Regulation of protein phosphorylation in pancreatic acini

Distinct effects of Ca^{2+} ionophore A23187 and 12-*O*-tetradecanoylphorbol 13-acetate

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Regulation of protein phosphorylation in isolated pancreatic acini by the intracellular messengers Ca^{2+} and diacylglycerol was studied by using the Ca^{2+} ionophore A23187 and the tumour-promoting phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate. As assessed by two-dimensional polyacrylamide-gel electrophoresis, the phorbol ester (1 μM) and Ca^{2+} ionophore (2 μM) altered the phosphorylation of distinct sets of proteins between M_r 83000 and 23000 in mouse and guinea-pig acini. The phorbol ester increased the phosphorylation of four proteins, whereas the ionophore increased the phosphorylation of two proteins and, in mouse acini, decreased the phosphorylation of one other protein. In addition, the phorbol ester and ionophore each caused the dephosphorylation of two proteins, of M_r 20000 and 20500. Administered together, these agents reproduced the changes in phosphorylation induced by the cholinergic agonist carbamoylcholine. The effects of the phorbol ester and ionophore on acinar amylase release were also studied. In mouse pancreatic acini, a maximally effective concentration of phorbol ester (1 μM) produced a secretory response that was only 28% of that produced by a maximally effective concentration of carbamoylcholine, whereas the ionophore (0.3 μM) stimulated amylase release to two-thirds of the maximal response to carbamoylcholine. In contrast, in guinea-pig acini, the phorbol ester and carbamoylcholine evoked similar maximal secretory responses, whereas the maximal secretory response to the ionophore was only 35% of that to carbamoylcholine. Combination of phorbol ester and ionophore resulted in a modest synergistic effect on amylase release in both species. It is concluded that cholinergic agonists act via both diacylglycerol and Ca^{2+} to regulate pancreatic protein phosphorylation, but that synergism between these intracellular messengers is of limited importance in stimulating enzyme secretion.

INTRODUCTION

Acetylcholine, acting via muscarinic receptors, is a major physiological regulator of pancreatic acinar cell secretion, ion transport and metabolism (Goldfine & Williams, 1983). An early event in acetylcholine action (within 1 min) is an increased turnover of membrane phosphatidylinositides (Putney *et al.*, 1983). Studies conducted in pancreas and other tissues indicate that the primary reaction elicited by acetylcholine and other agonists is the hydrolysis of plasma-membrane phosphatidylinositol 4,5-bisphosphate to 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (Banschbach *et al.*, 1981; Berridge *et al.*, 1983; Putney *et al.*, 1983; Thomas *et al.*, 1983). These two products subsequently activate separate regulatory pathways in the cell interior. Further work by Nishizuka and colleagues suggests that diacylglycerol regulates protein phosphorylation by increasing the Ca^{2+} affinity of a Ca^{2+} - and phospholipid-dependent protein kinase, called 'protein kinase C' (Nishizuka, 1984). Involvement of protein kinase C in pancreatic secretion is implicated by studies demonstrating the secretagogue-like action of tumour-promoting phorbol esters such as TPA, which can substitute for diacylglycerol in sensitizing protein kinase C to Ca^{2+} (Gunther, 1981; De Pont & Fleuren-Jakobs, 1984; Singh, 1985). A regulatory role for phosphatidylinositol metabolism in Ca^{2+} mobilization was suggested by Streb *et al.* (1983), who reported that inositol 1,4,5-trisphosphate stimulated the release of Ca^{2+}

from a non-mitochondrial store in permeabilized pancreatic acini. Considerable evidence supports the belief that intracellular Ca^{2+} is an important mediator of acetylcholine action in the pancreas (Schulz & Stolze, 1980; Williams, 1980; Burnham & Williams, 1984a). Like the diacylglycerol pathway, the Ca^{2+} pathway also may regulate protein phosphorylation, but by means of calmodulin-dependent protein kinases and phosphatases (Cohen, 1982).

Cholinergic analogues have been shown to alter the phosphorylation of specific proteins in isolated pancreatic acini and pancreatic slices (Burnham & Williams, 1982; Freedman & Jamieson, 1982a; Roberts & Butcher, 1983). However, the role of protein kinase C and calmodulin-dependent enzymes in mediating these changes in phosphorylation *in situ* remains to be established. In the present study, therefore, the relative importance of the diacylglycerol and Ca^{2+} pathways in eliciting specific phosphorylation events was examined in pancreatic acini by using TPA and the Ca^{2+} ionophore A23187. In addition, we evaluated the possibility of a synergistic relationship between these two pathways in stimulating pancreatic enzyme secretion, which has been reported in other secretory systems (Kaibuchi *et al.*, 1983; Knight & Baker, 1983; Kajikawa *et al.*, 1983; Katakami *et al.*, 1984). TPA and A23187 separately altered the phosphorylation of sets of proteins that were very similar in mouse and guinea-pig acini, and together they mimicked the effects on phosphorylation of the cholinergic agonist

Abbreviation used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

carbamoylcholine. However, TPA and A23187 exerted a relatively small synergistic effect on acinar amylase release, and the relative extent to which each of these agents singly stimulated secretion varied considerably between the two species studied.

EXPERIMENTAL

Materials

Benzamidine, dithiothreitol, 2-mercaptoethanol, carbamoylcholine, TPA, phorbol 12,13-dibutyrate and ionophore A23187 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; *sn*-1,2-dioctanoylglycerol was from Avanti Polar Lipids Inc., Birmingham, AL, U.S.A.; bovine serum albumin (fraction V) was from Miles Laboratories, Elkhart, IN, U.S.A.; carrier-free [32 P]P_i was from Amersham Corp., Arlington Heights, IL, U.S.A.; Ampholines were from LKB, Gaithersburg, MD, U.S.A.; Nonidet P-40 was purchased from Particle Data Lab., Elmhurst, IL, U.S.A.; Ultrapure grade of urea was from Schwarz/Mann, Spring Valley, NY, U.S.A. Other chemicals for electrophoresis gels and solutions were purchased either from Sigma or from Bio-Rad Laboratories, Richmond, CA, U.S.A. Cholecystokinin octapeptide was given by Squibb Institute, Princeton, NJ, U.S.A. The ionophore A23187 was dissolved and stored in ethanol at 4 °C and used at a final ethanol concentration of 0.25% (v/v); TPA was dissolved in dimethyl sulfoxide and used at a final dimethyl sulfoxide concentration of 0.25% (v/v). Neither ethanol nor dimethyl sulfoxide at these concentrations had any noticeable effect by themselves on 32 P labelling of proteins or amylase secretion.

Preparation and incubation of pancreatic acini

White Swiss male mice weighing 18–24 g were starved overnight before use. Isolated pancreatic acini were prepared by collagenase digestion by the procedure of Williams *et al.* (1978), except that bovine serum albumin (2 mg/ml) replaced hyaluronidase and chymotrypsin during enzymic digestion of the pancreas. Male albino guinea pigs weighing 200–250 g were starved overnight before preparation of pancreatic acini by a similar procedure (Schultz *et al.*, 1980).

Preparation and stimulation of 32 P-labelled pancreatic acini followed the procedure of Burnham & Williams (1982), except that the [32 P]P_i concentration during the labelling period was 200–250 μ Ci/ml. A 5 min stimulation period was chosen, as this was the time at which maximal changes in protein phosphorylation were observed in mouse acini (Burnham & Williams, 1982). Procedures for incubation and stimulation of unlabelled pancreatic acini were as described previously (Williams *et al.*, 1978). Amylase released into the medium by unlabelled acini was assayed by the method of Jung (1980) and expressed as the percentage of total cellular amylase released during the incubation period (Williams *et al.*, 1978).

Preparation of total particulate and soluble fractions and procedure for two-dimensional gel electrophoresis

After stimulation, 1 ml samples of 32 P-labelled pancreatic acini were centrifuged in an Eppendorf micro-centrifuge, and the cell pellet was resuspended by sonication in 1 ml of homogenization buffer [0.25 M-sucrose/10 mM-Hepes (pH 7.0)/2 mM-EDTA/0.1 M-NaF/2 mM-sodium pyrophosphate/1 mM-benzamidine]

(Burnham & Williams, 1982). After centrifugation at 100000 g for 60 min, the supernatant (soluble fraction) was adjusted to 9 M-urea by the addition of solid urea, 2% (v/v) Nonidet P-40 and 5% (v/v) 2-mercaptoethanol. Where indicated, the pellet (total particulate fraction) was resuspended in 1 ml of basic urea solution (9.3 M-urea/5 mM-K₂CO₃, pH 11) by sonication for 15 s on ice (Roberts *et al.*, 1984). The resuspended material was immediately neutralized by addition of 50 μ l of Hepes (120 mg/ml), pH 5.5, followed by addition of Nonidet P-40 (final concn. 2%) and 5 mg of dithiothreitol. The [32 P]phosphoprotein pattern of total particulate material suspended in basic urea was similar to that of material suspended in O'Farrell (1975) lysis buffer, although the amount of certain phosphoproteins was clearly increased by the former method. Samples were stored at –40 °C before electrophoresis.

Portions of samples (30–50 μ g of protein) were submitted to two-dimensional gel electrophoresis exactly as described by O'Farrell (1975). Isoelectric-focusing tube gels were equilibrated in SDS sample buffer for 90 min, and SDS/polyacrylamide-gel electrophoresis in the second dimension was performed on a 10–18% acrylamide exponential gradient. After staining with 0.2% Coomassie Brilliant Blue R in 50% (w/v) trichloroacetic acid and destaining, the gels were subjected to autoradiography as described previously (Burnham & Williams, 1982).

In order to examine phosphoproteins of basic pI, samples were also submitted to non-equilibrium pH-gradient gel electrophoresis (O'Farrell *et al.*, 1977). Electrophoresis in the first dimension was performed for 1600–2000 V·h in Ampholines pH 3.5–10.

The M_r of phosphoproteins was determined by using a series of protein standards (Burnham & Williams, 1982), which were loaded in a separate well on the second-dimension gel. In each experiment, a standard pH gradient was determined from an isoelectric-focusing tube gel loaded with O'Farrell (1975) lysis buffer. Because of the exponential acrylamide gradient, second-dimension slab gels after destaining were trapezoidal in shape. Thus the position of a particular phosphoprotein on the standard pH gradient was approximated by measuring the horizontal distance of the phosphoprotein from the nearest end of the gradient on the second-dimension gel.

Densitometric analysis of autoradiographs

The amount of 32 P associated with phosphoproteins was determined by scanning autoradiographs with a Zeineh Soft Laser Densitometer (Biomed Instruments Chicago, IL, U.S.A.) and measuring peak height in densitometric tracings. In order to determine whether test agents altered overall protein labeling, the peak heights of eight reference phosphoproteins not clearly affected by test agents were also measured. On the basis of six experiments, the average height of the reference peaks in treated samples did not vary significantly from control ($P > 0.05$, Student's unpaired *t* test; range = 80–150% of control). In order to correct for slight inter-sample variations in protein labelling, the heights of peaks clearly affected by test agents were normalized relative to the average height of reference peaks expressed as percentages of control. Percentage changes in peak height closely matched percentage changes in peak area. For example, for phosphoproteins 1, 3, 6, 7 and 9 (three experiments each), linear-regression analysis of peak height versus peak area showed a correlation coefficient of 0.91. Only

those autoradiographic spots were measured that had an absorbance within the region of a linear relationship between absorbance and radioactivity (Burnham & Williams, 1982).

RESULTS

Effects of carbamoylcholine, TPA and A23187 on acinar protein phosphorylation

The effects of carbamoylcholine TPA, and Ca^{2+} ionophore A23187 on acinar protein phosphorylation

were investigated by using the two-dimensional gel-electrophoresis system of O'Farrell (1975), which gives a much greater resolution of phosphoproteins than was achieved previously (Burnham & Williams, 1982). Carbamoylcholine consistently altered the phosphorylation of eight proteins in guinea-pig pancreatic acini (Fig. 1a). In mouse acini, changes in phosphorylation of nine proteins were observed with carbamoylcholine (Fig. 1b), eight of which had apparent M_r and pI values very similar to those of affected proteins in guinea-pig acini (Table 1). In both species, these proteins were all

Table 1. M_r and pI of pancreatic acinar proteins undergoing alterations in phosphorylation in response to carbamoylcholine

Each value is the mean of three experiments; in all cases, S.E.M. was 5% of mean value or less. Acini were incubated with 200–250 μCi of $[^{32}\text{P}]\text{P}_i/\text{ml}$ for 60 min, stimulated for 5 min with carbamoylcholine (100 μM , guinea pig; 3 μM , mouse), and soluble proteins were submitted to two-dimensional polyacrylamide-gel electrophoresis. Autoradiographs of gels were used to determine the M_r and pI of proteins which showed alterations in ^{32}P labelling.

Protein . . .		1	2	3	4	5	6	7	8	9
Guinea pig	$M_r (\times 10^{-3})$	83.2	68.9	61.9	39.6	27.6	22.9	20.2	20.1	
	pI	4.46	5.44	5.90	6.22	4.93	6.25	6.27	6.44	
Mouse	$M_r (\times 10^{-3})$	77.4	69.5	63.0	35.1	27.9	22.7	20.5	19.8	24.7
	pI	4.46	5.37	5.84	6.24	5.10	6.26	6.25	6.40	5.59

Fig. 1. Autoradiographs of soluble proteins obtained from guinea-pig (a) and mouse (b) pancreatic acini

Acini were incubated with 200–250 μCi of $[^{32}\text{P}]\text{P}_i/\text{ml}$ for 60 min, stimulated for 5 min with carbamoylcholine (CCh: 100 μM , guinea pig; 3 μM , mouse), and soluble proteins were submitted to two-dimensional polyacrylamide-gel electrophoresis. The isoelectric-focusing dimension is indicated (IEF), with positions of maximal equilibrium pH values; SDS/polyacrylamide-gel electrophoresis dimension is indicated (SDS) with positions of M_r standards. Proteins that undergo an alteration in phosphorylation in response to carbamoylcholine are numbered and indicated by arrows.

Table 2. Relative changes in phosphorylation of mouse pancreatic acinar proteins in response to carbamoylcholine, TPA and A23187

^{32}P -labelled mouse pancreatic acini were stimulated for 5 min with $3\ \mu\text{M}$ -carbamoylcholine (CCh), $1\ \mu\text{M}$ -TPA, $2\ \mu\text{M}$ -A23187, or TPA and A23187 together, and soluble proteins were submitted to two-dimensional polyacrylamide-gel electrophoresis. Values [$\pm$ S.E.M. (n)] represent change as percentage of control in ^{32}P labelling of acinar proteins described in Table 1.

Protein	CCh	TPA	A23187	TPA + A23187
1	327 $\pm$ 48 (5)	401 $\pm$ 36 (6)	126 $\pm$ 15 (7)	376 $\pm$ 18 (5)
2	197 $\pm$ 43 (4)	550 $\pm$ 132 (5)	99 $\pm$ 29 (6)	229 $\pm$ 32 (4)
3	313 $\pm$ 66 (4)	1015 $\pm$ 110 (6)	104 $\pm$ 14 (6)	1109 $\pm$ 279 (4)
4	304 $\pm$ 78 (4)	469 $\pm$ 52 (6)	80 $\pm$ 21 (5)	520 $\pm$ 108 (5)
5	660 $\pm$ 205 (5)	145 $\pm$ 12 (5)	798 $\pm$ 37 (5)	475 $\pm$ 88 (4)
6	365 $\pm$ 46 (6)	160 $\pm$ 11 (6)	374 $\pm$ 51 (7)	298 $\pm$ 25 (5)
7	34 $\pm$ 4 (6)	54 $\pm$ 4 (6)	39 $\pm$ 4 (7)	29 $\pm$ 9 (5)
8	38 $\pm$ 6 (6)	62 $\pm$ 5 (6)	43 $\pm$ 3 (7)	47 $\pm$ 8 (5)
9	28 $\pm$ 7 (6)	83 $\pm$ 5 (6)	26 $\pm$ 4 (7)	34 $\pm$ 9 (4)

Fig. 2. Autoradiographs of soluble proteins obtained from mouse pancreatic acini

Acini labelled with [^{32}P]P_i were incubated for 5 min with TPA ($1\ \mu\text{M}$), A23187 ($2\ \mu\text{M}$) or TPA and A23187 together. Proteins that undergo an alteration in phosphorylation relative to control (not shown) are indicated by arrows and numbered according to the nomenclature of Fig. 1. In the panel depicting the combined effects of TPA and A23187, the spot located between proteins 4 and 6 at approx. M_r 28000 was not observed in other experiments, and is presumed to be an artefact.

located in the acinar soluble fraction. Over the range of pI values resolved on the first-dimension gel (pH 4.5–6.8), no additional effects of carbamoylcholine on protein phosphorylation were noted in the total particulate fraction for either species. In order to resolve phosphoproteins of basic pI, acinar samples also were submitted to non-equilibrium pH-gradient gel electrophoresis (O'Farrell *et al.*, 1977) in the first dimension instead of isoelectric focusing. The only additional effect of carbamoylcholine noted in either species was an increase in phosphorylation of a basic M_r 32000–33000 particulate protein. This protein is most probably ribosomal protein S6, previously shown to be phosphorylated in response to various pancreatic regulatory agents (Burnham & Williams, 1982; Freedman & Jamieson, 1982b; Jahn & Söling, 1983). Treatment of mouse and guinea-pig acini with cholecystokinin octapeptide (0.3–1 nM) produced effects on phosphorylation of soluble proteins similar to those of carbamoylcholine (results not shown).

The relative effects of carbamoylcholine, TPA, A23187, and TPA and A23187 together, on phosphorylation of the nine acinar proteins described in Table 1 were then studied. In mouse acini, $3\ \mu\text{M}$ -carbamoylcholine increased the phosphorylation of proteins 1–6 and decreased the phosphorylation of proteins 7–9 (Table 2, Fig. 1b). The effects of $1\ \mu\text{M}$ -TPA and $2\ \mu\text{M}$ -A23187 on phosphorylation of proteins 1–6 were generally distinct in that TPA produced sizable increases in phosphorylation of proteins 1–4, whereas A23187 increased the phosphorylation of proteins 5 and 6. In addition, A23187 induced the dephosphorylation of proteins 7–9. TPA also caused these dephosphorylation events, particularly with regard to proteins 7 and 8, although to a lesser extent (Table 2, Fig. 2). No additional effects of TPA and A23187 on phosphorylation not seen with carbamoylcholine were consistently observed. The complete phosphorylation pattern obtained with carbamoylcholine was reproduced when mouse acini were treated with a combination of TPA and A23187 (Table 2, Fig. 2).

Alterations in phosphorylation of the eight guinea-pig acinar proteins induced by $100\ \mu\text{M}$ -carbamoylcholine followed the same pattern as that of mouse proteins 1–8, except that increased phosphorylation of protein 4 appeared to be considerably greater than in mouse (Fig. 1). In guinea pig $1\ \mu\text{M}$ -TPA and $2\ \mu\text{M}$ -A23187 also produced the same distinct effects on phosphorylation of proteins 1–6 as were observed in mouse acini. Although

Fig. 3. TPA and A23187 concentration–response relationships for amylase release by guinea-pig (top panels) and mouse (bottom panels) pancreatic acini

Left panels: A23187 was present at 0.1 μM (guinea pig) or 0.03 μM (mouse). Right panels: TPA was present at 10 nM for both species. Broken line indicates the amount of amylase released if the effects of TPA and A23187 were additive. The effects of carbamoylcholine (CCh) at 30 μM (guinea pig) or 3 μM (mouse) are also shown. Each value is the mean $\pm$ S.E.M. for three to five experiments.

dephosphorylation of proteins 7 and 8 was not observed in all experiments, this effect tended to occur in response to TPA as well as to the ionophore. Treatment of mouse and guinea-pig acini with a lower concentration of A23187 (0.1 μM) produced similar changes in phosphorylation as were observed with 2 μM -ionophore, although these changes tended to be smaller and were not reproduced in all experiments (results not shown).

Effects of TPA and A23187 on acinar amylase release

Fig. 3 shows the effects of TPA and Ca^{2+} ionophore A23187 on amylase release in mouse and guinea-pig pancreatic acini. In guinea-pig acini, the phorbol ester increased amylase release at 10 nM, and at 1 μM produced a maximal secretory response which was equivalent to that evoked by a maximally effective concentration of carbamoylcholine. In contrast with results with guinea-pig acini, TPA was a relatively weak secretagogue in mouse pancreatic acini. A maximally effective concentration of 1 μM -TPA produced a secretory response which was only 28% of that produced by a maximally effective concentration of carbamoylcholine (Fig. 3). Similar results were obtained in mouse acini by using either phorbol 12,13-dibutyrate (1 nM–1 μM) or synthetic diacylglycerol (dioctanoylglycerol, 3–100 $\mu\text{g}/\text{ml}$) (results not shown). Compared with TPA, A23187 in mouse acini was a considerably stronger secretagogue, a maximally effective concentration of 0.3 μM producing a secretory

response two-thirds of that produced by a maximally effective concentration of carbamoylcholine (Fig. 3). In guinea-pig acini, the reverse relationship was observed. A maximally effective concentration of A23187 (2 μM) stimulated amylase release to only 35% of the maximal response to carbamoylcholine (Fig. 3).

In both mouse and guinea-pig acini the combination of ionophore and TPA resulted in a modest synergistic effect on amylase release (Fig. 3). The amount of amylase released from guinea-pig acini in the presence of a submaximal concentration of A23187 and various concentrations of TPA was only 125–150% of that expected if the effects of these agents were additive. Although the amount of amylase released in excess of an additive effect appeared to be greater in mouse acini, the full secretory response to carbamoylcholine was not reproduced by a combination of A23187 and TPA at any of the phorbol ester concentrations studied (Fig. 3). In guinea-pig acini, the full secretory response to carbamoylcholine was reproduced by a submaximal concentration of TPA and relatively high concentrations of A23187 (0.3–2 μM). Over the range of A23187 concentrations studied, the synergistic response amounted at maximum to 185% of that expected for an additive effect. In mouse acini, combination of TPA with 0.3–2 μM -A23187 stimulated secretion slightly less than a maximally effective concentration of carbamoylcholine, and the amount of amylase released was 110–170% of that expected for an additive effect (Fig. 3).

DISCUSSION

The results of this study extend to exocrine pancreas the original observation made in platelets (Kaibuchi *et al.*, 1983) that tumour-promoting phorbol esters and the Ca^{2+} ionophore A23187 have generally distinctive effects on protein phosphorylation, and that these agents together mimic the full effect on phosphorylation of a physiological regulator. In pancreatic acini of guinea pig and mouse, TPA consistently induced several increases in protein phosphorylation, presumably through a pharmacological stimulation of protein kinase C. In contrast, A23187 caused both increases and decreases in acinar protein phosphorylation. These effects most probably involve Ca^{2+} and are mediated by one or more calmodulin-dependent protein kinases and phosphatases, which have been identified and characterized in pancreas (Gorelick *et al.*, 1983; Burnham & Williams, 1984b; Burnham, 1985). The mechanism by which TPA induces the dephosphorylation of proteins 7 and 8 is unclear, although inactivation of a phosphatase inhibitor via phosphorylation by protein kinase C is conceivable (Cohen, 1982). The role of Ca^{2+} -regulated protein kinases and phosphatases and protein kinase C in mediating specific changes in phosphorylation in the intact pancreatic acinar cell has not been examined previously. In guinea-pig parotid gland, the involvement of protein kinase C in the carbamoylcholine-induced phosphorylation of ribosomal protein S6 was demonstrated by phosphopeptide mapping techniques (Padel & Söling, 1985).

Some of the changes in phosphorylation of soluble proteins observed here in guinea-pig and mouse acini have been reported for mouse acini by using single-dimension SDS/polyacrylamide gel electrophoresis (Burnham & Williams, 1982). Proteins 6, 7 and 8 are of similar M_r and undergo carbamoylcholine-induced alterations in phosphorylation similar to those for previously noted soluble proteins of M_r 23 000, 21 000 and 20 500 respectively. The last two proteins are of particular interest because their dephosphorylation most closely correlates on a temporal basis with the stimulation of enzyme secretion (Burnham & Williams, 1982), although the exact function of these proteins ($pI = 6.25\text{--}6.40$) remains unknown.

Although TPA and A23187 together matched the effect on acinar protein phosphorylation of carbamoylcholine in mouse and guinea pig, TPA and A23187 had only a modest synergistic effect on amylase release in either species. These latter findings are in contrast with the results of Nishizuka and colleagues obtained in platelets (Kaibuchi *et al.*, 1983). Aside from matching the full secretory response to thrombin, a combination of A23187 and diacylglycerol stimulated 5-hydroxytryptamine secretion to a value over 4-fold greater than that if the effects of these agents were additive. A similar synergism between Ca^{2+} and TPA or diacylglycerol, albeit to a lesser extent, has been noted in other secretory systems (Knight & Baker, 1983; Kajikawa *et al.*, 1983; Katakami *et al.*, 1984). The results of the present study therefore suggest that, contrasted with other secretory systems studied so far, an interaction between protein-phosphorylation pathways regulated by diacylglycerol and Ca^{2+} is not of major importance in producing a full secretory response in exocrine pancreas. It is possible that stimulation of

pancreatic secretion, in at least mouse and guinea pig, is primarily under the control of a single regulatory pathway. In guinea-pig pancreatic acini, TPA alone elicits a full secretory response, whereas the response to A23187 is about 33% of maximum (Gardner *et al.*, 1980; Gunther, 1981; the present paper); in mouse acini the secretory response to TPA is 28% of maximum, whereas A23187 elicits a much larger response, close to that induced by carbamoylcholine (Williams *et al.*, 1978; the present paper). Although the substrates of protein kinase C and Ca^{2+} -activated phosphorylation enzymes (i.e. protein kinases and phosphatases) are qualitatively similar in mouse and guinea-pig pancreas, there may be quantitative differences in terms of the relative amount of substrate or degree of its altered phosphorylation. Such differences would provide the basis for the pre-eminence of one regulatory pathway over another. Indeed, in guinea-pig acini, phosphorylation of protein 4 in response to carbamoylcholine is considerably greater than in mouse acini (Fig. 1). Since phosphorylation of this protein is increased by TPA, it could be responsible for the larger secretory effect induced by TPA in guinea pig. This hypothesis can be tested when the relevant protein substrates are purified and their regulation by phosphorylation is elucidated.

A lack of sizeable synergism between TPA and A23187 in stimulating pancreatic amylase release may not necessarily apply to other acinar cell functions. Carbamoylcholine and cholecystokinin inhibit internalization of epidermal growth factor by cultured mouse pancreatic acini, and this effect can be fully reproduced by 10 nM-TPA (Logsdon & Williams, 1984). However, at concentrations of TPA and A23187 that separately do not affect internalization of epidermal growth factor (0.1 nM and 1 μM respectively), a combination of these agents elicits approx. 50% maximal inhibition of internalization.

Synergistic effects of TPA and A23187 on amylase secretion have been shown for rabbit pancreatic acini stimulated in Ca^{2+} -free medium before measurement of secretion in the presence of Ca^{2+} (De Pont & Fleuren-Jakobs, 1984). Whether such a response could be elicited in acini stimulated in Ca^{2+} -containing medium was not reported. Synergism between TPA- and A23187-induced amylase release also has been shown for superfused segments of rat pancreas (Singh, 1985). However, we failed to repeat this observation with rat pancreatic acini, which yielded results similar to mouse acini (J. A. Williams, unpublished work). It is possible that the results obtained with pancreatic segments stemmed from enhanced 'washout' of digestive enzymes, owing to a stimulatory effect of TPA or A23187 (or both) on fluid and electrolyte secretion.

In conclusion, the results of the present study suggest that the intracellular messengers, Ca^{2+} and diacylglycerol, activate largely separate protein-phosphorylation pathways in exocrine pancreas. These pathways may interact in a synergistic manner in the regulation of digestive-enzyme secretion, although such an interaction appears to play a limited role in the production of a full secretory response. Furthermore, the relative importance of the two pathways shows considerable species dependence.

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REFERENCES

- Banschbach, M. W., Geison, R. L. & Hokin-Neaverson, M. (1981) *Biochim. Biophys. Acta* **663**, 34–45
- Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482
- Burnham, D. B. (1985) *Biochem. J.* **231**, 335–342
- Burnham, D. B. & Williams, J. A. (1982) *J. Biol. Chem.* **257**, 10523–10528
- Burnham, D. B. & Williams, J. A. (1984a) *J. Pediatr. Gastroenterol. Nutr.* **3**, Suppl. 1, S1–S10
- Burnham, D. B. & Williams, J. A. (1984b) *Am. J. Physiol.* **246**, G500–G508
- Cohen, P. (1982) *Nature (London)* **296**, 613–620
- De Pont, J. J. H. M. & Fleuren-Jakobs, A. M. M. (1984) *FEBS Lett.* **170**, 64–68
- Freedman, S. D. & Jamieson, J. D. (1982a) *J. Cell Biol.* **95**, 903–908
- Freedman, S. D. & Jamieson, J. D. (1982b) *J. Cell Biol.* **95**, 909–917
- Gardner, J. D., Walker, M. D. & Rottman, A. J. (1980) *Am. J. Physiol.* **328**, G458–G466
- Goldfine, I. D. & Williams, J. A. (1983) *Int. Rev. Cytol.* **85**, 1–38
- Gorelick, R. S., Cohn, J. A., Freedman, S. D., Delahunt, N. G., Gershoni, J. M. & Jamieson, J. D. (1983) *J. Cell Biol.* **97**, 1294–1298
- Gunther, G. R. (1981) *J. Biol. Chem.* **256**, 12040–12045
- Jahn, R. & Söling, H. D. (1983) *FEBS Lett.* **153**, 71–76
- Jung, D. H. (1980) *Clin. Chim. Acta* **100**, 7–11
- Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Takashi, F. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 6701–6704
- Kajikawa, N., Kaibuchi, K., Matsubara, T., Kikkawa, U., Takai, Y. & Nishizuka, Y. (1983) *Biochem. Biophys. Res. Commun.* **116**, 743–750
- Katakami, Y., Kaibuchi, K., Sawamura, M., Takai, Y. & Nishizuka, Y. (1984) *Biochem. Biophys. Res. Commun.* **121**, 573–578
- Knight, D. E. & Baker, P. F. (1983) *FEBS Lett.* **160**, 98–100
- Logsdon, C. D. & Williams, J. A. (1984) *Biochem. J.* **223**, 893–900
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- O'Farrell, P., Goodman, M. M. & O'Farrell, P. H. (1977) *Cell* **12**, 1133–1142
- Padel, W. & Söling, H. D. (1985) *Eur. J. Biochem.* **151**, 1–10
- Putney, J. W., Burgess, G. M., Halenda, S. P., McKinney, J. S. & Rubin, R. S. (1983) *Biochem. J.* **212**, 483–488
- Roberts, M. L. & Butcher, R. R. (1983) *Biochem. J.* **210**, 353–359
- Roberts, R. M., Baumbach, G. A., Buhi, W. C., Denny, J. B., Fitzgerald, L. A., Babelyn, S. F. & Horst, M. N. (1984) in *Molecular and Chemical Characterization of Membrane Receptors* (Venter, J. C. & Harrison, L. C., eds.), pp. 61–113, Alan R. Liss, New York
- Schultz, G. S., Sarras, M. P., Gunther, G. R., Hull, B. E., Alicea, H. A., Gorelick, F. S. & Jamieson, J. D. (1980) *Exp. Cell Res.* **130**, 49–62
- Schulz, I. & Stolze, H. H. (1980) *Annu. Rev. Physiol.* **42**, 127–156
- Singh, J. (1985) *FEBS Lett.* **180**, 191–195
- Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) *Nature (London)* **306**, 67–69
- Thomas, A. P., Marks, J. S., Coll, K. E. & Williamson, J. R. (1983) *J. Biol. Chem.* **258**, 5716–5725
- Williams, J. A. (1980) *Am. J. Physiol.* **238**, G269–G279
- Williams, J. A., Korc, M. & Dormer, R. L. (1978) *Am. J. Physiol.* **235**, E517–E524

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