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Pituitary Adenylate Cyclase-Activating Polypeptide Induces the Voltage-Independent Activation of Inward Membrane Currents and Elevation of Intracellular Calcium in HIT-T15 Insulinoma Cells*

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ABSTRACT

The secretion of insulin by pancreatic β-cells is controlled by synergistic interactions of glucose and hormones of the glucagon-related peptide family, of which pituitary adenylate cyclase-activating polypeptide (PACAP) is a member. Here we show by simultaneous recording of intracellular calcium ion ($[Ca^{2+}]_i$) and membrane potential that both PACAP-27 and PACAP-38 depolarize HIT-T15 cells and raise $[Ca^{2+}]_i$. PACAP stimulation can result in membrane depolarization by two distinct mechanisms: 1) PACAP reduces the membrane conductance and increases membrane excitability; and 2) PACAP activates a pronounced inward current that is predominantly a Na^+ current, blockable by La^{3+}, and which exhibits a reversal potential of about −28 mV. Activation of this current does not require membrane depolarization, because the response is observed when cells are held under voltage clamp at −70 mV. This current may result from the cAMP-dependent activation of nonspecific cation channels because the current is also observed in response to forskolin or membrane-permeant analogs of cAMP.

We also suggest that PACAP raises $[Ca^{2+}]_i$ and stimulates insulin secretion by three distinct mechanisms: 1) depolarization activates Ca^{2+} influx through L-type voltage-dependent calcium channels, 2) mobilization of intracellular Ca^{2+} stores, and 3) entry of Ca^{2+} via voltage-independent Ca^{2+} channels. These effects of PACAP may play an important role in a neuro-entero-endocrine loop regulating insulin secretion from pancreatic β-cells during the transition period from fasting to feeding. (*Endocrinology* **136:** 1530–1536, 1995)

PITUITARY adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide structurally related to the peptide family consisting of glucagon, secretin, vasoactive intestinal peptide, and others (1). Two isoforms of PACAP, PACAP-27 and PACAP-38, which share the same 27 N-terminal amino acids, are derived by tissue-specific posttranslational processing of a 176-amino acid precursor (2). In the endocrine pancreas, PACAP is produced and secreted from nerve endings that innervate the islets of Langerhans (3), and PACAP is an extremely potent stimulator of glucose-dependent insulin secretion (4). Multiple isoforms of the PACAP receptor (PACAP-R) have been isolated and sequenced, including, in insulin-secreting β-cells, the type 3 PACAP-R (PACAP-R3), which is homologous to the rat vasoactive intestinal peptide$_2$ receptor (5, 6). The type 1 PACAP-R has been shown to stimulate both cAMP accumulation (EC_{50} 3 nM) and [^{3}H]inositol phosphate production (EC_{50}, 20 nM) in the gonadotrope-derived αT3–1 cell line (7). Similarly, when expressed in *Xenopus* oocytes, PACAP-R3 can couple to phospholipase-C as well as to adenylyl cyclase (5), but such dual coupling of PACAP receptors has yet to be demonstrated in insulinoma cells or pancreatic β-cells. However, the observation that nitrendipine blocks PACAP-induced elevation of intracellular calcium ion ($[Ca^{2+}]_i$) and insulin secretion (4) suggests that mobilization of intracellular Ca^{2+} stores through activation of phospholipase-C is not the primary signaling pathway of PACAP in β-cells and that stimulation of cAMP production may play a more dominant role.

In perfused rat pancreas, the two isoforms of PACAP, PACAP-27 and PACAP-38, induce insulin secretion in a glucose-dependent manner. PACAP-38 is more potent than PACAP-27 at 5.5 mM extracellular glucose, but the two forms are equally potent at 8.3 mM glucose (8). In rat β-cells, both PACAP-27 and PACAP-38 potentiate glucose-dependent insulin secretion at concentrations as low as 10^{-14}-10^{-13} M (4). These actions of PACAP are reported to be mediated through an increase in the activity of L-type voltage-dependent calcium channels (VDCCs), because nitrendipine, a specific blocker of L-type channels, abolishes the stimulatory effects of PACAP on both $[Ca^{2+}]_i$ and insulin secretion. However, the effects of PACAP on membrane currents or membrane potential were not examined (4).

As PACAP stimulates cAMP production, it is of interest to determine what effect it might exert on ion channels that are known to be regulated by this cyclic nucleotide, either through direct effects of cAMP on channel gating or via effects mediated by cAMP-dependent protein phosphorylation. Recent studies suggest that cAMP (and Ca^{2+}) activate a nonspecific cation channel (Ca-NS channels) in the rat insulinoma cell line, CRI-G1 (9). The activation of this current in isolated inside-out membrane patches does not appear to be mediated by protein kinase-A (PKA) and requires non-

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physiological cytoplasmic $[Ca^{2+}]$ (>0.1 mM) (9, 10) (the $[Ca^{2+}]_i$ required in the intact cell may be much lower). The activation of this current *in vivo* would be expected to cause membrane depolarization and a rise in $[Ca^{2+}]_i$, thereby triggering insulin secretion.

Glucagon-like peptide-1 (GLP-1) is a potent incretin hormone produced in and released from entero-endocrine cells in the small intestine in response to the ingestion of nutrients. The stimulation of insulin secretion by GLP-1 appears to be mediated by increases in cAMP levels, which potentiate glucose-induced closure of ATP-sensitive K-channels (ATP-K channels) (11). This closure leads to membrane depolarization and activation of VDCCs, resulting in a rise of $[Ca^{2+}]_i$, which triggers insulin secretion. GLP-1 is a member of the group of peptides related in structure to PACAP, and the glucose-dependent insulinotropic actions of PACAP might be expected to result from a similar mechanism of action. In addition to these effects, cAMP has been reported to enhance the current through L-type Ca^{2+} channels, by slowing their inactivation, and to have direct stimulatory effects on exocytosis (12).

The aim of the present study was to examine further the signal transduction pathway of PACAP in HIT-T15 cells using fura-2 fluorescence ratio measurements of $[Ca^{2+}]_i$ combined with perforated patch recording (13) of the membrane potential or membrane current. We show that PACAP induces depolarization of HIT-T15 cells by reducing the membrane conductance, raising the excitability of cells, and voltage-independent activation of an inward current that is predominantly carried by Na^+. Depolarization of the cell will activate VDCCs and produce a rise in $[Ca^{2+}]_i$. However, PACAP also raises $[Ca^{2+}]_i$ in cells held under voltage clamp, negative to the threshold for activation of VDCCs, by mobilization of intracellular stores and activation of a Ca^{2+} release-activated Ca^{2+}-current (I_{CRAC}).

As PACAP is reported to be the most potent insulinotropic peptide known (4), we propose that PACAP may play an important role in a neuro-entero-endocrine loop to potentiate insulin secretion during the fasting to feeding transition, before intestinal incretins, such as GLP-1, have an effect.

Materials and Methods

HIT-T15 cells (passages 64–78), obtained from the American Type Culture Collection (Rockville, MD), were cultured in F-12 medium containing 10 mM glucose and supplemented with 10% horse serum, 2.5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were transferred onto glass coverslips coated with 1 mg/ml of Concanavalin-A (type V, Sigma Chemical Co., St. Louis, MO), which facilitates adherence of the cells to the glass. Experiments were performed 2–4 days postplating.

Measurement of $[Ca^{2+}]_i$

HIT-T15 cells were prepared for measurement of $[Ca^{2+}]_i$ by washing three times with PBS to remove culture medium and then placed in 5 ml extracellular recording solution (see below) containing 0.8 mM glucose, 2% fetal bovine serum, 0.03% Pluronic F-127, and 5 μM fura-2/AM (Molecular Probes, Eugene, OR). Cells were loaded in this solution for 1.5–2 h at room temperature (20–22 C). Loading HIT-T15 cells at room temperature, rather than at 37 C, substantially reduces the degree of intracellular compartmentalization of fura-2 (14). Glass coverslips with fura-2-loaded adherent cells formed the base of a recording chamber mounted on a Peltier temperature-controlled (32 C) stage of a Zeiss IM35 microscope (Zeiss, Thornwood, NY) equipped with a dual excitation video imaging system (IonOptix Corp., Milton, MA). $[Ca^{2+}]_i$ was estimated at 1-sec intervals from the ratio of 510-nm emission fluorescences resulting from excitation by 350- and 380-nm wavelength light from the following equation (15): $[Ca^{2+}]_i = K_d\beta(R - R_{min})/(R_{max} - R)$, where K_d is the dissociation constant of fura-2 (224 nM), β is the ratio of 380 nm induced fluorescences of free/bound fura-2, R is the measured ratio of 350 nm/380 nm fluorescences, and R_{min} and R_{max} are 350 nm/380 nm fluorescence ratios in zero $[Ca^{2+}]$ and saturating Ca^{2+} concentrations, respectively. Values for β, R_{min}, and R_{max} were obtained by *in vitro* calibrations using penta-potassium fura-2.

Electrophysiology

Cells were bathed in extracellular recording solution containing 138 mM NaCl, 5.6 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM HEPES-NaOH (pH 7.4). D-Glucose was added to this solution, as specified in the text. In some experiments, Na^+-free solutions were produced by substituting NaCl with *N*-methyl-D-glucamine (NMG), and the pH was adjusted to 7.4 using HCl. Nominally Ca^{2+}-free solutions were produced by substituting $MgCl_2$ for $CaCl_2$. The resting potential of cells was measured under current clamp, or the cells were held under voltage clamp, using the perforated patch (13, 16) configuration. Patch pipettes pulled from Kimax-51 glass capillaries (Kimble Glass Inc., Vineland, NJ) were fire polished and tip-dipped in a solution of 95 mM K_2SO_4, 7 mM $MgCl_2$, and 5 mM HEPES-NaOH (pH 7.4), then back-filled with the same solution containing nystatin (240 μg/ml). The pipette was connected to an EPC-9 (Heka Electronics, Lambrecht, Germany) patch clamp amplifier, and the access resistance and cell capacitance were monitored after seal formation. The series resistance was compensated for by 60–80%. The pipette solution did not contain fura-2 and was nominally Ca^{2+} free. A sudden rise in $[Ca^{2+}]_i$ and a simultaneous decrease in fluorescence intensity provided a useful marker for accidental rupture of the cell membrane, whereupon the experiment was terminated. Test solutions were applied by pneumatic pressure ejection from micropipettes using a General Valves Picospritzer II (Fairfield, NJ). Forskolin, 8-bromo-cAMP (8Br-cAMP), and PACAP-27 and -38 were obtained from Sigma Chemical Co. (St. Louis, MO). Sp-cAMPs was obtained from BioLog (La Jolla, CA).

Results

Both PACAP-27 (Fig. 1, A and B) and PACAP-38 (Fig. 1C) induced rises in $[Ca^{2+}]_i$ of HIT-T15 cells preequilibrated in a fixed concentration (2 mM) of glucose, consistent with the presence of PACAP-R3 receptors in these cells (5). Responses to PACAP were of variable duration and were either transient (Fig. 1A) or prolonged (Fig. 1, B and C). Similar responses were seen after application of forskolin (100 μM), 8Br-cAMP (1 mM), or Sp-cAMPs (100 μM; Fig. 1D). By analogy with the effects of the related peptide, GLP-1, on rat β-cells (11), PACAP might be expected to increase $[Ca^{2+}]_i$ by potentiating the glucose-dependent closure of ATP-K channels, resulting in membrane depolarization and opening of VDCCs. The membrane conductance of a HIT-T15 cell was monitored in perforated patch voltage clamp by holding the cell at −80 mV and applying 1-sec steps of ±20 mV. In this experiment, 10 nM PACAP-27 produced about a 50% decrease in membrane conductance, with only a small effect on the holding current (I_h). This observation suggests that glucose-dependent inhibition of the ATP-sensitive K current by PACAP contributes to its ability to generate membrane depolarization (see below) and a consequent rise in $[Ca^{2+}]_i$, as previously shown for GLP-1 (11).

Simultaneous recordings of membrane potential and

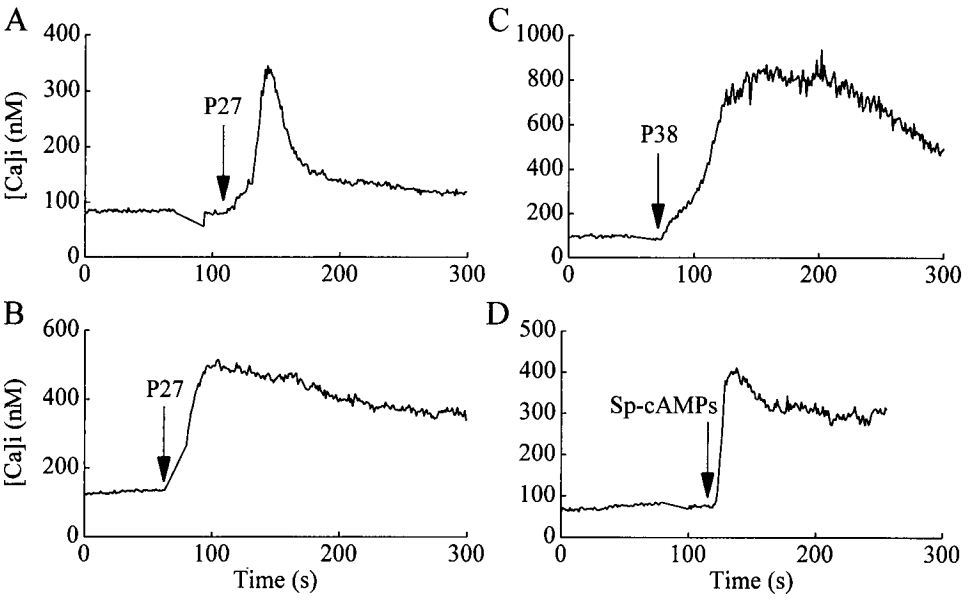

FIG. 1. Both PACAP-27 and PACAP-38 induce rises in $[Ca^{2+}]_i$ in HIT cells. A and B, The rise in $[Ca^{2+}]_i$ in HIT cells after application of 2 nM PACAP-27 for 10 sec. C, A record from a HIT cell that responded to the application of 10 nM PACAP-38 for 10 sec. These responses illustrate the variability of the time course of the effect of changes in $[Ca^{2+}]_i$ on PACAP stimulation, which may be transient (A) or prolonged (B and C). Similar changes in $[Ca^{2+}]_i$ could be elicited by the application of membrane-permeant cAMP analogs, such as Sp-cAMPs (100 μM; D). The cells were bathed in 2 mM glucose saline.

$[Ca^{2+}]_i$ in current-clamped HIT-T15 cells show that PACAP-27 causes membrane depolarization, the triggering of action potentials, and a simultaneous rise in $[Ca^{2+}]_i$ (Fig. 3, A and B). However, the same cell held under voltage clamp at −70 mV, negative to the threshold for activation of VDCCs (17), responded to a second application of PACAP-27 with a pronounced inward current (Fig. 3C) and a much larger and more prolonged rise in $[Ca^{2+}]_i$ (Fig. 3D). This type of response was observed more frequently than that illustrated in Fig. 2. The larger rise in $[Ca^{2+}]_i$ observed under voltage clamp, compared to current clamp, in this cell might be explained by the increased electrical driving force for cation entry through open channels, because under voltage clamp recording conditions, the membrane potential was held at −70 mV, whereas under current clamp recording conditions, the membrane potential shifts to about −20 mV.

Removal of extracellular Ca^{2+} (Ca^{2+}_o) during the response to PACAP reversibly abolished the rise in $[Ca^{2+}]_i$ (Fig. 4A).

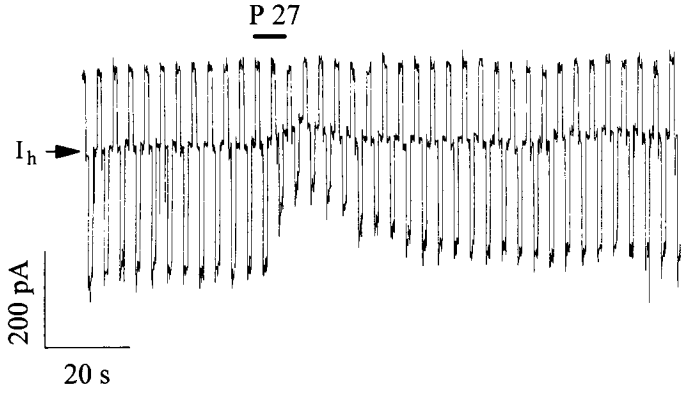

FIG. 2. PACAP causes an initial reduction in membrane conductance. A HIT cell was held under perforated patch voltage clamp at −80 mV, and 1-sec steps of ±20 mV were applied to monitor membrane conductance. Application of 10 nM PACAP-27 to the cell from a puffer pipette caused a decrease in membrane conductance, similar to the effects of the related peptide, GLP-1, which was ascribed to the closure of ATP-sensitive K^+-channels (8).

Interestingly, PACAP produced no rise in $[Ca^{2+}]_i$ in the presence of Ca^{2+}_o, but in the absence of Na^+_o (Fig. 4B). A similar dependence on Na^+_o has also been demonstrated for the PACAP-related peptide, GLP-1, in rat islets (18). The Na^+_o dependence of the rise in $[Ca^{2+}]_i$ in response to PACAP is likely to be explained at least in part by the inhibition of the inward current, which has a large Na^+ component (see below, Fig. 6A).

La^{3+} is frequently used as a blocker of a wide range of voltage-dependent and -independent Ca^{2+} channels. If the effect of PACAP on $[Ca^{2+}]_i$ results from activation of such channels, then La^{3+} might be expected to inhibit the PACAP-induced inward current. To test this possibility, HIT-T15 cells were held under voltage clamp at −70 mV, and ±10 mV voltage steps were applied to monitor membrane conductance. An inward current, increase in membrane conductance (Fig. 5A), and rise in $[Ca^{2+}]_i$ (Fig. 5B) were observed in response to PACAP-27. Direct application of 100 μM La^{3+} from a puffer pipette caused a reversible decrease in both the inward current and membrane conductance (Fig. 5A), indicating that at least one component of the current is blocked by La^{3+}. The PACAP-27-activated current was not completely blocked by La^{3+}, which did, however, completely block the rise in $[Ca^{2+}]_i$ in response to a voltage step from −70 to 0 mV (data not shown), indicating that VDCCs were fully blocked.

The current activated by PACAP is also activated by forskolin, 8Br-cAMP, and Sp-cAMPs. To obtain an estimate for the reversal potential of the current, cells were dialyzed with Cs^+ (substituted for K^+ in the pipette solution) in the perforated patch configuration. Voltage ramps of 100-msec duration from −70 to 30 mV were applied, and the currents in response to these ramps before stimulation with 8Br-cAMP were subtracted from currents during the response to stimulation with 8Br-cAMP to obtain the current-voltage relation of the evoked current. These currents had an apparent reversal potential of −28.0 ± 2.3 mV (n = 4). This value for the

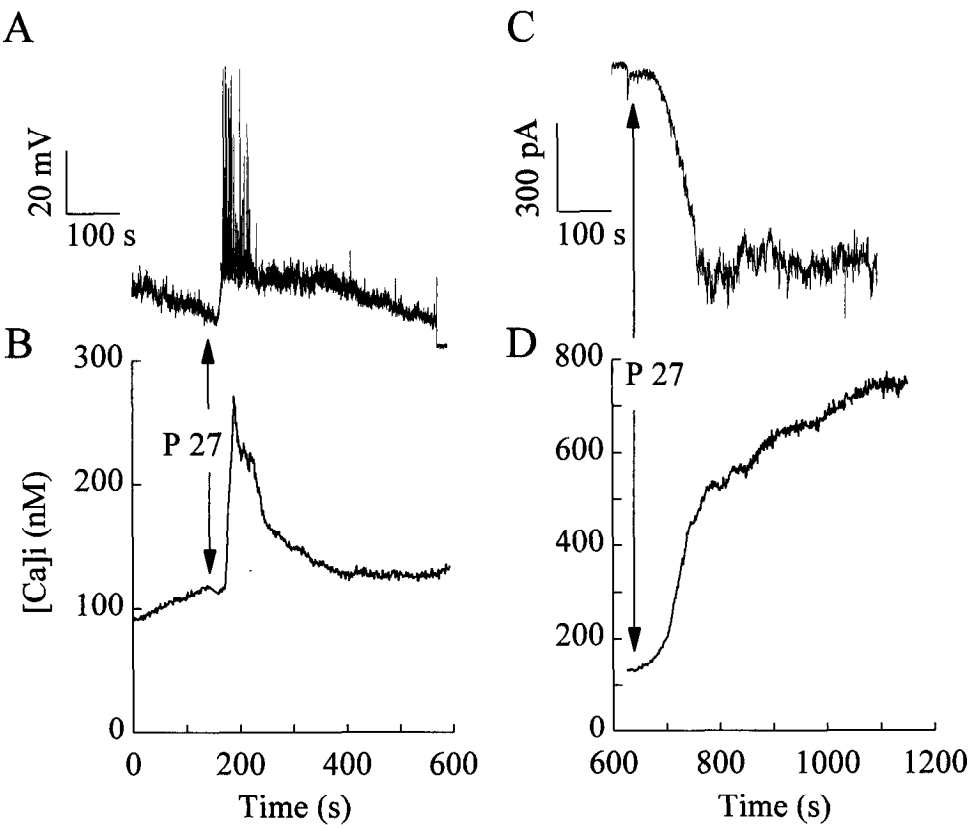

FIG. 3. Simultaneous records of $[Ca]_i$ and membrane potential or membrane current. Records from a single HIT cell bathed in 2 mM glucose. Initially, the fura-2-loaded cell was under perforated patch current clamp and responded to the application of 10 nM PACAP-27 (P27) by depolarizing and firing action potentials (A), accompanied by a rise in $[Ca^{2+}]_i$ (B). When the same cell was voltage clamped at −70 mV, application of PACAP-27 induced a large inward current (C) and a rise in $[Ca^{2+}]_i$ (D).

reversal potential is consistent with the activation of nonspecific cation channels.

In the presence of physiological ion gradients, the main charge carrier through such nonspecific cation channels would be expected to be Na^+. A HIT-T15 cell stimulated with 1 mM 8Br-cAMP developed a large inward current and an increase in membrane conductance (Fig. 6A). During the response, an extracellular solution in which Na^+ had been substituted by NMG was applied, which substantially and reversibly reduced the current and membrane conductance (Fig. 6A). The current evoked by 8Br-cAMP could also be inhibited by La^{3+} (Fig. 6B).

PACAP produced a marked increase in the Mn^{2+} permeability of HIT cells (Fig. 7). A 60-sec pulse of Mn^{2+} (Mn^{2+} was substituted for Ca^{2+} of normal bath solution) applied to a single cell from a puffer pipette had no effect on the 350/380 nm fluorescence ratio (Fig. 7A) or raw fluorescence intensity (Fig. 7B) before stimulation of the cell with PACAP-38. Stimulation with PACAP induced a rise in $[Ca^{2+}]_i$ (increased fluorescence ratio), and the subsequent application of a 60-sec pulse of Mn^{2+} led to quenching of the fura-2 fluorescence (Fig. 7B). Mn^{2+}-permeant channels believed to be associated with capacitative Ca^{2+} entry (I_{CRAC}) have been reported in HIT-T15 cells (14), RINm5F cells (19), and mouse β-cells (20). However, it remains to be determined whether the activation of an I_{CRAC} explains the increased Mn^{2+} permeability observed after treatment of HIT cells with PACAP.

Discussion

In this report we demonstrate that in HIT-T15 cells, the biologically active isoforms of PACAP induce membrane depolarization through at least two distinct mechanisms. First, PACAP reduces the membrane conductance and increases membrane excitability in a manner similar to that previously described for GLP-1 (11). This effect most likely results from a potentiation by PACAP of the inhibitory effect of glucose on ATP-K channels. Secondly, PACAP increases membrane conductance and activates a pronounced inward current that is predominantly a Na^+ current, blockable by La^{3+}, and exhibits a reversal potential of about −28 mV. We propose that the inward current results from activation of ion channels similar to the cAMP- and Ca^{2+}-gated nonspecific cation channels (Ca-NS channels) reported in CRI-G1 insulinoma cells (9).

PACAP also induced a rise in $[Ca^{2+}]_i$ by several distinct mechanisms. One process results from the effects on ATP-K and Ca-NS channels, as described above, leading to membrane depolarization and activation of L-type VDCCs. The Ca^{2+} current through VDCCs may also be enhanced by cAMP-dependent protein phosphorylation (12). A second mechanism by which PACAP raised $[Ca^{2+}]_i$ was observed under conditions in which HIT-T15 cells were voltage clamped at −70 mV (and also −120 mV; data not shown), thereby preventing the opening of VDCCs (17). Although the threshold for activation of VDCCs can shift to more negative membrane potentials under conditions of pharmacological

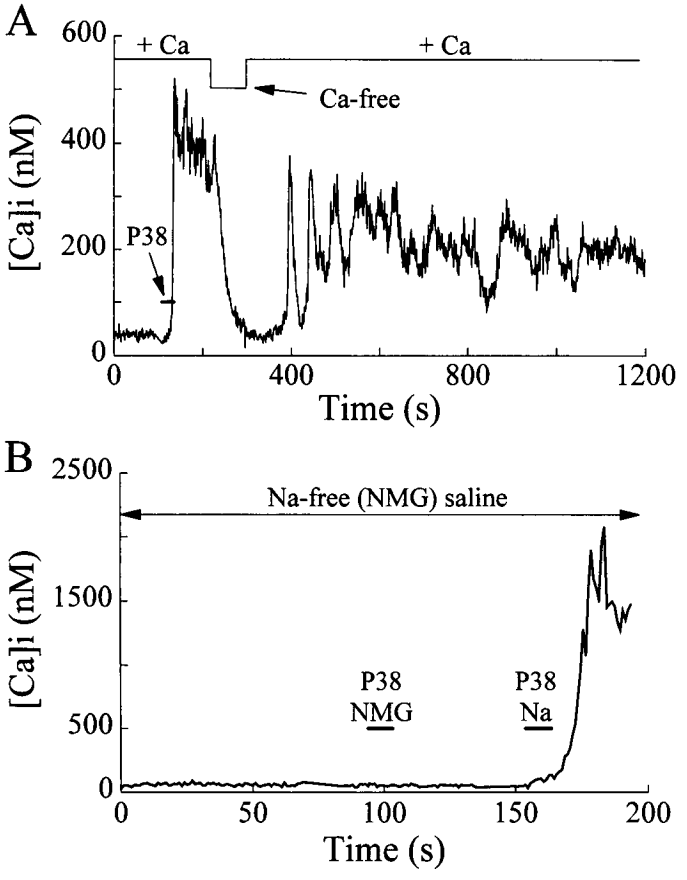

FIG. 4 The PACAP-induced rise in $[Ca^{2+}]_i$ is dependent on extracellular Ca^{2+} and Na^+. A, The rise in $[Ca^{2+}]_i$ of a HIT cell in response to a 10-sec pulse of 2 nM PACAP-38 is reversed by switching the bath solution to one that is nominally calcium free. Returning the cell to a bath solution containing Ca^{2+} caused $[Ca^{2+}]_i$ to rise again, indicating that the conductance mechanism remains open. All solutions contained 0.8 mM glucose. Similar results were obtained in all five cells tested. B, The sodium dependence of PACAP. A HIT cell was bathed in sodium free (NMG-substituted) saline and initially showed no rise in $[Ca^{2+}]_i$ in response to a 10-sec pulse of 2 nM PACAP 38 (P38) in Na-free NMG saline. In the continued presence of Na-free bathing saline, the same cell showed a large rise in $[Ca^{2+}]_i$ in response to a 10-sec pulse of 2 nM PACAP 38 in sodium-containing (138 mM Na^+) saline. All solutions contained 0.8 mM glucose. Similar effects were seen in all five cells tested using the same protocol.

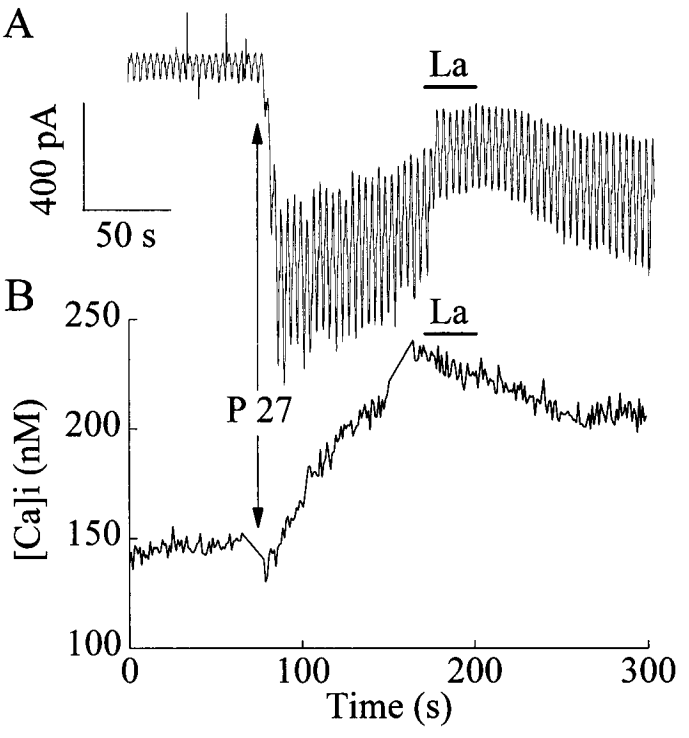

FIG. 5. PACAP-27 increases membrane conductance, which is blocked by La^{3+}. A HIT cell was held under perforated patch voltage clamp at −70 mV, and ±10 mV steps were applied to monitor membrane conductance. Application of 10 nM PACAP-27 to the cell induced an inward current and increased the membrane conductance, both of which were reduced by La^{3+} (A). The inward current was accompanied by a rise in $[Ca^{2+}]_i$ (B). $[Ca^{2+}]_i$ started to recover toward normal levels after the application of La^{3+} despite incomplete block of the PACAP-induced current and conductance increase.

stimulation (21), these shifts would not be expected to be large enough to significantly activate VDCCs at −70 mV. Therefore, the ability of PACAP to raise $[Ca^{2+}]_i$ under conditions of voltage clamp indicates the existence of a Ca^{2+} signaling mechanism involving voltage-independent Ca^{2+} channels and/or the release of Ca^{2+} from intracellular stores. These observations contrast with a previous report suggesting that the mechanism by which PACAP raises $[Ca^{2+}]_i$ in β-cells is by increasing the activity of L-type VDCCs (4).

We observed that PACAP was without effect on $[Ca^{2+}]_i$ under conditions in which the extracellular solution contained Ca^{2+}, but not Na^+. This suggests that mobilization of intracellular Ca^{2+} by PACAP might be initiated by the entry of Na^+ through Ca-NS channels and/or effects of cAMP on organellar Ca^{2+} stores. The elevation of $[Na^+]_i$ in β-cells is known to trigger Ca^{2+} release from intracellular stores (22). It has also been demonstrated that the secretory granules form a storage site for intracellular Ca^{2+}, and that this Ca^{2+} pool is rapidly altered by stimulation with glucose and by intracellular factors, notably cAMP and Na^+_i (23). Furthermore, cAMP reportedly stimulates $^{45}Ca^{2+}$ efflux from β-cell secretory granules (24). Therefore, ample precedent exists supporting the concept of an important influence of Na^+ and cAMP on β-cell Ca^{2+} signaling systems unrelated to VDCCs. Although the mechanism by which cAMP mobilizes Ca^{2+} in β-cells remains to be determined, cAMP-dependent phosphorylation of the inositol trisphosphate (IP_3) receptor by PKA can increase Ca^{2+} release (25, 26). However, there is at present no direct evidence that the type 3 IP_3 receptor, located in the membrane of insulin secretory granules (27), is a substrate for PKA (28).

We also report that stimulation of HIT-T15 cells by PACAP results in the activation of a Mn^{2+}-permeant divalent cation influx pathway. This was demonstrated by the quenching of intracellular fura-2 fluorescence by externally applied Mn^{2+}. Quenching is unlikely to reflect entry of Mn^{2+} via VDCCs, as it is well established that Mn^{2+} is a broad spectrum blocker of this class of Ca^{2+} channels. Instead, we propose that the entry of Mn^{2+} reflects the activation of a Ca^{2+}-release-activated Ca^{2+}-current (I_{CRAC}) (14, 19). This is consistent with the proposed ability of PACAP to mobilize intracellular Ca^{2+}

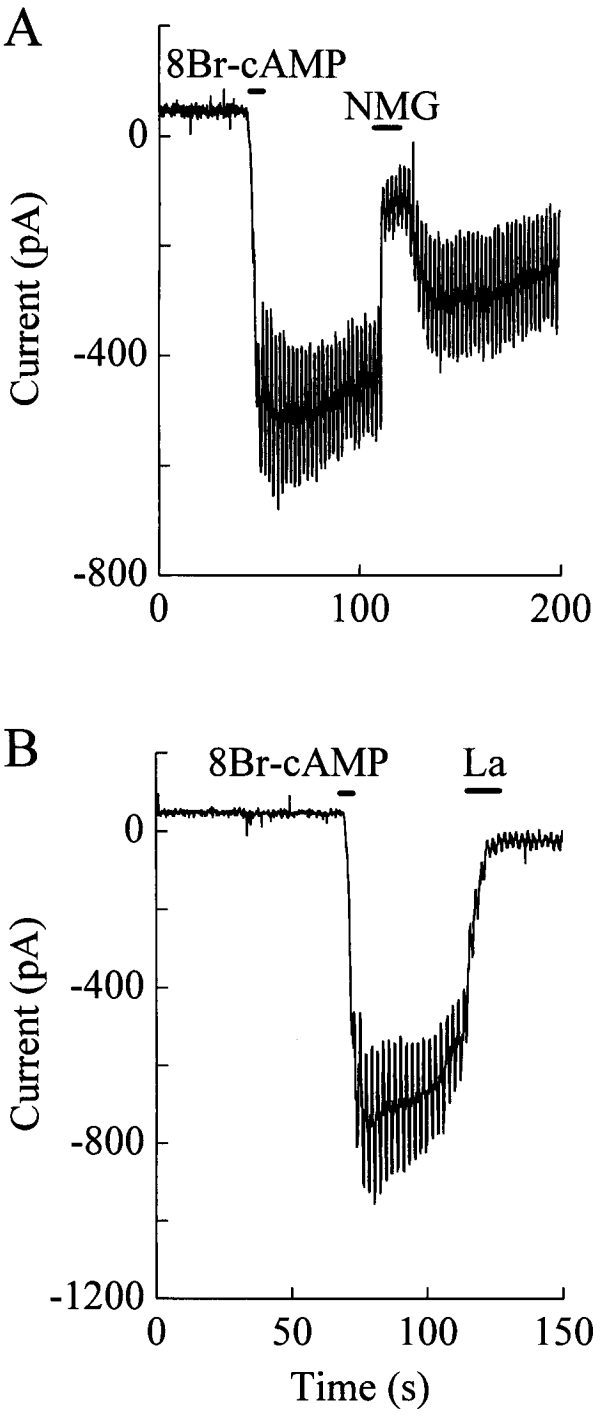

FIG. 6. 8Br-cAMP activates a current permeant to Na^+ and blocked by La^{3+}. A HIT cell bathed in 0.8 mM glucose saline was held at −70 mV in perforated patch voltage clamp, with 750-msec steps of ±10 mV applied. The cell initially had a very low membrane conductance until it was stimulated with 1 mM 8Br-cAMP (indicated by *bar*). A large inward current and increase in membrane conductance were observed in response to 8Br-cAMP (A). During the response, a pulse of Na^+-free saline (substituted by NMG) was applied (*bar*), and current and conductance were both reduced (A), suggesting that a component of the current was carried by Na^+. A different HIT cell, under the same recording conditions as those defined in A, was stimulated by 8Br-cAMP, generating an inward current and increase in conductance, which was inhibited by the application of 250 μM La^{3+} (B).

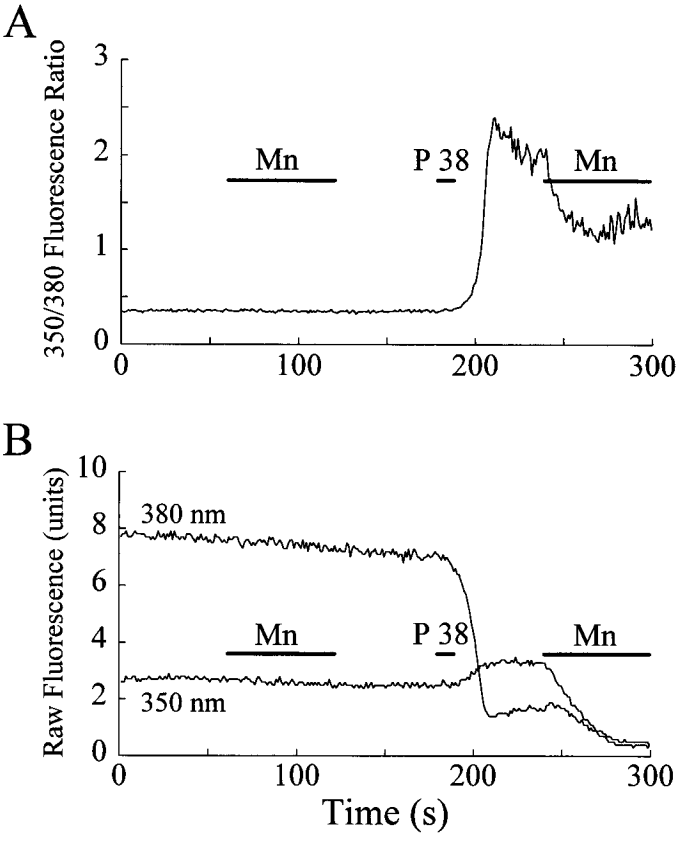

FIG. 7. Activation of a Mn^{2+}-permeant conductance after PACAP stimulation. A HIT cell, bathed in 0.8 mM glucose saline, showed no quenching of fura-2 fluorescence during a 60-sec pulse of Mn^{2+} before stimulation with a 10-sec pulse of 2 nM PACAP-38 (P38), but showed marked quenching after PACAP stimulation. Periods of application are indicated by *bars*. The Mn^{2+}-saline was applied from a puffer pipette and prepared by substitution of Mn^{2+} for Ca^{2+} (2.6 mM).

stores, because the depletion of such stores is known in some cell types to activate I_{CRAC}.

The PACAP-R3, present in HIT cells, couples to phospholipase-C, generation of IP_3, and activation of protein kinase-C (PKC) when expressed in oocytes (5). Although such coupling has yet to be demonstrated for the native receptor in β-cells or insulinoma cells, it remains possible that an effect of PACAP on IP_3 production or PKC activity contributes to the observed rise in $[Ca^{2+}]_i$ reported here. Activation of I_{CRAC} in RINm5F cells has been observed after stimulation of PKC (19), and PKC can also phosphorylate IP_3 receptors (29). Hence, regulation of intracellular Ca^{2+} stores and activation of I_{CRAC} may represent an important point of convergence, at which signal transduction cross-talk occurs between the cAMP and PLC/PKC/IP_3 signaling systems in β-cells.

The physiological role of PACAP remains to be fully clarified. PACAP immunoreactivity has been observed in nerve endings within the pancreas and was occasionally found within the islets (3). These observations suggest that PACAP may have local paracrine effects *in vivo*. We propose that a neuro-entero-endocrine loop may exist whereby feeding stimulates the local release of PACAP within pancreatic islets, thereby triggering insulin secretion. This release would

provide a rapid augmentation of insulin secretion before the intestinal incretins come into play.

In summary, both isopeptides of PACAP cause the voltage-independent activation of an inward current, which we suggest is carried through Ca-NS channels. PACAP also decreases the resting membrane conductance, probably through inhibition of ATP-K channels. These effects result in depolarization of the cell and activation of L-type VDCCs. Furthermore, there are additional mechanisms by which $[Ca^{2+}]_i$ rises in a membrane potential-independent manner. Ca^{2+} entry through voltage-independent Ca^{2+} channels, including I_{CRAC}, may contribute to the rise in $[Ca^{2+}]_i$, and the elevation of intracellular cAMP and/or Na^+ levels may stimulate Ca^{2+} release from intracellular stores. Acting in concert, these various Ca^{2+} signaling pathways are expected to exert important stimulatory influences on pancreatic insulin secretion.

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