A Wortmannin-sensitive Signal Transduction Pathway Is Involved in the Stimulation of Insulin Release by Vasoactive Intestinal Polypeptide and Pituitary Adenylate Cyclase-activating Polypeptide*

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Vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating polypeptide-27 (PACAP-27), and PACAP-38 stimulated insulin release with EC50 values of 0.15, 0.15, and 0.06 nM respectively, as expected for the VIP/PACAP3 receptor subtype. Secretion was stimulated promptly and peaked at 6–10 min. At 30 min, the secretion rate was still 2-3-fold higher than the control rate. The peptides increased cyclic AMP and [Ca2+]i transiently so that at 30 min they had returned to control values. Therefore, an additional signal is required to explain the prolonged stimulation of release. The prolonged effects, but not the acute effects of VIP and PACAP on insulin release were inhibited by low concentrations of wortmannin, a phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor. While wortmannin inhibited PI 3-kinase activity in cell lysates, no activation by the peptides was seen. Therefore, the wortmannin-sensitive pathway is either dependent on basal PI 3-kinase activity, or another target for wortmannin is responsible for inhibition of the peptide-stimulated secretion. It is concluded that the acute stimulation of insulin release by VIP and PACAP is mediated by increased cyclic AMP and [Ca2+]i, whereas the sustained release is mediated by a novel wortmannin-sensitive pathway.

Vasoactive intestinal polypeptide (VIP)1 and PACAP (pituitary adenylate cyclase-activating polypeptide) are members of a family of peptides that includes secretin, glucagon, and glucagon-like peptide-1 with diverse actions in several cell types (1, 2). Among a large array of biological effects, they stimulate the secretion of amylase (3, 4), the release of hypophysiotropic hormones such as prolactin (5, 6), and lower the systemic blood pressure (7). Additionally they augment insulin secretion (8, 9) and are thought to do this by interaction with the VIP/PACAP-3 receptor (10), which has been cloned (11). This receptor binds VIP and PACAP with similar affinities and has a selective tissue distribution. PACAP exists in two amidated forms: PACAP-27 and the C-terminally extended variant PACAP-38 (12). The N-terminal 1-28 sequence of PACAP-38 shows 68% sequence homology with VIP (13). PACAP is an extremely potent modulator of insulin secretion and has been reported to augment glucose-stimulated insulin release from rat pancreatic islets at a concentration of 0.1 pM (14). Both VIP and PACAP are localized in the gastrointestinal tract, and the pancreas and VIP- and PACAP-like immunoreactivity was observed in capillaries and nerve fibres terminating on pancreatic islets (15, 16). PACAP-specific staining has been detected even within the islet in rat (14).

The VIP/PACAP family of peptides are thought to couple positively to the adenyl cyclase system in their target cells. In some cell types, they increase [Ca2+]i. In accordance with this, in single cell studies in the presence of 8.3 mM glucose, 80% of rat pancreatic β-cells responded to PACAP with a rise in [Ca2+]i, a rise that was blocked by nitrendipine (14). However, in the mouse β-cell, VIP-stimulated insulin secretion was not associated with a detectable rise in cyclic AMP levels (17), a finding that suggests the possibility of an action independent of cyclic AMP and association of activation of PKA. Furthermore, in AtT20 cells, the stimulation of β-endorphin secretion by VIP was unaffected by the stable expression of mutated regulatory subunits of the cyclic AMP-dependent protein kinase, an expression that blocked the effects of isoproterenol and analogs of cyclic AMP (18). The authors concluded that VIP could stimulate β-endorphin secretion by a mechanism that did not involve cyclic AMP-dependent protein kinase.

In view of these data, we have reinvestigated the mechanisms of action of these peptides to stimulate insulin release. The HIT-T15 β-cell line was used, and careful attention was paid to the temporal aspects of the second messenger responses and their effect on insulin secretion. The results showed that the peptides increase cyclic AMP and [Ca2+]i levels only transiently, but they exert a prolonged stimulatory effect on insulin secretion. The prolonged stimulation of insulin secretion by VIP and PACAP is manifest after [Ca2+]i and cyclic AMP levels have returned to basal values. It is unlikely therefore that either cyclic AMP or raised [Ca2+]i is responsible for the prolonged stimulation of release. Finally, it was found that the prolonged release induced by VIP and PACAP, but not the release induced by forskolin or glucose, was selectively inhibited by low concentrations of wortmannin. Wortmannin, a microbial metabolite found in a variety of fungal species, is known to be an inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase) with IC50 values between 2 and 4 nM (19). At higher concentrations, wortmannin has been linked to the inhibition of phospholipase D (20) and myosin light chain kinase (21, 22), whereas no effect on cAMP-dependent protein kinase was detected at concentrations of wortmannin up to 10 μM (23). The selective inhibition of PACAP- and VIP-stimulated insulin release by low concentrations of wortmannin points to a novel signal transduction pathway in the action of these peptides.
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EXPERIMENTAL PROCEDURES

Cell Culture—HIT-T15 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a 95% air plus 5% CO2 atmosphere. The experiments were performed during passages 72-80.

Insulin Secretion—Static incubation experiments were carried out in Krebs-Ringer bicarbonate (KRB) buffer of the following composition: 129 mM NaCl, 5 mM NaHCO3, 4.8 mM KCl, 1.2 mM KH2PO4, 10 mM HEPES, bovine serum albumin at 0.1%, and the indicated glucose concentrations at pH 7.4. HIT cells were preincubated for 30 min at 37 °C. After removing the preincubation buffer, they were then exposed for another 30 min to fresh KRB buffer containing the test agents. Samples of the incubation medium were collected, centrifuged, and stored at −20 °C until radioimmunoassay was performed using a charcoal separation method.

For the measurement of insulin release under perfusion conditions, approximately 3 × 106 cells were placed in each of 4 chambers of a perfusion apparatus. Experiments were performed in KRB buffer at 37 °C at flow rates of 1 ml/min. The cells were preincubated for 50 min for stabilization of the secretion rates and then exposed to test and control conditions as appropriate. Samples were collected at 1-min intervals and kept at −20 °C until assay.

Cytosolic Free Calcium (Ca2+).—HIT cell suspensions were incubated for 30 min in KRB (pH 7.4) in the presence of 1 μM fura-2-acetoxymethyl ester at 37 °C under continuous shaking. The fura-2-loaded cells were washed 3 times with modified KRB buffer. 3 ml of the final cell suspension were placed in each quartz cuvette. During the experiments, the cell suspension was maintained at a temperature of 36–37 °C and continuously stirred while in the spectrofluorometer (Perkin-Elmer LS-5). Excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm were used. The cytosolic free Ca2+ concentration was calculated according to the formula by Tsien et al. (24).

Membrane Potential—Qualitative changes in membrane potential were fluorometrically monitored using the fluorescent dye bisoxonol. About 2 × 106 cells were suspended in a cuvette, and bisoxonol was added at a final concentration of 100 nM. The signal was allowed to stabilize for about 20 min before the test agents were added. The signals were monitored using a Perkin-Elmer LS-5 fluorescence spectrophotometer at wavelengths of 540 and 580 nm for excitation and emission, respectively.

Measurement of Cellular Cyclic AMP Levels—HIT cells were preincubated for 30 min at 37 °C in KRB buffer. The solution was replaced by KRB buffer containing the test agents, and the incubations were carried out for the indicated time periods. The reaction was terminated by removing the buffer and adding 0.25 ml ice-cold 6% trichloroacetic acid. The cells were scraped free, and the cell suspension was centrifuged at 2500 × g for 10 min at 4 °C. The supernatants were extracted 4 times with ether, evaporated to dryness, and then reconstituted in acetate buffer. Cyclic AMP content was determined by radioimmunoassay. The cell pellet was solubilized in 0.1 M NaOH, and the protein content was determined by the Bradford assay (25).

Immunoprecipitation and PI 3-Kinase Assay—Approximately 2 × 106 HIT-T15 cells/well were incubated for the indicated times in the presence or absence of glucose, VIP, PACAP, and wortmannin as appropriate at 37 °C. Then, the cells were washed twice with ice-cold phosphate-buffered saline and lysed by rocking at 4 °C for 20 min in a buffer of the following composition: 20 mM HEPES, 100 mM NaCl, 1% Triton X-100, 10 mM sodium orthovanadate, 50 mM NaF, and 2 mM EDTA (pH 7.2) supplemented with 10 μg/ml each apotinin and leupeptin. Immunoprecipitation and kinase assay were performed as described by Kaplan et al. (26) with modifications. The lysates were clarified by spinning at 11,000 rpm for 15 min in an Eppendorf centrifuge, and PI 3-kinase was immunoprecipitated with anti-p85 polyclonal antibody for 2 h (at 4 °C) and collected on Pansorbin (Calbiochem) over 1 h. Aliquots (250 μl) of the immunoprecipitates were resuspended in kinase buffer (50 mM Tris-HCl and 5 mM MgCl2 (pH 7.5)). The PI 3-kinase reaction was started by addition of 10 μg of sonicated phosphatidylinositol, 50 μM ATP, and 30 μCi of [γ-32P]ATP to the kinase buffer. The assays were performed at room temperature and stopped with 1 ml of ice-cold TCA after 5 min. Lipids were extracted from the liposomal organic phase after the addition of CHCl3/Methanol (1:1), washed, and spotted on a thin-layer chromatography plate (EM Science), which had been impregnated with potassium oxalate in methanol/water (2:3) and preactivated by heating at 110 °C for 60 min before spotting. The lipids were resolved by chromatography in CHCl3/Methanol/Ammonium Hydroxide/water (86:70:6:4), and radioactivity was detected by autoradiography.

Phospholipid Labeling and Extraction—The PI3 production was measured essentially according to the method described by Traylor-Kaplan et al. (27). HIT-T15 cells were suspended at a density of 107 cells/ml in the labeling medium consisting of 50 mM Tris-HCl, pH 7.4, 136 mM NaCl, 4.9 mM KCl, and 4 mM glucose (buffer A). 32P-orthophosphate (HCl free 500 μCi/ml) was added, and the cells were incubated at 37 °C for 60 min. The cells were washed twice with the buffer A (containing 1 mM CaCl2) and resuspended at 5 × 105 cells/ml. Aliquots (250 μl) of the cells were incubated at 37 °C for the indicated times with or without the addition of the neuropeptides. Cells were then quenched by the addition of 1.55 ml of chloroform, methanol, 8% HClO4, and 3% containing the antioxidant butylated hydroxytoluene (0.63 mg/ml) and carrier amounts of phosphonitroisotides, followed by vigorous stirring. Another 0.5 ml of chloroform and 0.5 ml of 8% HClO4 were added to separate the organic phase, which was then washed with chloroform-saturated 1% HClO4 before being dried. The lipids were dissolved in chloroform/methanol (95:5) and spotted on an impregnated thin-layer chromatography plate (see above). The plate, after being developed in chloroform/methanol/acetic acid/water (30:20:24:14), was dried and subjected to autoradiography.

Results—Wortmannin, forskolin, TPA, VIP, and fura-2 acetoxy-methyl ester were obtained from Sigma. PACAP-27 and PACAP-38 were purchased from Peninsula Laboratories (Belmont, CA). The fluorescent probe bisoxonol was from Molecular Probes, Eugene, OR. The cyclic AMP, 32P radioimmunoassay kits, [32P]orthophosphate, and [γ-32P]ATP were obtained from DuPont NEN. The anti-p85 polyclonal antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY).

Statistical Analysis—Results are presented as means ± S.E. Statistical analysis was by Student’s t test for paired and unpaired data as appropriate.

RESULTS

Concentration-Response Characteristics for the Actions of VIP and PACAP on Insulin Release—In Fig. 1 are shown the concentration-response characteristics for the action of VIP and PACAP to stimulate insulin release in the HIT-T15 cell line. The experiments were carried out under static incubation conditions over 30 min. The two left-hand bars in Fig. 1 show the basal rate of insulin release in the presence of 0.2 mM glucose and the stimulated rate with 4.0 mM glucose, respectively. The cells responded to 4 mM glucose with a 215% increase in the secretion rate. The right-hand bars illustrate the effects of VIP on insulin secretion in the presence of 4 mM glucose, at concentrations from 0.01 to 100 nM. Insulin secretion was stimulated at 0.1 nM, with maximal effect at 10 nM and maintained at maximum stimulation through 100 nM. Fig. 1B are shown the responses to PACAP-38. Again, the left-hand bars show the effects of 0.2 and 4.0 mM glucose, the latter inducing a 330% increase relative to 0.2 mM glucose. PACAP-38 was slightly more potent than VIP, and a small but significant increase in insulin release was obtained at 0.01 μM (p < 0.05). Stimulation was near maximal at 1 μM and maximal at 10 and 100 μM. PACAP-27 was slightly less potent than PACAP-38 and had similar concentration-response characteristics to VIP (data not shown).

Glucose Dependence of the Peptide Responses—To determine whether glucose is required for the stimulation of insulin release by VIP and PACAP, insulin release was measured both in the absence of glucose and in the presence of a low concentration of glucose (0.2 mM). The results demonstrated a clear dependence (see Fig. 2). Only minimal effects of VIP and PACAP were detected in the absence of glucose. While 0.2 mM glucose did not stimulate insulin release above the rate of that seen in the absence of glucose, the peptides caused a marked stimulation when 0.2 mM glucose was present. The stimulation was comparable, in -fold terms, to that seen in the presence of 4 mM glucose.

Temporal Profile of the Stimulation of Insulin Release by VIP
Insulin release in response to VIP and PACAP was also examined under perifusion conditions to determine the pattern and duration of the responses. The results of these experiments are shown in Fig. 3. VIP and PACAP, both used at maximally effective concentrations of 10 nM, stimulated insulin release in the presence of 4 mM glucose. The responses were prompt, and release rates reached peak values after approximately 10 min. The elevated rates of release declined only slightly after reaching these peak values and were still 2–2.5 times the rate with 4 mM glucose alone after 30 min of exposure to the peptides. Removal of the peptides from the perifusate caused a rapid decrease in the rates of insulin secretion and a return to control values after 10 min.

Concentration-Response Characteristics for the Actions of VIP and PACAP on Cellular Cyclic AMP Levels—Both VIP and PACAP-38 raised cellular cyclic AMP levels. As can be seen from Fig. 4, PACAP-38 was slightly more potent than VIP, a finding in accord with the relative potencies of these two peptides on insulin secretion. In Fig. 4A, the effects of PACAP-38 over the range of 0.01–100 nM. The left two bars illustrate insulin release in the presence of 0.2 and 4 mM glucose alone. The measurements were made under static incubation conditions over 30 min. The results are expressed as means ± S.E. n = 3–5.

Actions of VIP and PACAP on the Intracellular Free Calcium Concentration ([Ca2+]i)—The temporal effects of VIP, PACAP, and forskolin on [Ca2+]i, can be seen from the cellular fura-2 fluorescence traces shown in Fig. 5. Addition of the two peptides, in these cases at 10 nM, was followed by prompt but small increases in [Ca2+]i, which peaked at around 30 s. In all cases, the peak was followed by a decrease lasting for about 1–1.5 min and which usually took the [Ca2+]i to a nadir slightly below its starting value. Subsequently, the [Ca2+]i increased to values just above basal where they remained for less than 10 min. At all times after 10 min [Ca2+]i was at control values. Thus, the effects of the two peptides were similar in that the responses were small, biphasic, and of short duration. Forskolin, tested because of its ability to raise intracellular cyclic AMP levels by activation of adenyl cyclase, also increased [Ca2+]i transiently. The 1,9-dideoxy-derivative of forskolin, which lacks activity on adenyl cyclase (28), failed to affect [Ca2+]i. Thus, the effect of VIP and PACAP to increase [Ca2+]i is likely due to their ability to raise the cellular cyclic AMP content. However, as the increase in cyclic AMP caused by forskolin is large and prolonged (as described later), the transience and pattern of the increase in [Ca2+]i must be due to the channels or regulatory components other than cyclic AMP.

Neither the peptides nor forskolin had any effect on [Ca2+]i.
in the presence of 1 or 3 μM nitrendipine, an L-type Ca\(^{2+}\) channel blocker (3 μM shown in Fig. 5). Additionally, the response was unaffected by depletion of intracellular Ca\(^{2+}\) stores by thapsigargin (data not shown). These results suggest that the increase in [Ca\(^{2+}\)]\(_i\) is due entirely to activation of L-type voltage-dependent Ca\(^{2+}\) channels and increased Ca\(^{2+}\) entry. Because of this apparent reliance on the voltage-dependent channels, measurement of the effects of VIP and PACAP on membrane potential were made using the potential-sensitive indicator bisoxonol. Only a very slight and again transient effect to depolarize the membrane was detectable (data not shown). The concentration-response characteristics of the effects of VIP and PACAP on [Ca\(^{2+}\)]\(_i\), measured at the peak of the response, are shown in Fig. 6. VIP caused a small increase in [Ca\(^{2+}\)]\(_i\) at 0.1 nM and a near maximal increase at 1 nM. The maximum effect was seen at 10 nM. Interestingly, the peak value for [Ca\(^{2+}\)]\(_i\) decreased from the maximum value as the concentration of VIP was increased further to 100 nM. The results with PACAP-38 were similar to those with VIP except that PACAP was more potent. PACAP-38 gave a much larger increase than VIP at 0.1 nM and peaked at 10 nM. As was the case with VIP, an increase in PACAP-38 from 10 nM to 100 nM caused the [Ca\(^{2+}\)]\(_i\) to decrease from the peak value.

Time Course Studies on the Effects of VIP and PACAP to Raise Cyclic AMP Levels—It appears unlikely that the small and transient effects of the peptides on [Ca\(^{2+}\)]\(_i\) can account for the prolonged effect of VIP and PACAP on insulin secretion, as revealed in the perifusion studies in Fig. 3. Consequently, we determined the effects of VIP and PACAP on cyclic AMP levels over time. The data for PACAP are shown in Fig. 7A. PACAP caused a rapid increase in cyclic AMP content, which was close to maximal after 30 s. Maximal values were reached at 1–5 min, after which the cyclic AMP content decreased. Cyclic AMP content had returned to basal values by 30 min despite the continued presence of the peptide. The effects of VIP (not shown) paralleled those of PACAP.

In view of the importance of the findings that both [Ca\(^{2+}\)]\(_i\) and cyclic AMP levels were at basal levels at 30 min, a time at which insulin secretion was still stimulated by VIP and PACAP, a second series of experiments was performed to confirm that cyclic AMP levels had indeed returned to basal after 30 min of exposure to VIP and PACAP. These data are shown in Table I. It is clear that at 30 min, the peptides no longer have any effect on the cyclic AMP content of the cells.

In contrast to the transient responses to the peptides, forskolin had a prolonged effect on cellular cyclic AMP levels. As
can be seen from Fig. 7B, the increase in cyclic AMP caused by forskolin reached a peak at 5 min and only slightly declined thereafter. At 30 min, cyclic AMP levels remained highly elevated.

Given that insulin secretion at 30 min is still strongly stimulated by both VIP and PACAP and that the peptides no longer have any effects on 

**TABLE I**

Effect of VIP and PACAP on cAMP content at 30 min

| Additions                              | cAMP content (pmol/mg protein) |
|----------------------------------------|-------------------------------|
| 4 mM glucose (control)                 | 19.1 ± 1.8                    |
| +PACAP-27 (10 nM)                      | 19.3 ± 1.0                    |
| +VIP (10 nM)                           | 19.5 ± 1.7                    |

Under control conditions, in the presence of 4 mM glucose, VIP caused a prompt stimulation of insulin secretion that peaked after 10 min and declined slowly thereafter. The rate of insulin release in the down-regulated cells in 4 mM glucose was less than that of the control cells by approximately 40%. In these cells, stimulation by VIP was essentially similar to that in the controls except that both the starting level and the subsequent peak values were lower.

Treatment with TPA for 24 h reduces the insulin content of β-cells (insulin content of control cells was 250 ± 10 ng/10^6 cells, and that of the down-regulated cells was 147 ± 11 ng/10^6 cells, i.e. a 41% reduction). Thus it is possible that the reduced insulin secretion seen was due to the decrease in insulin content. Indeed, when the rate of insulin secretion is normalized for the decreased insulin content, as shown in Fig. 8B, there is no difference in the secretion rates for control or down-regulated cells. It seems unlikely, therefore, that PKC is responsible for the prolonged peptide stimulation of insulin secretion.

Effect of Wortmannin on VIP- and PACAP-stimulated Insulin Secretion—Another approach to the possibility that a presently unrecognized signal transduction pathway might be mediating the persistent stimulation of insulin secretion by VIP and PACAP was to study the effect of wortmannin, an inhibitor of PI 3-kinase. The potency of wortmannin is such that at low concentrations the inhibitor is currently thought to be selective for PI 3-kinase. At higher concentrations (μM), wortmannin inhibits other kinases, including myosin light chain kinase. From the results of the 30-min static incubation expe-
stimulated by 4 mM glucose. Thus wortmannin selectively inhibited VIP-stimulated insulin secretion to near basal levels by 20 min. Because of the possibility that these results were due to a slow onset of action of wortmannin, perhaps by slow diffusion to its site of action (although this seems unlikely), the experiments were repeated under conditions such that the test cells were preincubated with wortmannin for 60 min prior to the stimulation by peptide. This was done to preclude any concerns about possible delayed access of wortmannin to its site of action. The results obtained under these conditions with VIP and wortmannin are presented in Fig. 11. Under control conditions, 10 nM VIP stimulated insulin secretion as anticipated with a rapid increase in secretion rate to a peak value and then a small decline to a plateau after 25 min. At the 30-min point, the secretion rate was still 3 times that of the untreated (4 mM glucose) controls. In the presence of wortmannin, and despite long exposure to the drug, the response to VIP was still unaffected for the first 10 min. At that time, the rate of VIP-stimulated insulin release stopped increasing, inhibition of release began, and the release rate was approaching basal values after a further 10 min. Thus the inhibitory effect of wortmannin was exerted on the persistent secretion stimulated by VIP and not on the acute stimulation. This is in accord with an action of VIP (and PACAP) to stimulate insulin secretion acutely by means of the prompt but transient increases in cyclic AMP and [Ca$^{2+}$], and to cause persistent secretion by virtue of a wortmannin-sensitive pathway.

The next studies performed in the course of this work were aimed at the measurement of PI 3-kinase activity. The results of typical experiments are shown in Fig. 12. In Fig. 12A, PI 3-kinase activity is shown after incubation of the cells with 4 mM glucose alone (lane 1) and with PACAP-38 and VIP (lanes 3 and 5). No stimulation of PI 3-kinase activity by PACAP or VIP was detected in this and three similar experiments. Wortmannin (100 nM) markedly inhibited PI 3-kinase activity in the presence of glucose, PACAP, and VIP (lanes 2, 4, and 6). In Fig. 12B, PI 3-kinase activity is shown in the presence of both 0.2 and 4 mM glucose (lanes 1 and 4). There was no difference in PI 3-kinase activity in the two glucose concentrations (n = 3). PACAP-38 and VIP, both of which stimulate insulin secretion in the presence of either of these glucose concentrations, did not change the activity of PI 3-kinase (lanes 2, 3, 5, and 6). Finally, in panel C, the effects of different concentrations of wortmannin (3-100 nM) on PI 3-kinase activity in the presence of 4 mM glucose...
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FIG. 11. The effect of 100 nM wortmannin on insulin secretion (4 mM glucose) and 10 nM VIP-stimulated secretion. Wortmannin was added 50 min prior to zero time on the figure. The experiments were carried out under perfusion conditions, and the results were expressed as pg of insulin/10^6 cells/min (mean ± S.E.). n = 5. control; ○, VIP; ■, WT control; ◦, WT + VIP.

glucose can be seen. Wortmannin caused a concentration-dependent inhibition of the enzyme (n = 3). Near total inhibition was achieved by 100 nM wortmannin (n = 9). In another approach for testing the effects of PACAP and VIP on PI 3-kinase activity, HIT cells were labeled with ^32P-O_4 and then treated with PACAP, VIP, or, as a control, 4 mM glucose alone. Phosphoinositides were extracted from the cells, separated by thin-layer chromatography, and autoradiographed. No differences in the ^32P-labeling patterns, indicative of increased PI 3-kinase activity, were detected, n = 6.

DISCUSSION

The results of these studies show that the effects of VIP and PACAP to stimulate insulin secretion are the result of a complex set of interconnected second messenger systems. Both peptides interact with receptors that are positively linked to adenyl cyclase to raise the cellular cyclic AMP content. This, in turn, increases the activity of the β-cell L-type voltage-dependent Ca^{2+} channels and increases [Ca^{2+}]i. The increased [Ca^{2+}]i, and cyclic AMP then synergize to stimulate insulin secretion. It seems likely from our data that both VIP and PACAP interact with the same receptor on the β-cell, because the simultaneous addition of both peptides did not increase the rate of insulin release to levels higher than the addition of either one alone (data not shown). Furthermore, from the relative potencies of the peptides, it can be assumed that the HIT-T15 cell expresses the VIP type 2/PACAP type 3 receptor. This assumption is in accord with the observation that mRNA for this receptor subtype is expressed at moderate levels in the HIT-T15 (hamster) cells, RINm5f (rat) cells and in rat pancreatic islets and at high levels in the MIN6 (mouse) cell (11).

The effects of the peptides were rapid, and increases in cyclic AMP were detected after only a 30-s exposure, the earliest time point measured. Increased cyclic AMP levels in RINm5f cells, and increased adenyl cyclase activity in RINm5f and RIN14B in response to VIP have been reported previously (29, 30). The increase in [Ca^{2+}]i, peaked also at 30 s. In the presence of a stimulatory concentration of glucose (4 mM), the peptides induced a prompt increase in insulin secretion. It is obvious that any agent that increases both cyclic AMP and [Ca^{2+}]i, under these conditions will stimulate insulin secretion. However, it was noted in these studies that the effects of VIP and PACAP to stimulate insulin secretion were large and prolonged, whereas the effects of the peptides on cyclic AMP content and on [Ca^{2+}]i were transient. Additionally, the effects on [Ca^{2+}]i were quite small. After a 30-min exposure to the peptides, there was a complete dissociation between the stimulated rate of insulin release and the levels of cyclic AMP and [Ca^{2+}]i. At this time, while insulin release was still strongly stimulated by the peptides, both the cyclic AMP content and [Ca^{2+}]i, had returned to basal values. These data point to the existence of at least one additional signal transduction mechanism in which insulin secretion is maintained at stimulated levels. In seeking this additional mechanism, we were unable to find evidence that would implicate the activation of PLC or PKC as mechanisms by which the peptides might increase secretion rates. Neither down-regulation of PKC nor inhibition of PKC (data not shown) blocked the response to the peptides. Additionally, the increase in [Ca^{2+}]i, in response to VIP and PACAP was abolished by nitrendipine, and this would not have occurred if there was activation of PLC and mobilization of intracellular Ca^{2+}. While this is in agreement with data obtained in rat islets, where the increase in [Ca^{2+}]i in response to PACAP was also blocked by nitrendipine (14), this result con-
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trasts with a previous report in the HIT-T15 cell, where it was suggested that PACAP was acting also through mobilization of intracellular Ca\(^{2+}\) (31). Indirect evidence for a coupling of PACAP to the PLC pathway was obtained in Xenopus oocytes transfected with PACAP type 3 receptors. These cells responded to PACAP with activation of calcium-activated Cl\(^{-}\) currents (11), a phenomenon that was also observed with glucagon-like peptide-1 in transfected COS-7 cells (32). These results may be due to excessively high levels of receptor expression.

The major finding in the present study is that the sustained stimulation of insulin release by VIP and PACAP is extremely sensitive to wortmannin, a known inhibitor of PI 3-kinase. The best two characterized isoforms of PI 3-kinase are heterodimers consisting of an 85-kDa regulatory and a 110-kDa catalytic subunit (33), which are stimulated by a variety of growth factor receptors. This includes translocation and tyrosine phosphorylation of the 85-kDa subunit (34) and there are re-meric G protein linked receptors without the need for tyrosine phosphorylation of the 85-kDa subunit (34) and there are reports that PI 3-kinase can be directly regulated by G protein \(\alpha\) and \(\beta\) subunits (35, 36). The lipid products of this enzyme reaction, Ptdlns[3,4,5]P\(_3\) and Ptdlns[3,4]P\(_2\), have been implicated in mitogenesis, cell transformation, and importantly in exocytosis (37, 38). It was because of the latter that we investigated the effects of wortmannin on insulin secretion. Our measurements of PI 3-kinase activity show that wortmannin inhibits the enzyme over the same range as it inhibits PACAP- and VIP-stimulated insulin secretion. However, no stimulation by the peptides or by glucose was detected. The lack of effect of glucose was not unexpected as wortmannin had no effect on glucose-stimulated release. Therefore, from the data presented, there are at least three possibilities with respect to the nature of the wortmannin-sensitive pathway, which is linked to the stimulation of exocytosis. The first is that basal levels of PI 3-kinase activity are required for the provision of downstream intermediates necessary at some point of convergence with the VIP/PACAP signaling pathway. The second is that there may be a specific “exocytosis-associated” PI 3-kinase activity, which is undetectable by the techniques used here because of the preponderance of the other PI 3-kinase activities. The third possibility, despite PI 3-kinase being the only enzyme thus far reported to be inhibited by wortmannin at these low concentrations, is that the wortmannin effect is exerted on another enzyme which is critical in the stimulus-secretion pathway for these two peptides and not on PI 3-kinase. However, if a PI 3-kinase is involved in this pathway, it is noteworthy that in retinal pigment epithelial cells, VIP stimulated the phosphorylation of pp60\(^{src}\), an oncogene protein kinase that is known to phosphorylate PI 3-kinase (39). Thus a potential signal transduction pathway exists between the VIP receptor and pp60\(^{src}\), which leads to PI 3-kinase. Subsequent steps to increased exocytosis are unknown and need to be investigated.

From the patterns of release seen during peptide stimulation in control and wortmannin-treated cells, the temporal effects of the different components of the stimulatory mechanisms can be observed. It appears that the initial response, the prompt up-swing of stimulated insulin release is due to the combined effects of increased [Ca\(^{2+}\)], which can trigger release, cyclic AMP, which enhances the stimulated level of release, and the wortmannin-sensitive signal. It is interesting that cyclic AMP, well known to be a potentiator of release rather than an initiator (40, 41), appears to be responsible for the increased channel activity that results in increased [Ca\(^{2+}\)], and enhances release by at least two mechanisms under these conditions. After the increased rate of release by VIP in the presence of wortmannin, the increased rate of release is not sustained and decays in a manner consistent with the short time course of the effects of VIP and PACAP on cyclic AMP and [Ca\(^{2+}\)]. It supports also the idea that the wortmannin-sensitive pathway is primarily involved in the prolongation of the stimulation of insulin release by these two peptides. Neither the nature of this wortmannin-sensitive pathway nor the mechanism by which the occupied VIP/PACAP receptors activate the pathway is known. The activation is not secondary to the elevation of cyclic AMP or to the increase in [Ca\(^{2+}\)], because the effect of forskolin on insulin release, which mimics the actions of the peptides on cyclic AMP and [Ca\(^{2+}\)], is not inhibited by wortmannin. However, the wortmannin-sensitive pathway is receptor-mediated, because the prolonged stimulation of release decays rapidly to base-line values after removal of the peptides. The link between the receptor and this novel wortmannin-sensitive mechanism by which stimulated insulin secretion is maintained remains to be determined. It could be mediated by \(\alpha\) or \(\beta\) subunits of G\(_p\), which is activated by VIP and PACAP. It could conceivably be mediated by a heterotrimeric G protein not currently known to be associated with the VIP-2/PACAP-3 receptor. While the low concentrations of wortmannin used to inhibit the responses of the peptides are in accord with an action on PI 3-kinase, there may well be other kinases in the cell that have a similar sensitivity to wortmannin and which are, perhaps, essential components of this \(\beta\)-cell stimulus-secretion pathway.

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