Pancreatic acinar-cell desensitization alters InsP_3 production and Ca^{2+} mobilization under conditions where InsP_3 receptor remains intact

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Desensitization of rat pancreatic acinar cells with 0.1 mM carbamoylcholine (Cch) or 0.5 nM caerulein (CAE), a cholecystokinin (CCK) agonist, altered the subsequent secretory responses to these two agonists. Changes in receptor affinities, shifts in receptor populations, receptor internalization and phosphorylation are the major modifications affecting the muscarinic and CCK receptors in response to desensitization. In this study, post-receptor alterations were examined in order to explain the altered enzyme secretion. Cch or CAE desensitization resulted in decreased Ca^{2+} release in response to CAE and Cch respectively. Under desensitizing conditions, the biochemical and pharmacological properties of the InsP_3 receptor were not affected. Control and desensitized acini had similar B_{max} and K_D values. The Ca^{2+} -channel property of the InsP_3 receptor was not

affected, either, since Ca^{2+} release in response to increasing concentrations of InsP_3 remained comparable in both groups of saponin-permeabilized acini. Finally, the quantities of InsP_3 formed in response to Cch and CAE, measured by InsP_3 radio-receptor assay, were significantly decreased in the Cch- and CAE-desensitized groups, and these decreases were not due to increased InsP_3 turnover. These new data indicate that desensitization of acinar cells with Cch and CAE causes post-receptor modifications resulting in decreased InsP_3 formation and decreased intracellular Ca^{2+} mobilization. It is suggested that the attenuated Ca^{2+} response is related to a decreased formation of InsP_3 from PtdInsP_2 hydrolysis and that phospholipase C could be the immediate target of this regulation.

INTRODUCTION

Cholecystokinin (CCK) and acetylcholine are generally believed to stimulate enzyme secretion from pancreatic acinar cells by activating phospholipase C (PLC). This stimulation increases the levels of inositol 1,4,5-trisphosphate (InsP_3) and diacylglycerol. In turn, these second messengers activate Ca^{2+} mobilization and protein kinase C (PKC), respectively (Gardner and Jensen, 1987; Hootman and Williams, 1987; Matozaki and Williams, 1989). It is now well documented that the secretory response of the pancreatic acinar cells is changed by short- and long-term exposures to secretagogues that act through the PLC intracellular pathway (Larose et al., 1983; Otsuki and Williams, 1983).

Recently, rat pancreatic amylase secretion has been shown to be desensitized by short-term exposure to the muscarinic agonist carbamoylcholine (Cch) and the synthetic CCK agonist caerulein (CAE) (Asselin et al., 1987; Morisset et al., 1991). This desensitization decreased the potency of Cch and CAE in inducing amylase release. For the Cch-treated acini, desensitization was classified as homologous, since it did not significantly modify the release of amylase induced by CAE (Asselin et al., 1987). This Cch-induced desensitization was irreversible for up to 3 h, and could be explained in part by a shift in the high-affinity muscarinic-receptor population towards a low-affinity population (Asselin et al., 1987). Desensitization of CCK-treated acini was classified as heterologous, since it modified the release of amylase induced by several pancreatic secretagogues or activators whose actions are mediated by the release of intracellular Ca^{2+} (CCK, Cch, bombesin, A23187). On the other hand, this treat-

ment did not alter the stimulation caused by secretagogues whose actions are mediated by cyclic AMP (vasoactive intestinal peptide, secretin) (Abdelmoumene and Gardner, 1980). The desensitization induced by CCK was reversible, and appeared to result from modifications at post-receptor loci (Abdelmoumene and Gardner, 1980). After chronic or acute CCK treatments *in vivo*, Williams and colleagues concluded that the observed modifications of amylase release from isolated pancreatic acini in response to CCK or Cch were not the result of any significant change in the pharmacological properties of the CCK or muscarinic receptors (Otsuki and Williams, 1983; Otsuki et al., 1984).

Increases in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) represent important intracellular signalling steps leading to enzyme secretion by pancreatic acinar cells (Ishiguro et al., 1992). These variations in $[\text{Ca}^{2+}]_i$ are controlled by a receptor-associated mechanism involving a G-protein that activates a PLC which finally produces InsP_3 . The InsP_3 receptor is an important element of this mechanism, and it functions as an InsP_3 -sensitive Ca^{2+} -release channel located on specialized portions of the endoplasmic reticulum (Streb et al., 1983; Ferris et al., 1989; Berridge, 1993). Indeed, many investigators have provided direct evidence that the pharmacological and the biological properties of the InsP_3 receptor could be modified by typical desensitization conditions (after exposure to Cch or CCK) (Willems et al., 1989; Wojcikiewicz and Nahorski, 1991).

This study was therefore undertaken to establish whether short-term desensitization of the pancreatic acinar cells with Cch or CAE alters the process of intracellular Ca^{2+} mobilization and,

Abbreviations used: CAE, caerulein; $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concn.; Cch, carbamoylcholine; CCK, cholecystokinin; PK, protein kinase; PLC, phospholipase C; SBTI, soybean trypsin inhibitor.

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if so, to determine whether the alteration is due to changes in the InsP_3 receptor or to variations of InsP_3 production. In order to test these possibilities, acinar cells were desensitized with one stimulus (Cch), and the subsequent evaluations of Ca^{2+} mobilization, InsP_3 -receptor properties and InsP_3 production were performed in response to a second secretagogue, CAE, acting through a different receptor which activates the same effector system. In the counterpart, CAE served as the desensitizing agent in experiments where responses to Cch were evaluated.

MATERIALS AND METHODS

Materials

Purified collagenase (963 units/mg) was purchased from Worthington Biochemicals (Freehold, NJ, U.S.A.) and the Phadebas amylase test kit was from Pharmacia Diagnostics (Piscataway, NJ, U.S.A.). CAE was a gift from Professor R. de Castiglione, Farmitalia, Italy. $[\text{H}]\text{InsP}_3$ (40–60 Ci/mmol) and InsP_3 (trilithium salt) were respectively obtained from Amersham Radiochemical (Arlington Heights, IL, U.S.A.) and LC Services Corp. (Woburn, MA, U.S.A.). Fura-2 (free acid) and Fura-2AM (acetoxymethyl ester) were obtained from Calbiochem (La Jolla, CA, U.S.A.). Anion-exchange resin AG1-X8 (formate form) was from Bio-Rad (Richmond, CA, U.S.A.). All other drugs and chemicals were from commercial sources. The incubation medium (pH 7.4) for acini was prepared as previously described (Larose et al., 1983) and contained 24.1 mM Hepes, 98 mM NaCl, 6 mM KCl, 2.1 mM KH_2PO_4 , 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 11.4 mM glucose, 0.01 % (w/v) trypsin inhibitor, 2.5 % (v/v) amino acid mixture, 2.0 % (v/v) glutamine, 1.0 % (v/v) essential vitamin mixture, 1.0 % (w/v) BSA, 0.5 mM CaCl_2 and 1.2 mM MgCl_2 .

Tissue preparation

Experiments were performed on pancreases from overnight-fasted male Sprague-Dawley rats (240–270 g). The rats were killed by decapitation, and the pancreases were immediately removed and trimmed of fat and mesentery. A suspension of acini was prepared as reported by Peikin et al. (1978). Acini were suspended in the incubation medium for the different studies.

Treatment of acini

Desensitization of the pancreatic acini by Cch or CAE was performed as described by Abdelmoumene and Gardner (1980). Briefly, dispersed acini, resuspended in 80 ml of freshly oxygenated medium, were first incubated with or without 0.1 mM Cch or 0.5 nM CAE, for 60 min at 37 °C. Acini were then washed with 2×100 ml of incubation medium by successive centrifugations at 800 g for 60 s and resuspended in 80 ml of fresh incubation medium for the different studies.

Amylase secretion

After desensitization, control and treated acini from two pooled pancreases were evaluated for their secretory ability by establishing a complete dose-response curve of amylase release to Cch (0.1 μM –1 mM). Amylase secretion was determined as previously described by Larose et al. (1983), using Phadebas reagent. Amylase release was measured during 15 min and expressed as the percentage of total amylase activity initially present (content). The concentration of secretagogue that produced half-maximal stimulation of amylase release was defined as the EC_{50} .

Measurement of $[\text{Ca}^{2+}]$

Ca^{2+} -mobilization studies were performed on acini prepared from one pancreas (0.8–1.8 mg of protein) which had been loaded with Fura-2AM (3.3 μM) at 37 °C for 30 min in the presence of 10 nM MK-329 for the CAE-treated acini and with 10 μM atropine for the Cch-treated acini. These CCK- and acetylcholine-receptor antagonists were used to facilitate replenishment of the intracellular Ca^{2+} pools after the desensitization period. The acini were washed and resuspended in 12 ml of fresh incubation medium containing the respective antagonist. After loading, the preparations were kept on ice until used in order to prevent the resensitization process. Ca^{2+} mobilization was evaluated by establishing dose-response curves to Cch (50 nM–0.1 mM for the CAE-pretreated acini) and to CAE (1.0 pM–1.0 nM for the Cch-pretreated acini), 2 ml of acini being used for each drug concentration. Fluorescence (excitation at 340 and 380 nm, emission at 510 nm) was monitored with an Hitachi F-2000 spectrofluorimeter. Intracellular Ca^{2+} mobilization was calculated from the F_{max} and F_{min} values, obtained by adding 10 μM ionomycin and 10 mM EDTA respectively. The equation used was $[\text{Ca}^{2+}] = 224 \text{ nM} \times (F - F_{\text{min}}) / (F_{\text{max}} - F)$, as described by Grynkiewicz et al. (1985).

Binding of $[\text{H}]\text{InsP}_3$

For the InsP_3 -binding assay, pancreatic acini from eight pancreases (four control, four desensitized) were first homogenized with ten strokes of a Dounce homogenizer (loose pestle), followed by 20 strokes with the tight pestle, in a medium containing 110 mM KCl, 10 mM NaCl, 5 mM KH_2PO_4 , 1 mM dithiothreitol, 2 mM EDTA, 25 mM Tris/HCl, pH 7.6 and 0.01 % (w/v) soybean trypsin inhibitor (SBTI). After centrifugation at 500 g for 15 min, the supernatant was centrifuged at 35000 g for 20 min. The pellet was resuspended in the same medium. Proteins were measured by the method of Lowry et al. (1951), and the microsomes were kept on ice until the binding assay. The microsomes (0.7–1.1 mg of protein) were incubated in a buffer solution containing 25 mM Tris/HCl, pH 7.6, 100 mM KCl, 20 mM NaCl, 5 mM KH_2PO_4 , 1 mM EDTA, 0.01 SBTI and 0.1 % (w/v) BSA. Incubations were performed for 60 min at 0 °C in a final volume of 500 μl , with $[\text{H}]\text{InsP}_3$ (15000 c.p.m.) and selected concentrations of unlabelled InsP_3 . Non-specific binding was determined in the presence of 1 μM InsP_3 . Incubations were terminated by centrifugation at 15000 g for 15 min. The supernatant, which contained the free ligand, was removed, and the microsomal pellet containing the receptor-bound radioactivity was analysed by liquid-scintillation counting. The maximal number of binding sites (B_{max}) was calculated by the equation $B_{\text{max}} = \text{IC}_{50} \times L/T$, as described by DeBlasi et al. (1989), where L represents the quantity of $[\text{H}]\text{InsP}_3$ (c.p.m.) specifically bound in the absence of unlabelled InsP_3 and T represents the total quantity of $[\text{H}]\text{InsP}_3$ (15000 c.p.m.) used in the assay.

Permeabilization of acini and measurements of Ca^{2+} release

For Ca^{2+} mobilization induced by known concentrations of InsP_3 , freshly prepared and washed acini from 16 pancreases (eight control, eight desensitized) (exogenous Ca^{2+} was removed with 2 mM EGTA) were transferred to permeabilization buffer containing 20 mM NaCl, 100 mM KCl, 5 mM MgCl_2 , 20 units/ml creatine kinase, 10 $\mu\text{g/ml}$ oligomycin, 10 μM Ruthenium Red, 10 $\mu\text{g/ml}$ BSA, 0.1 % SBTI, 20 mM Hepes, pH 7.2, and 35 $\mu\text{g/ml}$ saponin (10 min at 37 °C). After saponin treatment, less than 25 % of the cells excluded Trypan Blue.

Release of Ca^{2+} was monitored at 37 °C in 2 ml of permeabilization buffer and evaluated with Fura-2. Each cuvette contained acini equivalent to 7.0 mg of acinar cell proteins. After addition of Fura-2 acid (1 μM), fluorescence was recorded at 510 nm (slit 10) after excitation at 340 nm (slit 10) with a Hitachi F-2000 spectrofluorimeter as previously described (Poitras et al., 1993). Ca^{2+} uptake was initiated by addition of 100 mM phosphocreatine and 3 mM ATP. Each record was calibrated by the addition of a known amount of Ca^{2+} (CaCl_2) to the mixture. The actual free Ca^{2+} concentration of the medium was calculated from the F_{max} and F_{min} values obtained by adding excess Ca^{2+} and EGTA respectively. The equation used was $[\text{Ca}^{2+}] = 224 \text{ nM} \times (F - F_{\text{min}}) / (F_{\text{max}} - F)$.

Mass measurements of InsP_3

For the mass measurement of InsP_3 contents in control and treated acini, InsP_3 was measured by a radio-receptor binding assay as previously described by Matozaki and Williams (1989). Briefly, 600 μl of pancreatic acini prepared from two control or two desensitized pancreases (2.0–3.0 mg of acinar cell proteins plus the incubation medium) were incubated at 37 °C for 10 s with various concentration of CAE (for the Cch-pretreated acini) or Cch (for the CAE-pretreated acini). The incubation was terminated by adding an equal volume of cold trichloroacetic acid (final concn. 10%, v/v). After centrifugation at 1000 g for 15 min at 4 °C, 500 μl of the supernatant was extracted with $5 \times 2.0 \text{ ml}$ of water-saturated diethyl ether and then neutralized with 20 μl of 1 M NaHCO_3 . A 100 μl portion of the extracted supernatant was analysed for InsP_3 contents by using bovine adrenal-cortex microsomes prepared as described previously for the pancreatic acini, but at pH 8.5. The binding conditions were also the same as those used for the InsP_3 receptor with the pancreatic acini. To estimate the possible degradation of InsP_3 by the microsomal membrane-bound 5-phosphatase after the extraction and neutralization procedures, a 500 μl sample was incubated for 15 min at 37 °C in the presence of 1 mg of bovine adrenal-cortex microsomes. After the incubation, the microsomes were removed by centrifugation (1 min, 13000 g) and 100 μl of the extract supernatant was analysed by the radio-receptor binding assay. Under these conditions, the non-specific binding of InsP_3 was around 300 c.p.m. when 1 μM unlabelled InsP_3 was used to displace specific binding.

InsP_3 degradation measurements

To assess InsP_3 turnover after desensitization by Cch and CAE, a mixture of [^3H] InsP_3 (25000 c.p.m.) and ATP (final concn. 1 mM) was incubated with 200 μl of homogenates (3–4 mg of protein) prepared from control and desensitized acini at 37 °C in a final volume of 357 μl . The acini homogenates from two pancreases per group were prepared as described under 'Binding of [^3H] InsP_3 ' with the incubation medium used by Larose et al. (1983). After different time periods, the incubations were mixed with 143 μl of 5% HClO_4 (final concn.). Samples were kept on ice for 30 min and centrifuged at 13000 g for 5 min at 4 °C. HClO_4 was extracted from the supernatants with 600 μl of a 1:1 mixture of Freon/tri-*n*-octylamine (Downes et al., 1986). After centrifugation (1 min at 13000 g), 400 μl was applied to AG1-X8 columns (1 ml wet bed volume) and inositol phosphates were sequentially eluted with the following eluents: $5 \times 3 \text{ ml}$ of water (for inositol); $4 \times 3 \text{ ml}$ of 0.2 M ammonium formate/0.1 M formic acid (for InsP_1); $5 \times 3 \text{ ml}$ of 0.5 M ammonium formate/0.1 M formic acid (for InsP_2); $6 \times 3 \text{ ml}$ of 0.8 M ammonium formate/0.1 M formic acid (for InsP_3); $5 \times 3 \text{ ml}$ of

1.0 M ammonium formate/0.1 M formic acid (for InsP_4 s). The radioactive content of each fraction (3 ml) was evaluated by liquid-scintillation counting.

RESULTS

Effects of Cch and CAE pretreatments on amylase release

The effects of first incubating rat pancreatic acini with Cch at the supramaximal concentration of 0.1 mM caused a shift to the right in the dose-response curve to Cch during a second incubation, as shown in Figure 1(a), a sign of lost acinar cell sensitivity, i.e. desensitization. Indeed, the EC_{50} value of $12.8 \pm 1.8 \mu\text{M}$ from the dose-response curve of Cch-pretreated acini is significantly different from the $0.57 \pm 0.05 \mu\text{M}$ value of the control group. The efficacy of Cch was not affected. In a similar fashion, as shown in Figure 1(b) CAE at 0.5 nM, also a supramaximal concentration for secretion, desensitized the subsequent secretory response to CAE, with EC_{50} values of $100 \pm 1 \text{ pM}$ for the CAE-pretreated acini, significantly different from the control values at $33 \pm 5 \text{ pM}$. A concentration of 0.5 nM CAE was used for desensitization, to avoid any residual effect of CAE observed at higher concentrations (Abdelmoumene and Gardner, 1980).

Measurement of Ca^{2+} mobilization induced by Cch and CAE

Variations in Ca^{2+} affect several biological functions such as enzyme secretion from pancreatic acini. In the desensitization process, such variations in Ca^{2+} mobilization might be regulated

Figure 1 Effects of first incubating pancreatic acini with Cch and CAE on amylase release induced by increasing concentrations of Cch and CAE during a second incubation

Acini were first preincubated without (●) or with 0.1 mM Cch (▲) (a) or 0.5 nM caerulein (▲) (b) for 60 min at 37 °C. The acini were then washed, resuspended in a fresh standard incubation medium and incubated for 15 min with increasing concentrations of Cch (a) or CAE (b) for measurement of amylase release as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three separate experiments.

Figure 2 Effects of first incubating pancreatic acini with Cch or CAE on the increase in $[Ca^{2+}]_i$ induced by 1 nM CAE or 0.1 mM Cch during a second incubation

Acini were first preincubated without (left panels, **a** and **b**), or with 0.1 mM Cch (right panel, **a**) or 0.5 nM CAE (right panel, **b**) for 60 min at 37 °C. The acini were then washed, resuspended in fresh medium and incubated with 3.3 μ M Fura-2AM and 10 μ M atropine (**a**) and 10 nM MK-329 (**b**) for 30 min at 37 °C. These Fura-2-loaded acini were washed, resuspended in fresh medium and re-incubated with 1 nM CAE (**a**) or 0.1 mM Cch (**b**) for measurement of $[Ca^{2+}]_i$. Tracings in the left and right panels represent a single experiment which is representative of five others. (**c**) Control acini were incubated with Fura-2AM as described above. EGTA (10 mM) was added to the loaded acini before their subsequent incubation with 50 pM CAE. Results represent the ratio between the two excitation wavelengths 340 nm/380 nm, and are representative of two other experiments.

during post-receptor events. In Figures 2 and 3, Ca^{2+} mobilization was evaluated under resting and stimulated conditions in control and Cch- or CAE-pretreated acini. During these experiments, atropine or MK-329 was used during the Fura-2 loading period to avoid any residual effects of Cch or CAE, respectively. Furthermore, these specific antagonists allowed the intracellular Ca^{2+} pools to be replenished after the desensitization period. As shown in Figures 2(a) and 2(b), resting $[Ca^{2+}]_i$ was 100 nM in both control and Cch- or CAE-pretreated acini. In response to 1 nM CAE (Figure 2a), $[Ca^{2+}]_i$ rose and reached a peak at 1900 nM in the control and at 1000 nM in Cch-pretreated acini within 5 s, for a net increase of 1800 nM and 900 nM respectively. In Figure 2(b), desensitization by 0.1 mM Cch had the same effect on $[Ca^{2+}]_i$. Indeed, the $[Ca^{2+}]_i$ rose and reached a peak at 800 nM in control acini, compared with 400 nM in CAE-pretreated acini. These elevations were not sustained, and decreased to a plateau

Figure 3 Effects of first incubating pancreatic acini with Cch or CAE on variations in $[Ca^{2+}]_i$ induced by increasing concentrations of CAE and Cch, respectively, during a second incubation

(**a**) Acini were first incubated without (●) or with 0.1 mM Cch (▲) for 60 min at 37 °C. The acini were then washed, resuspended in fresh medium and incubated with 3.3 μ M Fura-2AM and 10 μ M atropine for 30 min at 37 °C. These Fura-2-loaded acini were washed, resuspended in fresh medium and re-incubated with increasing concentrations of caerulein for measurement of $[Ca^{2+}]_i$. This typical experiment is representative of five others. (**b**) Acini were first incubated without (●) or with 0.5 nM CAE (▲) for 60 min at 37 °C. The acini were treated as described in (**a**) and were re-incubated with increasing concentrations of Cch for measurement of $[Ca^{2+}]_i$. This typical experiment is representative of five others.

level which was dependent on the presence of extracellular Ca^{2+} and the occupation of the membrane-bound receptor by the agonist (results not shown). Control studies were also performed to ascertain and confirm that Ca^{2+} mobilization was of cellular origin. As shown in Figure 2(c), when extracellular Ca^{2+} is chelated with 10 mM EGTA, 50 pM CAE is still able to release intracellular Ca^{2+} . The increases in $[Ca^{2+}]_i$ induced by CAE or Cch in control and pretreated pancreatic acini are shown in Figure 3. In Cch-pretreated acini, CAE caused a dose-dependent rise in the $[Ca^{2+}]_i$, but with much less efficacy (Figure 3a); similarly, in CAE-pretreated acini, Cch also caused a dose-dependent increase in $[Ca^{2+}]_i$, and also with less efficacy than in untreated acini (Figure 3b).

InsP₃ binding to its receptor

As possible modifications in the binding properties of the InsP₃ receptor might account for the decreases in Cch- and CAE-induced Ca^{2+} mobilization, the effects of Cch and CAE pretreatments on InsP₃ binding were examined. As shown in Figure 4(a), [³H]InsP₃ binding to membranes which had been prepared from untreated acini (control) and from Cch-pretreated acini was displaced by increasing concentrations of InsP₃, yielding IC₅₀ and B_{max} values of 10.6 ± 1.4 nM and 230 ± 43 fmol/mg of protein for the control acini and 11.2 ± 2.9 nM and 232 ± 34 fmol/mg of protein for the Cch-pretreated acini. Comparable results were obtained in CAE-pretreated acini (Figure 4b), with IC₅₀ and B_{max} values of 9.7 ± 1.3 nM and

Figure 4 Effects of first incubating pancreatic acini with Cch or CAE on the competitive binding of $[^3\text{H}]\text{InsP}_3$ to pancreatic acini microsomes

Acini were first incubated without (●) or with 0.1 mM Cch (▲) or 0.5 nM-CAE (▲) for 60 min at 37 °C. The acini were then washed, resuspended in fresh medium and homogenized as described in the Materials and methods section. Pancreatic acini microsomes (0.7–1.1 mg of protein) were incubated at 0 °C in a medium containing $[^3\text{H}]\text{InsP}_3$ (15000 c.p.m.) and increasing concentrations of unlabelled InsP_3 . After 60 min, the incubation was stopped by centrifugation as indicated in the Materials and methods section. The data are expressed as values relative to total binding observed in the absence of unlabelled InsP_3 and corrected for non-specific binding. Results are means $\pm$ the experimental variation of duplicate values. Similar results were obtained with 10 different microsomes preparations. IC_{50} and B_{max} values obtained in the control and Cch-pretreated acini (a) were 10 nM and 220 fmol/mg of protein and 11.8 nM and 230 fmol/mg of protein respectively. In (b), those values were, for control and CAE-pretreated acini, 9.7 nM and 196 fmol/mg of protein and 10.4 nM and 185 fmol/mg of protein respectively.

164 ± 77 fmol/mg of protein for the control acini and 8.7 ± 1.3 nM and 158 ± 57 fmol/mg of protein for the CAE-pretreated acini. These data clearly indicate that the pancreatic acinar-cell InsP_3 receptors were not affected by Cch and CAE desensitization; these data also agree with those reported from our previous characterization of this receptor (Servant et al., 1994).

Effects of Cch and CAE pretreatments on Ca^{2+} -releasing activity of InsP_3

Since the binding properties of the InsP_3 receptor were not affected by the Cch and CAE pretreatments, the biological Ca^{2+} -channel properties of this receptor might have been affected during the desensitization process. Therefore, the channel properties of this receptor were examined in permeabilized acini in which Ca^{2+} mobilization was induced by increasing concentrations of InsP_3 . As shown in Figure 5(a), at a Ca^{2+} concentration of $3.5 \mu\text{M}$ in the presence of the inhibitors of mitochondrial Ca^{2+} uptake (Ruthenium Red and oligomycin), permeabilized rat pancreatic acini displayed a rapid and ATP-dependent uptake of Ca^{2+} when incubated at 37 °C. Indeed, the ambient Ca^{2+} concentration decreased to about 5 nM, indicating a very high

Figure 5 Effects of first incubating pancreatic acini with Cch or CAE on InsP_3 -induced Ca^{2+} release in permeabilized pancreatic acini

Permeabilized pancreatic acini (7.0 mg of protein) were incubated at 37 °C, and their Ca^{2+} uptake and release were monitored with Fura-2 (free acid) as described in the Materials and methods section. (a) shows a typical fluorescence trace, in which the Ca^{2+} taken up by an ATP-dependent mechanism is partially released upon addition of $15 \mu\text{M}$ InsP_3 , 3 mM ATP, $1 \mu\text{M}$ ionomycin (IO), 4 nmol CaCl_2 (Ca), 1 mM CaCl_2 (Ca^*) and 10 mM EGTA. This trace is representative of several similar experiments in which Ca^{2+} movements were studied in the presence of different concentrations of InsP_3 . (b) represents a dose-response curve of InsP_3 -induced Ca^{2+} release from acini that were first preincubated without (●) or with 0.1 mM Cch (▲) for 60 min at 37 °C. The experiment was performed as described in (a). The amount of Ca^{2+} released (nmol/7.0 mg of protein) by increasing concentrations of InsP_3 is plotted as a function of InsP_3 concentration. This experiment gave an EC_{50} of $1.5 \mu\text{M}$ in both groups and is representative of three similar experiments. (c) represents a dose-response assay of InsP_3 -induced Ca^{2+} release from acini that were first preincubated without (□) or with 0.5 nM CAE (▨) for 60 min at 37 °C. The experiment was performed as described in (b) and is representative of three separate experiments.

accumulation (4.29 nmol) of Ca^{2+} per mg of acini protein. In both Cch- and CAE-pretreated acini this Ca^{2+} uptake was not affected, indicating that the Ca^{2+} -ATPase was not modified under these desensitization conditions (results not shown). Addition of InsP_3 ($15 \mu\text{M}$) caused an immediate release of 6.3 nmol of Ca^{2+} , or 900 pmol of Ca^{2+} per mg of protein, followed by a slow re-uptake (Figure 5a). A subsequent stimulation with $15 \mu\text{M}$ InsP_3 evoked a diminished response, indicating a possible rapid desensitization of the InsP_3 receptor or probably a re-uptake of the mobilized Ca^{2+} in InsP_3 -insensitive pools. The immediate release of accumulated Ca^{2+} in response to $1 \mu\text{M}$ ionomycin

Figure 6 Effects of first incubating pancreatic acini with Cch or CAE on the formation of InsP_3 induced by increasing concentrations of CAE and Cch respectively, during a second incubation

(a) Bovine adrenal-cortex microsomes (1.0 mg of protein) were incubated at 0 °C in a medium containing $[\text{H}]\text{InsP}_3$ (10000 c.p.m.) and increasing concentrations of unlabelled InsP_3 . After 30 min, the incubation was stopped by centrifugation as described in the Materials and methods section. The data are expressed as values relative to total binding observed in the absence of unlabelled InsP_3 (1570 c.p.m.) and corrected for non-specific binding (405 c.p.m.). Results are means $\pm$ the experimental variation of duplicate values. $\circ$, Displacement of the $[\text{H}]\text{InsP}_3$ by a sample of acini cellular extract that was first stimulated with 1 μM Cch for 10 s; Δ , the same sample of cellular extract which was treated with membrane-bound 5-phosphomonoesterase as described in the Materials and methods section. (b) and (c): Acini were first pre-incubated without ($\bullet$) or with 0.1 mM Cch (b) or 0.5 nM CAE (c) ($\blacktriangle$) for 60 min at 37 °C. The acini were then washed, resuspended in fresh medium, and InsP_3 formation was induced by increasing concentrations of CAE for the Cch-pretreated acini (b) and of Cch for the CAE-pretreated acini (c) as described in the Materials and methods section. The amount of InsP_3 formed (pmol/mg of protein) is plotted as a function of increasing concentrations of Cch or CAE. Results are means $\pm$ the experimental variation of duplicate values. In each case, similar results were obtained with at least three different acini preparations.

supports the vesicular nature of the Ca^{2+} -sequestering compartment. In comparison, the release of Ca^{2+} by a maximal concentration of InsP_3 (15 μM) mobilized only a small portion of the sequestered Ca^{2+} , as compared with the huge release caused by ionomycin. These data support the concept of two Ca^{2+} pools in the pancreatic acini, one sensitive and the other insensitive to InsP_3 . As shown in Figure 5(b), InsP_3 released Ca^{2+} dose-dependently in non-treated and Cch-treated acini. In both groups the EC_{50} was 1.5 μM , with comparable dose-response curves.

Figure 7 Effects of first incubating acini with Cch or CAE on the degradation of InsP_3

(a) and (b): Acini were preincubated without (a) or with 0.1 mM Cch (b) for 60 min at 37 °C. The acini were then washed, resuspended in a fresh medium and homogenized as described in the Materials and methods section. Pancreatic acini homogenates (3–4 mg of protein) were incubated at 37 °C in a medium containing $[\text{H}]\text{InsP}_3$ (25000 c.p.m.) and ATP (1 mM) for the indicated time periods. The incubation was stopped by addition of HClO_4 , and the supernatants containing the $[\text{H}]\text{inositol}$ phosphates were applied to AG1-X8 columns; the inositol phosphates were sequentially eluted by addition of ammonium formate/formic acid mixtures of increasing ionic strengths as described in the Materials and methods section: $\blacktriangle$, InsP_3 ; Δ , InsP_2 ; $\square$, InsP_1 ; $\blacksquare$, InsP ; $\circ$, inositol. The amount of inositol phosphates (fmol/mg of protein) is plotted as a function of time. (c) and (d): Acini were preincubated without (c) or with 0.5 nM CAE (d) for 60 min at 37 °C. The acini were treated as described in (a) and (b). Results are means $\pm$ the experimental variation of duplicate values and are representative of three separate experiments.

Similar results were also obtained with CAE-pretreated acini (Figure 5c), indicating that the Ca^{2+} -channel properties of the InsP_3 receptor were not modified during the desensitization process induced by Cch and CAE.

Effects of Cch and CAE pretreatments on InsP_3 production

Several controls had to be performed to validate the InsP_3 -binding assay before it could be used to evaluate production of acinar-cell InsP_3 in response to Cch and CAE. Indeed, it is known that ATP can compete for the InsP_3 -binding sites (Willcocks and Nahorski, 1988). In order to eliminate or evaluate any potential ATP interference, the initial extracts were treated with hexokinase in the presence of glucose to remove all the ATP. This treatment did not affect $[\text{H}]\text{InsP}_3$ displacement, and indicated that endogenous ATP was not interfering (results not shown). To ascertain further that endogenous InsP_3 was the only agent responsible for $[\text{H}]\text{InsP}_3$ displacement, the extracts were treated with membrane-bound 5-phosphomonoesterase, which catalyses the degradation of InsP_3 to $\text{Ins}(1,4)\text{P}_2$ (Connolly et al., 1985; Rana et al., 1986). This compound does not interfere in the binding assay. As shown in Figure 6(a), the $[\text{H}]\text{InsP}_3$ bound to its receptor is totally displaced by increasing concentrations of InsP_3 . However, the displacement observed in response to one acini extract ($\circ$) totally disappeared when the extract was exposed to the membrane-bound 5-phosphomonoesterase (Δ).

This observation strongly strengthens the validity of the assay and confirms InsP_3 as the only displacement agent. The assay was then used to measure InsP_3 formation in response to Cch and CAE in control acini as well as in desensitized acini by Cch or CAE. As shown in Figure 6(b), CAE caused a dose-dependent increase in InsP_3 production with a maximal effect at 1 nM; in Cch-pretreated acini, a net decrease in InsP_3 production was seen under basal conditions, whereas the production in response to CAE was tremendously decreased. As indicated in Figure 6(c), InsP_3 production in response to Cch exhibited a dose-response curve comparable with that of CAE, with a maximal response at 0.5 mM. In CAE-pretreated acini, the basal InsP_3 production remained suppressed and the response to Cch was significantly decreased.

Effects of Cch and CAE pretreatments on InsP_3 turnover

Since the decrease in InsP_3 production observed after Cch and CAE desensitization may result from increased InsP_3 turnover, this possibility was investigated. As shown in Figures 7(a) and 7(b), $[^3\text{H}]\text{InsP}_3$ disappeared at the same rate in control and Cch-pretreated acini over the 60 s studied. As a result of InsP_3 degradation, InsP , InsP_2 and InsP_4 were formed, and again there was no significant difference in the rate of production of these metabolites between the control and the Cch-desensitized groups. When the acini were desensitized by CAE, the degradation of InsP_3 and the production of InsP , InsP_2 and InsP_4 was similar in both the control (Figure 7c) and the desensitized groups (Figure 7d). These results clearly indicate that an increase in InsP_3 turnover does not occur during the desensitization period, and therefore cannot account for the decreased InsP_3 production observed after Cch and CAE pretreatments.

DISCUSSION

The phenomenon of diminished response of an organ or gland subsequent to the initial action of an agent has been observed in many cellular systems (Jun and Triggle, 1981; Decoster et al., 1984). This response can be viewed as a protective cellular regulatory mechanism that can modulate the cell response to wide variations in the agonist concentration.

The present study confirms previous observations on short-term desensitization of rat pancreatic acini with the muscarinic agonist, Cch, and the analogue of cholecystokinin, CAE. Indeed, our present data on amylase release agree with those of Abdelmoumene and Gardner (1980), Hootman et al. (1986) and Asselin et al. (1987) with Cch as the desensitizing agent, and also with those of Abdelmoumene and Gardner (1980) and Morisset et al. (1991) with CAE. The physiological response of the acinar cell to this desensitization resulted in significant shifts to the right of the dose-response curves for amylase release.

Previous studies have also indicated that pancreatic acinar-cell desensitization by Cch and CAE caused important modifications of the muscarinic-receptor properties (Asselin et al., 1987; Larose et al., 1983) and phosphorylation of the CCK receptor (Klueppelberg et al., 1991). However, it is not clear that these receptor modifications are the sole mechanism responsible for the altered secretory responses. In the present study, we have focused our attention on other possible sites of adaptation to the two most potent pancreatic secretagogues known to activate phosphoinositide hydrolysis. Our data provide new findings on Ca^{2+} mobilization, the InsP_3 receptor, the capacity of the InsP_3 receptor to release Ca^{2+} , and finally on InsP_3 production.

Our first approach was to determine if intracellular Ca^{2+} mobilization had been affected by either Cch or CAE desensi-

tization; indeed, failure to increase $[\text{Ca}^{2+}]_i$ to a certain level may impair enzyme secretion. In control acini, Cch and CAE caused dose-dependent rises in $[\text{Ca}^{2+}]_i$, with EC_{50} values comparable with those previously reported by Ansah et al. (1986) and Saluja et al. (1989). The EC_{50} values obtained for $[\text{Ca}^{2+}]_i$, 200 pM and 2 μM in response to CAE and Cch respectively, are slightly higher than those reported for secretion, at 10 pM and 0.6 μM for CAE and Cch respectively (Morisset et al., 1991; the present study, Figure 1; Asselin et al., 1987).

In this study, Cch and CAE desensitization significantly decreased the efficacy of both agonists to release intracellular Ca^{2+} . Such modifications cannot be ascribed to altered receptors, since desensitization by 0.1 mM Cch did not significantly affect the subsequent secretory response to CAE (Asselin et al., 1987). A long-term treatment *in vivo* with a supramaximal dose of CCK-8 did not modify amylase release measured *in vitro* in response to Cch (Otsuki et al., 1984). The absence of receptor modifications thus points towards post-receptor alterations.

The decreased content of Ca^{2+} stores cannot explain the decreased Ca^{2+} mobilization that we observed, since, after desensitization by Cch, the cells were incubated in the presence of atropine, and those desensitized by CAE were treated with MK 329; such a procedure has been shown to replenish Ca^{2+} stores rapidly (Schulz, 1980). A potential candidate responsible for this decreased release of Ca^{2+} may certainly be the InsP_3 receptor and its Ca^{2+} channel. It is now well established that the InsP_3 receptor is a substrate for many protein kinases, including PKA (Supattapone et al., 1988) PKC, Ca^{2+} /calmodulin-dependent PK II (Ferris et al., 1991) and cyclic-GMP-dependent PK (Komalavilas and Lincoln, 1993). It has also recently been suggested that the InsP_3 receptor has intrinsic PK activity and is able to autophosphorylate (Ferris et al., 1992). Even though the InsP_3 receptor is a good substrate for phosphorylation, the functional significance of such an event is still poorly understood. For example, the phosphorylation of the InsP_3 receptor may be stimulatory or inhibitory, and in isolated rat hepatocytes it is proposed that the InsP_3 receptor in native membranes is inhibited and that Ca^{2+} - and cyclic-AMP-dependent PK may act to relieve this inhibition (Joseph and Ryan, 1993). On the contrary, phosphorylation of cerebellar InsP_3 receptor by PKA did not markedly alter $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ -binding characteristics, but shifted the dose-response curve of InsP_3 -mediated $^{45}\text{Ca}^{2+}$ release to the right (Supattapone et al., 1988). In both systems, however, phosphorylation of the receptor was specific to PKA, with no effect from PKC and Ca^{2+} /calmodulin-dependent PK II PKs. Our study, however, clearly indicates that acinar-cell desensitization by Cch and CAE did not affect the biochemical and pharmacological properties of the InsP_3 receptor, although InsP_3 -receptor phosphorylation was not evaluated. The B_{max} and K_d values are comparable in both control and treated acini. Furthermore, the Ca^{2+} -channel property of the receptor was not affected, since the EC_{50} values for InsP_3 -induced Ca^{2+} release as well as maximal Ca^{2+} outputs were identical in control and desensitized acini.

Willems et al. (1989) demonstrated that desensitization of rabbit pancreatic acini by Cch (0.1 mM), CCK-8 (0.1 μM) and phorbol 12-myristate 13-acetate (1 μM) led to an inhibition of InsP_3 -induced Ca^{2+} release. In their study, they reported a rightward shift and a decreased maximal response, which were suggesting that the affinity of the receptor for InsP_3 , as well as the number of InsP_3 receptors for InsP_3 -operated Ca^{2+} channels, was decreased. Our data clearly demonstrate that, under comparable desensitizing conditions by Cch, neither the InsP_3 receptor nor its Ca^{2+} -channel property was affected by Cch or CAE. The stability of the InsP_3 receptor and its Ca^{2+} -channel activity seem to be

dose-dependent. Indeed, Wojcikiewicz and Nahorski (1991) have reported that chronic exposure of SH-SY5Y human neuroblastoma cells to 2 mM Cch for 24 h caused a net decrease in the potency and efficacy of InsP_3 to induce $^{45}\text{Ca}^{2+}$ mobilization. This was associated with a decreased number of InsP_3 receptors, without modification of their affinity. These data clearly indicate that the InsP_3 receptor can be the target of reactions initiated by desensitization, and that its properties and functions are indeed susceptible to the intensity of the phenomenon.

Therefore, under 'mild' desensitization conditions, in which neither the InsP_3 receptor nor its Ca^{2+} -channel properties have been affected, how can we explain the decreased Ca^{2+} release in response to CAE and Cch when the cells were respectively desensitized by Cch and CAE? The observation that Cch and CAE pretreatments resulted in subsequent decreases in total $\text{Ins}(1,4,5)\text{P}_3$ formation in response to CAE and Cch, respectively, strongly suggests that the decreased Ca^{2+} release in the desensitized cells, under our experimental conditions, may simply result from decreased availability of InsP_3 . Again, our data do not agree with those recently published by Vinayek et al. (1993), who were unable to observe any difference between control and 10 mM Cch-desensitized acini in their capacity to release InsP_3 in response to increasing concentrations of CCK-8. However, comparison of both studies suggests a lack of sensitivity in their assay, as they were able to detect significant increases in InsP_3 production only at 1 nM CCK-8, whereas a maximal response was obtained at this CAE concentration in our assay. Also, their data are expressed as 'percent maximal'; this makes it virtually impossible to visualize differences in the efficacy of a hormone or a drug. It is noticeable that dose-response curves for InsP_3 production and for intracellular Ca^{2+} increase are not exactly superimposable. This is probably the reflection of the complex mechanism of cellular Ca^{2+} regulation which involves both release of Ca^{2+} from intracellular pools and influx of Ca^{2+} from the extracellular milieu. Depending on the agonist used, the relative contribution of each of these two sources of Ca^{2+} to the global intracellular Ca^{2+} increase may vary significantly. It is thus not surprising that InsP_3 production (which is mostly associated with intracellular Ca^{2+} release) does not always parallel global intracellular Ca^{2+} increase.

The decreased content of $\text{Ins}(1,4,5)\text{P}_3$ may be the consequence of modifications which occurred before InsP_3 binding to its receptor. The immediate receptor-G-protein effect (or coupling) thus becomes the potential target. Lohse (1993) has recently reported modifications of G-proteins upon desensitization, and Ryu et al. (1990) have also presented evidence of PLC- β_1 phosphorylation resulting in a decreased activity. Finally, the activation of InsP_3 3-kinase and InsP_3 5-phosphatase during the desensitization process could possibly account for InsP_3 decreased. However, this last possibility can be ruled out, since our data clearly indicated that desensitization by Cch or CAE did not affect the rate of InsP_3 degradation and the rate of production of inositol phosphate metabolites.

In summary, these new data on InsP_3 -receptor properties and its Ca^{2+} -channel activity, as well as those on InsP_3 formation, clearly indicate that desensitization of the pancreatic acinar cell by Cch or CAE, under our experimental conditions, involves modifications at post-receptor loci. Our data also point out that intracellular Ca^{2+} mobilization has been severely affected and that this may be related to decreased PLC- β activity, since InsP_3

levels were significantly decreased under conditions where InsP_3 turnover and InsP_3 -receptor activity were unaltered.

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