Biochem. J. (1985) **227**, 743–751
Printed in Great Britain

Protein kinase activity associated with pancreatic zymogen granules

Daniel B. BURNHAM,* Phyllis MUNOWITZ,* Niels THORN† and John A. WILLIAMS*†‡
**Departments of Physiology and Medicine, University of California, San Francisco, CA 94143, U.S.A., and †Institute of Physiology C, University of Copenhagen, Copenhagen, Denmark*

(*Received 10 September 1984/9 January 1985; accepted 18 January 1985*)

Purified zymogen granules were prepared from rat pancreas by using an iso-osmotic Percoll gradient. In the presence of [γ-^{32}P]ATP, phosphorylation of several granule proteins was induced by Ca^{2+}, most notably a M_r-13000 protein, whereas addition of cyclic AMP was without effect. When phosphatidylserine was also added, Ca^{2+} increased the phosphorylation of additional proteins, with the largest effect on a protein of M_r 62000. Purified granules were also able to phosphorylate exogenous substrates. Ca^{2+}-induced phosphorylation of lysine-rich histone was enhanced over 3-fold in the presence of phosphatidylserine, and cyclic AMP-activated protein kinase activity was revealed with mixed histone as substrate. The concentrations of free Ca^{2+} and cyclic AMP required for half-maximal phosphorylation of both endogenous and exogenous proteins were 1–3 μM and 57 nM respectively. Treatment of granules with 0.25 M-KCl resulted in the release of phosphatidylserine-dependent kinase activity into a high-speed granule supernatant. In contrast, granule-protein substrates of Ca^{2+}-activated kinase activity were resistant to KCl extraction, and in fact were present in purified granule membranes. Kinase activity activated by cyclic AMP was not extracted by KCl treatment. It is concluded that phosphorylation of integral membrane proteins in the zymogen granule can be induced by one or more Ca^{2+}-activated protein kinases. Such a reaction is a potential mechanism by which exocytosis may be regulated in the exocrine pancreas by Ca^{2+}-mediated secretagogues.

Secretion of pancreatic digestive enzymes involves a process of controlled fusion of zymogen granules with the luminal plasma membrane of the acinar cell. This process is triggered by secretagogues utilizing Ca^{2+}, cyclic AMP and possibly diacylglycerol as intracellular messengers (Gardner, 1979; Williams, 1980; Burnham & Williams, 1984*b*). All of these messengers interact with and activate specific protein kinases, which have been shown to be present in acinar cells (Jensen & Gardner, 1978; Gorelick *et al.*, 1983; Burnham & Williams, 1984*a*). Moreover, pancreatic secretagogues induce changes in the phosphorylation of specific proteins *in situ* in acinar cells, although the intracellular localization and function of these proteins is generally unknown (Burnham & Williams, 1982; Freedman & Jamieson, 1982; Roberts & Butcher, 1983). One possible site of messenger-regulated protein phosphorylation during pancreatic stimulus–secretion coupling is the zymogen granule.

Previous studies have demonstrated phosphorylation of several proteins in a membrane preparation of isolated zymogen granules, although the addition of cyclic AMP, the only intracellular messenger tested, did not consistently alter membrane protein phosphorylation (Lambert *et al.*, 1974; Macdonald & Ronzio, 1974). Cyclic AMP-activated kinase activity was revealed, however, if exogenous substrate (histone) was used (Lambert *et al.*, 1974; Lewis & Ronzio, 1979). Furthermore, the addition of cyclic AMP to a mixture of zymogen granules and postmicrosomal supernatant resulted in a 5-fold increase in granule-associated protein kinase activity, which could be removed by 0.15 M-NaCl (Lewis & Ronzio, 1979). In the present study, therefore, highly purified zymogen granules were prepared by using an iso-osmotic Percoll gradient and studied for regulation by Ca^{2+} and cyclic AMP of protein kinase activity with endogenous and exogenous substrates. The

Abbreviation used: SDS, sodium dodecyl sulphate.

‡ To whom reprint requests should be addressed.

nature of association of protein kinases and substrate proteins with the granule was explored by treatment of granules with 0.25M-KCl and preparation of purified zymogen-granule membranes.

Materials and methods

Materials

Phenylmethanesulphonyl fluoride, histone types II-S and III, phosphatidylserine, theophylline and ATP were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; bovine brain calmodulin was from Calbiochem–Behring, La Jolla, CA, U.S.A.; [γ-^{32}P]ATP (15–20Ci/mmol) was from Amersham, Arlington Heights, IL, U.S.A.; enzyme-grade sucrose was from Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.; and Percoll was from Pharmacia, Uppsala, Sweden. Chemicals for electrophoresis gels and solutions, including silver-staining reagents, were purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. Trifluoperazine dihydrochloride was a gift from Smith, Kline and French, Philadelphia, PA, U.S.A. A stock solution of phosphatidylserine (0.5mg/ml) was prepared fresh for each experiment by suspension in 20mM-Tris/HCl (pH7.5) by sonication (Burnham & Williams, 1984*a*).

Preparation of zymogen granules

Initial experiments were carried out in Copenhagen with a Wistar-derived outbred strain. Later experiments, including all phosphorylation studies, were carried out in San Francisco with Sprague–Dawley rats. Pancreases were removed from two or three starved male rats, placed in ice-cold 0.9% NaCl, trimmed of fat and connective tissue and weighed. All subsequent operations were carried out at 2–4°C. The pancreas was cut into 1–2mm pieces with scissors in 20ml of 0.3M-sucrose containing 10mM-Mes, pH6.0, and 1mM-EGTA (henceforth abbreviated as SME). For phosphorylation studies, 100μM-phenylmethanesulphonyl fluoride was also included in the SME. Homogenization was carried out at 2000rev./min in a Thomas size B Teflon/glass homogenizer by four up-and-down strokes, followed by filtration through two layers of medical gauze. This homogenate was centrifuged at 250***g*** for 5min to obtain the pellet P_1 fraction, and the supernatant was re-centrifuged at 1400***g*** for 7min to obtain pellet P_2 and supernatant S_2. In some experiments, S_2 was centrifuged at 100000***g*** for 60min to obtain a cytosol fraction. P_2 was resuspended in 2ml of SME and added to 50ml of 50% Percoll (1 vol., plus 1 vol. of SME) which had been dialysed overnight against SME. The mixture was put into four 13ml Ultraclear tubes and centrifuged at 30000rev./min in a Beckman Spinco Ti50 or Ti70.1 rotor for 30min.

After centrifugation, 0.5ml fractions were pumped from the bottom of the tube and collected for measurement of refractive index, protein and amylase. Gradients without biological material were used for characterization of the density profile by refractive index or with density beads, and for blank values in the protein analysis. Alternatively the bottom of the tube was punctured with a 23-gauge needle and the dense white zymogen-granule band collected directly. To remove Percoll, the collected granule bands were centrifuged at 1400***g*** for 8min, the pellet was resuspended in 4ml of SME and re-centrifuged at 1400***g***. This final granule pellet was then used for marker-enzyme analysis or studies of protein phosphorylation. In the latter studies the granule pellet was suspended in SME to 2mg of protein/ml.

Protein kinase assay

The reaction mixture (final volume 110μl) contained the following: 25mM-Pipes (pH7.0), 10mM-$MgCl_2$, 0.1mM-dithiothreitol, 0.1mM-phenylmethanesulphonyl fluoride, 0.1mM-leupeptin, 0.2mM-EGTA (minus Ca^{2+}) or 0.2mM-EGTA and various amounts of $CaCl_2$ to give the indicated concentrations of Ca^{2+} ('plus Ca^{2+}'), 15μM-[γ-^{32}P]ATP, 40μg of granular protein, and where indicated 1μg of calmodulin or 5μg of phosphatidylserine. The amount of $CaCl_2$ added to the reaction mixture to give a desired free Ca^{2+} concentration was calculated by an iterative Gauss–Newton program on a Hewlett–Packard 9825 computer. This program considered binding of Ca^{2+} by ATP as well as EGTA and competition for binding by Mg^{2+} ions (Burnham & Williams, 1984*a*). Cyclic AMP, at concentrations indicated, was added in the absence of added Ca^{2+}. Theophylline (5mM) did not affect the absolute increase or sensitivity to cyclic AMP of cyclic AMP-induced protein labelling and thus was routinely not used.

After preincubation of the mixture for 30s at 30°C, the reaction was initiated by addition of [γ-^{32}P]ATP. Incubation was routinely performed for 1min and terminated by addition of 50μl of SDS 'stop' solution [30mM-Tris/HCl (pH7.4), 15% (v/v) glycerol, 9% (w/v) SDS, 0.05% Bromophenol Blue and 5mM-ATP] and heating in boiling water for 5min. After cooling, 50μl of 8% (v/v) 2-mercaptoethanol was added and samples were stored at −40°C overnight before analysis by SDS/polyacrylamide-gel electrophoresis. Methods for gel electrophoresis, densitometric analysis of autoradiographs and determination of M_r of ^{32}P-labelled proteins have been discussed previously (Burnham & Williams, 1982).

In experiments using exogenous protein substrates, the above reaction mixture was used with the addition of 50μg of histone type II or III. The reaction was routinely terminated after 2min by the addition of 4ml of ice-cold 5% trichloroacetic acid containing 1.5% $Na_4P_2O_7$ and 1% NaH_2PO_4. The samples were then measured for trichloroacetic acid-insoluble radioactivity after collection on Millipore filters (Lam & Kasper, 1979; Burnham & Williams, 1984*a*). In the absence of exogenous substrate, cyclic AMP or Ca^{2+} (with or without added phosphatidylserine or calmodulin) did not significantly alter total labelling of trichloroacetic acid-insoluble material.

Under the conditions used, phosphorylation of granular and exogenous protein substrates by cyclic AMP- or Ca^{2+}-activated kinase activities was a linear function of time for at least 3min at 30°C. Results of kinase assays using granular or exogenous substrates are representative of at least two and usually three or four experiments.

Preparation of zymogen-granule membranes

This was done by the method of Meldolesi *et al.* (1971) as modified by Paquet *et al.* (1982). Granules (4–6mg of protein) were suspended in 3.5ml of lysis buffer [25mM-Hepes (pH8.2), 0.1M-KCl, 0.1mM-phenylmethanesulphonyl fluoride] and incubated for 30min at 4°C. The lysed granules were then layered over 0.5ml of 0.3M-sucrose, which in turn overlaid 0.5ml of 1.0M-sucrose. After centrifugation for 45min at 243000*g* in a Beckman SW50.1 rotor, the granule membranes were collected at the 0.3M/1.0M-sucrose interface, suspended in 5ml of 0.25M-NaBr, and re-centrifuged at 243000*g* for 60min. The resulting pellet was suspended in a minimal volume of lysis buffer (100–200μl) and prepared for analysis by SDS/polyacrylamide-gel electrophoresis. The granular contents were obtained from the top layer of the sucrose step gradient and concentrated 6–7-fold (Centricon 10; Amicon, Danvers, MA, U.S.A.). In general, 5mg of granules yielded 15–30μg of membrane protein.

Preparation of granule membranes after ^{32}P labelling followed the above procedure, except that (*a*) EDTA and NaF were present during granule lysis at 4mM and 50mM and in the sucrose and NaBr solutions at 0.1mM and 50mM respectively, and (*b*) the granules were briefly sonicated by a probe-type sonicator (Braun-Sonic, 75W, 10s) before the first centrifugation step to ensure complete lysis.

KCl extraction of granules

Granules (2–3mg of protein) were suspended in 2ml of SME extraction solution containing 0.1mM-leupeptin, 0.1mM-phenylmethanesulphonyl fluoride and 0.25M-KCl and immediately centrifuged at 1400*g* for 8min. The resultant pellet (KCl-extracted granules) contained approx. 70–80% of the initial granular protein and was suspended in SME plus 0.1mM-phenylmethanesulphonyl fluoride before assay for protein kinase activity. The clear supernatant was centrifuged at 243000*g* for 60min to obtain the KCl supernatant. In experiments where granules were labelled with ^{32}P before KCl extraction, the extraction solution contained, in addition, 50mM-NaF and 2mM-EDTA. After centrifugation at 3000*g* for 8min, the pellet was suspended in SME containing 50mM-NaF to 0.4mg of protein/ml and prepared for analysis by SDS/polyacrylamide-gel electrophoresis.

Analytical and other procedures

Protein was determined by the fluorescamine reaction (Böhlen *et al.*, 1973) or by use of the Bio-Rad protein reagent (Bradford, 1976) with bovine serum albumin as standard. For the gradient profiles, a blank value obtained from a similar Percoll gradient was determined and subtracted. For the washed purified granules no correction was made, as the amount of residual Percoll was not quantifiable.

Amylase was determined with Procion-Yellow-labelled starch as substrate (Jung, 1980) after appropriate dilution with SME containing 1mg of bovine serum albumin/ml. Addition of Triton X-100 (0.1%) to samples and standards had no effect on the results and was not routinely used. DNA was measured fluorimetrically after reaction with diaminobenzoic acid (Hinegardner, 1971). Glutamate dehydrogenase, an enzyme marker for the mitochondrial matrix, was determined as described by Schmidt (1974) in the presence of 0.1% Triton X-100, 1mM-leucine and 1mM-ADP. Rotenone-insensitive NADH dehydrogenase, an enzyme marker for microsomal fractions, was determined as described by Takesue & Omura (1970). β-Glucuronidase and acid phosphatase lysosomal markers were assayed with umbelliferyl β-D-glucuronide and 4-methylumbelliferyl dihydrogen phosphate as substrates (Price & Dance, 1967). K^+-stimulated *p*-nitrophenyl phosphatase was assayed by the method of Hootman & Philpott (1979).

Electron microscopy was carried out on zymogen granules and other fractions that were pelleted in and fixed overnight with 1.5% glutaraldehyde/1% paraformaldehyde in 0.08M-cacodylate buffer, pH7.4. Pellets were postfixed in 2% OsO_4, dehydrated and embedded in Polybed 812. Thin sections were cut with a diamond knife and examined at 60kV in a Zeiss EM-9 electron microscope.

Results

Preparation of zymogen granules

When rat pancreas was homogenized in iso-osmotic SME, a crude granule pellet (P_2) could be prepared by differential centrifugation. In this pellet amylase was enriched 2.9-fold, but the mitochondrial enzyme glutamate dehydrogenase was also concentrated, and significant amounts of DNA, NADH dehydrogenase and β-glucuronidase remained (Table 1). When this pellet was centrifuged in a self-forming Percoll gradient, a heavy white band was observed at a density of 1.14g/ml just above the bottom of the tube, and one or two broader, more translucent, bands were observed at the top of the tube, at a density of 1.03–1.04g/ml. Profiles of protein and amylase in the gradient revealed two peaks, with amylase almost entirely associated with the denser band and protein split about equally. Electron micrographs revealed that the bottom band contained purified zymogen granules (Fig. 1), whereas the top band contained a mixture of mitochondria, endoplasmic reticulum and assorted membranous debris (results not shown). Measurement of DNA and enzyme markers showed a high degree of granule purity, with amylase enriched 8-fold over the homogenate and 250–500-fold relative to DNA, glutamate dehydrogenase and NADH dehydrogenase (Table 1). In separate experiments measuring the activity of the plasma-membrane marker enzyme K^+-stimulated *p*-nitrophenyl phosphatase, no measurable activity was observed in the granule fraction. Of the various markers, β-glucuronidase and acid phosphatase (results not shown) were most prominent in the granule fraction, although these data may reflect small amounts of these lysosomal enzymes in zymogen granules as previously described by electron-microscope histochemistry (Scheele, 1982). Recovery of amylase in the purified granule fraction was $13.7 \pm 1.5\%$ ($n = 6$) of that in the homogenate. Each granule preparation routinely yielded 2–3mg of protein.

Zymogen-granule protein kinase activity: endogenous protein substrates

^{32}P labelling of several granular proteins was observed for purified zymogen granules that had been incubated with [γ-^{32}P]ATP in the absence of added Ca^{2+} or cyclic AMP and submitted to SDS/polyacrylamide-gel electrophoresis and autoradiography. However, the addition of Ca^{2+} selectively caused a large increase in phosphorylation of a protein of M_r 13000 (Fig. 2*a*). Labelling of proteins of M_r 29000 and 21000 was also increased in the presence of Ca^{2+}. Addition of calmodulin and Ca^{2+} produced no effects in addition to those observed with Ca^{2+} alone (Fig. 2*b*). Phosphatidylserine plus Ca^{2+}, besides producing changes seen with Ca^{2+} alone, increased the

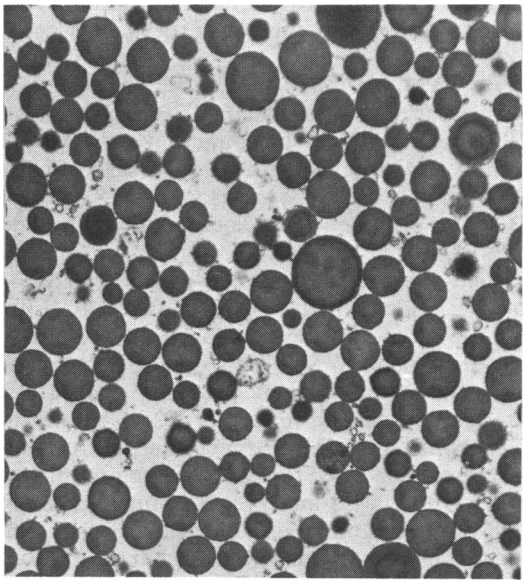

Fig. 1. *Electron micrograph of purified zymogen granules* Magnification ×28000.

Table 1. *Relative specific activities of markers in purified zymogen granules and in fractions separated by differential centrifugation*

Relative specific activity is the ratio of the percent of marker to the percent of protein in a given fraction. Values are means ± S.E.M.

Marker	No. of expts.	Relative specific activity			
		P_1	P_2	S_2	Granules
Amylase	6	1.53±0.08	2.90±0.13	0.67±0.02	8.19±0.52
Glutamate dehydrogenase	3	1.13±0.21	2.60±0.15	0.63±0.03	0.033±0.006
DNA	5	8.63±0.57	0.46±0.08	0.04±0.01	0.022±0.008
NADH dehydrogenase	3	0.54±0.22	0.73±0.08	1.08±0.07	0.016±0.003
β-Glucuronidase	4	1.12±0.04	0.17±0.03	0.93±0.03	0.17±0.02

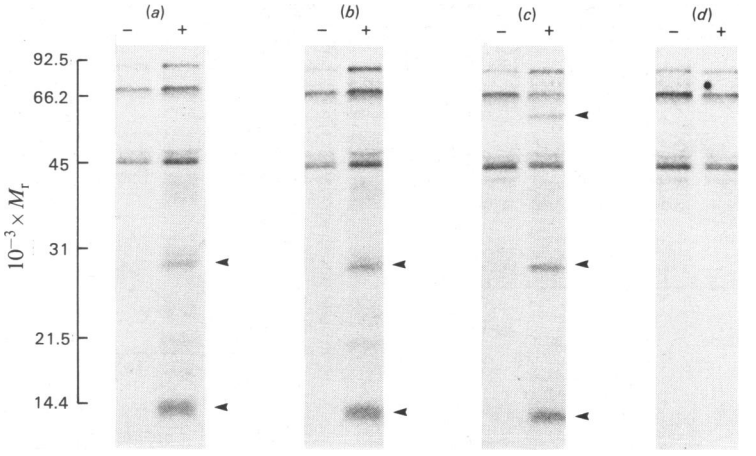

Fig. 2. *Autoradiographs of purified zymogen granules*
Granules were incubated for 1 min with [^{32}P]ATP in the presence (+) or absence (−) of 300 μM-Ca^{2+} (*a–c*) or 1 μM-cyclic AMP (*d*). (*a*) and (*d*), no additions; (*b*), +calmodulin; (*c*), +phosphatidylserine. Arrows: bands altered by activators.

phosphorylation of additional proteins, the most apparent being of M_r 62000 (Fig. 2*c*). Other changes not clearly shown in Fig. 2(*c*) include increased phosphorylation of proteins of M_r 15000 and 18000. Addition of cyclic AMP had no consistent effects on granule-protein phosphorylation (Fig. 2*d*). The phenothiazine trifluoperazine (100 μM) completely abolished Ca^{2+}-induced phosphorylation of granule proteins in the presence or absence of phosphatidylserine or calmodulin. ^{32}P-labelling of granules in the presence of cytosol (10–20 μg of protein) revealed no additional Ca^{2+}- or cyclic AMP-induced phosphorylated proteins relative to the sum of those induced with granules and cytosol alone (results not shown).

Zymogen-granule protein kinase activity: dose–response relationships and exogenous protein substrates

The Ca^{2+}-sensitivity of granule protein kinase activity was investigated by use of a Ca^{2+}–EGTA buffer system. Over the range of free Ca^{2+} concentrations believed to exist intracellularly, increased phosphorylation of the M_r-13000 protein was maximal at 10–30 μM-Ca^{2+}, in the presence or absence of calmodulin (Fig. 3). In four experiments, calmodulin did not significantly alter the concentration of Ca^{2+} at which half-maximal phosphorylation of the M_r-13000 protein was achieved, being 1.3 μM- and 1.5 μM-Ca^{2+} in the absence and presence of calmodulin respectively. Phosphorylation of the M_r-62000 protein induced by Ca^{2+} in the presence of phosphatidylserine was maximal at 3–10 μM-Ca^{2+} (Fig. 4). In the absence of phosphatidylserine, maximal Ca^{2+}-induced phosphorylation of this protein was only 25–30% of

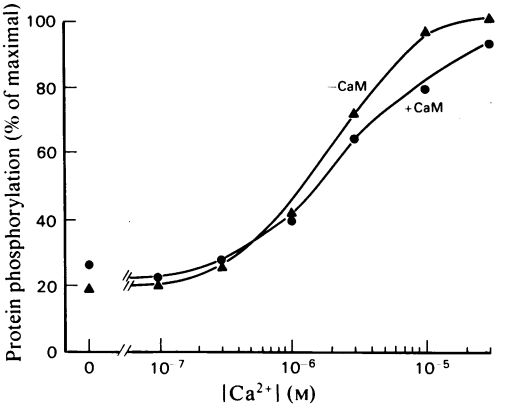

Fig. 3. *Phosphorylation of the M_r-13000 granule protein*
Ca^{2+}-activated kinase activity was measured in the presence and absence of calmodulin (CaM) as a function of concentration of Ca^{2+}. Values were obtained by densitometric scans of autoradiographs from four separate experiments. No significant differences exist for phosphorylation in the presence or absence of calmodulin at any concentration of Ca^{2+}.

that in the presence of phosphatidylserine. In addition to the M_r-62000 granular protein, phosphatidylserine-dependent kinase activity could also use as substrate exogenous lysine-rich (type III) histone (Fig. 4). As with the endogenous substrate, Ca^{2+}-induced phosphorylation of the histone was largely dependent on phosphatidylserine. In four experiments, the concentration of Ca^{2+} required for half-maximal histone phosphorylation in the presence or absence of phosphatidylserine

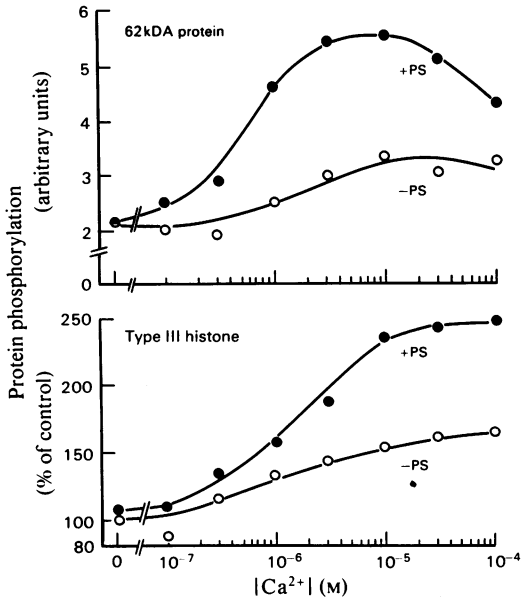

Fig. 4. *Phosphorylation of the M_r-62000 granule protein and type III histone*
Phosphatidylserine (PS)-dependent Ca^{2+}-activated kinase activity was measured as a function of concentration of Ca^{2+}. In the presence of histone, control kinase activity was 1.71 pmol of P_i incorporated/min per mg of protein.

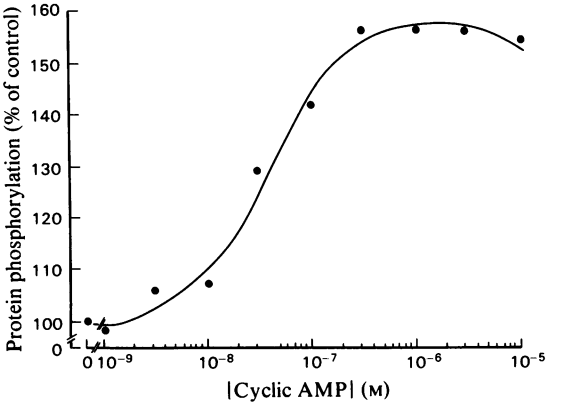

Fig. 5. *Phosphorylation of type II histone*
Cyclic AMP-activated kinase activity was measured as a function of the concentration of cyclic AMP. Control kinase activity was 1.82 pmol of P_i incorporated/min per mg of protein.

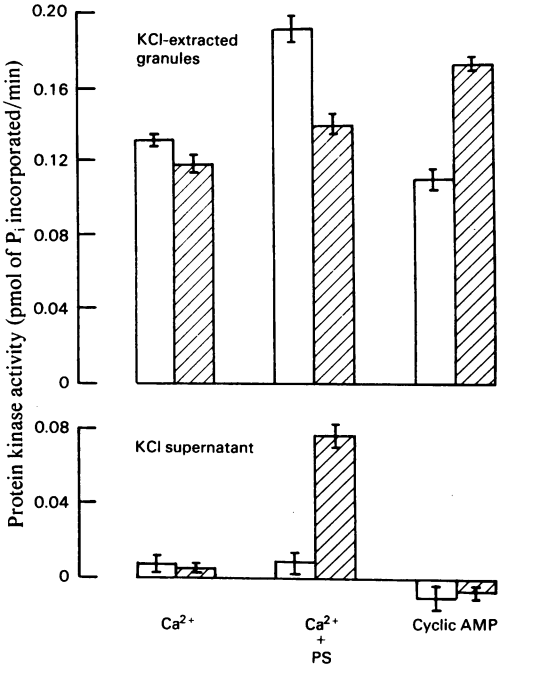

Fig. 6. *Protein kinase activity in KCl-extracted granules and KCl supernatant*
Kinase activity was measured in the absence (□) and presence (▨) of 300 μM-Ca^{2+}, with or without phosphatidylserine (PS), or of 1 μM-cyclic AMP. Each value is the mean ± S.E.M. of quadruplicate determinations.

was 1.15 ± 0.28 and 3.05 ± 0.99 μM respectively. Corresponding values for phosphorylation of the M_r-62000 protein were not significantly different (i.e. 2.4 ± 0.9 μM- and 8.3 ± 4.0 μM-Ca^{2+}; $n = 4$). Addition of exogenous 1,2- or 1,3-diolein had no effect on the Ca^{2+}-sensitivity of the phosphatidylserine-dependent protein kinase.

Although cyclic AMP did not induce any apparent changes in phosphorylation of granule proteins, addition of mixed (type II) histone to the kinase reaction mixture revealed that cyclic AMP-stimulated kinase activity was associated with purified zymogen granules (Fig. 5). Maximal histone phosphorylation induced by cyclic AMP was achieved at 0.3–1 μM-cyclic AMP, and half-maximal phosphorylation was achieved at 56.8 ± 10.2 nM-cyclic AMP ($n = 4$).

KCl extraction of zymogen granules

In order to examine the nature of the association between Ca^{2+}-activated and cyclic AMP-activated protein kinases and zymogen granules, purified granules were extracted with a high-ionic-strength KCl solution (Fig. 6). Ca^{2+}-activated protein kinase activity was completely abolished in KCl-extracted granules, whether measured in the presence or absence of phosphatidylserine. In fact, in the presence of phosphatidylserine Ca^{2+} caused a consistent decrease in protein labelling; the reason for this decrease is at present unclear. By contrast, cyclic AMP-activated protein kinase

activity was still present in KCl-extracted granules, although specific activity was decreased by 35%. A high-speed supernatant obtained from KCl-extracted granules contained Ca^{2+}-activated protein kinase activity which was completely dependent on phosphatidylserine (Fig. 6). Relative to total kinase activity induced by Ca^{2+} and phosphatidylserine in starting granule material, this soluble activity represented a 90% recovery and 13-fold enrichment (i.e. 23.9pmol of P_i incorporated/min per mg of protein) of the phosphatidylserine-dependent protein kinase(s). Cyclic AMP-activated kinase activity was not detected in the high-speed granule supernatant (Fig. 6).

In addition to examining the effect of KCl treatment on granule protein kinase activity, the effect of such treatment on the granule proteins phosphorylated by granule-associated kinases was also investigated (Fig. 7). As expected, KCl-extracted granules did not exhibit any Ca^{2+}-induced protein phosphorylation in the presence of phosphatidylserine. In fact, phosphorylation of several granule proteins by Ca^{2+}-independent protein kinases was also abolished or decreased in extracted granules (e.g. proteins of M_r 72000 and 90000; see Fig. 7*b*). However, if granules were incubated with [γ-^{32}P]ATP and phosphatidylserine in the presence or absence of added Ca^{2+} before KCl treatment, the Ca^{2+}-induced phosphorylation of the M_r-62000 and -13000 proteins, as well as of other proteins, was preserved (Fig. 7*c*). Similarly, if purified membranes were made from ^{32}P-labelled granules, Ca^{2+}-induced phosphorylation of these same substrates was still observed in the membrane preparation and in fact enhanced relative to other phosphoproteins, although phosphorylation of the M_r-62000 protein was consistently decreased (Fig. 7*d*). Attempts to demonstrate Ca^{2+}-induced protein phosphorylation with purified granule membranes in the reaction mixture were unsuccessful.

Phosphatidylserine-dependent Ca^{2+}-activated protein kinase activity could also be completely extracted from granules into high-speed-supernatant material if KCl and EGTA were replaced by 20mM-EDTA in the extraction solution. Moreover, EDTA-treated granules did not display Ca^{2+}-induced phosphorylation of granule proteins in the presence or absence of phosphatidylserine or calmodulin (results not shown).

Constituent proteins of intact zymogen granules, granule contents and granule membranes

Fig. 8 shows proteins from intact zymogen granules, granule contents and granule membranes that have been resolved by SDS/polyacrylamide-gel electrophoresis and submitted to both Coomassie Blue and silver staining. Major protein bands present in intact granules as expected were also present in the granule contents (Figs. 8*a* and 8*b*). Several of the major bands present in intact granules and granule contents were present in the granule membrane preparation (Fig. 8*c*), and most probably represented secretory enzymes that had not been completely removed from the membranes by NaBr treatment. Two proteins of M_r 92000 and 13000, however, were not apparent in the granule or granule-contents preparations and presumably are of membrane origin. Additional bands which

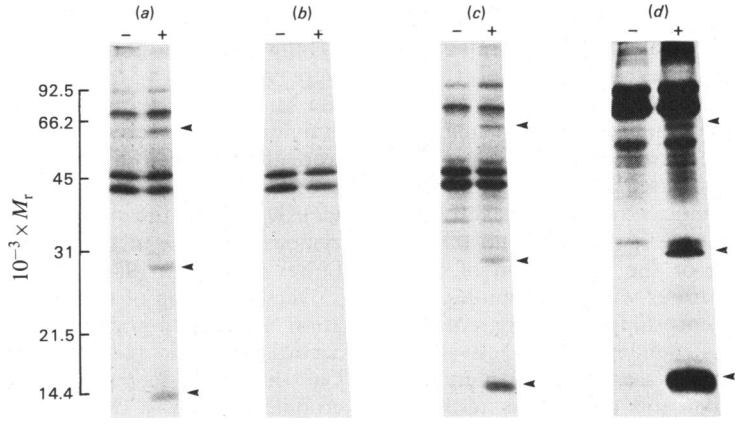

Fig. 7. *Autoradiographs of KCl-extracted granules and granule membranes*
Zymogen granules were incubated for 1 min with [^{32}P]ATP and phosphatidylserine in the presence (+) or absence (–) of 300μM-Ca^{2+}. (*a*), Intact purified granules; (*b*), granules extracted with KCl before ^{32}P-labelling; (*c*), granules extracted with KCl after ^{32}P-labelling; (*d*), granule membranes prepared after ^{32}P-labelling of intact granules. Arrows: bands altered by Ca^{2+}.

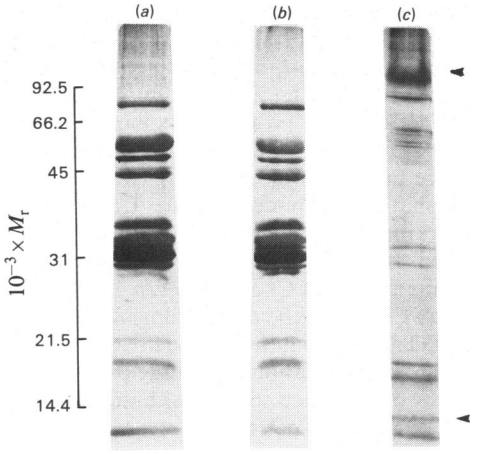

Fig. 8. *SDS/polyacrylamide-gel electrophoresis of proteins from purified zymogen granules*

(*a*) Intact granules; (*b*) granule contents; (*c*) granule membranes. Gels were stained with Coomassie Blue and then silver-stained. Arrows: M_r-92000 and -13000 membrane proteins.

were unique to the membrane preparation had apparent M_r values of 17000 and 29000. Resolution of additional proteins was either obscured by the presence of residual secretory enzymes or impaired owing to their low concentration in the membrane preparation.

Discussion

This study reports the preparation of highly purified zymogen granules from rat pancreas by using a self-forming Percoll density gradient. Preparation of secretory granules with Percoll has been reported for other tissues (Gratzl *et al.*, 1980, 1981; Carty *et al.*, 1980; Andersson & Abrahamsson, 1983), as well as pancreas (De Lisle *et al.*, 1984). The basic aims of this study were (*a*) to evaluate whether protein kinase activity regulated by either of the intracellular messengers, Ca^{2+} or cyclic AMP, was associated with the purified granules, (*b*) to determine whether such activity utilized granule proteins as substrates and (*c*) to investigate the nature of the association of the kinases and their substrates with the granules.

Several lines of evidence support the contention that a phospholipid-dependent Ca^{2+}-activated protein kinase, similar to the enzyme originally described by Nishizuka and co-workers termed protein kinase C (Takai *et al.*, 1979; Kishimoto *et al.*, 1980), is associated with purified zymogen granules. First, Ca^{2+}-induced phosphorylation of a M_r-62000 granule protein was largely dependent on the presence of phosphatidylserine. Second, phospholipid-dependent kinase activity also utilized as substrate lysine-rich histone, which has been routinely used to demonstrate the presence of protein kinase C in other systems (Takai *et al.*, 1979). Third, Ca^{2+}-activated histone kinase activity, which was extracted from intact granules by KCl or EDTA treatment, was totally dependent on exogenous phosphatidylserine. At present, it is not clear if in addition to the phospholipid-dependent kinase one or more types of Ca^{2+}-activated kinases dependent on calmodulin are also associated with the purified granules. Exogenous calmodulin did not alter the Ca^{2+}-sensitivity or specificity for granule-protein substrates of Ca^{2+}-activated kinase activity measured in the absence of added phospholipid. One possible explanation for these observations is that the granules contain one or more calmodulin-dependent kinases similar to phosphorylase kinase in that calmodulin is a tightly associated subunit of the enzyme (Shenolika *et al.*, 1979). Alternatively, the phospholipid-dependent enzyme is the only Ca^{2+}-activated kinase present in granules and can utilize endogenous phospholipid (LeBel & Beattie, 1984) to support submaximal activity in the absence of added phosphatidylserine. Further investigation is required to evaluate these two mechanisms.

In any event, it is clear that, from results of KCl and EDTA treatment, the phospholipid-dependent kinase(s) is relatively easily dissociated from the granule membrane, whereas cyclic AMP-activated kinase activity was resistant to KCl extraction. In contrast, the granule proteins that were substrates of Ca^{2+}-activated kinase activity were still associated with granules after KCl treatment, and even after preparation of granule membranes. These observations strongly suggest that the granule substrates are integral proteins of the zymogen-granule membrane. In fact, relative to intact granules, granule-membrane preparations were enriched in a M_r-13000 protein, which was of the same M_r as the principal substrate of Ca^{2+}-activated kinase activity. Membrane preparations were also enriched in a M_r-92000 protein which is presumably the GP2 glycoprotein, described previously as being the main protein constituent of the zymogen-granule membrane (Ronzio *et al.*, 1978; LeBel & Beattie, 1984).

Demonstration of Ca^{2+}-dependent phosphorylation of zymogen-granule membrane proteins is in accord with one possible mechanism by which Ca^{2+}-mediated secretagogues promote secretory-granule exocytosis. On the basis of the activation pathway proposed for protein kinase C (Nishizuka & Takai, 1980), increased diacylglycerol production, and possibly elevated cytosolic Ca^{2+} concentration, resulting from secretagogue–receptor interaction would lead to the activation of phos-

pholipid-dependent protein kinase present in the cytoplasm or on the zymogen-granule membrane. Both phosphatidylinositol breakdown and an increase in cytosolic Ca^{2+} have previously been demonstrated in pancreatic acinar cells (Putney *et al.*, 1983; Ochs *et al.*, 1983). Subsequent phosphorylation of specific granule membrane proteins might promote granule migration to and/or fusion with the apical plasma membrane. Although granule proteins did not undergo cyclic AMP-induced phosphorylation *in vitro*, such a reaction may still be involved in the action of cyclic AMP-mediated secretagogues in the intact cell. Further work is required to determine whether zymogen-granule protein phosphorylation occurs *in situ* in response to pancreatic secretagogues.

This work was supported by NIH grants AM32994 to J.A.W. and AM33335 to D.B. and by a grant for co-operative studies from NATO to J.A.W. and N.T.

References

Andersson, T. & Abrahamsson, H. (1983) *Anal. Biochem.* **132**, 82–88

Böhlen, P., Stein, S., Dairman, W. & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155**, 213–220

Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254

Burnham, D. B. & Williams, J. A. (1982) *J. Biol. Chem.* **257**, 10523–10528

Burnham, D. B. & Williams, J. A. (1984*a*) *Am. J. Physiol.* **246**, G500–G508

Burnham, D. B. & Williams, J. A. (1984*b*) *J. Pediatr. Gastroenterol. Nutr.* **3**, *Suppl.* **1**, 1–10

Carty, S. E., Johnson, R. G. & Scarpa, A. (1980) *Anal. Biochem.* **106**, 438–445

De Lisle, R. C., Schulz, I., Tryrakowski, T., Haase, W. & Hopfer, U. (1984) *Am. J. Physiol.* **246**, G411–G418

Freedman, S. D. & Jamieson, J. D. (1982) *J. Cell Biol.* **95**, 903–917

Gardner, J. D. (1979) *Annu. Rev. Physiol.* **41**, 55–66

Gorelick, F. S., Cohn, J. A., Freedman, S. D., Delahunt, N. G., Gershoni, J. M. & Jamieson, J. D. (1983) *J. Cell Biol.* **97**, 1294–1298

Gratzl, M., Torp-Pedersen, C., Dart, D., Treiman, M. & Thorn, N. A. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1615–1628

Gratzl, M., Krieger-Braver, H. & Ekerdt, R. (1981) *Biochim. Biophys. Acta* **649**, 355–366

Hinegardner, R. T. (1971) *Anal. Biochem.* **39**, 197–201

Hootman, S. R. & Philpott, C. W. (1979) *Anat. Rec.* **193**, 99–130

Jensen, R. T. & Gardner, J. D. (1978) *Gastroenterology* **75**, 806–817

Jung, D. H. (1980) *Clin. Chim. Acta* **100**, 7–11

Kishimoto, A. T., Takai, Y., Mori, T., Kikkawa, U. & Nishizuka, Y. (1980) *J. Biol. Chem.* **255**, 2273–2276

Lam, K. S. & Kasper, C. B. (1979) *Biochemistry* **18**, 307–311

Lambert, M., Camus, J. & Christophe, J. (1974) *FEBS Lett.* **49**, 228–232

LeBel, D. & Beattie, M. (1984) *Biochim. Biophys. Acta* **769**, 611–621

Lewis, D. S. & Ronzio, R. A. (1979) *Biochim. Biophys. Acta* **583**, 422–433

Macdonald, R. J. & Ronzio, R. A. (1974) *FEBS Lett.* **40**, 203–206

Meldolesi, J., Jamieson, J. D. & Palade, G. E. (1971) *J. Cell Biol.* **49**, 109–129

Nishizuka, Y. & Takai, Y. (1980) in *Protein Phosphorylation* (Rosen, O. M. & Krebs, E. G., eds.), vol. 8, pp. 237–250, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Ochs, D. L., Korenbrot, J. I. & Williams, J. A. (1983) *Biochem. Biophys. Res. Commun.* **117**, 122–128

Paquet, M. R., St.-Jean, P., Roberge, M. & Beaudoin, A. R. (1982) *Eur. J. Cell Biol.* **28**, 20–26

Price, R. S. & Dance, N. (1967) *Biochem. J.* **105**, 877–883

Putney, J. W., Burgess, G. M., Halenda, S. P., McKinney, J. S. & Rubin, R. P. (1983) *Biochem. J.* **212**, 483–488

Roberts, M. L. & Butcher, F. R. (1983) *Biochem. J.* **210**, 353–359

Ronzio, R. A., Kronquist, K. E., Lewis, D. S., Macdonald, R. J., Mohrlok, S. H. & O'Donnell, J. J. (1978) *Biochim. Biophys. Acta* **508**, 65–84

Scheele, G. A. (1982) in *The Secretory Granule* (Poisner, A. & Trifaro, J., eds.), pp. 213–246, Elsevier Biomedical Press, Amsterdam

Schmidt, E. (1974) in *Methoden der enzymatischen Analyse* (Bergmeyer, H. U., ed.), 3rd edn., pp. 689–696, Verlag Chemie, Weinheim/Bergstr.

Shenolikar, S., Cohen, P. T. W., Cohen, P., Nairn, A. C. & Perry, S. V. (1979) *Eur. J. Biochem.* **100**, 329–337

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. & Nishizuka, Y. (1979) *J. Biol. Chem.* **254**, 3692–3695

Takesue, S. & Omura, T. (1970) *J. Biochem.* (*Tokyo*) **67**, 259–266

Williams, J. A. (1980) *Am. J. Physiol.* **238**, G269–G279