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# Secretin stimulates cyclic AMP and inositol trisphosphate production in rat pancreatic acinar tissue by two fully independent mechanisms

([Tyr$^{10,13}$]secretin/[Tyr$^{10,13}$,Phe$^{22}$,Trp$^{25}$]secretin/cytosolic free $Ca^{2+}$/cholera toxin/phorbol 12-myristate 13-acetate)

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**ABSTRACT** **In rat pancreatic acinar tissue adenylate cyclase is stimulated by low concentrations of secretin, while higher concentrations also activate phosphatidylinositol bisphosphate hydrolysis. By the use of the secretin analogues [Tyr$^{10,13}$]secretin and [Tyr$^{10,13}$,Phe$^{22}$,Trp$^{25}$]secretin, we have shown that substitution of tyrosine for leucine at positions 10 and 13 was sufficient to reduce the ability of the peptide to stimulate the production of inositol trisphosphate and the increases in cytosolic free calcium, while the ability to stimulate cAMP is little affected and the peptide remained a full agonist. Incubation with cholera toxin caused increases in cAMP, which were maximal after 30 min. Cholera toxin treatment also resulted in a marked reduction of secretin-stimulated inositol trisphosphate production, but this required a much more prolonged treatment (150–240 min), suggesting that different cholera toxin substrates were involved. Activation of protein kinase C with the phorbol ester phorbol 12-myristate 13-acetate had no effect on secretin-induced cAMP formation, nor was secretin-stimulated inositol trisphosphate formation altered by further increases in cAMP. These results indicate that the mechanisms by which secretin stimulates adenylate cyclase and activates phospholipase C in acinar tissue are completely independent.**

In the rat exocrine pancreas, binding of low concentrations of secretin to specific high-affinity receptors on acinar tissue leads to activation of adenylate cyclase (1), cAMP accumulation (2), and enzyme secretion (2, 3). However, with increasing concentrations of secretin the rate of cAMP accumulation becomes maximal when the rate of enzyme secretion is only half-maximal, or even less (2, 3). We have recently shown that at these higher concentrations of secretin there is also evidence of phosphatidylinositol bisphosphate (PtdIns$P_2$) hydrolysis with rapid production of inositol trisphosphate (Ins$P_3$) and diacylglycerol (3), each of which is believed to play a role in stimulus-secretion coupling (4). These observations argue against the previously held idea that within a single tissue a peptide hormone cannot activate both the adenylate cyclase and the PtdIns$P_2$ systems. It is known that hormones that either stimulate or inhibit the adenylate cyclase system do so through interaction with well-defined GTP-binding proteins, guanine nucleotide stimulatory ($G_s$) and inhibitory ($G_i$) factors (5). Recent work has shown that in several different cell systems (6–17), including the exocrine pancreas (18–20), agonists that activate phospholipase C activity and initiate PtdIns$P_2$ hydrolysis also interact with GTP-binding proteins. Although these proteins are not yet as well characterized as those linked to the adenylate cyclase system, they do mark one point where the control mechanisms of the different second messenger pathways are similar.

The aim of the present investigation was 2-fold. The first was to determine whether the two signal pathways stimulated by secretin are separate at the receptor level. For this purpose, we have utilized secretin analogues with different amino acid substitutions in the middle and COOH-terminal portions of the molecule, regions that are not critical for cAMP generation (21, 22). Second, we have investigated whether the two second messenger systems activated by secretin are independent of each other at the postreceptor level.

## MATERIALS AND METHODS

Dispersed pancreatic acini were prepared by collagenase digestion from male Wistar rats (180–200 g) fed ad lib as described (23). For measurements of inositol phosphates, acini were suspended (≈10 mg of acinar protein per ml) in a modified Krebs–Ringer solution, buffered to pH 7.4 with 12.5 mM Hepes, containing 0.1% human serum albumin (23). Acini were preincubated in the presence of 2 μM *myo*-[2-$^3$H]inositol (specific radioactivity, 16.5 Ci/mmol; 1 Ci = 37 GBq) for 2 hr as described (3). In experiments in which cholera toxin was used, acini were suspended in RPMI 1640 medium containing glucose (100 mg/ml) and buffered to pH 7.4 with 25 mM Hepes. Acini were then placed in spinner culture bottles with or without cholera toxin (3 μg/ml) and the usual concentration of *myo*-[2-$^3$H]inositol and were maintained in an incubator at 37°C for variable times as indicated in the text. Acini were washed and resuspended in fresh buffer (≈2.5 mg of acinar protein per ml). Incubations (final vol, 1 ml) were started by addition of the agonists and were terminated by acidification with ice-cold trichloroacetic acid [final concentration, 10% (wt/vol)]. Extracts were washed with ether and then applied to Dowex 1 × 8 columns and the fractions containing Ins$P_1$, Ins$P_2$, and Ins$P_3$ were collected separately as described by Berridge *et al.* (24). Radioactivity in the various fractions was determined by liquid scintillation spectrometry. For measurements of cytosolic free calcium ($[Ca^{2+}]_i$), acini were suspended (≈10 mg of acinar protein per ml) in RPMI 1640 medium, buffered to pH 7.4 with 25 mM Hepes containing 0.5% human serum albumin. $[Ca^{2+}]_i$ was determined by loading acini with quin-2/AM (final concen-

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Abbreviations: PtdIns$P_2$, phosphatidylinositol bisphosphate; Ins$P_3$, inositol trisphosphate; $[Ca^{2+}]_i$, cytosolic free calcium; $G_s$ and $G_i$, GTP-binding proteins associated with stimulation and inhibition of adenylate cyclase, respectively; VIP, vasoactive intestinal polypeptide; IBMX, 3-isobutyl-1-methylxanthine; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; PMA, phorbol 12-myristate 13-acetate.

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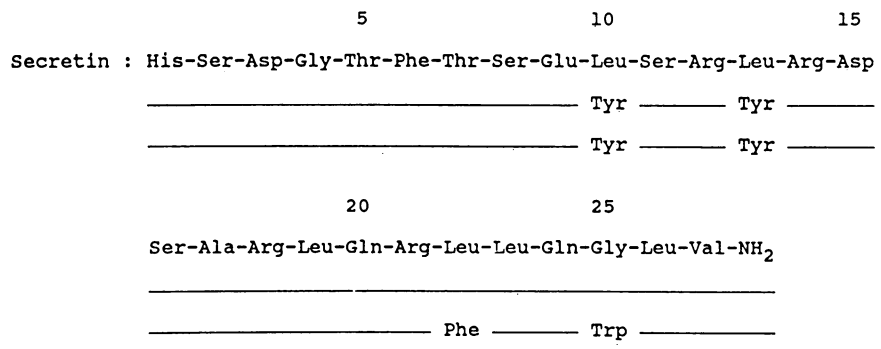


FIG. 1. Amino acid sequence of secretin and secretin analogues [Tyr$^{10,13}$]secretin and [Tyr$^{10,13}$,Phe$^{22}$,Trp$^{25}$]secretin.

tration, 50 $\mu$M) for 30–35 min as described in detail (25). The fluorescence signal was calibrated at the end of each trace and, finally, $[Ca^{2+}]_i$ was estimated according to equation 1 of Tsien *et al.* (26).

For measurements of cAMP, samples were extracted with ethanol and dried. Reconstituted samples were assayed with a commercially available kit (C1S; Oris Industrie SA, Gif-sur-Yvette, France). Protein was measured by the method of Bradford (27). Cholera toxin (lot 105F-0429), phorbol 12-myristate 13-acetate (PMA), 3-isobutyl-1-methylxanthine (IBMX), and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) were purchased from Sigma. [Tyr$^{10,13}$]secretin and [Tyr$^{10,13}$,Phe$^{22}$,Trp$^{25}$]secretin were synthesized by the solid-phase method (28, 29) and purified to homogeneity by reverse-phase HPLC on $C_{18}$ silica columns. Very weak binding of the analogues to glucagon receptors of liver membranes has already been reported briefly (30). Synthetic secretin and vasoactive intestinal polypeptide (VIP) were bought from Bachem (Bubendorf, Switzerland). The source of other materials was as described (3).

## RESULTS

**Structural Requirements in the Secretin Molecule for Stimulation of PtdIns$P_2$ Hydrolysis.** The two secretin analogues used have a tyrosine substituted for leucine at both positions 10 and 13 and the second analogue has, in addition, phenylalanine and tryptophan substitutions for leucine and glycine at positions 22 and 25, respectively (Fig. 1). These are aromatic residues that occur in glucagon, a peptide hormone showing considerable homology with secretin. It is clear that with these changes in the secretin molecule, the ability to stimulate Ins$P_3$ production is practically abolished, and the ability to cause increases in $[Ca^{2+}]_i$ is eliminated (Table 1). Results with carbachol (1 $\mu$M), which stimulates enzyme secretion to a similar degree as secretin (0.2 $\mu$M), are shown for comparison. The substitutions in the secretin molecule, which seem to eliminate significant hydrolysis of PtdIns$P_2$, appear to have little effect on cAMP production, either in the absence (Table 2) or in the presence of the phosphodiesterase inhibitor IBMX (Table 3). At low concentrations (1 nM), the analogue was ≈50% as active as secretin, and at higher concentrations it was a full agonist. This finding would be consistent with previous reports that the configuration of the $NH_2$-terminal portion of secretin, preserved in the analogues used in the present investigation, is important for stimulation of cAMP formation (21, 22). Thus, substitutions in the secretin molecule that replace leucine with tyrosine at positions 10 and 13 are sufficient to greatly reduce the capacity of secretin to activate PtdIns$P_2$ hydrolysis while having little effect on cAMP accumulation in pancreatic acinar cells. The changes in conformation of secretin caused by these aromatic residues are not yet known.

**Effect of VIP on Stimulation of cAMP Production and Ins$P_3$ Formation.** VIP also stimulates cAMP production in the exocrine pancreas. The dose response for VIP-induced cAMP formation is similar to that of secretin (Table 2). However, VIP does not stimulate Ins$P_3$ production at concentrations of 1 $\mu$M (Fig. 2) or at 0.1 or 0.01 $\mu$M (not shown). When VIP (1 $\mu$M) was added to secretin (0.2 $\mu$M) [a submaximal concentration with respect to Ins$P_3$ formation (3)], it did not modify secretin-induced Ins$P_3$ production (Fig. 2). The above findings would suggest that the GTP-binding protein, $G_s$, implicated in the stimulation of adenylate cyclase by both VIP and secretin is not directly involved in the events leading to secretin-induced PtdIns$P_2$ hydrolysis.

**Effect of Cholera Toxin on cAMP and Ins$P_3$ Production.** The effects of cholera toxin on cAMP formation were maximal after short incubation periods with the toxin. Thus, the increases seen after 60 and 90 min of pretreatment with the toxin were not greater than those seen at 30 min (Fig. 3 *Left*). By contrast, there was no effect on Ins$P_3$ production when the incubation period with the toxin was 90 min or less (Fig. 3 *Right*). However, when incubation times with the toxin were extended to 2.5–4 hr, there was a marked reduction in secretin-stimulated Ins$P_3$ production. The reduction was of

Table 1. Effect of secretin, secretin analogues, and carbachol on Ins$P_3$ production and changes in $[Ca^{2+}]_i$ in pancreatic acini of the rat

| | Ins$P_3$, % control | $[Ca^{2+}]_i$, nM | |
|---|---|---|---|
| | | Basal | Maximal |
| Secretin (0.2 $\mu$M) | 276 ± 48 (5) | 120 ± 10 | 218 ± 14 (3) |
| [Tyr$^{10,13}$]Secretin (0.2 $\mu$M) | 121 ± 4 (5) | 136 ± 18 | 144 ± 14 (3) |
| [Tyr$^{10,13}$,Phe$^{22}$,Trp$^{25}$]Secretin (0.2 $\mu$M) | 116 ± 7 (5) | 136 ± 5 | 137 ± 12 (7) |
| Carbachol (1 $\mu$M) | 309 ± 68 (5) | 130 ± 6 | 261 ± 13 (4) |

Ins$P_3$ was measured 1 min after addition of the agonists and results are given as percentage of control (without agonist). $[Ca^{2+}]_i$ was measured using the fluorescence indicator quin-2; results are shown as basal and maximal $[Ca^{2+}]_i$, which occurred within 20–30 sec after addition of agonist. Secretin and its analogues were tested at 0.2 $\mu$M and carbachol at 1 $\mu$M, a concentration that stimulates enzyme secretion to a similar degree as secretin (0.2 $\mu$M) (not shown). Results are expressed as mean ± SEM of the number of observations shown in parentheses.

Table 2. Effect of secretin, secretin analogue, and VIP on cAMP content of rat pancreatic acini

| | cAMP, pmol per mg of acinar protein | | |
|---|---|---|---|
| | 1 nM peptide | 10 nM peptide | 0.1 μM peptide |
| Secretin | 15.3 ± 2.0 | 17.2 ± 3.1 | 18.3 ± 3.3 |
| [Tyr$^{10,13}$,Phe$^{22}$,Trp$^{25}$]Secretin | 12.5 ± 0.9 | 16.0 ± 3.7 | 19.2 ± 1.2 |
| VIP | 18.8 ± 0.9 | 18.9 ± 0.4 | 21.1 ± 1.7 |

Acini were incubated for 30 min in the presence of the peptide. Basal cAMP content was 10.1 ± 0.7 pmol per mg of acinar protein. Results are expressed as mean ± SEM of duplicate estimations in three independent experiments.

the order of 75% at 15 s and 60% at 60 s (Fig. 4). By contrast, there was no significant effect of cholera toxin, after preincubation for 2.5–4 hr, on carbachol-stimulated Ins$P_3$ production, the results being 390% ± 53% vs. 327% ± 27% of control at 1 min for untreated and cholera toxin-treated tissue, respectively ($n$ = 11; four independent experiments). The differences in the time course for effects on cAMP and on secretin-induced Ins$P_3$ production point to a cholera toxin substrate other than $G_s$ being implicated in secretin-induced PtdIns$P_2$ hydrolysis.

**Effect of cAMP on Ins$P_3$ Production.** In other tissues, it has been shown that cAMP alone can alter Ins$P_3$ production (31, 32). However, we have just shown that in the exocrine tissue, VIP-stimulated cAMP production is not associated with increases in Ins$P_3$ (see above). Therefore, this showed that increases in cAMP alone could not stimulate Ins$P_3$ formation in exocrine tissue. Furthermore, when cAMP formation stimulated by secretin was accentuated by addition of 0.1 mM IBMX, there was no alteration in secretin-induced Ins$P_3$ formation (Table 4), nor did 5 mM 8-Br-cAMP alter carbachol-induced Ins$P_3$ production (data not shown).

**Effect of Pretreatment with PMA on cAMP Accumulation.** The phorbol ester PMA was used to see whether activation of protein kinase C alone would alter subsequent secretin-induced second messenger formation. Pretreatment of acini for 10 min with 1 μM PMA had no effect on secretin (10 pM to 1 μM)-induced cAMP formation measured both at 10 and at 30 s (not shown). However, PMA pretreatment did cause a reduction in secretin-induced Ins$P_3$ production (Fig. 5).

## DISCUSSION

Since the low-affinity secretin receptors are only occupied at the higher concentrations of secretin that induce PtdIns$P_2$ hydrolysis (3), it seems highly probable that it is only the low-affinity receptor that is linked to phospholipase C activation. The possibility that the receptors linked to the adenylate cyclase and PtdIns$P_2$ second-messenger systems are different has been strengthened by the use of secretin analogues. Thus, tyrosine for leucine substitutions at positions 10 and 13 were sufficient to practically eliminate secretin-induced changes in both Ins$P_3$ and $[Ca^{2+}]_i$, while having almost no effect on cAMP formation. These substitutions are at a distance from the $NH_2$-terminal amino acids 1–4, which are essential for the secretin-induced cAMP response (21, 22). The present study strongly suggests, therefore, that the middle portion of the secretin molecule is important for binding to the low-affinity receptor and/or activation of phospholipase C.

The results obtained with the secretin analogues suggest that the secretin-induced signals for activation of adenylate cyclase and PtdIns$P_2$ hydrolysis are separate from each other as early as the receptor level. Experiments we have undertaken to probe the second messenger systems at more distal points show that they remain separate. We have demonstrated that a cholera toxin substrate is involved in the events associated with secretin-stimulated Ins$P_3$ production. However, that this is the $G_s$ protein of the adenylate cyclase system seems unlikely for several reasons. Firstly VIP, like secretin, stimulates adenylate cyclase through the $G_s$ protein, and yet VIP had no effect on PtdIns$P_2$ hydrolysis at high concentrations where VIP-stimulated cAMP levels were similar to those of secretin. Furthermore, when VIP was added to secretin there was no modification of secretin-induced Ins$P_3$ formation, although the concentration of secretin (0.2 μM) was submaximal for Ins$P_3$ production (3). Thirdly, the time course of cholera toxin-induced changes in cAMP, which depends on ADP-ribosylation of $G_s$ (33–35), was different from that associated with reduction in secretin-induced Ins$P_3$ production. It appears that the protein involved in adenylate cyclase activation is the protein most readily ribosylated by cholera toxin (34, 35). However, when ribosylation of this protein is completed, either by exposure to low concentrations of cholera toxin for a longer period or to high concentrations for a shorter period, then ribosylation of other proteins occurs (34, 35). This is a possible explana-

Table 3. Effect of IBMX on stimulation of cAMP by secretin and secretin analogues

| | cAMP, pmol per mg of acinar protein | |
|---|---|---|
| | − IBMX | + IBMX |
| Secretin | 22.1 ± 1.8 | 119 ± 8.1 |
| [Tyr$^{10,13}$]Secretin | 20.3 ± 4.1 | 111 ± 5.0 |
| [Tyr$^{10,13}$,Phe$^{22}$,Trp$^{25}$]Secretin | 23.1 ± 5.1 | 111 ± 10 |

Acini were incubated for 30 min with or without 0.1 mM IBMX. Basal cAMP content was 12.1 ± 0.9 pmol per mg of acinar protein. Results are expressed as mean ± SEM of duplicate estimations in four independent experiments. Peptide concentration was 0.2 μM.

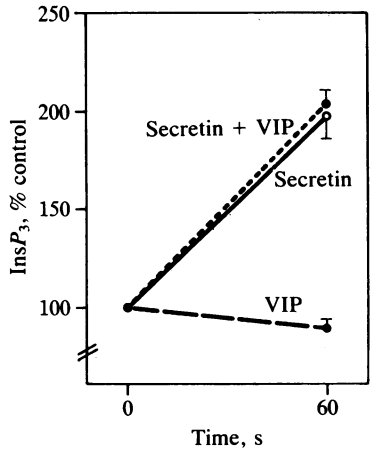


FIG. 2. Effect of VIP on secretin-stimulated Ins$P_3$ production. Acini were preincubated with *myo*-[$^3$H]inositol for 2 hr. Secretin (0.2 μM) and/or VIP (1 μM) was added at time 0. Measurements of Ins$P_3$ were made at time 0 and at 1 min. Results are expressed as a percentage of control values (no agonist), and are given as the mean ± SEM ($n$ = 5) (in each case).

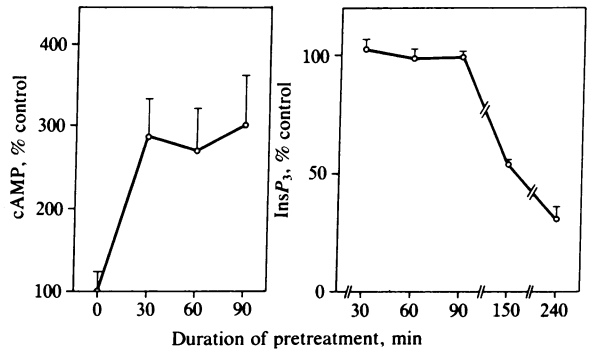


FIG. 3. Time course of the effect of cholera toxin pretreatment on cAMP content and Ins$P_3$ production in pancreatic acini. (*Left*) All acini (including controls) were preincubated for 90 min in the absence or presence of cholera toxin (3 $\mu$g/ml) added for the times indicated. Acini were washed, resuspended in buffer containing 1 mM IBMX, and incubated for 10 min. In the presence of the phosphodiesterase inhibitor, cAMP content of control acini was 29 ± 9 pmol per mg of acinar protein. Results are given as the mean ± SEM of duplicate estimations in four independent experiments. (*Right*) Acini were preincubated with cholera toxin (3 $\mu$g/ml) for the times indicated, and labeled with *myo*-[$^3$H]inositol. Acini were then resuspended in fresh buffer and Ins$P_3$ production measured following addition of secretin 0.2 $\mu$M for 1 min. In each case, the increment above control values (no secretin) was measured and the secretin-induced increase of Ins$P_3$ with cholera toxin pretreatment expressed as a percentage of the increase in the absence of cholera toxin. The results show the mean ± SEM of three, six, three, three, and nine estimations for 30-, 60-, 90-, 150-, and 240-min cholera toxin pretreatment, respectively.

tion for the late effect of cholera toxin on Ins$P_3$ formation. A similar phenomenon has been reported for substrates of pertussis toxin (36). Others have shown that GTP-binding protein(s) are involved in PtdIns$P_2$ hydrolysis in pancreatic acinar cells stimulated by carbachol and the cholecystokinin analogue cerulein (19). However, experiments probing the effect of cholera toxin on cholecystokinin analogue-induced PtdIns$P_2$ hydrolysis have yielded conflicting results (19, 20). On the basis of susceptibility to cholera toxin, our studies also indicate that the GTP-binding protein involved in carbachol-induced Ins$P_3$ formation (19) is different from the cholera toxin substrate (presumably another GTP-binding

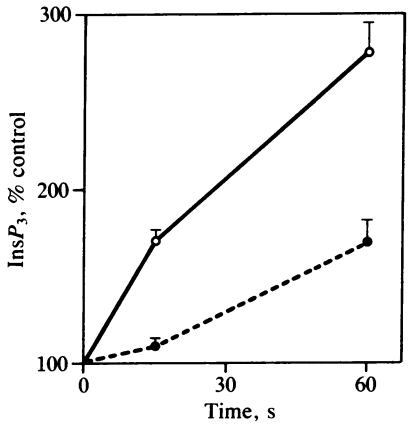


FIG. 4. Effect of cholera toxin pretreatment on the kinetics of secretin-stimulated Ins$P_3$ production. Acini were preincubated for 150–240 min in the presence of cholera toxin (3 $\mu$g/ml) and *myo*-[$^3$H]inositol (●). Following this, acini were resuspended in fresh buffer and secretin (0.2 $\mu$M) was added for 15 sec or 1 min. Results are expressed as percentage of the relevant control samples (○) (no secretin) and are the mean ± SEM of nine estimations in each case, from three independent experiments. Cholera toxin pretreatment significantly reduced secretin-stimulated Ins$P_3$ production both at 15 sec ($P < 0.001$) and 1 min ($P < 0.001$).

Table 4. Effect of IBMX on secretin-induced Ins$P_3$ production in pancreatic acini

| Secretin | IBMX | Ins$P_3$, % control |
|---|---|---|
| 0.2 $\mu$M | None | 165 ± 24 |
| 0.2 $\mu$M | 0.1 mM | 176 ± 11 |

Ins$P_3$ production was measured 1 min after addition of secretin. IBMX was added 15 s before secretin. Results are mean ± SEM of seven or eight observations from three independent experiments.

protein) involved in secretin-induced PtdIns$P_2$ hydrolysis. In addition, the time course studies suggest, as do the studies of Imboden *et al.* on a human T-cell line (37), that the GTP-binding proteins involved in PtdIns$P_2$ breakdown are different from the $G_s$ protein of the adenylate cyclase system.

These results indicate that the signals generated by secretin, which on the one hand lead to cAMP accumulation and on the other hand cause PtdIns$P_2$ hydrolysis, are independent of each other at the level of the (putative) GTP-binding proteins involved and probably also at the receptor level. However, in some tissues, events subsequent to PtdIns$P_2$ hydrolysis can be modified by changes in cAMP, as can levels of cAMP be altered on protein kinase C activation. Thus, protein kinase C activation by PMA is associated with enhancement of cAMP in some tissues (38–41), acute inhibition in others (42–44), and either a delayed inhibitory effect (45) or no effect (46) in others. The reported influences of the adenylate cyclase pathway on the PtdIns$P_2$ pathway have been equally diverse. Thus, cAMP has been shown to potentiate increases in Ins$P_3$ in hepatocytes (31) and to inhibit Ins$P_3$ production in thymocytes (32). Forskolin (but not 8-Br-cAMP) inhibited Ins$P_3$ production in permeabilized pancreatic acinar cells (20). In addition to modulation of the activity of one second messenger system by second messengers from another system, feedback within the same system has been observed. Thus, protein kinase C activation results in reduced Ins$P_3$ production under some (47–51), but not all (52), conditions. In our experiments, protein kinase C activation by PMA had no effect on secretin-stimulated cAMP levels either at low concentrations of secretin where PtdIns$P_2$ hydrolysis is not activated by secretin, or at high concentrations of secretin. Similarly, cAMP had no acute effect on Ins$P_3$ production whether tested by addition of either VIP or IBMX to incubations with secretin. Thus, in the exocrine pancreas we found no evidence for rapid modulation of one second messenger system by the other. We did, however,

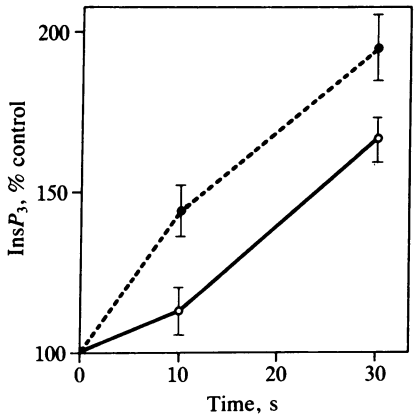


FIG. 5. Effect of pretreatment with PMA on secretin-induced Ins$P_3$ formation. Acini were preincubated with *myo*-[$^3$H]inositol and resuspended in fresh buffer. PMA (1 $\mu$M) was added for 10 min following which secretin (0.2 $\mu$M) was added for either 10 or 30 sec. Results are expressed as mean ± SEM. PMA pretreatment caused a reduction in secretin-stimulated Ins$P_3$ production at 30 sec ($P < 0.05$; $n = 12$; four independent experiments). ●, Control; ○, PMA treated.

find negative feedback within the PtdIns$P_2$ pathway, in that protein kinase C activation by PMA was associated with reduced Ins$P_3$ production.

In summary, therefore, we have found that the two second messenger pathways activated by secretin in the rat acinar tissue are probably linked to two different secretin receptors in the plasma membrane. In this respect, these findings show some analogy with those recently reported for the effects of glucagon (a member of the same peptide family) in the hepatocyte (53). Furthermore, the results suggest that two different cholera toxin substrates (possibly GTP-binding proteins) are involved in secretin stimulation of pancreatic acinar tissue, and that there is no evidence of interaction between the two signal transduction systems on the generation of second messengers.

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