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# Competitive inhibition by procaine of carbachol-induced stimulus-secretion coupling in rat pancreatic acini

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**1** Procaine (0.03–10 mM) inhibited carbacbol (CCh)-induced amylase release from rat isolated pancreatic acini in a competitive manner. Kinetic analysis of the relation between CCh concentrations and the amount of amylase released in the presence of various procaine concentrations indicated that procaine caused competitive inhibition with the affinity constant ($pA_2$) value of 5.00 ± 0.08.

**2** Receptor binding assay confirmed that procaine (0.01–10 mM) competitively inhibited [N-methyl-$^3$H]-scopolamine chloride ([$^3$H]-NMS) binding to its receptor with binding affinity ($pK_i$) of 4.63 ± 0.10.

**3** Procaine transformed CCh-evoked $[Ca^{2+}]_i$ dynamics: the initial rise in $[Ca^{2+}]_i$ followed by a gradual decay during continuous stimulation with 3 μM CCh was transformed by 0.3 mM procaine to the oscillatory $[Ca^{2+}]_i$ dynamics, which resembled the response to 0.3 μM CCh in the absence of procaine. The initial phase of $[Ca^{2+}]_i$ oscillation corresponded to the initial phase of CCh-induced amylase release in isolated perfused acini.

**4** Procaine (0.3–3 mM) did not inhibit the secretory response to cholecystokinin octapeptide (CCK-8) in isolated incubated acini. A higher concentration of procaine (10 mM) caused weak but significant inhibition of the response to only limited concentrations of CCK-8, 30 and 100 pM. Procaine lower than 10 mM was ineffective on [$^{125}$I]-BH-CCK-8 binding, although procaine (10 mM) caused weak but significant inhibition of the binding.

**Keywords:** Exocrine secretion; pancreas; procaine; amylase release; $[Ca^{2+}]_i$; carbachol; cholecystokinin (CCK)

## Introduction

The term 'stimulus-secretion coupling' originally expressed the sequence of events set in motion by acetylcholine (ACh) which results in the release of catecholamines from the adrenal medulla. This term has since been applied to a variety of secretory systems including the exocrine pancreas (Kanno, 1972). A cardinal intracellular signal in the coupling is the increase in cytoplasmic concentration of calcium ion, $[Ca^{2+}]_i$. Information on $[Ca^{2+}]_i$ dynamics in various types of cells has become available following the development of the bioluminescent probe, aequorin (Cobbold, 1989 for reference) and of $Ca^{2+}$-sensitive fluorescent probes (Grynkiewicz *et al.*, 1985; Tsien *et al.*, 1985). Spatial and temporal $[Ca^{2+}]_i$ dynamics in stimulus-secretion coupling of rat pancreatic acinar cells have been monitored by a digital image analysing technique using Fura-2 (Kanno *et al.*, 1989; Habara & Kanno, 1991).

An approach to elucidate the cardinal role of $[Ca^{2+}]_i$ dynamics in the coupling may be pharmacological analysis with local anaesthetics, since it was reported that the local anaesthetic, tetracaine, reduced both acetylcholine-induced $^{45}Ca^{2+}$ uptake and catecholamine secretion in the chromaffin cells of the adrenal gland (Douglas & Kanno, 1967; Rubin *et al.*, 1967; Rubin, 1970). In the present study, recordings were made of $[Ca^{2+}]_i$ dynamics and of amylase release evoked by stimulation with carbachol (CCh) or the C-terminal octapeptide of cholecystokinin (CCK-8). In addition, binding assays for muscarinic or cholecystokinin (CCK) receptors were used to elucidate the mechanism of the inhibitory action of procaine on stimulus-secretion coupling, which is activated by binding of CCh or CCK-8 to respective receptors in the rat pancreatic acinar cell.

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## Methods

Male rats (Sprague-Dawley) of 200–250 g b.w. were fasted overnight and used for the experiments.

### *Isolation and incubation of pancreatic acini*

HEPES-buffered Ringer Solution (HR; pH 7.4) was used for preparing, incubating, and perfusing isolated acini. The composition of the standard solution was as follows (mM): NaCl 118, KCl 4.7, $NaH_2PO_4$ 1.0, $MgCl_2$ 1.13, $CaCl_2$ 2.5, D-glucose 5.5, HEPES 10.0. The solution was supplemented with soybean trypsin inhibitor (0.1 mg ml$^{-1}$), bovine serum albumin (2 mg ml$^{-1}$), Eagle's minimal essential amino acids medium, and 2.0 mM L-glutamine. The solution was gassed with 100% $O_2$. Pancreatic acini were prepared by the method of Hootman *et al.* (1986) with slight modifications as reported previously (Habara *et al.*, 1986). The isolated acini were preincubated for 30 min prior to the experiments. The dose-response curves were obtained by stimulating acini with increasing concentrations of CCh or CCK-8 in the absence or the presence of various concentrations of procaine for 30 min at 37°C. After 30 min stimulation with secretagogue, 1 ml aliquot was taken from the flask and centrifuged at 12,000 × *g* for 20 s. The supernatant was kept on ice for amylase determination.

### *Perfusion*

An aliquot of acinar suspension was loaded onto a Millipore filter held in a plastic holder (Imamura *et al.*, 1983) and was perfused at a rate of 1 ml HR min$^{-1}$ with a Perista Minipump. Initial collections of perfusate were made at 20 min intervals for 60 min and subsequent collections at every 2 min thereafter. Acini were continuously stimulated with CCh in the absence or the presence of 0.3 mM procaine.

### Receptor binding assay

The possible antagonism of procaine with muscarinic receptors was examined by use of a specific muscarinic antagonist, [$^3$H]-NMS (2.94 TBq mmol$^{-1}$), as described in previous reports (Hootman *et al.*, 1986; Habara *et al.*, 1986). Acini were incubated with 0.5 nM [$^3$H]-NMS in a total volume of 5 ml of HR in the presence of varying concentrations of atropine or procaine or carbachol for 60 min at 37°C. The binding reaction was then stopped by pouring the medium onto Whatman GF/A glass fibre filters in a vacuum filtering manifold and rinsed three times with 5 ml of chilled 0.9% NaCl. Filters were then placed in scintillation vials, extracted for several hours in a cocktail consisting of 10% Solvable in Atomlight, and counted for radioactivity with an Aloka liquid scintillation spectrometer. The ability of procaine to interact with pancreatic acinar CCK receptors was characterized by use of [$^{125}$I]-BH-CCK-8 (81.4 TBq mmol$^{-1}$) as described in a previous paper (Miller *et al.*, 1981). Acini were incubated with 13 pM [$^{125}$I]-BH-CCK-8 in a total volume of 1 ml of HR in the presence of various concentrations of CCK-8 and procaine for 30 min at 37°C. The binding reaction was then terminated as described in [$^3$H]-NMS experiments. Saline containing 0.2% BSA was used for rinsing solution. Radioactivity of the filters was measured with an Aloka gamma counter.

### Fura-2-AM loading and digital image analysis

Fura-2-AM loading and measurement of fluorescence ratio with double excitation wavelengths were carried out as reported previously (Kanno *et al.*, 1989). In brief, acini were loaded with a final concentration of 10 μM Fura-2-AM for 40 min at 37°C with mild shaking. After pelleting and rinsing, acini resuspended in fresh HR were transferred onto a Cell-Tak coated non-fluorescent glass cover slide mounted in a Sykus-Moore chamber and allowed to attach to the cover slide for several minutes. The chamber was then installed on a stage of a modified inverted microscope (TMD-2, Nikon, Tokyo, Japan) equipped with an inlet of perfusing solution and an outlet by suction. At 5 s intervals, a pair of fluorescence images of the emissions (510 nm) formed by excitation at 340 and 380 nm were obtained with a silicon intensified target (SIT) camera and data processing was carried out with an Argus 50 (Hamamatsu Photonics, Hamamatsu, Japan) as described previously (Kanno *et al.*, 1989; Habara & Kanno, 1991). Regulation of excitation wavelengths was controlled with a personal computer which is on-line with a filter exchange unit and SIT camera.

### Measurement of amylase release

Amylase activity present in incubation or perfusion media was determined by the modified method of Bernfeld using soluble Zulkowsy starch as substrate (Kanno, 1975). Amylase activity was expressed as a percentage of the total enzyme activity initially present in acinar cells.

### Affinity constant analysis

Schild plots and standard analysis for linear regression were adopted for the analysis of antagonism in the functional studies (Arunlakshana & Schild, 1959). In the binding studies, the p$K_i$ values are negative logarithms of $K_i$ values which were derived from the $IC_{50}$ value, according to the following equation (Cheng & Prusoff, 1973), $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] and $K_d$ are the concentration of [$^3$H]-NMS ( = 0.5 nM) and dissociation constant ( = 180 pM), respectively. The $K_d$ value of [$^3$H]-NMS was employed from the results of Hootman *et al.* (1991), because the pancreatic acini were prepared according to their method.

### Statistics

The results are presented as mean ± s.e.mean. Statistical analyses were carried out by Student's *t* test.

### Materials

Procaine hydrochloride and HEPES (N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) were purchased from Nacalai Tesque (Kyoto, Japan); collagenase (purified; CLSPA) was from Worthington Biochemical (Freehold, NJ, U.S.A.); soybean trypsin inhibitor (type 1-S), bovine serum albumin (Fraction V) and carbamylcholine chloride (carbachol, CCh) were from Sigma Chemical (St. Louis, MO, U.S.A.); cholecystokinin octapeptide (CCK-8) was from Peptide Institute (Minoh, Japan); Eagle's minimal essential amino acids medium (50 × concentrate) was from Flow Laboratories (Irvine, Scotland); Cell-Tak from Collaborative Research (Bedford, MA, U.S.A.); soluble Zulkowsky starch was from Merck (Darmstadt, Germany); Fura-2-AM from Dojindo Laboratories (Kumamoto, Japan). [N-methyl-$^3$H]-scopolamine chloride ([$^3$H]-NMS), [$^{125}$I]-Bolton & Hunter-labelled cholecystokinin octapeptide (BH-CCK-8), Solvable, and Atomlight were from Du Pont/New England Nuclear (Boston, MA, U.S.A.). Millipore filter (type SM, pore size 5 μm) was from Nihon Millipore Kogyo (Yonezawa, Japan).

## Results

### Effect of procaine on amylase release induced by CCh or CCK-8 stimulation in isolated incubated acini

The effect of procaine on secretagogue-induced amylase release was examined in isolated incubated acini. Figure 1 illustrates the relation between the concentrations of CCh or CCK-8 and the level of amylase release in the absence or the presence of various concentrations of procaine. When the procaine concentration was increased stepwise from zero to 10 mM, the dose-response relation for CCh was shifted to the right in a concentration-dependent manner. The maximal responses to CCh in the presence of various procaine concentrations were not significantly different from the maximal response to CCh alone. In contrast to CCh, the CCK-8-

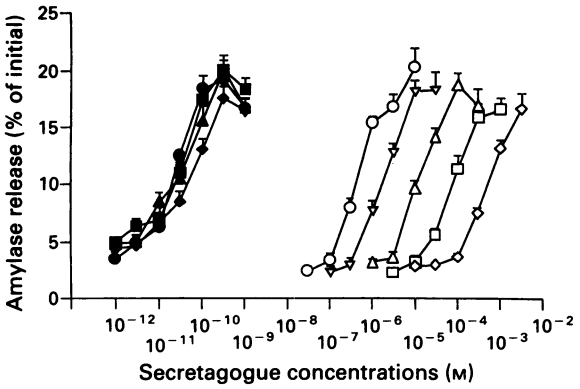


**Figure 1** Dose-response relation of amylase release induced by carbachol (CCh) or cholecystokinin octapeptide (CCK-8) in the absence or the presence of procaine. CCh or CCK-8 at different concentrations as indicated was added to the incubation media and amylase released over 30 min was determined. Each value represents the mean ± s.e.mean obtained from 6 (CCh) or 5 (CCK-8) experiments. Each symbol represents the value obtained in following conditions: CCh without procaine (○); CCh with 0.03 mM procaine (▽); CCh with 0.3 mM procaine (△); CCh with 3 mM procaine (□); CCh with 10 mM procaine (◇); CCK-8 without procaine (●); CCK-8 with 0.3 mM procaine (▲); CCK-8 with 3 mM procaine (■); CCK-8 with 10 mM procaine (◆).

induced secretory response was not affected by procaine (0.3–3 mM), but amylase release induced by CCK-8 (30 pM or 100 pM) stimulation was significantly decreased by 10 mM procaine. The dose-response curve was slightly shifted downward in the presence of 10 mM procaine (Figure 1).

The inhibitory effect of procaine on CCh-stimulated amylase release was further analysed by a method of Arunlakshana & Schild (1959) in Figure 2. The $pA_2$ for procaine was $5.00 \pm 0.08$ ($n = 6$). The slope of the line in Figure 2 was $0.98 \pm 0.04$ ($n = 6$), which was not significantly different from unity. On the contrary, kinetic analysis of the inhibitory action of procaine on CCK-8 induced amylase release was also carried out, but it did not give us a definite conclusion as to the mode of inhibition.

### *Effect of procaine on [$^3$H]-NMS or [$^{125}$I]-BH-CCK-8 binding*

A view that procaine may compete with CCh at the receptor site was further confirmed by examining the inhibitory effect of procaine on [$^3$H]-NMS binding to isolated incubated acini. The results presented in Figure 3 show that procaine competitively inhibited [$^3$H]-NMS binding. The $pK_i$ value for procaine calculated from binding studies was $4.63 \pm 0.10$ ($n = 3$). This value was not significantly different from the $pA_2$ value ($P > 0.05$). The data depicted in the Figure 3 were calculated according to Hill equation and yielded a Hill coefficient of $1.07 \pm 0.06$ ($n = 3$) which was not significantly different from unity.

Procaine concentrations of less than 10 mM were ineffective on [$^{125}$I]-BH-CCK-8 binding, although 10 mM procaine caused weak but significant inhibition of the binding.

### *Effect of procaine on secretagogue-induced amylase release and temporal dynamics of [$Ca^{2+}$]$_i$ in acini*

The effect of procaine on $[Ca^{2+}]_i$ dynamics induced by secretagogues at various concentrations was examined in isolated perfused acini and the corresponding effect on the time course of the secretory response was compared in the other preparations. In the absence of procaine, 0.3 μM CCh induced $[Ca^{2+}]_i$ oscillation (Figure 4b) and sustained amylase release (Figure 4a). Procaine (0.3 mM) inhibited $[Ca^{2+}]_i$ dynamics and secretory response induced by continuous stimulation with 0.3 μM CCh (Figure 4c and d).

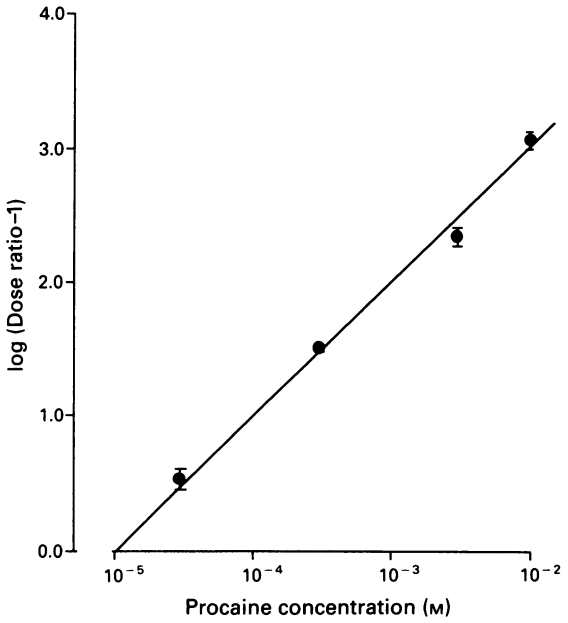


**Figure 2** Schild plot of procaine inhibition of amylase release. Dose-ratios for procaine were calculated from Figure 1. Values are the mean ± s.e.mean of 6 experiments.

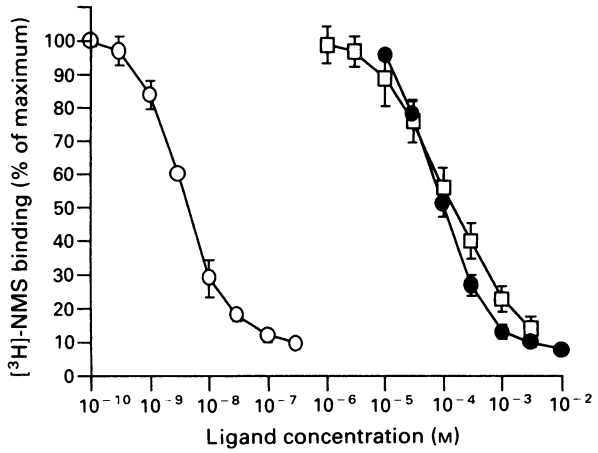


**Figure 3** Effect of procaine on [N-methyl-$^3$H]-scopolamine ([$^3$H]-NMS) binding to acini. Acini were incubated in HR containing 0.5 nM [$^3$H]-NMS for 60 min at 37°C in the presence of varying concentrations of atropine (○), procaine (●), or carbachol (CCh) (□). The results were represented as a percentage of maximum. Values are the mean ± s.e.mean of 3 experiments in which duplicate measurements were carried out.

When isolated pancreatic acini were stimulated with 3 μM CCh, $[Ca^{2+}]_i$ reached peak levels, on which small oscillatory spikes were superimposed, within 2 min after the initiation of the stimulation, and gradually returned to the stable level (Figure 5b). This pattern of $[Ca^{2+}]_i$ was transformed by 0.3 mM procaine to $[Ca^{2+}]_i$ oscillation, which resembled the response to 0.3 μM CCh in the absence of procaine (Figure 5d). Amylase release was rapidly increased and reached the peak level within 4 min after the initiation of stimulation and was maintained at a stable level during stimulation (Figure 5a). The initial phase of $[Ca^{2+}]_i$ oscillation corresponded to the initial phase of amylase release (Figure 5c).

When the preparation was stimulated with 100 μM CCh, $[Ca^{2+}]_i$ rose rapidly to the peak level followed by sharp decay, and amylase release increased to an initial phase followed by second plateau phase. Addition of 0.3 mM procaine prolonged the CCh-evoked $[Ca^{2+}]_i$ increase and initial phase of amylase release.

Procaine (0.3 mM) was ineffective by itself on $[Ca^{2+}]_i$ dynamics and secretory response, and did not inhibit the responses to CCK-8 (10–1000 pM). However, procaine at a higher concentration, 10 mM, caused weak inhibition of the responses to CCK-8. When isolated pancreatic acini were stimulated with CCK-8 at a lower concentration, 10 or 30 pM, $[Ca^{2+}]_i$ increased with oscillatory spikes and amylase release was gradually increased followed by a plateau phase. Addition of 10 mM procaine attenuated the $[Ca^{2+}]_i$ oscillation, and decreased both the rate of rise in amylase release and the level of plateau phase. When the preparation was stimulated with CCK-8 at a higher concentration, 100 or 1000 pM, $[Ca^{2+}]_i$ rapidly reached the peak level followed by a rapid decay, and amylase release was increased rapidly to reach the initial transient phase followed by gradual decay. Addition of 10 mM procaine induced slight inhibition of the $[Ca^{2+}]_i$ increase and delayed the declining phase of the secretory response to CCK-8. Procaine alone (10 mM) was ineffective on both responses.

## Discussion

### *Cholinoceptor*

The present study carried out in the isolated incubated preparations of rat pancreatic acini gave us the following

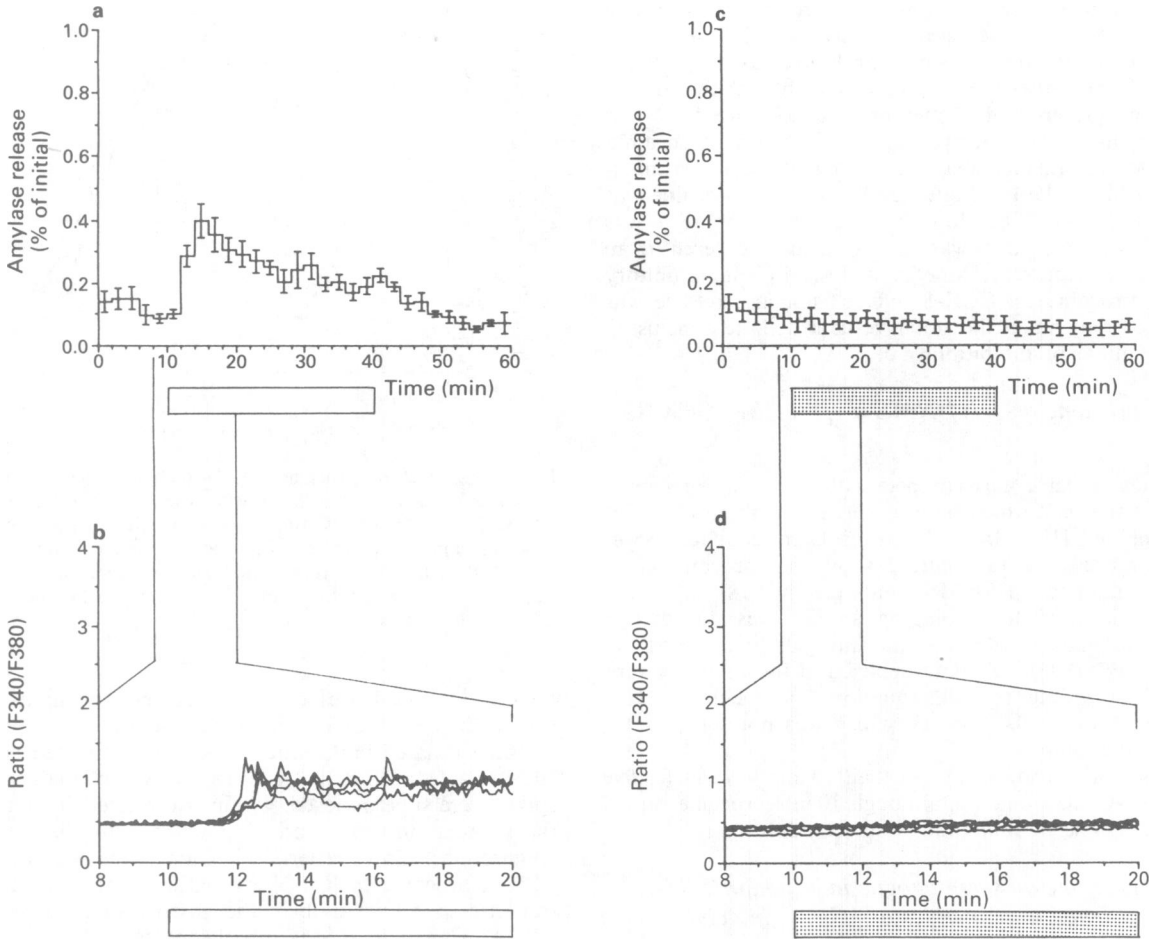


**Figure 4** Effect of 0.3 mM procaine on 0.3 μM carbachol (CCh)-induced amylase release and $[Ca^{2+}]_i$ dynamics in isolated perfused rat pancreatic acini. (a) Time course of amylase release induced by continuous stimulation with 0.3 μM CCh. The values are the mean ± s.e.mean obtained from 5 experiments. Open horizontal bar indicates the period of CCh perfusion. (b) Shows the changes in $[Ca^{2+}]_i$ measured in four different regions of the same acinus, and is part of a continuous recording of $[Ca^{2+}]_i$ dynamics (indicated by the thin lines) at a high magnification of time scale in order to distinguish the $[Ca^{2+}]_i$ dynamics during the perfusion with CCh. $[Ca^{2+}]_i$ is expressed as fluorescence ratio (F340/F380). Similar records were obtained in three other experiments. (c) Time course of amylase released induced by 0.3 μM CCh in the presence of 0.3 mM procaine. The values are the mean ± s.e.mean obtained from 5 experiments. (d) Time course of $[Ca^{2+}]_i$ dynamics induced by perfusion with 0.3 μM CCh in the presence of 0.3 mM procaine. Stippled horizontal bar indicates the perfusion period with CCh and procaine. Similar records were obtained in three other experiments. Other explanations as in (b).

results; (1) the maximal responses to CCh in the presence of various procaine concentrations were not significantly different from the maximal response to CCh alone (Figure 1); (2) the dose-response relation for the CCh-induced amylase release showed a parallel shift to the right by increasing doses of procaine (Figure 1); and (3) the slope of the Schild plots thus obtained was not significantly different from unity (Figure 2). These results are compatible with a view that procaine is a competitive antagonist for the muscarinic receptor in pancreatic acinar cells. This view was further strengthened by the receptor binding experiments, indicating that the $pK_i$ value was not significantly different from the $pA_2$. The coincidence of two values, $pK_i$ and $pA_2$, indicates that the main mechanism of procaine inhibition on CCh-induced pancreatic secretion may certainly be at the muscarinic receptor level, and that the drug may cause little, if any, inhibitory effects on the $Ca^{2+}$ entry step and on the steps between $[Ca^{2+}]_i$ increase and exocytosis. Furthermore, the Hill coefficient calculated from the results of the receptor binding experiments was 1.07 ± 0.06, which was not significantly different from unity, indicating that the mode of procaine binding with the receptor is single order. This conclusion coincides with that obtained by characterizing muscarinic cholinoceptors on rat pancreatic acini (Dehaye *et al.*, 1984).

The conclusion that procaine is a competitive muscarinic antagonist in the pancreatic acinar cells is compatible with previous findings obtained in other types of cells. Richelson *et al.* (1978) concluded by measuring CCh-induced guanosine 3':5'-cyclic monophosphate (cyclic GMP) formation in cultured neuroblastoma clone of mouse that local anaesthetics, including procaine, were apparently competitive inhibitors of CCh. Sharkey *et al.* (1988) concluded from a binding assay using [$^3$H]-quinuclidinyl benzilate that local anaesthetics including procaine could act as antimuscarinic agents in heart and brain of rat. Hisayama *et al.* (1989) suggested that local anaesthetics including procaine may interact directly with the muscarinic $M_2$ receptor sites in a competitive manner in smooth muscle of guinea-pig. In addition, the $pK_i$ value of 4.63 is similar to the value obtained in heart ($pK_i = 5.5$) or medulla ($pK_i = 4.4$) or taenia caecum ($pK_i = 5.03$), all of which have $M_2$ type muscarinic receptors (Sharkey *et al.*, 1988; Hisayama *et al.*, 1989).

### *CCK receptor*

The present experiments demonstrated that procaine at a much higher concentration (10 mM) caused weak but significant inhibition of [$^{125}$I]-BH-CCK-8 binding, whereas the

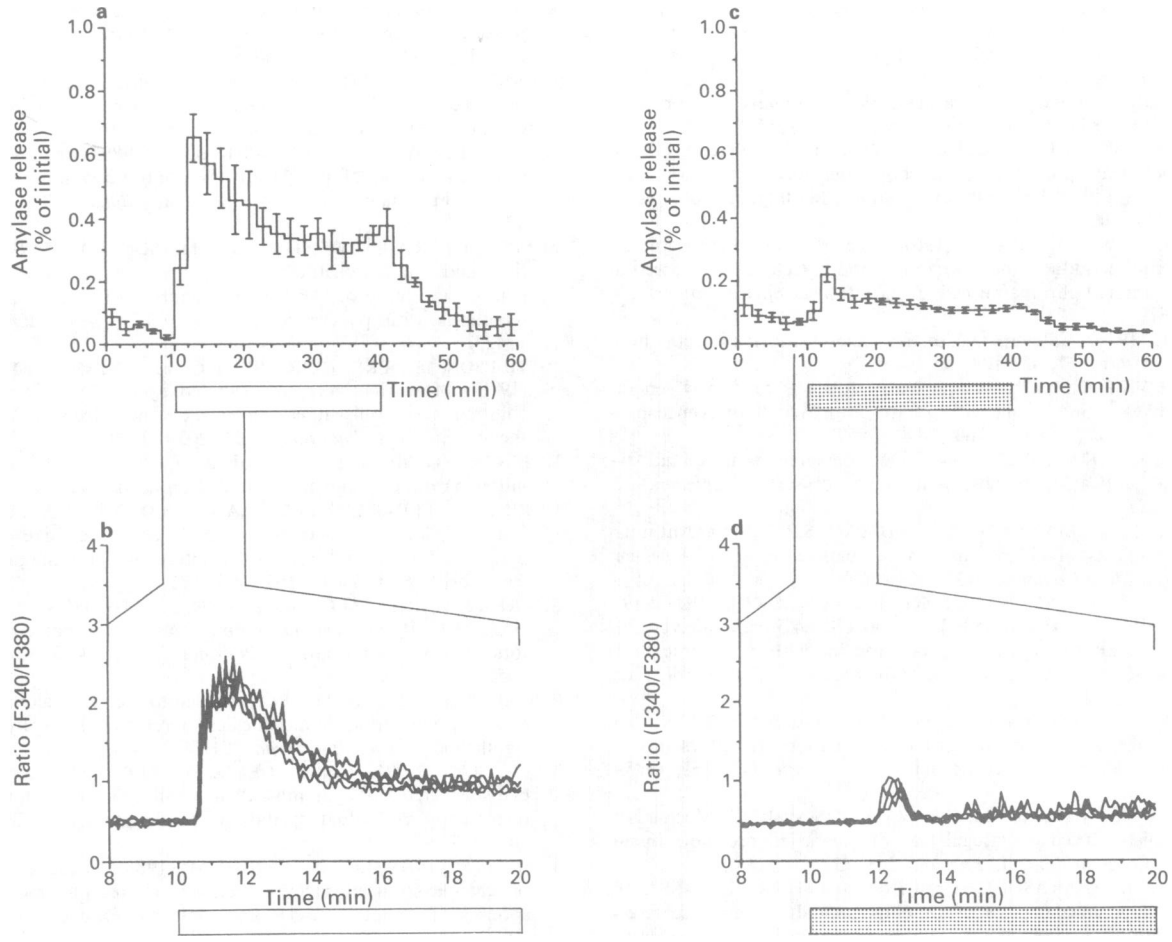


**Figure 5** Effect of 0.3 mM procaine on 3 μM carbachol (CCh)-induced amylase release and $[Ca^{2+}]_i$ dynamics in perfused acini. Symbols and explanations except CCh concentration as in Figure 4.

drug at concentrations lower than 10 mM was without effect on binding. The weak inhibitory effect of procaine on CCK-8-induced secretory response was further confirmed in isolated incubated acini: procaine (0.3–3 mM) failed to inhibit the secretory response evoked by CCK-8 stimulation at various concentrations. A higher concentration of procaine (10 mM) caused a weak but significant inhibition of the response to only limited concentrations (30 and 100 pM) of CCK-8.

The weak inhibitory effect of procaine at a much higher concentration may not be entirely due to the disturbance by procaine at the binding sites of CCK-8 but may also be due to action on the steps between receptor binding and exocytosis. Precise mechanisms of the inhibitory action of procaine on CCK-8-induced responses remained to be elucidated, although the following views have been proposed in various preparations; (1) local anaesthetics including procaine may inhibit the $Ca^{2+}$-induced $Ca^{2+}$ release in various preparations (Thorens & Endo, 1975; Endo, 1977; Yagi *et al.*, 1985) and (2) local anaesthetics including procaine may interfere with $Na^+$ channel, $Ca^{2+}$ channel, various membrane-associated proteins including adenylate cyclase, guanylate cyclase, calmodulin-sensitive proteins, ion-pumping enzymes ($Na^+/K^+$-ATPase and $Ca^{2+}/Mg^{2+}$-ATPase), phospholipase $A_2$, and phospholipase C (for reference see Butterworth & Strichartz, 1990; Charlesworth *et al.*, 1992).

### *Correlation between $[Ca^{2+}]_i$ dynamics and time course of amylase release*

A cardinal intracellular signal in stimulus-secretion coupling is the increase in $[Ca^{2+}]_i$, the dynamics of which may correlate with the time course of secretory responses to secretagogues. To analyse the correlation, however, it should be noted that the $[Ca^{2+}]_i$ dynamics were recorded from a single acinar cell, whereas the secretory response was the mean of a population of many cells. The mean of asynchronous oscillatory $[Ca^{2+}]_i$ dynamics induced by continuous stimulation with CCh at a low concentration (0.3 μM) in different acini may correspond to a gradual increase in secretory response followed by a plateau phase (Figure 4). The dual phase of $[Ca^{2+}]_i$ dynamics, an initial rapid rise followed by gradual decay, caused by continuous stimulation with CCh at a higher concentration (3 μM) apparently resembled the dual phase of the time course of the secretory response (Figure 5). The pattern of $[Ca^{2+}]_i$ dynamics caused by stimulation with 3 μM CCh in the absence of procaine was transformed to the pattern caused by stimulation with 0.3 μM CCh in the solution containing 0.3 mM procaine. These results again support the view that the $[Ca^{2+}]_i$ dynamics correlated with the secretory response, and that procaine inhibits the muscarinic receptor and, in turn, decreases the CCh-induced $Ca^{2+}$ entry.

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