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Effects of a cytosolic protein on the interaction of rat pancreatic zymogen granules in vitro

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Photon correlation spectroscopy has been used to study the kinetics of aggregation of isolated rat pancreatic zymogen granules in vitro by monitoring time-dependent changes in mean particle size derived from the photon count autocorrelation function, $g^2(\tau)$. Isolated granules were stable in isotonic sucrose (pH 5.4–7.0). At pH 6.0 they maintained a mean diameter of 1225 ± 18 nm with a polydispersity index of 0.199 ± 0.007. The mean granule diameter showed a limited decrease (approx. 20%) with increasing pH within the range 5.4–7.0, but the polydispersity index was unaltered. At pH > 7.0 granule instability was indicated by a rapid reduction in total photon counts. In solutions of monovalent cations ($[M^+] > 10$ mM) and divalent cations ($[M^{2+}] > 0.5$ mM) zymogen granules aggregated at a rate dependent upon both ion and granule concentration. These effects were consistent with the bimolecular nature of the interaction mechanism and were clearly distinguishable from the limited size changes associated with osmolarity. At concentrations of Na^+ or K^+ salts > 50 mM granule aggregation was accompanied by anion-dependent solubilisation. A soluble protein fraction separated from the pancreatic acinar cell cytosol by gel filtration reduced the mean diameter and polydispersity index of zymogen granules suspended in isotonic sucrose, inhibited cation-induced aggregation and stabilised granules to solubilisation induced by raising pH > 7.0 or exposure to high ionic strength media. The inhibitory effects of this protein were apparent at concentrations $\leqslant 10\ \mu g \cdot ml^{-1}$ (i.e. at inhibitor: granule protein ratios < 1:20) and could not be mimicked by bovine serum albumin, the Ca^{2+}-binding proteins calmodulin and troponin C ($\leqslant 100\ \mu g \cdot ml^{-1}$), nor the highly negatively charged polymer polyglutamate ($\leqslant 10\ \mu g \cdot ml^{-1}$). Inhibitory activity was also absent from fractions of rat liver cytosol prepared identically to pancreatic acinar cytosol. These observations are consistent with the presence in pancreatic acinar cells of a specific cytosolic granule stabilisation factor (or factors) that normally restricts zymogen granule interaction and may therefore play an important role in the regulation of granule mobility and exocytosis.

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Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; Mes, 4-morpholineethanesulphonic acid; $[M^{n+}]_i$, intracellular free cation concentration; PMSF phenylmethylsulphonyl fluoride.

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Introduction

The secretion of digestive enzymes by exocytosis from pancreatic acinar cells involves controlled fusion between the membranes of the zymogen granules and the apical region of the cell. The process is triggered by secretagogue activation of membrane receptors which initiates a change in cellular membrane conductance [1,2] and genera-

tion of one or more of a variety of putative second messengers such as Ca^{2+}, inositol phosphates, diacylglycerol or cyclic GMP [3–7]. The precise role of any of these intracellular mediators in the mobilisation of zymogen granules and the exocytotic extrusion of their contents is, however, still unknown. Attempts to elucidate the various stages of the secretory process by subcellular fractionation have revealed that, when compared to storage granules isolated from other types of secretory cell, isolated zymogen granules are less stable at physiological ionic strength and display an optimal stability in non-electrolyte solutions at pH 4.5–6.0 [8,9]. Since from measurements of total ionic content the intracellular milieu of pancreatic acinar cells appears to be similar to that of other cells, i.e. $[K^+]_i$ approx. 140 mM, $[Na^+]_i$ approx. 50 mM, pH 6.8–7.0 [10], it might be anticipated that in vivo the zymogen granule population would be unstable and undergo spontaneous lysis. Electron microscopic studies, however, have indicated no dissolution of granules in intact cells, even when maximally stimulated [11]. The differences in zymogen granule behaviour in vivo and in vitro have been ascribed by De Lisle et al. [12] to damage during the isolation procedure which renders the granules susceptible to ionic dissolution of the contents. An alternative possibility is that a cytoplasmic granule stabilisation factor normally restricts granule interaction in vivo and therefore plays an important role in the regulation of granule mobilisation and exocytosis. O'Connor and Matthews [13] have described some evidence for such a factor in cells of the endocrine pancreas. We report here the isolation and partial purification of a soluble protein factor in cells of the exocrine pancreas that stabilises zymogen granules to ion and pH-induced dissolution and potently inhibits granule aggregation in vitro.

The new technique of photon correlation spectroscopy based on dynamic laser light scattering has been used to investigate the kinetics of granule interaction, since this method allows time-dependent changes in granule dimension and aggregation to be monitored. The effects of osmolarity on granule size per se and the influence of pH and cations on zymogen granule interaction in the absence and presence of the cytosolic factor are described.

Methods

Zymogen granule preparation

Pancreases were excised from male Sprague-Dawley rats of 250–300 g and placed immediately in ice-cold Krebs-Henseleit solution (pH 7.4). After removal of accessory adipose tissue and blood vessels the pancreases were finely chopped in 10 volumes of cold 0.28 M sucrose solution and homogenised using three strokes in a Dounce homogeniser. Zymogen granules were obtained from the homogenised tissue essentially as described by Meldolesi et al. [14]. Nuclei and cellular debris were removed by centrifugation in a swing-out rotor at $300 \times g$ for 10 min and the supernatant was transferred to a fixed angle rotor and centrifuged at $700 \times g$ for 10 min. The resulting white zymogen granule pellet was washed free of the brown overlay of mitochondria, lysosomes etc. with 2×1 ml of 0.28 M sucrose, 5 mM Mes(pH 6.0) and resuspended in this medium to give a protein concentration of approx. 2 $mg \cdot ml^{-1}$. For light scattering measurements granules were routinely diluted into the suspension medium to give a granule protein concentration of approximately 200 $\mu g \cdot ml^{-1}$.

Cytosol fractionation

To obtain the pancreatic acinar cell cytosol fraction, PMSF was added at a final concentration of 0.1 mM to the supernatant from the zymogen granule preparation and the solution centrifuged at $100\,000 \times g$ in an ultracentrifuge for 60 min. The supernatant was dialysed overnight against 150 mM NaCl, 5 mM Mes (pH 6.0), concentrated to 1/40 of the original volume using Minicon B filters (Amicon) and subjected to gel filtration on a 60×1.5 cm column of Sephadex G-150 equilibrated with 150 mM NaCl, 5 mM Mes (pH 6.0). Cytosol fractions were eluted from the column in 2-ml aliquots of this buffer.

Biochemical analyses

The protein content of zymogen granule and cytoplasmic fractions was determined by the method of Bradford [15] using bovine serum albumin as a standard.

Cytochrome *c* oxidase was measured by the method of Hodges and Leonard [16] with the

modifications of Pacquet et al. [17]. *N*-Acetyl-β-glucosaminidase and NADPH-cytochrome *c* reductase were assayed by the methods of Findlay et al. [18] and Omura and Takesue [19], respectively, and α-amylase was determined according to the method of Rinderknecht et al. [20]. Enzyme activities were calculated as relative specific activity i.e. the ratio of the percent of enzyme marker to the percent of total protein in a given fraction, taking 100% activity as the activity in the cell homogenate.

Electron microscopy

Zymogen granules were fixed overnight at 4°C in 0.1 M sodium cacodylate buffer (pH 6.0) containing 2% glutaraldehyde, 2% paraformaldehyde and 2% sucrose and centrifuged at $8700 \times g$ for 2 min (Beckmann microfuge). Pellets were post-fixed with 2% osmium tetroxide for 2 h at room temperature, transferred to 0.05 M sodium maleate buffer (pH 5.2) and stained overnight with 2% uranyl acetate prior to dehydration in a graded ethanol series and embedding in Spurr's epoxy resin. Sections were cut with a diamond knife and stained with uranyl acetate and lead citrate before observation with a Philips EM 300 electron microscope.

Photon correlation spectroscopy

Particle size determinations. Coherent light from a 15 mW He-Ne laser (Spectra Physics Model 124A) was focussed at the centre of the sample (0.6 ml) contained in a glass cuvette maintained at a temperature of 37 ± 0.05°C in an optical index matching bath. Photon scattering was detected at 90° to the incident collimated laser beam by a photomultiplier tube (EMI D260E) equipped with an amplifier/discriminator. The digitized output of the photomultiplier tube was analysed with the 96 channel Autocorrelator (K7023) of a Malvern System 4300 Photon Correlation Spectrometer (Malvern Instruments Ltd., Malvern, U.K.) and on-line data fitting routines performed by an MPD 7023S microprocessor and printer [21].

The normalised autocorrelation function for quasi-elastic light scattering from a suspension of identical, freely diffusing particles is given by

$$g^2(\tau) = 1 + Ae^{-2\Gamma\tau} \tag{1}$$

where τ is the sample time in ms, A is an instrument optical constant, $\Gamma = DK^2$, and D is the diffusion coefficient. The scattering wave vector K is defined by

$$K = \frac{4\pi n}{\lambda} \sin \frac{\theta}{2} \tag{2}$$

and is constant for a given angle, θ, where n is the refractive index of the scattering medium and λ is the wavelength of the He-Ne laser light source (632.8 nm).

The gradient of the semi-log regression (determined by a least-squares polynomial fitting routine) from Eqn. 1, see Fig. 1, therefore yields D and from the Stokes-Einstein relationship

$$D = \frac{kT}{3\pi\eta d} \tag{3}$$

the particle diameter, d, can be determined, where k is Boltzmann's constant, T is temperature (K), η is medium viscosity. For each correlation function the time-averaged scattered light intensity, i.e. total photon counts, were also recorded for the correlation period. The computed quadratic function of the semi-log regression from Eqn. 1 also yielded an initial polydispersity index, $\mu_2/\bar{\Gamma}^2$ (see Refs. 22 and 23), for samples prior to aggregation.

Measurement of particle interaction and aggregation. If there are present initially in the scattering system n_0 monomers per unit volume and aggregation begins at time $t = 0$ then at $t > 0$ aggregates of 2, 3, 4, ..., j particles will be formed assuming that each particle encounter leads to lasting contact. At time t the number of j-fold particles will be given by

$$\sum_{j=1}^{\infty} n_j = \frac{n_0}{1 + 4\pi D n_0 t} \tag{4}$$

where D is the particle diffusion constant.

Now D, the average diffusion constant measured by photon correlation spectroscopy, is the average of the diffusion constants of all the particles in the suspension weighted by the intensity of the light scattered.

Thus for samples which are aggregating, i.e. are polydispersed, Eqn. 1 becomes:

$$g^2(\tau) = 1 + Ae^{-2\Gamma\tau} + \text{higher order terms} \tag{5}$$

The initial gradient of log $g^2(\tau)$ is now $\overline{D}K^2$ and $\overline{D}$ is the so-called z-averaged diffusion coefficient [22] i.e.

$$\overline{D} = \frac{\Sigma C_i D_i M_i}{\Sigma C_i M_i} \quad (6)$$

where for the ith species, C_i is the concentration, M_i is the mass and D_i is the diffusion coefficient. For completely mono-dispersed particles of diffusion coefficient, D, then $\overline{D} = D$, and higher order cumulants are small or negligible, but as aggregation proceeds, i.e. the number of j-mers increase (see Eqn. 4), then $\overline{D}$ becomes smaller than D. This progressive decrease in $\overline{D}$ will be reflected by the Stokes-Einstein relationship as a time-dependent increase in the mean particle diameter, $\bar{d}$. Measurements of mean particle diameter can be therefore be used as a sensitive measure of the interaction and aggregation of zymogen granules.

For particle aggregation studies using laser-light scattering techniques the optimal size range of the initial monomer is defined by

$$\lambda/4 < d/2 < \lambda$$

where λ is the wavelength of the incident radiation and d is the particle diameter [24].

Statistical analysis

The standard error of the mean (S.E.) was calculated and the significance of difference between means determined using Student's t-test.

Results

Granule characteristics and purity

Zymogen granules were obtained in high yield, approx. 2 mg granule protein/gram pancreas, using the two stage centrifugation procedure described in Methods. Enzyme assays for subcellular organelles indicated that mitochondrial, lysosomal and microsomal contamination of the zymogen granule fraction was low (the relative specific activities of cytochrome c oxidase, N-acetyl-β-glucosaminidase and NADPH-cytochrome c reductase in the granule fractions were respectively 0.32 ± 0.03 ($n = 25$), 0.63 ± 0.07 ($n = 25$) and 0.25 ± 0.1 ($n = 7$), (mean $\pm$ S.E.)) and the fraction was enriched in the secretory enzyme α-amylase, containing $17.96 \pm 2.63\%$ ($n = 25$) of total cellular α-amylase. The purity of the granules was further confirmed by electron microscopy (Plate 1a).

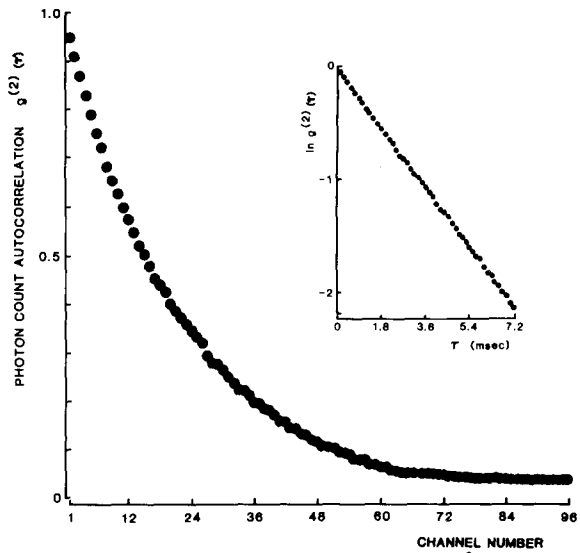
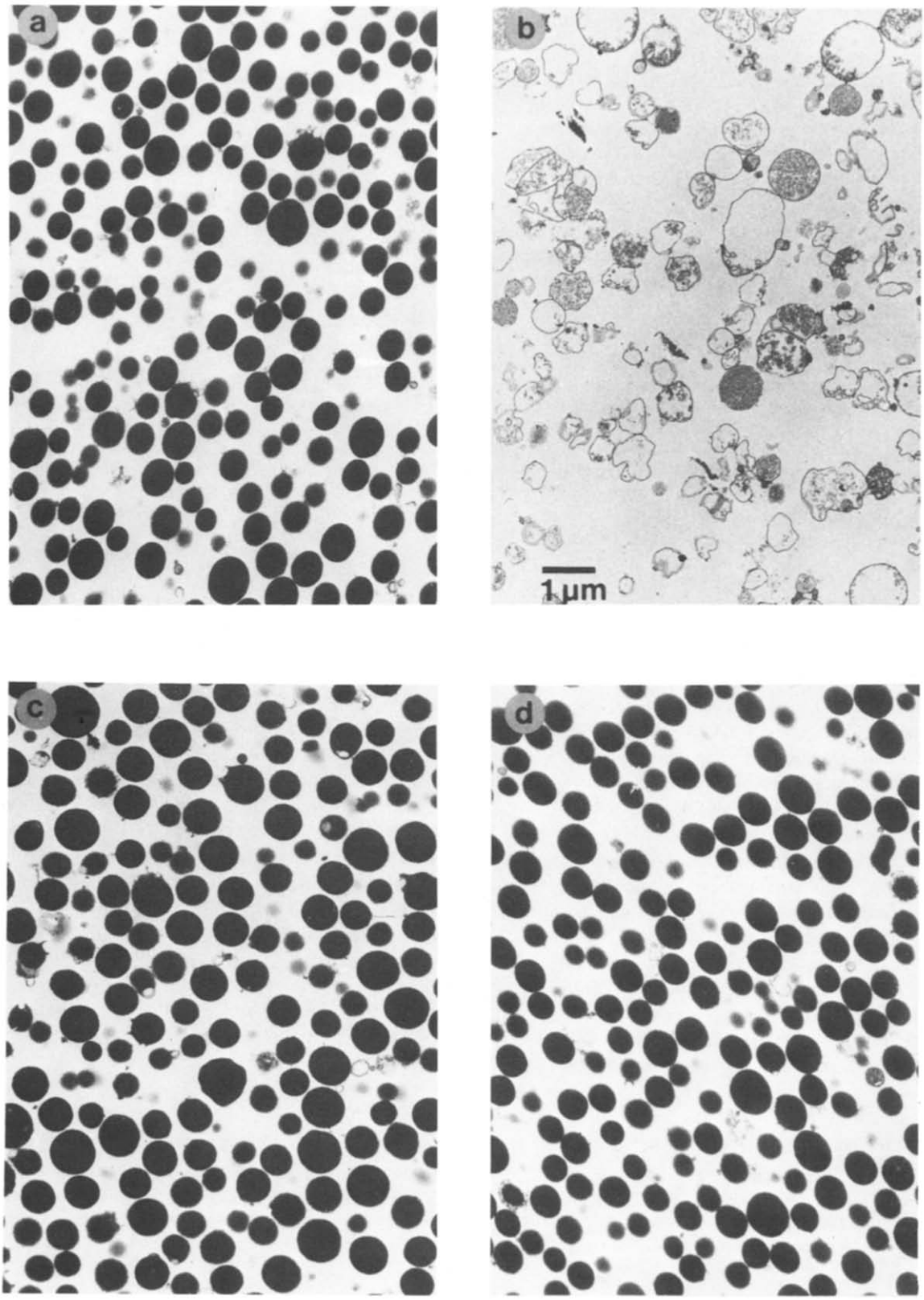


Fig. 1. Photon count autocorrelation function $g^2(\tau)$ of pancreatic zymogen granules suspended in 0.28 M sucrose, 5 mM Mes (pH 6.0) at a protein concentration of approx. 200 $\mu g \cdot m^{-1}$, measured at 37°C with a scattering angle of 90°. Time delay of each channel 150 μs; sample number 10^5. Inset: semi-log plot of $g^2(\tau)$ as a function of sample time.

The diffusion coefficient and mean diameter of zymogen granules were routinely determined from the photon count autocorrelation function (Fig. 1). The mean diameter of granules suspended in 0.28 M sucrose, 5 mM Mes (pH 6.0) was 1225 ± 18 nm (mean $\pm$ S.E.) ($n = 27$). This hydrodynamic value agrees with previously published estimates of zymogen granule diameter from electron mi-

Plate 1. Electron micrographs of zymogen granules isolated from rat pancreas. Magnification ×10800. The bar in (b) represents 1 μm; the same scale is used in all plates. (a) Granules suspended in 0.28 M sucrose, 5 mM Mes (pH 6.0). (b) Granules suspended in 125 mM NaCl, 5 mM Mes (pH 6.0) at 37°C for 10 min. (c) Granules incubated with 5 mM $CaCl_2$ for 15 min in the presence of cytosolic inhibitory fraction at a protein concentration of 10 $\mu g \cdot ml^{-1}$. Zymogen granules were suspended in 0.28 M sucrose, 5 mM Mes (pH 6.0) at 37°C. (d) Granules incubated in 125 mM NaCl, 5 mM Mes (pH 6.0) at 37°C for 15 min in the presence of cytosolic inhibitory fraction at a protein concentration of 10 $\mu g \cdot ml^{-1}$.


a
b
1 µm
c
d

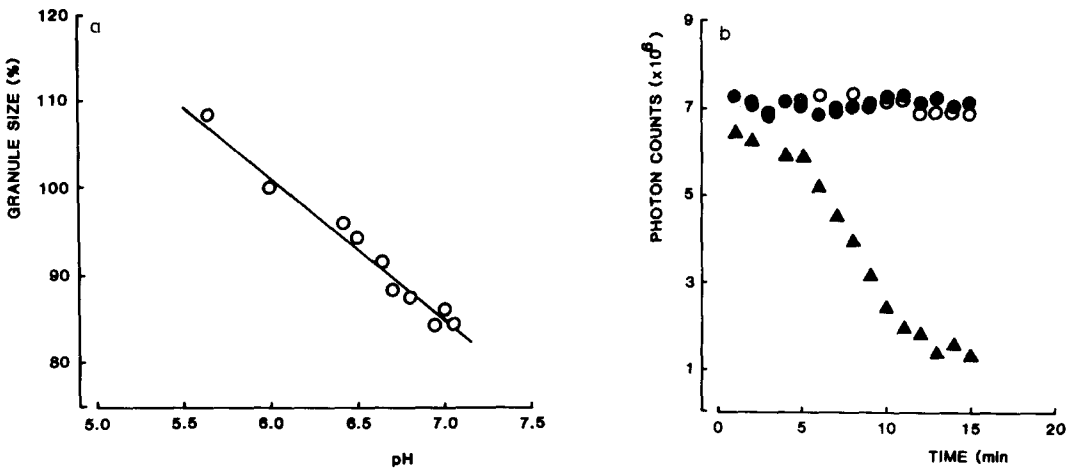


Fig. 2. Effect of pH on zymogen granules. (a) pH dependence of mean granule diameter. Zymogen granules were suspended in 0.28 M sucrose, 5 mM Mes at a protein concentration of 200 $\mu g \cdot ml^{-1}$ and granule diameter determined as the mean of 10 successive values calculated from the autocorrelation function as described in Methods. Granule size is expressed as a percentage of the mean diameter of granules measured at pH 6.0. The data are taken from four separate experiments. (b) Time dependence of zymogen granule stability monitored by total photon counts of granule suspensions buffered at pH 6.0 (○), pH 6.9 (●) and pH 7.3 (▲).

croscopy (924–1240 nm) [12,25]. The mean polydispersity index of zymogen granules suspended in sucrose/Mes was 0.199 ± 0.007. This value indicates that although the size range of the granules was greater than that of a homogeneous synthetic particle suspension (e.g., the mean diameter of polystyrene latex particles was 109 ± 0.4 nm with a polydispersity index of 0.073 ± 0.008), the range is similar to that shown by preparations of granules from other secretory cells [26].

Ionic dependence of granule stability

When compared with many other isolated secretory granule preparations, zymogen granules are relatively unstable under conditions approximating to normal intracellular ion concentrations and pH [8,9]. In 0.28 M sucrose, 5 mM Mes, at 37°C, however, the granules were stable in the laser beam over a pH range of 5.4 to 7.0. Increasing the pH within this range reduced the mean granule diameter (Fig. 2a) but the polydispersity index was unchanged. At pH > 7.0 the granules were unstable. The granule contents became solubilised and this phenomenon, assayed as α-amylase release from granules, correlated with a decrease in the total light scattering (photon counts) detected by the photomultiplier (Fig. 2b). Although the granules were relatively stable in sucrose solution between pH 5.4 and 7.0, they were routinely prepared and maintained at pH 6.0 before use, at which pH they were stable for > 90 min at 37°C.

When zymogen granules were suspended in 125 mM KCl, 5 mM Mes (pH 6.0), an immediate rapid, decrease in diffusion coefficient was observed. This was monitored continuously over the first 10 min by autocorrelation spectroscopy and used to determine the initial rate of increase in mean diameter of the granules. The rate of increase was dependent upon the concentration of KCl (Fig. 3) and showed a similar concentration dependence in NaCl, indicating that the response was not selective with respect to either cation. Measurement of the increase in mean diameter was limited to the first 10 min after raising the ionic strength by a decrease in total light scattering (photon counts) when $[M^+] > 50$ mM. The photon count reduction correlated with loss of α-amylase from the granules and the presence of empty vesicles in electron micrographs (Plate 1b), indicating that the granule contents are solubilised in high ionic strength media. For granules suspended in 125 mM solutions of Na^+ or K^+ salts containing 5 mM Mes (pH 6.0), both the rate of solubilisation and the aggregation rate were dependent upon the anion present. Thus for the

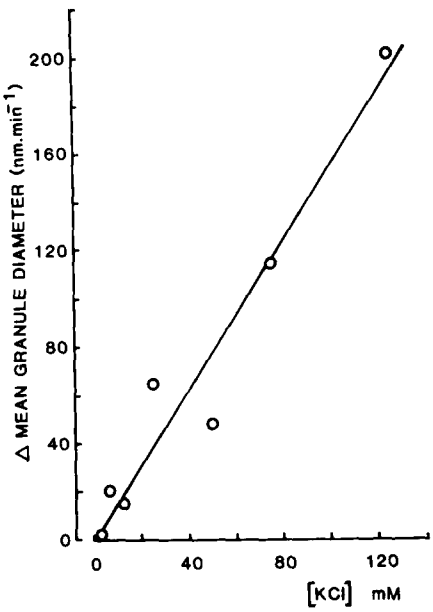


Fig. 3. Relationship between the rate of increase in mean diameter of zymogen granules and KCl concentration. Zymogen granules were suspended at a protein concentration of approx. 200 $\mu g \cdot ml^{-1}$ in 5 mM Mes (pH 6.0) with various concentrations of KCl. The rate of increase in mean granule diameter was determined from the values of granule diameter calculated for the first 10 minutes following exposure to ions. The results shown are from one experiment representative of five others.

following anion species the decreasing order of potency with respect to solubilisation was acetate > nitrate > chloride > bromide > isethionate > glutamate, which was strikingly similar to the increasing order of potency with respect to aggregation, viz. nitrate < acetate < chloride < isethionate < bromide < glutamate. The decrease in cation-induced aggregation rate in granule samples undergoing rapid solubilisation may be due to a decrease in photon counts reducing the accuracy of size determination. It is more probable, however, that solubilisation induced by permeant anions alters the light scattering properties of the granules and thus obscures the cation-induced aggregation under these conditions.

Effects of Ca^{2+}

Since a rise in intracellular [Ca^{2+}] is an early consequence of acinar cell stimulation by several secretagogues, the effects of Ca^{2+} on isolated zymogen granules were investigated. Addition of Ca^{2+} at concentrations > 0.5 mM to granules suspended in 0.28 M sucrose 5 mM Mes (pH 6.0), evoked a sustained increase in mean granule diameter which could not be reversed by the addition of EGTA. The mean rate of size increase

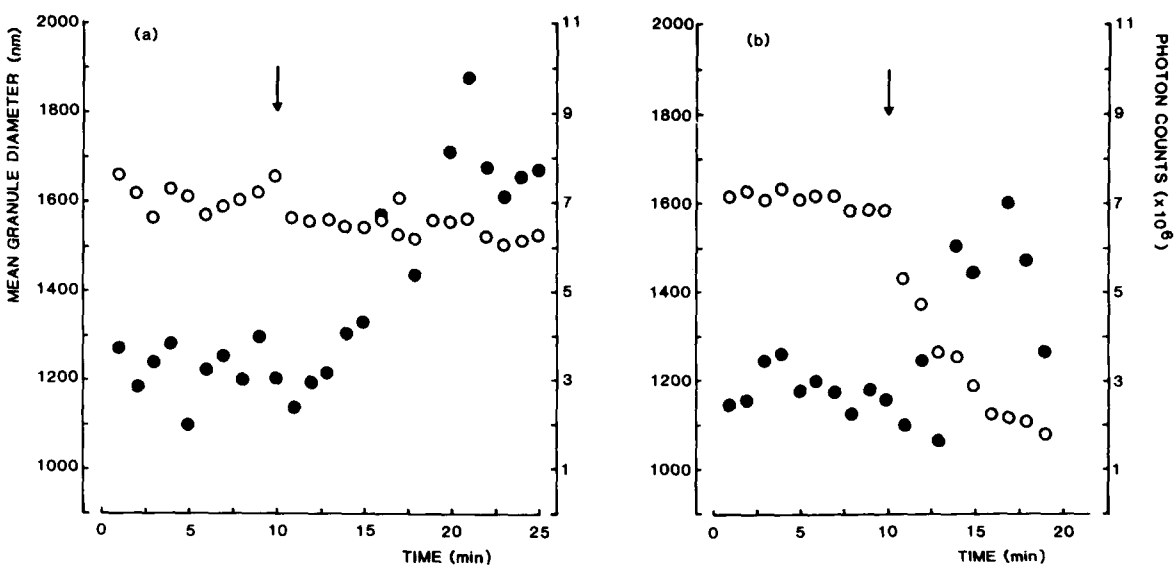


Fig. 4. Time dependence of the effect of Ca^{2+} on zymogen granule behaviour. The increase in mean granule diameter (●) and the change in total photon counts (○) induced by the addition of 5 mM $CaCl_2$ (at arrow) to zymogen granules (200 μg protein $\cdot ml^{-1}$) suspended in 0.28 M sucrose, 5 mM Mes were monitored continuously at (a) pH 6.0 and (b) pH 6.9. The results shown are taken from one experiment typical of at least five others.

induced by 5 mM Ca^{2+} was 54 ± 2 nm/min over the first 15 min (Fig. 4a). In contrast to the behaviour of granules exposed to high concentrations of monovalent cations, the Ca^{2+}-induced increase in diameter at pH 6.0 was not accompanied by a rapid decrease in total photon counts (< 0.5% min) and a large proportion of the granules appeared intact on electron microscopy. With increasing pH, however, both the rate of increase in diameter induced by Ca^{2+} and the stability of the granules were reduced until, at pH 7.0, 5 mM Ca^{2+} induced immediate breakdown of the granules, as evidenced by a rapid decrease in photon counts (Fig. 4b). Although Ca^{2+} is thought to be the major ion involved in the mechanism of secretion, the response of zymogen granules to divalent metal cations was not Ca^{2+}-specific. In decreasing order of potency in 0.28 M sucrose, 5 mM Mes (pH 6.0) Zn^{2+}, Mn^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+} and Mg^{2+} each increased mean granule diameter.

Osmotic effects

To determine whether the cation-induced increase in granule diameter represents aggregation or is due, at least in part, to osmotic swelling, the effect of osmolarity on granule diameter was measured using sucrose to adjust solution tonicity. As illustrated in Fig. 5, there was only a small change

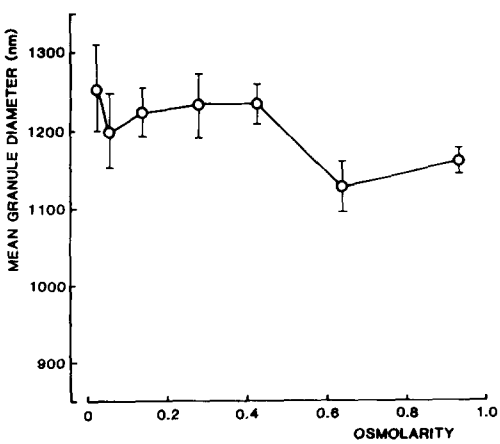


Fig. 5. Effect of osmolarity on zymogen granule diameter. The osmolarity of zymogen granule suspensions (approx. 200 μg protein · ml^{-1}) was controlled by changing the sucrose concentration in solutions containing 5 mM Mes (pH 6.0). The results are expressed as mean ± S.E. for four different experiments.

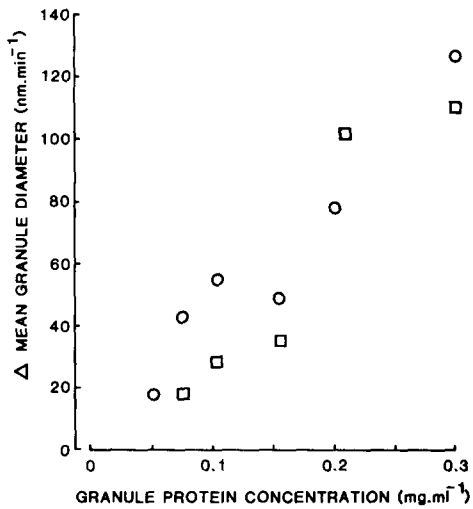


Fig. 6. Dependence of zymogen granule aggregation rate on granule concentration. Aggregation was induced by suspension of granules in 125 mM KCl (□) or by addition of 5 mM $CaCl_2$ to granules suspended in 0.28 M sucrose (○) and the aggregation rate determined as the increase in mean granule diameter measured over the first 10 minutes following exposure to ions. All solutions contained 5 mM Mes buffered at pH 6.0. Granule concentration is expressed as concentration of granule protein in mg · ml^{-1}.

(< 12%) in granule diameter in solutions ranging from 0 to 1 M sucrose, indicating that intact granules have only a very limited distensibility. Furthermore, if the ion-induced size increase were due simply to osmotic swelling it would be expected to be independent of granule concentration, but this is not so for either the K^+ or the Ca^{2+}-induced increase in mean granule diameter (Fig. 6). We therefore attribute the initial increase in mean granule diameter evoked by cations to aggregation. In the absence of aggregation the diffusion coefficient and mean granule diameter remained constant over a wide range of particle concentrations (0.04–0.4 protein · ml^{-1}), in agreement with quasi-elastic light scattering theory for non-constrained, non-interacting particles.

Granule stabilisation factor(s)

As noted in the Introduction, there is evidence that in some secretory cells granule mobilisation and exocytosis is controlled, at least in part, by soluble protein factors. To investigate whether such proteins exist in rat pancreatic acinar cells the 100 000 × *g* supernatant from homogenised rat

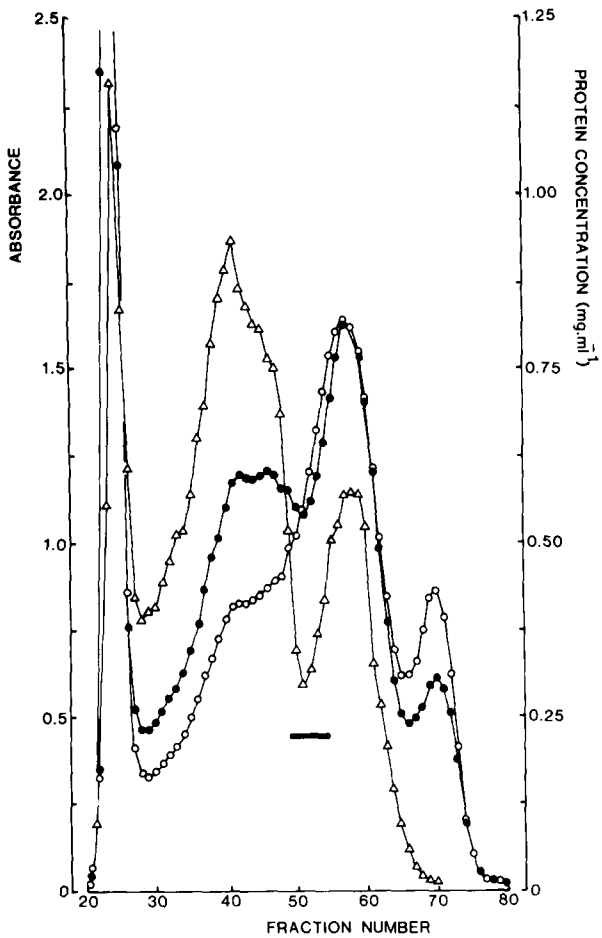


Fig. 7. A typical elution profile of pancreatic acinar cell cytosol fractionated on Sephadex G-150 (see Methods). 2-ml fractions were eluted in 150 mM NaCl, 5 mM Mes (pH 6.0). Absorbance at 280 nm (●); absorbance at 260 nm (○); protein concentration ($mg \cdot ml^{-1}$) (△). The bar indicates fractions that inhibited cation-induced aggregation of zymogen granules when added to granule suspensions at concentrations $\leq 10\ \mu g \cdot ml^{-1}$.

pancreas was fractionated on Sephadex G-150 as described in Methods. A typical elution profile of cytosolic components from the column is shown in Fig. 7. Fractions 48–54 consistently exhibited marked stabilising effects on granule suspensions and inhibited cation-induced aggregation at concentrations of approx. 10 $\mu g \cdot ml^{-1}$. The molecular weight range of proteins eluting from the column in these fractions was estimated, by comparison with fractionation of molecular weight markers, to be 20–27 kDa. All other fractions, or bovine serum albumin, when added at the same final protein concentration (5–200 $\mu g \cdot ml^{-1}$) were without effect on the ion-induced changes in granule laser light scattering. Identically prepared fractions from rat liver cytosol also showed no inhibition of cation-induced zymogen granule aggregation. The inhibitory fractions had four effects on zymogen granules: (1) the mean granule diameter in 0.28 M sucrose, 5 mM Mes (pH 6.0) was reduced by approx. 20%, from 1225 ± 18 nm to 998 ± 15 ($p < 0.001$) ($n = 27$); (2) the mean polydispersity index in 0.28 M sucrose was reduced from 0.199 ± 0.007 to 0.154 ± 0.004 ($P < 0.001$) ($n = 27$); (3) the cation-induced increase in mean granule diameter i.e., aggregation, was inhibited; and (4) the ion-induced decrease in total light scattering, i.e., photon counts, was markedly reduced. The effects of the inhibitory cytoplasmic fractions on Ca^{2+}- and KCl-induced aggregation are shown in Figs. 8 and 9, respectively, and in Plate 1c and 1d. The inhibition of the ionic effects on zymogen granules at pH 6.0 was also seen in the normal cellular pH range (pH 6.8–7.0). Preliminary attempts to purify further the inhibitory factor by DEAE-cellulose ion-exchange chromatography indicate that it is negatively charged at pH 6.0.

One way in which the inhibitory protein(s) might modulate the action of Ca^{2+} on zymogen granules is to act as a Ca^{2+} binding protein. However, using a method based on that of Cuatrecasas [27], there was no detectable $^{45}Ca^{2+}$

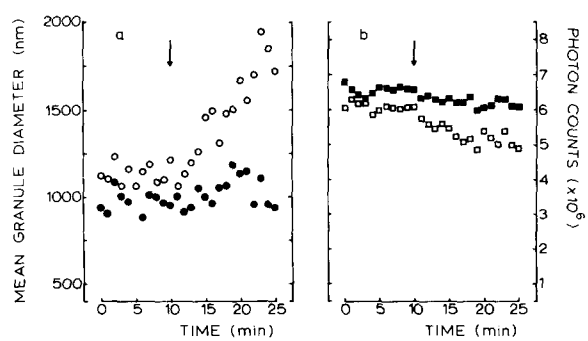


Fig. 8. Time dependence of the response of mean granule diameter (a) and photon counts (b) of zymogen granules to the addition of 5 mM $CaCl_2$ (at arrow) in the absence (open symbols) and presence (closed symbols) of cytosolic inhibitory fraction. Zymogen granules were suspended in 0.28 M sucrose, 5 mM Mes (pH 6.0). The final protein concentration of the inhibitory fraction was 10 $\mu g \cdot ml^{-1}$.

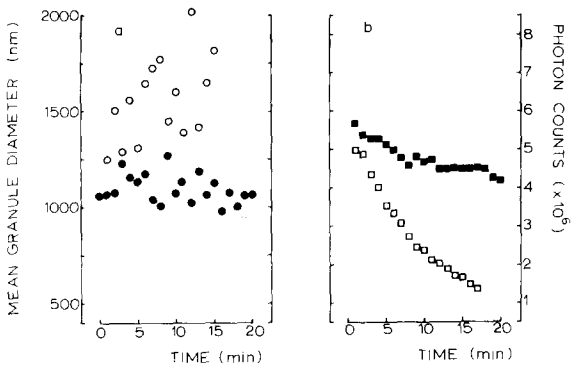


Fig. 9. Time dependence of the response of mean granule diameter (a) and photon counts (b) of zymogen granules suspended in 125 mM KCl, 5 mM Mes (pH 6.0) at $t = 0$, in the absence (open symbols) or presence (filled symbols) of cytosolic inhibitory fraction. The final protein concentration of the inhibitory fraction was 10 $\mu g \cdot ml^{-1}$.

binding to the protein in fractions 48–54. Furthermore, addition of purified calmodulin or troponin C ($\leqslant 5$ $\mu M \equiv 100$ $\mu g \cdot ml^{-1}$) did not reduce the mean diameter of zymogen granules suspended in sucrose, nor inhibit the effects of Ca^{2+} or KCl in increasing granule size (i.e., aggregation) or decreasing photon counts (i.e., solubilisation). Another possibility is that the negatively charged inhibitory protein may bind to the granule membrane and increase the electrostatic repulsion between adjacent granules. In each of three experiments, the addition of the polyanion, polyglutamate ($\leqslant 10$ $\mu g \cdot ml^{-1}$), had no effect on the properties or ion-induced responses of isolated zymogen granules. Any electrostatic effect of the inhibitory protein must therefore be dependent on specific adsorption to the granule surface.

Discussion

In this paper we describe the first isolation of a cytosolic factor that stabilises rat pancreatic zymogen granules. This factor reduced the hydrodynamic diameter of granules suspended in sucrose and inhibited cation-induced aggregation. The inhibitory factor also reduced the solubilisation of granule content when the pH was raised above 7.0 or the granules suspended in high ionic strength media. The aggregating effect of cations in the absence of inhibitory protein is most probably due to a decrease in the net negative surface charge of the zymogen granule membrane [28]. As divalent cations will be much more effective (> 50-fold) than monovalent cations in compressing the ionic double layer and reducing the interfacial potential energy barrier between individual zymogen granules, they will promote aggregation at much lower concentrations. This may result in fusion and loss of granule contents, but cannot be the only mechanism of solubilisation, since at pH > 7.0 loss of granule content occurred rapidly with no indication of prior aggregation. Furthermore, in 5 mM Mes (pH 6.0) in the presence of 125 mM sodium glutamate, aggregation proceeded rapidly without extensive solubilisation, while in the presence of 125 mM sodium acetate rapid solubilisation was accompanied by only a low rate of aggregation. These observations suggest that solubilisation results from permeation of ions across the granule membrane, a process determined by its selective permeability to anions.

The action of the cytoplasmic fraction in inhibiting the aggregation process cannot be attributed to simple electrostatic effects or cationic charge neutralisation lowering bulk phase electrolyte concentration, because the protein was eluted from the column in 150 mM NaCl and added to zymogen granules in this solution. Furthermore, the absence of detectable Ca^{2+} binding to the inhibitory factor and the inability of troponin C or calmodulin to mimic the inhibitory effects clearly indicates that granule fusion is not regulated by Ca^{2+} binding proteins alone. Although changes in viscosity may exert some effect it is likely that the inhibitory factor(s) undergoes adsorption to the zymogen granule surface and both spatially opposes the electrostatic action of cations and stabilises membrane structure; a polyanionic protein would be particularly effective in this regard. Such a protein could also reduce solubilisation by decreasing the exchange of free anions with, and permeation across, the granule membrane. The inability of bovine serum albumin or the highly negatively charged polymer, polyglutamate, to mimic the effects of the cytosolic factor points to an important specificity in the surface adsorption and aggregation-inhibition reaction. This specificity is also suggested by the absence from rat liver cytosol of an effective in-

hibitory factor. Thus, in addition to modulation of the ion-induced short-range interactions which would otherwise lead to wholesale aggregation within the acinar cell, the cytosolic factor(s) may have an important functional role to play when adsorbed to the granule surface or in close proximity to it.

The in vivo stability of zymogen granules in the resting acinar cell compared with their instability in an in vitro ionic environment approximating to that of the intracellular compartment has hitherto been rationalised by three proposals: (1) the granule isolation procedures may damage the granules, making them unstable in cytosol-like media [12]; (2) granule proteins may equilibrate across the granule membrane and thus be lost down the concentration gradient in vitro [29]; and (3) the activity of ions in the cytosol of acinar cells must be considerably lower than their concentrations [30]. The first proposal, based on the observation that zymogen granules prepared by density gradient centrifugation in Percoll are stable in isotonic NaCl or KCl at physiological pH, has been criticized by Rothman and Liebow [31]. They pointed out that, when prepared by other methods, more than 90% of granules would have to be damaged to explain their behaviour in ionic media although, in electron microscopy sections, they appear very similar to granules prepared in Percoll. We have found that extensive washing partially restored the response of granules prepared in Percoll to cation-induced aggregation without increasing their instability in isosmotic sucrose; this treatment also reduced a component of the total light scattering contributed by small particles (data not shown). We therefore suggest that the refractoriness of Percoll-prepared granules to isotonic NaCl or KCl can be attributed to the prevention of granule interaction by Percoll binding to the granule surface. Zymogen granules prepared by the two-step procedure described in Methods were very stable in sucrose solution in the pH range 5.4–7.0, at 37°C, as determined by the absence of any change in total photon counts for at least 30 min. Furthermore, this stability persisted upon dilution, a finding inconsistent with equilibration of granule proteins across the granule membrane, as proposed by Liebow and Rothman.

The third rationalisation, i.e. that the activity of ions in the cytosol is considerably lower than their concentrations, has been shown to be untenable. Intracellular free ion concentrations of 10.5 mM and 124 mM for Na^+ and K^+, respectively, have been recorded with ion-selective microelectrodes [32,33] and the intracellular pH has been estimated as 6.8–7.0 from 5,5-dimethyl-2,4-oxazolidinedione (DMO) partitioning experiments [10]. The $[Ca^{2+}]_i$ in resting acinar cells has also been shown to be similar to that in other cell types, i.e. approx. 150 nM, when measured by quin2 fluorescence [4,34]. Cellular compartmentalisation might permit maintenance of a specialised ionic microenvironment in the vicinity of the zymogen granules, which are largely confined to the apical region of the cell. There is, however, no convincing evidence for any cellular structure which would effectively isolate the mature granules of the apical region from cytosolic continuity with more distal regions [35]. The presence of a cytosolic protein factor, reported here to stabilise isolated zymogen granules against the aggregating effects of solutions of physiological ionic strength, would readily explain the maintenance of granule integrity observed in vivo and has several important implications for control of normal secretion. Further experiments are required to resolve the mechanism whereby the inhibitory effects of the protein may be counteracted during exocytosis: this may be regulated, directly or indirectly, by the phosphorylation of cytosolic or granule proteins that occurs when acinar cells are stimulated with a variety of ligands [36,37]. This process may be similar to the regulation of the inhibitory protein synapsin by Ca^{2+}-calmodulin dependent phosphorylation in neurosecretory cells [38].

Finally, the results of this study illustrate the advantages of photon correlation spectroscopy for investigating the kinetics of granule interaction. By use of this technique we have been able to distinguish between the limited changes in zymogen granule size resulting from osmotic swelling and the extensive changes due to granule aggregation upon exposure to cations. Furthermore, by continuously monitoring the total light scattering, the stability of the granules over the experimental period can be assessed under precisely controlled conditions.

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