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Secretin induces rapid increases in inositol trisphosphate, cytosolic Ca^{2+} and diacylglycerol as well as cyclic AMP in rat pancreatic acini

Elisabeth R. TRIMBLE,* Roberto BRUZZONE, Trevor J. BIDEN and Robert V. FARESE†

Institut de Biochimie Clinique, Centre Médical Universitaire, 1211 Geneva 4, Switzerland

Previous studies have shown that the dose–response relationship for secretin-stimulated cyclic AMP accumulation is different from that for secretin-stimulated enzyme secretion in the rat exocrine pancreas. Here we show that secretin concentrations of 10^{-10} M and higher stimulated a rise in cyclic AMP levels, with maximum effect on cyclic AMP accumulation being achieved already with 10^{-8} M-secretin. However, at this concentration of secretin, enzyme secretion rates were approximately half-maximal. Unexpectedly, at concentrations of secretin greater than 10^{-8} M there was evidence suggestive of phosphatidylinositol bisphosphate hydrolysis with rapid increases in inositol trisphosphate, cytosolic free calcium and diacylglycerol content of rat pancreatic acini. Furthermore, there was a dose–response relationship among secretin concentration (in the range 10^{-8} M–2×10^{-6} M), increases in inositol trisphosphate and increases in cytosolic free calcium ($[Ca^{2+}]_i$). Contrary to what has been previously believed, these results clearly indicate that in rat pancreatic acini secretin not only stimulates cyclic AMP accumulation but also raises inositol trisphosphate, $[Ca^{2+}]_i$ and diacylglycerol. Thus, two second messenger systems may play a role in the regulation of secretin-induced amylase release.

INTRODUCTION

Enzyme secretion from the exocrine pancreas is regulated to a large extent by hormones and neurotransmitters which have specific receptors on the acinar cells. Most of these receptors appear to be coupled with either the adenylate cyclase or phosphoinositide second messenger systems of stimulation-secretion coupling [1,2]. Activation of the latter system leads to hydrolysis of PIP_2 with the liberation of $InsP_3$ and diacylglycerol [2]. In turn, $InsP_3$ causes release of Ca^{2+} from the endoplasmic reticulum [3,4] and increases in diacylglycerol lead to activation of protein kinase C [5]. It has been commonly believed that secretin receptors are coupled exclusively to the adenylate cyclase system and that it has no effect on Ca^{2+} homeostasis within the acinar cell [1]. Secretin receptors of both high and low affinity exist [6,7], and a recent study has concluded that occupation of high affinity receptors in rat pancreatic acini leads to an increase in cyclic AMP levels and a low rate of enzyme secretion. By contrast, occupation of the low-affinity receptors appears to cause a more marked increase in enzyme secretion by a cyclic AMP-independent mechanism [7].

However, studies where cyclic AMP and amylase secretion have been investigated have tended to focus on time points relatively late (30 min) after the addition of the secretagogue [7]. Since changes in second messenger systems occur within seconds of addition of the agonist, and since these changes evolve with time, it was considered important to re-examine the relationship between cyclic AMP generation and amylase secretion during secretin stimulation. Therefore our first aim was to see if there was indeed a discrepancy between cyclic AMP accumulation and amylase secretion at earlier times in the events of stimulus–secretion coupling. The second aim was to see whether another second messenger system was triggered by secretin. Specifically, we have looked for evidence of PIP_2 hydrolysis. Thus, we measured diacylglycerol, $InsP_3$ and $[Ca^{2+}]_i$ in exocrine tissue. Our study indicates that secretin not only activates adenylate cyclase but also triggers PIP_2 hydrolysis in rat pancreatic acini.

EXPERIMENTAL

Preparation of isolated acini

Acini were prepared from fed male Wistar rats (190–220 g) by collagenase digestion as described in detail elsewhere [8].

Amylase secretion.

Acini were suspended in KRB/Hepes/HSA (12.5 mM-Hepes, 135 mM-NaCl, 4.8 mM-KCl, 1.0 mM-$CaCl_2$, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 5.0 mM-$NaHCO_3$, 0.1% human serum albumin) containing 5 mM-glucose,

Abbreviations used: PIP_2, phosphatidylinositol bisphosphate; $InsP_3$, inositol trisphosphate; $InsP_2$, inositol bisphosphate; $InsP_1$, inositol phosphate; KRB, Krebs–Ringer bicarbonate; HSA, human serum albumin; quin2/AM, quin2 acetoxymethylester; CCh, carbamoylcholine; TRH, thyrotropin releasing hormone; $[Ca^{2+}]_i$, cytosolic free calcium.

* To whom correspondence and reprint requests should be addressed.

† Permanent address: Veterans Administration Hospital, Department of Medicine, University of South Florida Medical School, Tampa, FL 33612, U.S.A.

pH 7.4, and preincubated at 37 °C for 30 min. After washing, the acini were resuspended in fresh buffer and incubated for the time indicated in the results section. Amylase was measured by the method of Bernfeld [9], and the amount released during the incubation period was expressed as a percentage of the total initial content.

Cyclic AMP measurement

Acini prepared as above were incubated for the times indicated in the Results section. Samples were extracted with ethanol and assayed with a commercially available kit (CIS, Oris Industrie SA, Gif-sur-Yvette, France).

Inositol phosphates

Acini were preincubated in KRB/Hepes/HSA for 2 h in the presence of 2 μM-*myo*-[2-^{3}H]inositol (sp. radioactivity 16.5 Ci/mmol; New England Nuclear, Dreieich, Germany). For the first 115 min the buffer contained 0.1 mM-Ca^{2+}, since it was noted in previous studies that labelling of polyphosphoinositides is enhanced in the presence of reduced extracellular calcium (R. V. Farese, unpublished work). At 5 min before the end of the preincubation period Ca^{2+} was adjusted to 1 mM. Acini were washed, resuspended in fresh buffer and incubated for the times indicated in the Results section. LiCl was not added to the incubation. Incubations were stopped by the addition of ice-cold trichloroacetic acid (final concentration 10%, w/v). After repeated washes with diethyl ether to remove trichloroacetic acid, samples were applied to Dowex-1 columns as described by Berridge *et al.* [10]. The fractions containing InsP_1, InsP_2 and InsP_3 were collected separately.

$[Ca^{2+}]_i$ measurements

Acini were suspended in RPMI 1640 buffered to pH 7.4 with 25 mM-Hepes and loaded with quin2/AM (Sigma Chemical Co.) for 30–35 min as previously described in detail [11]. Fluorescence measurements and calibration of fluorescence were carried out as described by Wollheim & Pozzan [12]. $[Ca^{2+}]_i$ was calculated according to eqn. (1) of Tsien *et al.* [13].

Diacylglycerol

These measurements were carried out essentially by the technique of Banschbach *et al.* [14]. After incubation with the agonist, acini were extracted with ice-cold chloroform/methanol (2:1, v/v). The chloroform layer was washed with water and then evaporated to dryness under a steady stream of N_2. The samples (resuspended in 50 μl of chloroform/methanol/water, 75:25:2, by vol.) were submitted to a first t.l.c. step to isolate the diacylglycerol. The purified diacylglycerol was then acetylated with [^{3}H]acetic anhydride (New England Nuclear) and rechromatographed. Diacylglycerol content was measured by comparison with similarly acetylated 1,2-diolein standards (Sigma Chemical Co.) after liquid-scintillation counting.

Protein was measured by the method of Bradford [15]. Synthetic secretin was bought from Bachem, Bubendorf, Switzerland, and CCh from Sigma.

RESULTS

The first objective was to re-examine the relationship between the dose–response for secretin-induced cyclic AMP formation and secretin-induced amylase secretion. At 10 s there was a dose-dependent increase in cyclic AMP content induced by secretin (Table 1). Maximal effects were observed with 10^{-7} M- and 10^{-6} M-secretin. At 30 s, however, the dose–response was less clear cut and at 10 min maximal cyclic AMP concentrations were found with a wide concentration range of secretin i.e. 10^{-9} M–10^{-6} M. By contrast, 10^{-8} M-secretin elicited only 50% of maximal amylase secretion. Amylase release was further increased in a dose-dependent manner by higher secretin concentrations (Table 1).

In view of the apparent discrepancy between the secretin dose–responses for cyclic AMP accumulation and amylase secretion with the higher concentrations of secretin, we investigated the possible involvement of the phosphatidylinositol pathway in secretin-induced stimulus–secretion coupling. On account of the difficulties involved in accurate measurements of small decreases in the PIP_2 pool, we measured the breakdown products of PIP_2, i.e. InsP_3 and diacylglycerol. These are the molecules of interest with respect to second messenger roles in stimulus–secretion coupling and can be more accurately measured than can PIP_2 hydrolysis. Fig. 1 shows that secretin stimulated InsP_3 production in a dose-dependent manner with a threshold at around 10^{-8} M-secretin. Results for secretin were compared with those for CCh, which previously has been shown to trigger PIP_2 hydrolysis in the exocrine pancreas [16–19]. At early time points the increase in InsP_3 stimulated by

Table 1. Effect of secretin on cyclic AMP accumulation in and amylase secretion by rat pancreatic acini

Values are means ± S.E.M. The numbers of independent experiments for measurement of cyclic AMP and amylase were five and seven respectively; *$P < 0.05$, †$P < 0.025$, ‡$P < 0.001$.

[Secretin] (M)	Cyclic AMP (pmol/mg of acinar protein) at: 10 s	30 s	10 min	Amylase secretion at 10 min (% of initial content)
0	4.1±0.6	–	3.9±0.1	3.1±0.3
10^{-11}	4.5±0.8	4.0±0.6	5.2±0.5	3.2±0.5
10^{-10}	4.7±0.6	4.9±0.9	6.6±0.8*	3.3±0.6
10^{-9}	6.5±1.2*	7.2±1.4	8.1±0.7†	4.9±0.5‡
10^{-8}	7.9±0.8‡	9.1±1.3†	8.9±0.8†	5.5±0.4‡
10^{-7}	11.6±2.1†	9.0±0.4‡	9.2±0.7†	8.2±1.1‡
10^{-6}	10.3±1.1†	8.6±0.4‡	8.8±0.6†	9.6±0.9‡

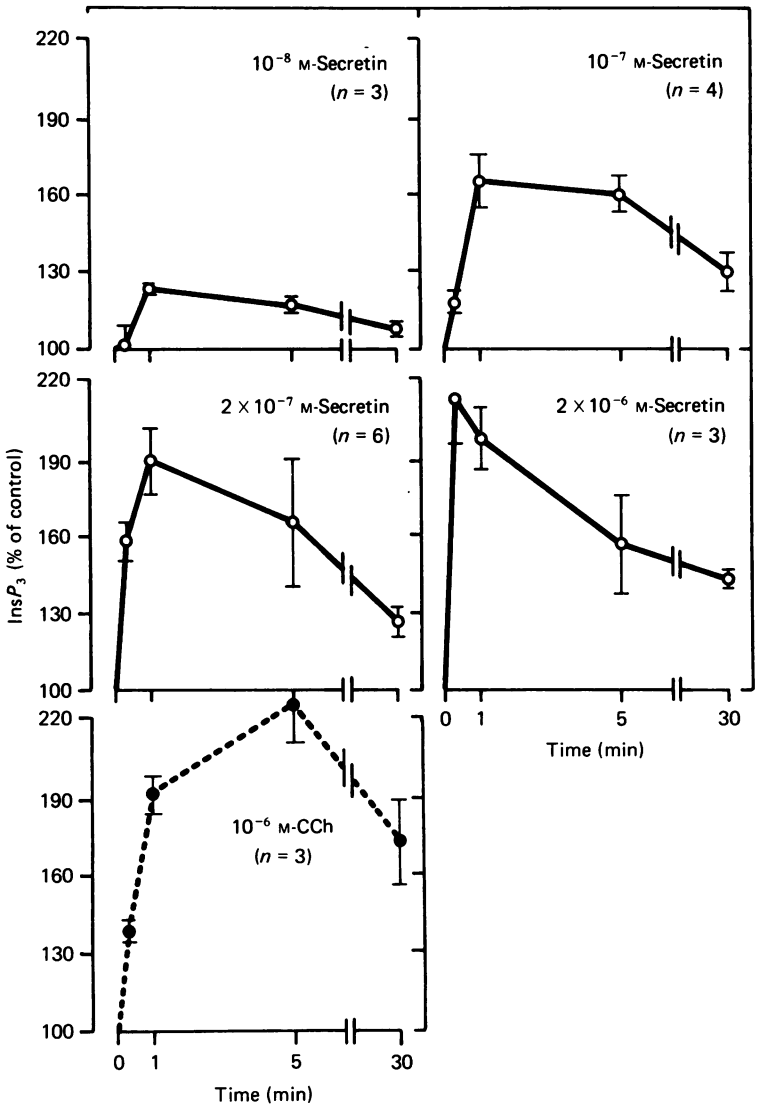

Fig. 1. Effect of secretin and CCh on turnover of $InsP_3$ in rat pancreatic acini

Acini were preincubated with *myo*-[^{3}H]inositol for 2 h and then incubated in the presence of secretin or CCh (LiCl not present). Results (c.p.m. of ^{3}H in $InsP_3$) are expressed as a percentage of control values. The samples were determined in triplicate and the results shown are the means ± S.E.M. from the numbers of independent experiments shown for each condition.

Table 2. $InsP_3$ at 15 s and maximal increase in $[Ca^{2+}]_i$

Values are means ± S.E.M. The numbers in parentheses indicate the number of independent experiments. The maximal rise in $[Ca^{2+}]_i$ occurred at approx. 20 s after addition of the agonist. Mean basal $[Ca^{2+}]_i$ was 124 ± 6 nM ($n = 9$). *$P < 0.05$, †$P < 0.025$, ‡$P < 0.001$.

Agonist	Concn. (M)	$InsP_3$ (% of basal)	$[Ca^{2+}]_i$ (% of basal)
Secretin	10^{-8}	102 ± 7 (3)	110 ± 5 (4)
	10^{-7}	119 ± 5 (4)†	157 ± 14 (4)*
	2×10^{-7}	158 ± 7 (6)†	183 ± 17 (3)*
	2×10^{-6}	215 ± 18 (3)†	222 ± 20 (3)*
CCh	10^{-6}	141 ± 5 (3)†	201 ± 7 (4)‡

10^{-6} M-CCh was similar to that achieved by secretin at 2×10^{-7} M and 2×10^{-6} M. Since LiCl was not present in the incubations, increases in $InsP_1$ and $InsP_2$ were small, being of the order of 10–20% (results not shown). It is likely that the $InsP_3$ fractions contained not only Ins(1,4,5)P_3 but also Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4 as well [20]. However, since it has been shown that Ins(1,4,5)P_3 is phosphorylated to Ins(1,3,4,5)P_4 which subsequently gives rise to Ins(1,3,4)P_3 [21], measurement of the total '$InsP_3$' fraction provides a sensitive indicator of PIP_2 hydrolysis. Ins(1,4,5)P_3 predominates at the very early time points [22,23] and it is this isomer which causes release of Ca^{2+} from the endoplasmic reticulum [3,4].

Therefore, measurements were made of $[Ca^{2+}]_i$ following stimulation with various concentrations of secretin. Table 2 shows that concentrations of secretin of 10^{-7} M and higher stimulated a dose-dependent rise in

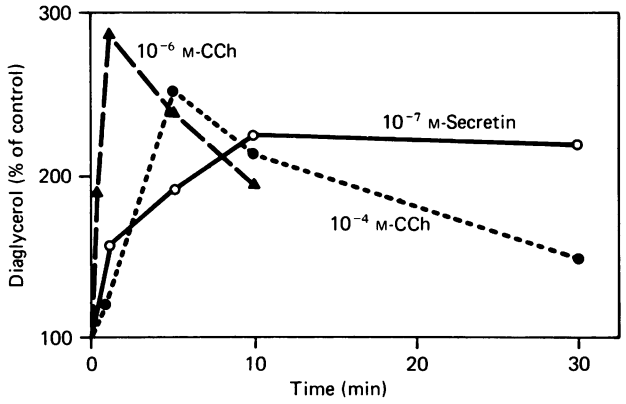

Fig. 2. Diacylglycerol content of rat pancreatic acini in the presence of secretin or CCh

Diacylglycerol was first separated from other lipids by t.l.c. The purified diacylglycerol and 1,2-diolein standards were acetylated with [^{3}H]acetic anhydride and rechromatographed. Basal values of diacylglycerol were 1.16 ± 0.27 μg/mg of acinar protein. One experiment for each condition is shown in the Figure, which shows a mean of triplicate determinations, typical of three experiments.

$[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ were transient, with basal values being re-established within 2 min in the continuous presence of secretin (results not shown). This is similar to what has been shown previously for cholecystokinin analogues and CCh [11,24]. Changes in $[Ca^{2+}]_i$, which were maximal at 20 s, were compared with those increases of InsP_3 found at 15 s. Table 2 shows that increases in $[Ca^{2+}]_i$ and InsP_3 occurred over a similar range of secretin concentrations.

Finally, diacylglycerol content of pancreatic acini was measured in a limited number of experiments. Both secretin and CCh caused increases in diacylglycerol content from early time points (Fig. 2). These data provide further evidence for the action of secretin in PIP_2 hydrolysis with the simultaneous generation of InsP_3 and diacylglycerol.

DISCUSSION

Until now the mechanism by which secretin stimulates both fluid and bicarbonate output [25] as well as enzyme secretion [26] has been believed to be intimately and exclusively linked with activation of adenylate cyclase. This study indicates that secretin triggers two second messenger systems with activation of PIP_2 hydrolysis as well as stimulation of adenylate cyclase. The present results show that except for a very early time point (10 s) there was an apparent discrepancy between secretin-induced cyclic AMP accumulation and secretin-induced amylase secretion. Indeed, several other groups have found a discrepancy between the cyclic AMP content and amylase release from pancreatic acini [7,27,28]. In particular, forskolin, which acts directly on the catalytic subunit of adenylate cyclase, was a rather weak agonist with respect to amylase release, in spite of marked increases in cyclic AMP content [27,28]. In the present study, a discrepancy was evident at secretin concentrations greater than 10^{-8} M. Interestingly, it was at these higher concentrations that increases in InsP_3 and diacylglycerol were found. The most likely explanation for their early, simultaneous increase is receptor-activated PIP_2 hydrolysis through phospholipase C [2]. The threshold concentration of secretin needed to trigger PIP_2 hydrolysis was greater than that needed for activation of adenylate cyclase activity. With 10^{-8} M-secretin, InsP_3 production was not increased at 15 s and there was no significant increase in $[Ca^{2+}]_i$, while cyclic AMP accumulation was nearly maximally stimulated. We therefore propose that activation of the phosphatidylinositol pathway may be involved in the dose-dependent increase in amylase release triggered by the higher concentrations of secretin used in this study. As expected, 10^{-6} M-CCh caused PIP_2 hydrolysis as measured by increases in InsP_3 and diacylglycerol. The percentages of total PIP_2 hydrolysed by 10^{-6} M-CCh [17] and 10^{-6} M-secretin [27] are probably quite small, since there is not a significant loss of radioactivity from PIP_2 previously labelled with ^{32}P; it is only at higher concentrations of CCh that a reduction in the PIP_2 pool becomes evident [17].

These results were intriguing because, although it is known that the two second messenger systems may interact [30–35], it has been generally assumed that for any single tissue a peptide hormone triggers a given response by activation of only one second messenger system. Thus, although vasopressin can activate both adenylate cyclase and PIP_2 hydrolysis, these events occur in different tissues. Acting via V_1 receptors in liver and vascular smooth muscle [36,37], vasopressin stimulates phosphoinositide metabolism and via V_2 receptors in renal tubules [36,38] it activates adenylate cyclase. In the case of TRH, only transient increases of cyclic AMP occur [39,40] and the events subsequent to PIP_2 hydrolysis are generally held to be those that are important with respect to stimulus–secretion coupling [41–43]. Recently, it has been shown that adrenocorticotrophin at low concentrations induces rises in InsP_3 and $[Ca^{2+}]_i$, while at higher concentrations it causes accumulation of cyclic AMP in rat adrenal cells [44]. The present results for secretin in the exocrine pancreas show a strong, sustained increase in cyclic AMP which at the higher concentrations is accompanied by marked increases in InsP_3, $[Ca^{2+}]_i$ and diacylglycerol.

In conclusion, we have demonstrated that, as well as cyclic AMP, other second messengers such as InsP_3, diacylglycerol and $[Ca^{2+}]_i$ are stimulated by secretin and must now be taken into account when considering the mechanism(s) by which secretin promotes amylase release from pancreatic acinar cells.

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