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Pancreatic amylase secretion and cytoplasmic free calcium

Effects of ionomycin, phorbol dibutyrate and diacylglycerols alone and in combination

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Both protein kinase C and Ca^{2+} may act in concert to bring about activation of secretion. This study examined the actions on pancreatic acini of ionomycin and phorbol dibutyrate, which selectively stimulate one or the other of these pathways; their stimulatory effects were compared with those of receptor agonists, such as carbachol and caerulein, which activate phospholipase C. The Ca^{2+} ionophore ionomycin produced a dose-dependent increase in amylase secretion and intracellular free Ca^{2+} (as measured by quin-2). The increase in amylase secretion elicited by carbachol or caerulein was accompanied by a small sustained increase in intracellular free Ca^{2+}, following an initial peak. However, the elevation in intracellular free Ca^{2+} produced by these receptor agonists for a given level of amylase secretion was less than that observed with ionomycin. Phorbol dibutyrate stimulated amylase secretion by a mechanism that was independent of extracellular Ca^{2+}, and no change in intracellular free Ca^{2+} was observed. Synergistic stimulatory effects of phorbol dibutyrate and ionomycin were observed, whether the phorbol ester was present before, or in combination with, ionomycin. Diacylglycerols containing unsaturated fatty acids (1,2-dioleoylglycerol and 1,3-dioleoylglycerol) also stimulated amylase secretion and exhibited synergistic effects on secretion with ionomycin. These findings suggest that complete activation of amylase secretion from the pancreas requires stimulation of both Ca^{2+}-dependent and protein kinase C-activated pathways.

Although secretagogue-induced phospholipase C activation in the pancreas was inferred from the early experiments of Hokin & Hokin (1964), the ability of the pancreatic secretagogues carbachol and caerulein to activate phospholipase C was established recently by measurement of the accumulation of water-soluble inositol phosphates in pancreatic acini (Hokin-Neaverson & Sadeghian, 1984; Rubin, 1984; Rubin *et al.*, 1984). The products of phospholipase C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate are inositol trisphosphate and diacylglycerol, both of which have putative second-messenger functions (for recent reviews, see Berridge & Irvine, 1984; Nishizuka, 1984; Rasmussen *et al.*, 1984; Takai *et al.*, 1984). Inositol trisphosphate has been found to mobilize intracellular Ca^{2+} in a number of cell types (Berridge & Irvine, 1984), including pancreatic acinar cells (Streb *et al.*, 1983). This increase in intracellular free $Ca^{2+}([Ca^{2+}]_i)$ may modulate calmodulin-dependent reactions, including protein phosphorylation (Rasmussen *et al.*, 1984). An increase in diacylglycerol content leads to activation of protein kinase C (Kishimoto *et al.*, 1980; Nishizuka, 1984). Both C kinase and Ca^{2+}/calmodulin-dependent protein kinase activities have been described in extracts of pancreatic acini (Wrenn, 1983; Burnham & Williams, 1984; Wrenn & Wooten, 1984). The Ca^{2+}- and diacylglycerol-dependent pathways may be activated separately by means of Ca^{2+} ionophores, which bypass receptors to elevate cellular Ca^{2+} directly (Kauffman *et al.*, 1980), and by phorbol esters, which substitute for diacylglycerol to activate protein kinase C directly (Castagna *et al.*, 1982; Niedel *et al.*, 1983).

Synergistic effects of Ca^{2+} ionophores and phorbol esters or diacylglycerol have been de-

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+}; PDBu, 4β-phorbol 12,13-dibutyrate.

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scribed in a number of systems, including rabbit pancreas (de Pont & Fleuren-Jakobs, 1984; Rasmussen *et al.*, 1984). In each case, stimulation was comparable with that observed with a receptor agonist. The aim of the present study, therefore, was to investigate whether optimal activation of pancreatic amylase secretion requires stimulation of both a Ca^{2+}-dependent pathway as well as protein kinase C.

Experimental procedures

Materials

Collagenase (chromatographically purified; 400 units/mg) was purchased from Worthington Biochemical Corp., Freehold, NJ., U.S.A. Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.) supplied the bovine serum albumin and Hepes. Soya-bean trypsin inhibitor, 1,2-dioleoylglycerol, 1,3-dioleoylglycerol, 1,2-distearylglycerol, carbachol and PDBu were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Peninsula Laboratories (Belmont, CA, U.S.A.) supplied the caerulein. A solution of essential amino acids (50 × concentrate) was purchased from Gibco Laboratories, Grand Island, NY, U.S.A. and quin-2 acetoxymethyl ester was obtained from Calbiochem–Behring, San Diego, CA, U.S.A. Nyosil 50 was purchased from William F. Nye Specialty Lubricants, New Bedford, MA, U.S.A. Ionomycin (Ca^{2+} salt) was a gift from Squibb Institute for Medical Research, Princeton, NJ, U.S.A. All other chemicals were obtained from Fisher Scientific Co., Fairlawn, NJ, U.S.A., or Sigma Chemical Co.

Preparation of pancreatic acini

Pancreatic acini were prepared from male Sprague–Dawley rats (150–200g) by a modification of the methods of Williams *et al.* (1978) and Halenda & Rubin (1982). The basic medium for these experiments was a Hepes-buffered Krebs–Henseleit medium, pH 7.4, containing Hepes (10mM), dextrose (0.2%), essential amino acids and soya-bean trypsin inhibitor (0.1mg/ml). Unless otherwise stated, the Ca^{2+} and Mg^{2+} concentrations were 1.28mM and 1.18mM respectively. The common bile duct was ligated at its juncture with the duodenum, and 10ml of the above medium containing low Ca^{2+} and Mg^{2+} (0.1mM), collagenase (20 units/ml), and bovine serum albumin (0.25%) was injected into the duct to distend the pancreas. The pancreas was rapidly removed, dissected free of extraneous tissue, then incubated for 10min in 5ml of the same medium, under an atmosphere of O_2 at 37°C with rapid shaking (300 cycles/min). The medium was replaced with fresh medium containing collagenase, and incubation was continued for an additional 20min. Acini were dispersed by vigorous shaking of the flask; a 20ml portion of the basic medium containing 4% (w/v) bovine serum albumin was added, and clumps of undigested tissue were removed by means of a Pasteur pipette. Acini, obtained by centrifugation for 5min at 50*g*, were again washed in medium containing 4% (w/v) bovine serum albumin. After resuspension in medium containing 1% (w/v) bovine serum albumin, acini were incubated at 37°C under O_2 and shaken at 150 cycles/min before use.

Amylase secretion

Acini (approx. 0.2mg of cell protein/ml) were incubated in 500μl portions of medium containing 0.1% bovine serum albumin at 37°C under O_2 and shaken at 300 cycles/min. The drugs used and times of incubation are given in the Results section. Dimethyl sulphoxide was employed to solubilize the ionomycin and PDBu; the final concentration of the solvent, 0.2%, in the medium had no effect on basal amylase release. The incubations were carried out in Microfuge tubes (1.5ml capacity) containing 100μl of silicone oil (Nyosil) below the medium to enable rapid separation of acini from medium. The diacylglycerols were solubilized in chloroform/methanol (9:1, v/v). Portions were dried under N_2, resuspended by sonication in incubation medium (250μl) containing dimethyl sulphoxide and incubation was initiated by the addition of acini suspended in 250μl of medium. The final concentration of dimethyl sulphoxide in these incubations was 1% (v/v), which had no effect on basal secretion. Nyosil was not included in these experiments; instead, the supernatant was rapidly removed after centrifugation. Incubations were terminated by centrifugation for 10s at 15600*g*, and amylase activity released into the medium was determined enzymically by the method of Bernfeld (1955). The total (acinar plus medium) amylase content was determined by lysis of a portion of cells with Triton X-100 (0.2%). Amylase secretion is expressed as a percentage of total content after subtraction of the amylase present in the medium at zero time. Incubations were performed in duplicate within each experiment, and the results are expressed as means ± S.E.M. for at least three different experiments.

Measurement of $[Ca^{2+}]_i$

Acini (approx. 10mg of cell protein/ml) were incubated for 30min in medium containing 0.1% bovine serum albumin and 100μM-quin-2/AM ester at 37°C under O_2 with rapid shaking (300 cycles/min). Acini were then washed three times by centrifugation (5min at 50*g*) and resuspended

in medium containing 0.1% bovine serum albumin. This time period allowed complete hydrolysis of the quin-2/AM ester taken up by the cells. Acini (2mg of cell protein/ml) were resuspended in medium containing 0.1% bovine serum albumin and maintained at 37°C under O_2. A 500μl portion (approx. 1mg of cell protein) was centrifuged for 10s at 15600*g* and resuspended in 2ml of medium containing 0.1% bovine serum albumin at 37°C for fluorescence measurements. Samples were continuously stirred and maintained at 37°C. All additions were made through a light-tight tube with no interruption in monitoring. Fluorescence measurements were performed by using a Perkin–Elmer (model 203) fluorescence spectrophotometer. The excitation and emission wavelengths were 340 and 480nm respectively. The autofluorescence of unloaded cells was unaffected by any of the additions. Basal and stimulated amylase release was unaffected by quin-2. Although acini produced interference in the signal, changes in the signal due to alterations in Ca^{2+} concentrations were still clearly delineated. The intracellular concentration of free Ca^{2+}, $[Ca^{2+}]_i$, was calculated by the method of Tsien *et al.*, (1982).

Results

Comparative effects of ionomycin and receptor agonists on $[Ca^{2+}]_i$ and amylase secretion

The $[Ca^{2+}]_i$ of unstimulated pancreatic acini, as determined by quin-2 fluorescence, was calculated to be 138±5nM (mean±S.E.M. of 18 determinations). Ionomycin caused an immediate increase in $[Ca^{2+}]_i$, which was sustained throughout the experiment (monitored up to 10min) (Fig. 1). The dose–response curve for the elevation of $[Ca^{2+}]_i$ by ionomycin corresponded to that for stimulated amylase release (Fig. 2); in each case, the half-maximal (EC_{50}) value was 0.5μM, and 3μM-ionomycin appears to be virtually maximal for both parameters. The maximum Ca^{2+} concentration measured was approx. 800–850nM, and further increases in the concentration of ionomycin had little additional stimulatory effect.

In contrast with the effects of ionomycin, Ca^{2+}-mobilizing receptor agonists produced a biphasic effect on $[Ca^{2+}]_i$, with an initial peak followed by a sustained phase (Fig. 1). Fig. 3 illustrates dose–response curves showing the effect of carbachol and caerulein on the initial peak and sustained $[Ca^{2+}]_i$ and on amylase secretion. After an initial dose-dependent peak, the sustained Ca^{2+} level reached a plateau at approx. 250nM.

Effects of PDBu

PDBu caused a dose-dependent stimulation of amylase secretion over a 30min incubation period (Fig. 4). The EC_{50} for stimulation was 10nM, and maximal stimulation was observed with 1μM-PDBu. The maximal stimulatory effect of PDBu (Fig. 4), however, was considerably less than the maximal effect observed with ionomycin (Fig. 2), or with carbachol or caerulein (Fig. 3). The time

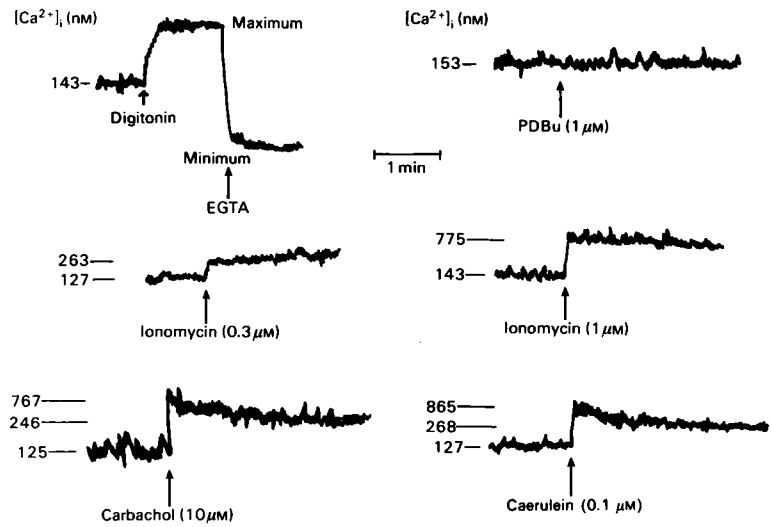

Fig. 1. *Effects of pancreatic secretagogues on the fluorescence of intracellular quin-2 in pancreatic acini*
Calibration of the signal and effects of ionomycin (which causes an immediate and sustained, dose-dependent increase) and the lack of effect of PDBu are shown. The effects of carbachol and caerulein are included for comparison. The estimated $[Ca^{2+}]_i$ values obtained from these individual traces are indicated. The arrows indicate the time at which agents were added. The traces are calibrated by the addition of digitonin, followed by EGTA, as described in the Experimental procedures section and illustrated in top left trace.

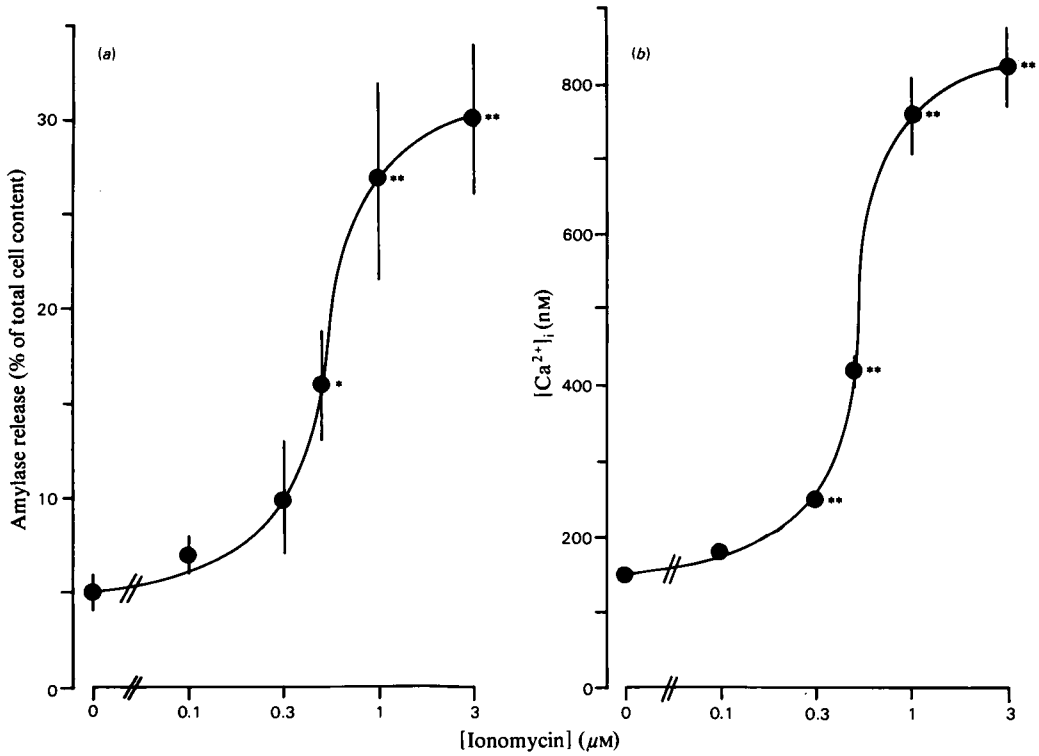

Fig. 2. *Dose–response of ionomycin on (a) amylase release and (b)* $[Ca^{2+}]_i$
Acini were incubated and processed for Ca^{2+} determinations and amylase secretion as described in the Experimental procedures section. The increase in $[Ca^{2+}]_i$ was measured 10s after the addition of ionomycin, although the increase was sustained throughout the period of measurement. Accumulation of amylase activity in the medium was measured over a 30min incubation period to enable a more precise measurement of secretion. Results are given as means ± S.E.M. for three to seven separate experiments. Significant differences from control values, as determined by analysis of variance, were: $*P<0.05$; $**P<0.01$.

Table 1. *Effects of phorbol dibutyrate and ionomycin alone and in combination on amylase secretion and* $[Ca^{2+}]_i$
Acini were incubated in the presence of either PDBu (1 μM) or ionomycin (0.3 μM), or PDBu plus ionomycin. Amylase release was determined after a 30min incubation, whereas $[Ca^{2+}]_i$ was determined from the sustained response. Results are means ± S.E.M. for n separate experiments, with the control value for amylase secretion (5.4 ± 1.0%) subtracted ($n=5$).

Agonist	Amylase secretion (% of total cell content) ($n=5$)	$[Ca^{2+}]_i$ (nM) ($n=5$)
None (control)	–	138 ± 5
PDBu	8.8 ± 1.1*	158 ± 30
Ionomycin	2.9 ± 1.2	250 ± 7*
PDBu plus ionomycin	20.1 ± 2.5*†	248 ± 9*‡

* Significantly different from control ($P<0.01$).
† Significantly greater than an additive effect of each stimulus alone ($P<0.05$).
‡ Not significantly different from ionomycin alone.

course of PDBu-stimulated amylase secretion is illustrated in Fig. 5. A 10min lag period was observed before the stimulatory effect of PDBu became manifest. There was a rapid rate of release between 10 and 15min, followed by a slower sustained effect.

To decrease extracellular Ca^{2+} without depleting intracellular stores, acini were washed in medium devoid of added Ca^{2+} plus EGTA (100 μM), then incubated in medium containing no added Ca^{2+} or EGTA. Under these conditions, PDBu (1 μM) increased amylase secretion from 6.2

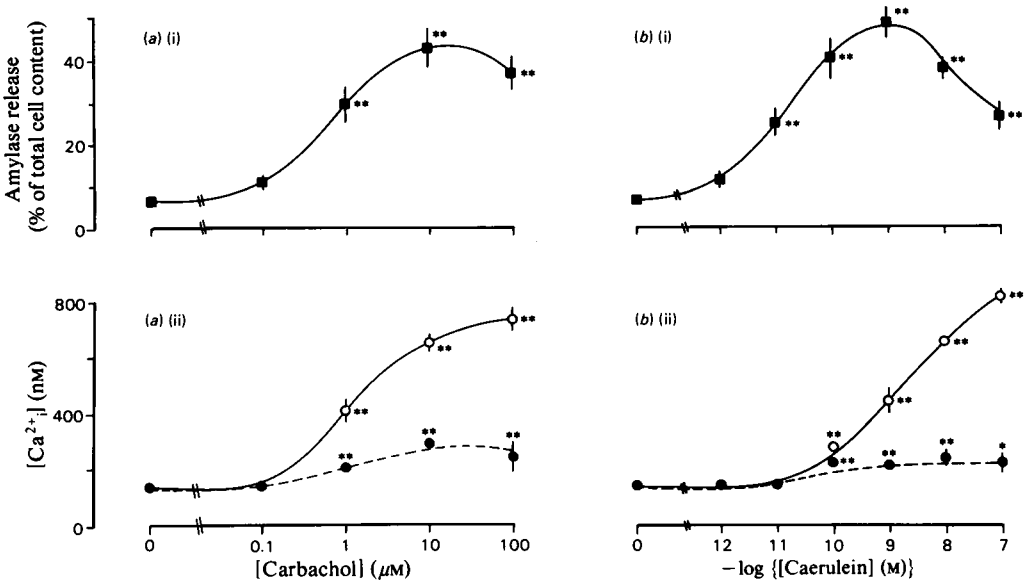

Fig. 3. *Dose-responses of (a) carbachol and (b) caerulein on (i) amylase release and (ii)* $[Ca^{2+}]_i$
Acini were incubated for 30 min and processed for amylase release. The initial peak $[Ca^{2+}]_i$ (○), measured 10 s after addition of agonist, and the sustained levels (●) measured 2 min later, are indicated. The results are means ± S.E.M. for three to eight experiments. Significant differences from control values, as determined by analysis of variance, are as follows: $*P<0.05$; $**P<0.01$.

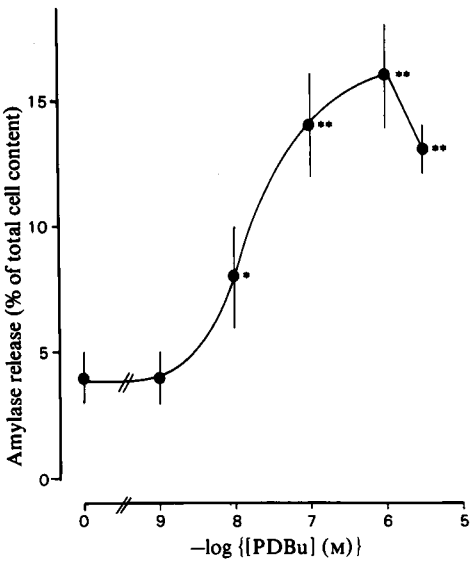

Fig. 4. *Dose-response of PDBu on amylase secretion*
Acini were incubated for 30 min and processed for amylase release as described in the Experimental procedures section. Results are means ± S.E.M. for seven separate experiments. Significant differences from control values, as determined by analysis of variance, are shown by: $*P<0.05$; $**P<0.01$).

(±0.9) to 15.6 (±3.2)% ($P<0.05$; $n=5$). This increase was similar in magnitude to the output observed in the presence of Ca^{2+} (Table 1). In addition to the lack of dependence of the secretory action of PDBu on extracellular Ca^{2+}, PDBu was unable to enhance $[Ca^{2+}]_i$ (Fig. 1 and Table 1). Results are shown only for a maximal stimulatory concentration of PDBu (1 μM), although the same lack of effect was observed throughout the dose range.

Synergistic effects of ionomycin and PDBu

Having demonstrated stimulatory effects of each secretagogue alone, the combined effects of ionomycin and PDBu were investigated by using a maximal stimulatory concentration of PDBu (1 μM) and 0.3 μM-ionomycin. This concentration of ionophore produced a small increase in secretion, and elevated $[Ca^{2+}]_i$ to a level comparable with the sustained level observed with carbachol and caerulein (250 nM) (compare Table 1 with Figs. 2 and 3). Incubation of acini in the presence of PDBu plus ionomycin abolished the lag phase observed with PDBu alone (Fig. 5) and caused a dramatic increase in amylase secretion (Table 1; Fig. 5). After 10 and 30 min, amylase secretion induced by both stimuli in combination was significantly greater than the additive effect of each stimulus alone (Fig. 5; Table 1). However, $[Ca^{2+}]_i$ in the presence of both stimuli was not significantly different from that observed with ionomycin alone (Table 1).

When acini were preincubated for 10 min with PDBu, a synergistic interaction was observed with

ionomycin (0.3 μM), whether or not PDBu was present during the subsequent incubation. Following preincubation with PDBu, amylase secretion evoked by ionomycin alone was comparable with that obtained from acini incubated for 30 min in the simultaneous presence of ionomycin and PDBu (Table 2). Similar results were observed for secretion over a 10 min incubation period (results not shown).

Effects of dioleolyglycerol

1,2-Dioleoylglycerol, a diacylglycerol containing two unsaturated fatty acids at positions 1 and 2, produced a dose-dependent stimulation of amylase secretion over a 30 min incubation period (Table 3). Significant stimulation of secretion was observed with 1,2-dioleoylglycerol at 200 and 400 μg/ml. In the presence of 0.3 μM-ionomycin the action of 1,2-dioleoylglycerol as a secretagogue was considerably enhanced at all concentrations tested (Table 3). Amylase secretion elicited by a combination of 1,2-dioleoylglycerol (at 200 and 400 μg/ml) plus ionomycin was significantly greater than an additive effect of each stimulus alone ($P<0.05$).

Table 3 compares the effects on amylase release of 1,2-dioleoylglycerol, 1,3-dioleoylglycerol (unsaturated fatty acids in positions 1 and 3), and 1,2-distearoylglycerol (saturated fatty acids at positions 1 and 2). 1,3-Dioleoylglycerol produced stimulatory effects comparable with those observed with 1,2-dioleoylglycerol, in that a modest but significant stimulatory effect was found with the dioleoylglycerol alone and synergistic effects were detected in the presence of ionomycin. On the other hand, 1,2-distearylglycerol at the same concentrations possessed no stimulatory effect, either alone or in the presence of ionomycin (Table 3).

Table 2. *Effect of preincubation with PDBu on ionomycin-induced amylase secretion*

Acini were preincubated for 10 min in the presence or absence of PDBu (1 μM), washed, and then incubated for 30 min in the presence or absence of ionomycin (0.3 μM), PDBu (1 μM) or ionomycin plus PDBu. Values are means ± S.E.M. for five separate experiments, with the control values subtracted for clarity. Control values are 4.0 ± 0.4% (control preincubation) and 8.6 ± 2.4% (PDBu preincubation). * $P<0.05$; ** $P<0.01$ for significance of differences from corresponding control values; and † $P<0.05$ for significance of differences from corresponding values from control preincubations.

Incubation (30 min) with:	Preincubation conditions	Amylase secretion (% of total cell content) ... Control	PDBu
Ionomycin		5.8 ± 1.3*	16.8 ± 2.6**†
PDBu		8.0 ± 1.0*	4.2 ± 1.4
Ionomycin plus PDBu		17.6 ± 1.7**	18.6 ± 3.2**

Table 3. *Effect of diacylglycerols and ionomycin on amylase secretion*

Incubations were carried out for 30 min. Basal values for secretion in each experiment have been subtracted for clarity; the mean basal value was 10.8 ± 1.4%. Values are means ± S.E.M. for five to eight experiments. Significant stimulation of secretion by diacylglycerol is depicted as * $P<0.05$; ** $P<0.01$ according to the paired Student's t test. Stimulation was also significantly enhanced by ionomycin alone ($P<0.05$).

Incubation with	Concn. (μg/ml)	Amylase secretion (% of total cell content) Control	Ionomycin (0.3 M)
Nothing (control)		0	9.6 ± 2.5
1,2-Dioleoylglycerol	100	0.1 ± 1.4	12.5 ± 2.1
	200	7.8 ± 1.9*	24.0 ± 3.4**
	400	13.3 ± 3.0**	31.3 ± 3.1**
1,3-Dioleoylglycerol	100	2.3 ± 1.0	13.8 ± 2.9
	200	6.7 ± 1.8*	23.2 ± 3.4**
	400	10.2 ± 3.2*	26.8 ± 4.0**
1,2-Distearoylglycerol	100	0.7 ± 0.3	9.2 ± 1.9
	200	0.0 ± 0.0	10.4 ± 2.6
	400	1.0 ± 0.3	12.2 ± 2.8

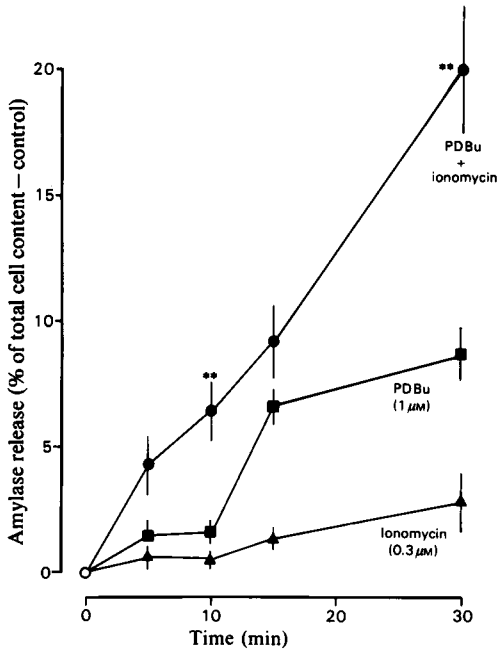

Fig. 5. *Temporal profile of amylase release stimulated with PDBu, ionomycin or PDBu plus ionomycin*
Acini were incubated with PDBu (1 μM), ionomycin (0.3 μM), or ionomycin plus PDBu for various time periods, and amylase release was determined as described in the Experimental procedures section. The control values at each time point have been subtracted for clarity. Data are represented as means ± S.E.M. for five separate experiments. Significantly greater than the additive effect of each stimulus alone, as determined by unpaired Student's *t* test, is shown by $**P<0.05$.

Discussion

Agonists that activate phospholipase C cause increases in tissue $[Ca^{2+}]_i$ and diacylglycerol content (Berridge & Irvine, 1984; Nishizuka, 1984). The aim of the present study was to investigate the two branches of this pathway in exocrine pancreas utilizing the Ca^{2+} ionophore ionomycin, the phorbol ester PDBu, and diacylglycerol, all of which bypass receptor activation. Neither ionomycin (1 μM) nor PDBu (1 μM) causes the activation of phospholipase C, measured as the accumulation of water-soluble inositol phosphates in pancreatic acini (Rubin *et al.*, 1984).

The resting $[Ca^{2+}]_i$ concentration in pancreatic acini was found to be 138 nM, which is similar to the previously reported value of 180 nM obtained by using the quin-2 procedure (Ochs *et al.*, 1983). The addition of ionomycin induced a rapid and sustained rise in $[Ca^{2+}]_i$, as expected. This rise paralleled ionomycin-induced elevations in amylase release with respect to time course (Halenda & Rubin, 1982) and ionophore concentrations, indicating that the stimulant action of ionomycin was mainly mediated by the rise in $[Ca^{2+}]_i$. At high concentrations of ionomycin, the $[Ca^{2+}]_i$ did not exceed 850 nM; this value corresponds to the Ca^{2+} set point for mitochondrial Ca^{2+} uptake in pancreatic acini, which was reported to be 660 nM (Streb & Schulz, 1983). Thus, in the exocrine pancreas, the mitochondrial uptake system may serve to buffer further increases in $[Ca^{2+}]_i$, as brought about, for example, by increasing concentrations of ionomycin.

In contrast with the maintained increase in $[Ca^{2+}]_i$ elicited by ionomycin, receptor agonists initially caused a large transient increase in $[Ca^{2+}]_i$, presumably due to inositol trisphosphate-induced mobilization of cellular Ca^{2+}. This transient increase was followed by a rapid decline to a lower sustained level, presumably due to Ca^{2+} influx into the acinar cell. The sustained increase in $[Ca^{2+}]_i$ brought about by ionomycin can support the secretory response, since the dose–response curves for an ionomycin-induced increase in $[Ca^{2+}]_i$ and amylase secretion were virtually superimposable. However, a lower concentration of ionomycin (0.3 μM), which produced a sustained increase in $[Ca^{2+}]_i$ (250 nM) comparable with that elicited by carbachol and caerulein, exhibited a negligible stimulatory effect on amylase secretion; in contrast, the receptor agonists, at these concentrations, elicited a powerful secretory response. This indicates that sustained elevation of $[Ca^{2+}]_i$ alone is insufficient to evoke a full secretory response. Although higher concentrations of ionomycin stimulated amylase secretion to an extent similar to that observed with Ca^{2+}-mobilizing receptor agonists, the sustained high $[Ca^{2+}]_i$ obtained with high ionomycin concentrations is unlikely to be physiologically relevant. Moreover, $[Ca^{2+}]_i$ at these supraphysiological levels may activate the C kinase in the absence of diacylglycerol (Nishizuka, 1984).

The ability of PDBu to stimulate amylase secretion with no discernible increase in $[Ca^{2+}]_i$ is in agreement with previous studies, which showed that pancreatic amylase secretion may be stimulated by phorbol myristate acetate with no observable effects on Ca^{2+} metabolism (Gunther, 1981; Knight & Koh, 1984). Moreover, the EC_{50} of PDBu induced stimulation of amylase release (10 nM) (cf. Fig. 4) corresponds to the values reported for the dissociation constant (8 nM) for PDBu binding to the C kinase and the activation constant (8 nM) for activation of C kinase (Nishizuka, 1984). Thus our present findings, taken together with previously obtained data, support the hypothesis that PDBu bypasses the requirement for activation of phospholipase C and

acylglycerol production to activate the C kinase directly. This hypothesis is strengthened by the observations that phorbol myristate acetate causes a translocation of the C kinase from the soluble fraction, concurrent with the enhancement of amylase secretion (Wooten & Wrenn, 1984).

The finding that the stimulatory effect of phorbol esters, which is independent of Ca^{2+}, is considerably smaller than that of Ca^{2+}-mobilizing receptor agonists indicates that activation of the C kinase alone is insufficient to sustain a maximal secretory response. This was confirmed by the finding that the addition of a threshold concentration of ionomycin abolished the lag phase and markedly enhanced the stimulatory action of PDBu. The synergistic effects of a phorbol ester and a calcium ionophore are similar to those reported in several other secretory systems (Kojima *et al.*, 1983; Yamanishi *et al.*, 1983; Zawalich *et al.*, 1983; Putney *et al.*, 1984). It is therefore apparent that activation of both the Ca^{2+} and protein kinase C components of the intracellular signalling system are required for an optimal secretory response, as exhibited by Ca^{2+}-mobilizing receptor agonists which activate phospholipase C. The finding that preincubation with PDBu was sufficient to produce a synergistic effect during a subsequent incubation with ionomycin in the absence of PDBu suggests that the preincubation period allowed sufficient PDBu to accumulate within the acinar cell to activate the C kinase, thus enabling the subsequent increase in Ca^{2+} to trigger the full secretory response. Alternatively, PDBu may elicit a persistent change in protein kinase C that allows this process to interact with Ca^{2+} to maximize the secretory response.

Additionally, both 1,2-dioleoylglycerol and 1,3-dioleoylglycerol stimulated amylase release and produced synergistic interactions with ionomycin, which are consistent with a direct effect of acylglycerol on protein kinase C. Similar synergistic effects have been reported for platelet secretion with a synthetic diacylglycerol [1-oleoyl-2-acetylglycerol] and ionomycin (Rink *et al.*, 1983). Relatively low concentrations of the synthetic diacylglycerol were required compared with the concentrations of dioleoylglycerol employed in the present study. This may be due to the greater hydrophobicity of dioleoylglycerol, which may impair its ability to reach the active site. The fact that the action of dioleoylglycerol was not shared by 1,2-distearylglycerol suggests that it was not due to non-specific hydrophobic effects. Diacylglycerols containing at least one unsaturated fatty acid at positions 1 or 2 are capable of activating protein kinase C, whereas diacylglycerols containing two saturated fatty acids are much less effective (Mori *et al.*, 1982). In this context, prolactin secretion from pituitary-tumour (GH_3) cells is stimulated by 1,2-dioleoylglycerol and 1,3-dioleoylglycerol, whereas, 1,2-distearoylglycerol is ineffective (Martin & Kowalchyk, 1984). The synergistic effects of dioleoylglycerol plus ionomycin, taken together with the lack of effect of 1,2-distearylglycerol, are therefore indicative of a direct action of the diacylglycerol analogues on protein kinase C. Under these experimental conditions, the requirement for endogenous acylglycerol production in the stimulation of amylase secretion is bypassed.

In conclusion, the present studies demonstrate that the activation of both the Ca^{2+}-dependent and C kinase pathways are required for an optimal secretory response in the exocrine pancreas. These experiments thus provide support for the general concept promulgated by Nishizuka (1984) that increased Ca^{2+} availability and protein kinase C-induced protein phosphorylation act synergistically to promote a full biological response during cell activation.

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