca^{2+} influx through channels or exchangers, in agreement with data obtained in rat hippocampal neurons (31). However, a number of reports indicate that PACAP activates a Ca^{2+} influx through various pathways, including Ca^{2+} channels (29, 42–44). In chromaffin cells from adult human adrenal glands, voltage-dependent Ca^{2+} channels have been described (45), but no electrophysiological studies have been performed in fetal cells. KCl depolarization, or application of nicotine, were able to induce an increase in $[Ca^{2+}]_i$ in human fetal chromaffin cells suggesting the presence of functional voltage-dependent Ca^{2+} channels. However, our results clearly show that these Ca^{2+} channels were not involved in PACAP-induced Ca^{2+} increase.

The second most important feature of the present data was that PACAP-responsive Ca^{2+} pools were insensitive to TG. It has been reported that TG triggers the release of Ca^{2+} from major nonmitochondrial Ca^{2+} stores (46), including the IP_3-sensitive Ca^{2+} pool (47). Our data conclusively demonstrate that preincubation of the cells with TG or chronic application of high concentrations of TG (8 μM) have no effect on the Ca^{2+} response triggered by PACAP-38. Previous experiments have shown that, in human fetal chromaffin cells, PACAP-38 triggered low production of IP_3 (16). However, the present results, using either the PLC inhibitor U73132 (35) or Xestospongin C, a blocker of IP_3 receptors (36), demonstrated that Ca^{2+} release from IP_3-sensitive Ca^{2+} stores did not contribute to the increase in $[Ca^{2+}]_i$ triggered by PACAP-38.

Several lines of evidence pointed toward involvement of the cAMP/PKA pathway in the $[Ca^{2+}]_i$ increase triggered by PACAP-38 in human fetal chromaffin cells. FSK, which directly activates adenylyl cyclase to produce cAMP, triggered an increase in Ca^{2+} that was similar in amplitude and kinetics to that induced by PACAP-38. Moreover, like PACAP-38, the response triggered by FSK was independent of the presence of Ca^{2+} in the external medium. Additionally, blocking PKA using H-89 or (R_p)-cAMPS inhibited the effects of both PACAP-38 and FSK on $[Ca^{2+}]_i$. Opposite results were found in hippocampal neurons where PACAP-induced Ca^{2+} response was not triggered by FSK or $(Bu)_2$cAMP and insensitive to H-89 (31). Cyclic AMP-sensitive Ca^{2+} stores have been described in numerous cell types, but their relationship with the IP_3- and the ryanodine/caffeine Ca^{2+} stores is still a matter of debate. In several cell types, it has been shown that the ryanodine receptor is subject to phosphorylation by several kinases, including PKA (48, 49). Our results clearly indicated that PACAP- and FSK-induced Ca^{2+} increases were sensitive to PKA-dependent phosphorylation. This, together with the effect of ryanodine/caffeine, could indicate that PACAP-38 mobilizes Ca^{2+} from caffeine-sensitive stores in human fetal chromaffin cells.

Application of caffeine to human fetal chromaffin cells triggered a moderate increase in $[Ca^{2+}]_i$ confirming the presence of caffeine-sensitive Ca^{2+} stores. In bovine adult chromaffin cells, PACAP released Ca^{2+} from a ryanodine/caffeine store (50), which was independent of IP_3, as observed for our cell model. However, it was also reported that Ca^{2+} rise was insensitive to (R_p)-cAMPS contrary to human fetal chromaffin cells where H-89, or (R_p)-cAMPS, completely inhibited both PACAP- and FSK-induced responses. The role and weightiness of the caffeine-dependent Ca^{2+} pools in chromaffin cells have been outlined by several authors (51) who report that caffeine-dependent Ca^{2+} pools release more Ca^{2+} than IP_3-dependent Ca^{2+} pools in permeabilized chromaffin cells (52, 53). The signaling pathway has not yet been fully characterized, although several possibilities have been proposed (54). More recently, secretory vesicles in neuroendocrine cells have been demonstrated to constitute a dynamic Ca^{2+} pool (38). Several features of the vesicle-Ca^{2+} stores are similar to those defined by PACAP-dependent stores. Indeed, these pools are insensitive to TG, are not mobilized by IP_3, and are sensitive to caffeine and ryanodine. As proposed by Mitchell *et al.* (38), cytosolic Ca^{2+} is pumped into the deep vesicles by an ATP-dependent P-type Ca^{2+} pump. Ca^{2+} is released by primed vesicles located near the plasma membrane when $[Ca^{2+}]_i$ concentrations increase due to Ca^{2+} channel opening (55). Our data indicate that treatment of chromaffin cells with Na_3VO_4 did not empty the PACAP-sensitive Ca^{2+} pools, as illustrated by identical levels of Ca^{2+} increase obtained before and after Na_3VO_4 application. However, the fact that the amplitude of the subsequent responses to PACAP-38 decreased as a function of the number of PACAP-38 applications indicated that re-pumping of Ca^{2+} may have been impaired by Na_3VO_4.

By using amperometry, we also demonstrated that human fetal chromaffin cells release catecholamine in response to PACAP-38. $[Ca^{2+}]_i$ increase is a requirement, as outlined by the absence of secretion in cells treated with PACAP (6–38) or (R_p)-cAMPS. Catecholamine secretion could be linked to the paracrine control of secretion of dehydroepiandrosterone sulfate and cortisol by fetal steroidogenic cells. Indeed, in cell cultures, PACAP-38 stimulates DHEA and cortisol secretion, an effect abolished by preincubation with the β-adrenoreceptor antagonist, propranolol (15).

In summary, stimulation of chromaffin cells with PACAP-38 induced a cAMP-dependent increase in intracellular Ca^{2+} whose properties and regulation exhibit particular features. This model has thus enabled the identification of a novel pathway in the regulation of cAMP-dependent Ca^{2+} release, which could be specific to fetal tissues. In addition to its role in catecholamine secretion, PACAP-38 could act as a survival factor for chromaffin cells, because, in contrast to fetal cells, chromaffin cells do not undergo apoptosis, either *in vivo* or *in*

vitro (56, 57). Recent studies conducted in knock-out mice for PACAP, revealed that PACAP was not essential for normal development of the adrenal gland, nor for basal catecholamine secretion; however, its presence was essential for adaptive responses. Indeed, mice lacking PACAP were unable to survive in response to a metabolic stress (58). Taken together, these results clearly indicate that PACAP is one of the important factors necessary in maintaining adequate interaction between catecholamine and steroid-producing cells, an interaction that occurs during normal development.

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PAC_1 Receptor Activation by PACAP-38 Mediates Ca^{2+} Release from a cAMP-dependent Pool in Human Fetal Adrenal Gland Chromaffin Cells

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