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Caerulein and carbamoylcholine stimulate pancreatic amylase release at resting cytosolic free Ca^{2+}

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1. Cytosolic free calcium concentrations ($[Ca^{2+}]_i$) and amylase secretion were measured in isolated rat pancreatic acini loaded with the intracellularly trapped fluorescent indicator quin2. 2. Both caerulein and carbamoylcholine caused a rapid increase in $[Ca^{2+}]_i$, with a maximal 3-fold increase at 10^{-9} M-caerulein and 10^{-4} M-carbamoylcholine. However, caerulein (10^{-12} M and 10^{-11} M) as well as carbamoylcholine (10^{-7} M) caused a significant stimulation of amylase release, while not inducing any detectable rise in $[Ca^{2+}]_i$. 3. Changes in $[Ca^{2+}]_i$ after addition of either secretagogue were transient and did not last more than 2–3 min. By contrast, when amylase secretion was monitored as a function of time, two distinct secretory phases could be observed upon addition of either carbamoylcholine (10^{-5} M) or caerulein (10^{-10} M). An initial, rapid phase (0–5 min) which caused a 6–7-fold increase above basal, followed by a sustained (5–30 min), but less marked, secretory rate (2–3-fold above basal). 4. Addition of atropine (10^{-4} M) 5 min after carbamoylcholine (10^{-5} M) (i.e. after termination of the rise in $[Ca^{2+}]_i$ and of the first secretory phase) did not cause any significant change in $[Ca^{2+}]_i$, while significantly inhibiting amylase secretion from 5 to 30 min to the same rate observed in the absence of the secretagogue. 5. These results show that caerulein and carbamoylcholine, two agents thought to activate secretion mainly through mobilization of Ca^{2+} from intracellular stores, are capable of eliciting amylase secretion independently of a concomitant rise in $[Ca^{2+}]_i$. Furthermore, with both secretagogues the rise in $[Ca^{2+}]_i$, when observed, was only transient, while the stimulation of amylase release was sustained.

INTRODUCTION

The role of calcium in the regulation of pancreatic enzyme secretion is well established, and has been extensively reviewed (Williams, 1980; Schulz, 1980; Gardner & Jensen, 1981). More recently, the availability of an intracellular fluorescent probe to measure cytosolic free calcium ($[Ca^{2+}]_i$) (Tsien *et al.*, 1982) has allowed a direct demonstration that secretagogues such as carbamoylcholine and caerulein cause an elevation of $[Ca^{2+}]_i$ in pancreatic acini (Ochs *et al.*, 1983; Pandol *et al.*, 1985*a*; Powers *et al.*, 1985). However, in these studies no attempt was made to correlate the kinetics of changes in $[Ca^{2+}]_i$ with those in enzyme secretion. This would have seemed essential, particularly in view of the fact that $[Ca^{2+}]_i$ had returned almost to resting levels after 2–3 min of stimulation (Pandol *et al.*, 1985*a*; Powers *et al.*, 1985). Moreover, several studies have questioned the assumption that a rise in $[Ca^{2+}]_i$ is a necessary and/or sufficient event for the stimulation of secretion in different cell types (Rink *et al.*, 1983; Pozzan *et al.*, 1983, 1984; Di Virgilio *et al.*, 1984; Kolesnick & Gershengorn, 1985). Therefore we have addressed the specific question as to whether an elevation of $[Ca^{2+}]_i$ is sufficient and/or necessary to activate and maintain secretion from isolated acini. To this end we have used caerulein and carbamoylcholine, two agents which are thought to cause secretion mainly by mobilization of Ca^{2+} from intracellular stores (Williams, 1980; Schulz, 1980; Gardner & Jensen, 1981). The data presented here support the concept that pathways independent from an elevation of $[Ca^{2+}]_i$ also regulate enzyme secretion in the exocrine pancreas.

EXPERIMENTAL

Pancreatic acini were prepared as previously described (Bruzzone *et al.*, 1985) from male Wistar rats (approx. 200 g) fed *ad libitum*. After isolation, acini were suspended in RPMI 1640 medium buffered to pH 7.4 at 37 °C with 25 mM-Hepes, containing 0.5% human serum albumin. Quin2/AM, dissolved in dimethyl sulphoxide, was added to the acinar suspension at a final concentration of 50 μM and then loaded for 30–35 min at 37 °C. Control acini were treated in parallel with equal amounts (0.5%, v/v) of dimethyl sulphoxide. After loading, quin2- and dimethyl sulphoxide-treated acini were centrifuged at 600 rev./min for 20 s and resuspended in fresh RPMI 1640 without albumin and kept at room temperature until use. The final cell concentration for fluorescence and secretion studies was $(4–8) \times 10^6$ cells/ml. Fluorescent measurements (excitation wavelength: 339 nm; emission wavelength: 492 nm) and calibration of quin2 fluorescence were performed as already described (Wollheim & Pozzan, 1984). Briefly, acini were pelletted and then resuspended in 2 ml of a modified Krebs–Ringer bicarbonate buffer, containing: 25 mM-Hepes, 5 mM-$NaHCO_3$, 1 mM-$CaCl_2$, 0.1 mM-EGTA and 5 mM-glucose. Measurements of fluorescence were made in glass cuvettes placed in a thermostatted cuvette holder, using a Perkin–Elmer fluorescence spectrophotometer (LS-3). The acinar suspension was continuously stirred with a magnetic stirrer. At the end of each individual trace the fluorescence signal was calibrated (Wollheim & Pozzan, 1984) and finally $[Ca^{2+}]_i$ was estimated according to eqn. (1) of Tsien *et al.* (1982). No changes in the

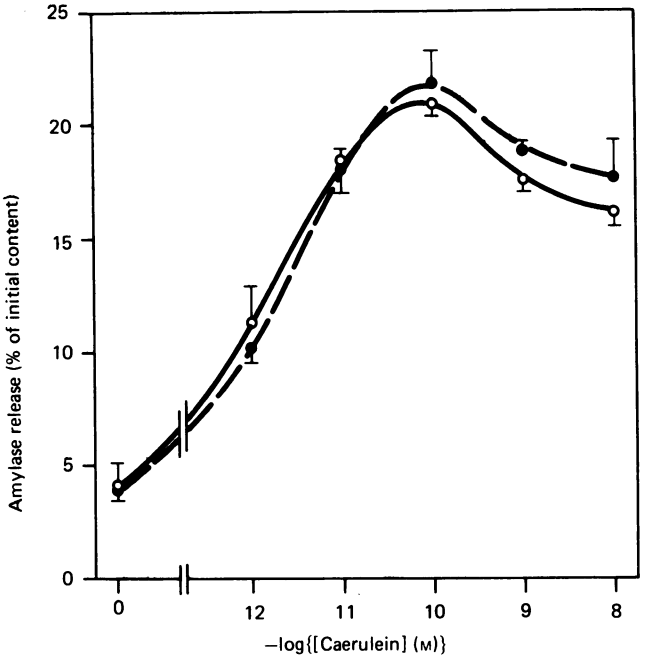

Fig. 1. Caerulein-induced amylase release from quin2-loaded (○) and control (●) rat pancreatic acini

Results are shown as means ± S.E.M. of four independent experiments.

autofluorescence of acini were noticed when any of the substances used in this study was tested. In some experiments, measurements of $[Ca^{2+}]_i$ were made using fura-2, a new Ca^{2+} indicator with increased brightness of fluorescence (Grynkiewicz *et al.*, 1985). Acini were loaded with 1 μM-fura-2/AM for 30 min and subsequently processed as described above for quin2. Fluorescence was recorded using excitation and emission wavelengths of 340 nm and 505 nm, respectively.

Amylase release was measured according to Bernfeld (1955), from either quin2-loaded or control acini incubated at 37 °C. At the beginning of the incubation (time 0) two 0.5 ml aliquots were taken from the acinar suspension, centrifuged at approx. 10000 rev./min for 20 s in a Beckman microcentrifuge and then amylase was determined in both pellet and supernatant to obtain total initial content. The incubation was terminated by centrifugation of two 0.5 ml aliquots of the test vials as described above and amylase secretion was measured in the supernatant. The amount of amylase measured in the supernatant at time 0 was then subtracted from all the results obtained at the end of the various incubation periods and amylase release was expressed as percentage of total initial content.

Results are expressed as means ± S.E.M. Statistical analysis was performed by Student's *t* test for paired data.

The materials and their sources were as described elsewhere (Bruzzone *et al.*, 1985). Quin2/AM was initially purchased from Amersham International, and later from Sigma Chemical Co. Fura-2/AM was purchased from Molecular Probes Inc., Junction City, OR, U.S.A.

RESULTS

The response of both quin2-loaded and control (dimethyl sulphoxide-treated) acini to increasing concentrations of caerulein were superimposable (Fig. 1). A detectable and already significant stimulation was observed at 10^{-12} M-caerulein. Maximal responses were

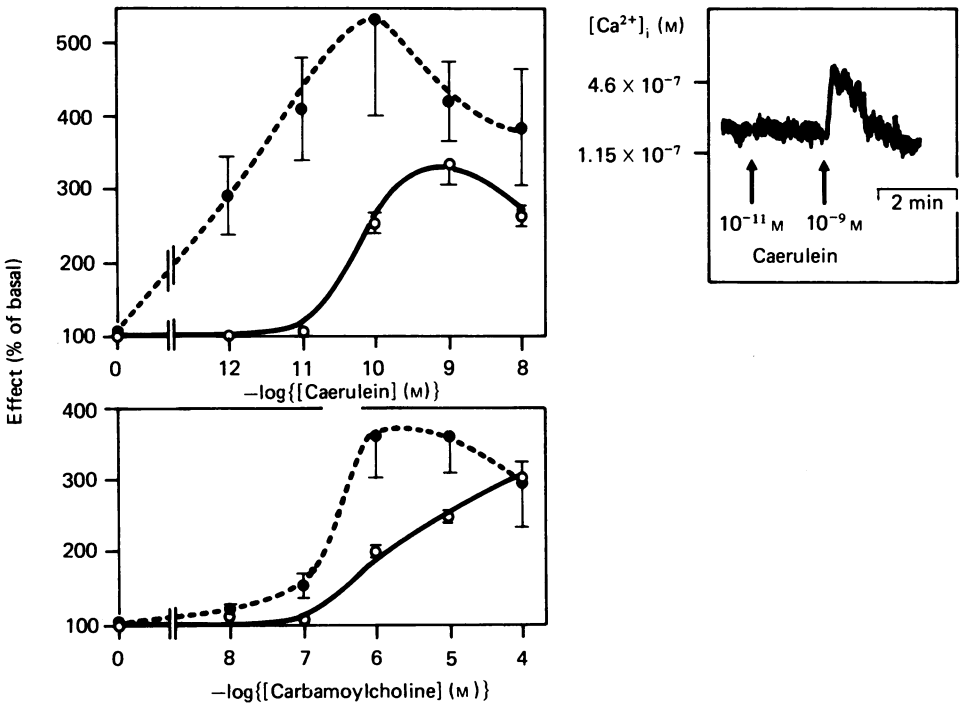

Fig. 2. Effect of caerulein and carbamoylcholine on $[Ca^{2+}]_i$ (○) and amylase (●) release from dispersed rat pancreatic acini

Results are shown as means ± S.E.M. of four to six independent experiments. A typical trace of $[Ca^{2+}]_i$ is shown in the inset.

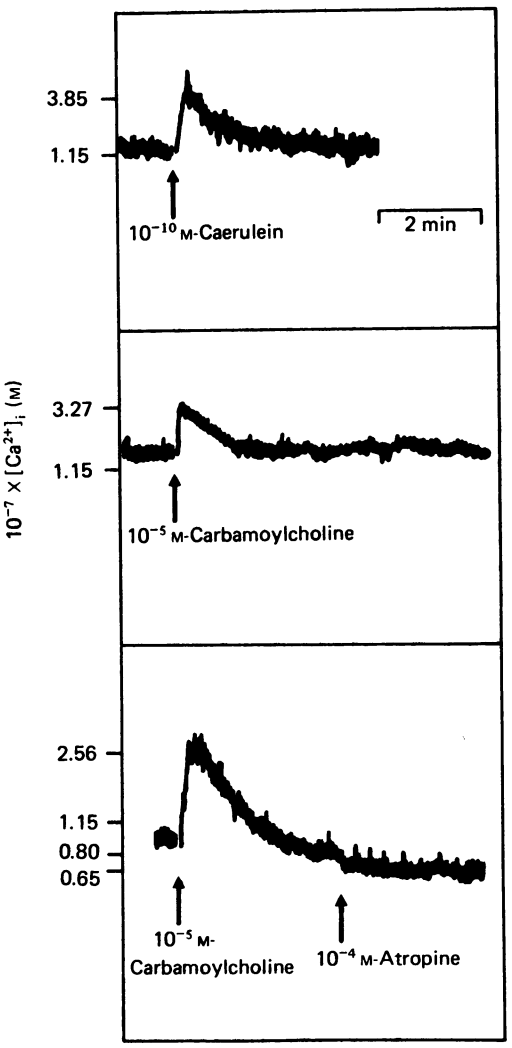

Fig. 3. Effect of caerulein (*a*), carbamoylcholine (*b*) and carbamoylcholine plus atropine (*c*) on $[Ca^{2+}]_i$

For mean values of resting $[Ca^{2+}]_i$ see the text. The mean peak values of $[Ca^{2+}]_i$ in response to 10^{-10} M-caerulein and 10^{-5} M-carbamoylcholine were 352 ± 23 nM ($n = 4$) and 296 ± 18 nM ($n = 4$), respectively.

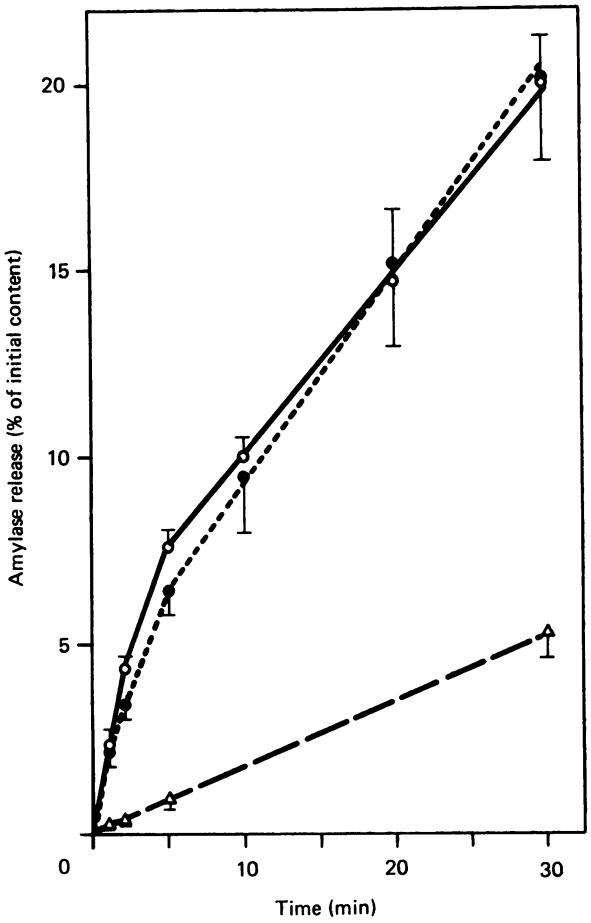

Fig. 4. Time course of amylase release from dispersed rat pancreatic acini

Caerulein (10^{-10} M; ○); carbamoylcholine (10^{-5} M; ●); control (△). Results are shown as means ± S.E.M. of four to six independent experiments.

obtained at 10^{-10} M-caerulein, and higher agonist concentrations (10^{-9} M- and 10^{-8} M-caerulein) evoked less amylase release. Carbamoylcholine also caused a dose-dependent increase in amylase release which was similar in quin2-treated and control acini (results not shown).

Experiments were carried out in parallel to measure $[Ca^{2+}]_i$, by monitoring quin2 fluorescence. In a large series of experiments, the mean resting $[Ca^{2+}]_i$ was 122 ± 3 nM ($n = 18$). Maximal increases in $[Ca^{2+}]_i$ were observed with 10^{-9} M-caerulein (444 ± 29 nM, $n = 6$) and 10^{-4} M-carbamoylcholine (345 ± 32 nM, $n = 6$). However, when the dose-responses for amylase release and quin2 fluorescence were compared (Fig. 2), two important features became apparent. First, maximal stimulatory doses for secretion and $[Ca^{2+}]_i$ did not coincide, i.e. there was a rightward shift of the dose–response curve for $[Ca^{2+}]_i$ when compared with that for secretion. Second, most importantly, caerulein (10^{-12} M and 10^{-11} M) as well as carbamoylcholine (10^{-7} M) were capable of inducing a significant ($P < 0.05$) release of amylase without causing any change in $[Ca^{2+}]_i$. This dissociation was particularly evident in the case of 10^{-11} M-caerulein, which elicited almost 90% of maximal secretion while not changing quin2 fluorescence (Fig. 2, inset). By contrast, 10^{-9} M-caerulein, while stimulating amylase release to the same exent as 10^{-11} M-caerulein, caused a prompt and marked elevation of $[Ca^{2+}]_i$ (Fig. 2, inset). This was also true when fura-2, a new Ca^{2+} indicator with higher quantum yield and absorption coefficient, was used to monitor $[Ca^{2+}]_i$ (results not shown). It should be noted that the amount of fura-2 trapped in this experiment was 0.015 nmol/10^6 cells, while the average quin2 content in the previous experiments was 0.1 nmol/10^6 cells, i.e. the buffering capacity provided by fura-2 was 7-fold lower. Furthermore, in response to those concentrations of either caerulein or carbamoylcholine which did cause a rise, $[Ca^{2+}]_i$ reached a peak value within 30–40 s, after which it returned to basal levels within only 2–3 min of stimulation and thereafter remained stable (Fig. 3). By contrast, amylase release after addition of the same doses of carbamoylcholine (10^{-5} M) and caerulein (10^{-10} M) remained significantly elevated over basal rates during a 30 min period (Fig. 4). Two distinct phases of secretion could be observed when the rate of amylase release/min

Table 1. Effect of atropine on amylase release from dispersed rat pancreatic acini

Amylase release is expressed as percentage of total content released/min during the first (0–5 min) and second (5–30 min) phase of stimulated secretion. Atropine was added at the concentration of 10^{-4} M. Results are given as means ± S.E.M. of four to six independent experiments. Statistical analysis was made by Student's *t*-test for paired data. $*P < 0.01$ versus carbamoylcholine (5–30 min) alone.

	Amylase release		
		5–30 min	
Additions	0–5 min	−Atropine	+Atropine
Control	0.19 ± 0.05	0.18 ± 0.03	0.25 ± 0.04
Carbamoylcholine (10^{-5} M)	1.30 ± 0.15	0.54 ± 0.03	0.25 ± 0.04*
Caerulein (10^{-10} M)	1.52 ± 0.11	0.50 ± 0.09	–

was calculated (Table 1). In the first phase, during the first 5 min of stimulation, the secretory rate in the presence of either carbamoylcholine or caerulein was 6–7-fold greater than in control acini. In the second phase, from 5 to 30 min, secretion was reduced with both secretagogues with respect to the first phase, but remained 2–3-fold greater than basal secretion (Fig. 4 and Table 1). When the incubation period was prolonged for another 30 min (30–60 min), carbamoylcholine-induced amylase release declined slightly, but the secretory rate/min remained significantly above basal (results not shown). Finally, in order to analyse further the dependence of amylase release on an elevation of $[Ca^{2+}]_i$, atropine was added 5 min after carbamoylcholine, i.e. after termination of the rise in $[Ca^{2+}]_i$. This did not cause any significant change in $[Ca^{2+}]_i$ (although a slight decrease in $[Ca^{2+}]_i$ was occasionally seen), but inhibited amylase release from 5 to 30 min to levels similar to basal rates of secretion (Fig. 3 and Table 1). As expected, when atropine was added before carbamoylcholine, both the rise in $[Ca^{2+}]_i$ and the stimulation of amylase release were completely blocked.

DISCUSSION

Caerulein (or its mammalian counterpart, cholecystokinin) and carbamoylcholine are among the most intensively used stimulants of pancreatic enzyme release. It is now generally accepted that upon binding to their specific receptors, both substances stimulate the hydrolysis of phosphatidylinositol bisphosphate to form inositol trisphosphate and diacylglycerol (Berridge & Irvine, 1984). The released inositol trisphosphate could function as a second messenger to mobilize Ca^{2+} from an intracellular store (Streb *et al.*, 1983) which has been identified as the endoplasmic reticulum (Prentki *et al.*, 1984; Streb *et al.*, 1984). It is generally believed that this rise in $[Ca^{2+}]_i$ in turn stimulates exocytosis. We have now demonstrated that both caerulein and carbamoylcholine can also stimulate enzyme release without affecting $[Ca^{2+}]_i$. The most striking dissociation between these two parameters was observed in the case of 10^{-11} M-caerulein. The obvious implication of this finding is the lack of correlation between the stimulation of amylase release and $[Ca^{2+}]_i$. However, since quin2 is also a high-affinity Ca^{2+} chelator, we then investigated whether this property could have resulted in buffering and hence, masking of small $[Ca^{2+}]_i$ transients elicited by this concentration of the secretagogue. To this end we used a newly synthesized Ca^{2+} indicator, fura-2 (Grynkiewicz *et al.*, 1985). This dye displays a much higher fluorescence intensity than quin2 (approx. 30 times on a molar basis), a property which permits a considerable decrease in intracellular dye loading and buffering of $[Ca^{2+}]_i$ transients (Grynkiewicz *et al.*, 1985). Indeed, in our experiments the amount of intracellular fura-2 was 7-fold lower than the average amount of trapped quin2. Under these conditions, 10^{-11} M-caerulein was still unable to cause any detectable rise in $[Ca^{2+}]_i$. However, this does not necessarily imply that inositol trisphosphate is not generated by this concentration of caerulein, but only suggests that it may be produced in small amounts which cannot trigger the release of the intracellular calcium pool. In fact, evidence that caerulein/cholecystokinin stimulates polyphosphoinositide metabolism and inositol trisphosphate production in pancreatic acini has been obtained using much higher (micromolar) concentrations (Rubin, 1984; Rubin *et al.*, 1984; Orchard *et al.*, 1984; Streb *et al.*, 1985; Pandol *et al.*, 1985*a*). Alternatively, since quin2 measures average cytosolic $[Ca^{2+}]_i$, it cannot be excluded that localized and transient increases in $[Ca^{2+}]_i$ occurred, but escaped detection.

The unexpected demonstration that picomolar concentratons of caerulein elicit a 300–400% increase of enzyme release by mechanism(s) independent of a rise in $[Ca^{2+}]_i$ is particularly intriguing. In fact, it has been recently shown that the amount of cholecystokinin released after a meal could account for the postprandial enzyme release in man. Furthermore, plasma cholecystokinin levels after a meal are in the same picomolar range (Beglinger *et al.*, 1985) as the caerulein concentrations which stimulate secretion at resting $[Ca^{2+}]_i$. We realize that caerulein, an amphibian peptide (Erspamer, 1970), is not present in human plasma, but it has been shown that, on a molar basis, caerulein and cholecystokinin-33 are equipotent in stimulating enzyme release (Beglinger *et al.*, 1984; Solomon *et al.*, 1984).

Another important finding in this study is that, even in the case of an elevation of $[Ca^{2+}]_i$, this is a transient event, in contrast with the sustained enzyme secretion. Interestingly, the $[Ca^{2+}]_i$ rise preceeded or was concomitant with the onset of the early fast phase of amylase release. This suggests that an elevation of $[Ca^{2+}]_i$ may play a role in triggering the initial phase of secretion. However, the question remains as to which mechanism(s)

are responsible for both the activation and the prolonged stimulation of secretion at resting $[Ca^{2+}]_i$. A role for cyclic AMP is highly unlikely, since cholecystokinin only raises cyclic AMP at micromolar concentrations (Long & Gardner, 1977). Vice versa, a role for protein kinase C (Nishizuka, 1984) as a possible mediator of Ca^{2+}-independent secretion has been suggested in other cell types (Rink *et al.*, 1983; Di Virgilio *et al.*, 1984; Pozzan *et al.*, 1984). Protein kinase C has now been characterized in pancreatic acini and evidence that activation of this enzyme by phorbol esters can stimulate enzyme secretion has been provided recently (Noguchi *et al.*, 1985). Since phorbol ester-induced secretion is not accompanied by an elevation of $[Ca^{2+}]_i$ (R. Bruzzone, unpublished work), the same pathway may be implicated in caerulein- and carbamoylcholine-stimulated amylase release at resting $[Ca^{2+}]_i$. In fact, preliminary observations in our laboratory have demonstrated that carbamoylcholine causes a sustained increase in diacylglycerol (Trimble *et al.*, 1985), the physiological activator of protein kinase C (Nishizuka, 1984). Furthermore, our results demonstrate that the prolonged phase of secretion needs a continuous agonist–receptor interaction, since addition of atropine after termination of the carbamoylcholine-induced $[Ca^{2+}]_i$ rise blocked the second phase of amylase release without affecting $[Ca^{2+}]_i$.

Taken together, these findings question a major role for an elevation of $[Ca^{2+}]_i$ as a necessary event in the stimulus–secretion coupling of caerulein- and carbamoylcholine-induced amylase release.

After submission of this paper, a report has appeared (Pandol *et al.*, 1985*b*) showing that carbamoylcholine, bombesin and cholecystokinin stimulate only a transient rise in $[Ca^{2+}]_i$ and that sustained enzyme secretion can occur at resting $[Ca^{2+}]_i$.

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