brief communication

Elastic behavior of zymogen granule membranes in response to changes in pH and pCa

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ABSTRACT In the process of secretion, the membrane of secretory granules is expected to change its elastic behavior. Elastic modulus of the membrane of zymogen granules, prepared from the rat pancreas acinar cell, was measured by an osmotic swelling method. The elastic modulus of the granule membrane at pCa 8 reduced from the maximal value of 230 dyn/cm at pH 6.0 to almost zero at pH 7.5. In a cytosol of an acinar cell, calcium ions play an important role as a second messenger in secretion. The elastic modulus of the granule membrane reduced in a sigmoidal fashion at pCa between 7.0 and 6.0. This range of pCa corresponds to a physiological rise of free Ca^{2+} concentrations in the cell cytosol when stimulated by external secretagogues. Reduction of the elastic modulus indicates that the state of the granule membrane switches to a more flexible one in which the granule is easy to appose to the cell plasma membrane and then swell as a final step of exocytosis.

INTRODUCTION

In biological cells, many signal-transduction steps are activated in response to external stimulation, and as a final result, the cell extends the effect to the outside of the cell or to other cells. Many efforts have been made to clarify this mechanism. Especially in secretion cells, a large amount of material is released as a response to small external stimulation. In this secretion process, there are many such steps as reception of second messenger signal, movement of a granule to a secretion site, apposition of the granule to a cell plasma membrane, fusion, and secretion of its content. Many of these steps have not necessarily been clarified so far (De Lisle and Williams, 1986; Holz, 1986). The final step of exocytosis of biological cells is essentially the fusion process between the vesicle (granule) and the plasma membrane. As a good model of this process, Cohen et al. (1982) have examined the effect of an osmolarity change on the fusion between phospholipid vesicles and a planar phospholipid bilayer in the following steps. Addition of vesicles on the *cis*-side, addition of divalent cations to enforce apposition of the vesicle to the planar bilayer, and application of the osmotic pressure to the vesicle or to the planar bilayer. In the same way, granules in the exocytotic process of such biological cells as exocrine, endocrine, and neuron are supposed to swell osmotically after apposition to the cell plasma membrane. In the mast cell, a substantial osmotic swelling of giant granules was observed under a video-enhanced optical microscope and also by a measurement of capacitance (Zimmerberg et al., 1987; Breckenridge and Almers, 1987). In the secretion process, the state of the granule membrane should switch to a more flexible one where the granule easily apposes to the cell membrane with sufficient area, and then easily swells in response to a small osmolarity change. Osmotic effects in membrane fusion during exocytosis have extensively been described in a recent review article (Brocklehurst and Pollard, 1988).

In this study, our interest is concentrated on the measurement of a change in a physical property of the membrane of zymogen granules isolated from the rat pancreas acinar cells. By use of an osmotic swelling method, the elastic modulus of the granule membrane was measured in similar ranges of pH and Ca^{2+} concentration ($[Ca^{2+}]$) to physiological ones in a step of amylase secretion. This method has successfully been used to obtain the membrane elastic modulus of the synthetic phospholipid vesicles (Li et al., 1986), and of the biological vesicles prepared from brush border of the rat small intestine (Miyamoto et al., 1988). The key step in this method is an accurate determination of sizes of submicroscopic vesicles. For this aim, a dynamic light-scattering method is the most powerful.

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MATERIALS AND METHODS

Preparation of granules

Zymogen granules were prepared from the pancreas of Sprague-Dawley rats (male, 150–300 g body weight) by the method of Meldolesi (1983) with slight modifications. Two to three rats were used per experiment.

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(1–3 g pancreas wet weight). The animals were anesthetized with ether vapor or injection of Na-penthobarbitol (1 mg/100 g body weight). The pancreas was quickly excised and put into an ice-cold Krebs-Henseleit solution (pH 7.4). The excised pancreas was trimmed free of fat and connective tissue (to remove adipose tissue and blood vessels). The pancreas was transferred into the 10 vols of an unbuffered 280-mM sucrose solution, and minced with fine scissors. The suspension was homogenized with three strokes in a Dounce (glass/Teflon) homogenizer (Ikemoto Rika Co. Ltd., Tokyo) operated at 3,000 rpm. Aliquots (6 ml each) were distributed in 12-ml conical glass tubes, and centrifuged at 1,000 g_{max} in a swinging-bucket rotor for 10 min. The supernatant was discarded, each pellet was overlaid with 5 ml of the unbuffered solution and resuspended manually by using a Teflon rod. Special care was paid to crush the small white mass rich in zymogen granules. These suspensions were centrifuged at 180 g_{max} for 12 min. The resultant supernatant was aspirated and filtered through 110-mesh nylon gauze into 15-ml round-bottomed corex tubes, and centrifuged at 1,000 g_{max} for 3.5 min. Zymogen granules were packed tightly as a thin, round white pellet covered by a loose tan layer rich in mitochondria. This tan layer was removed by gently pouring 1 ml of a buffered solution containing 280 mM sucrose and 5 mM MES (pH 6.5) along the tube wall and swirling carefully. The solution was carefully aspirated and discarded. Each pellet was resuspended on 3 ml of the buffered solution, and centrifuged at 180 g_{max} for 12 min. The supernatant was collected in a proper volume of the buffered solution. These purification steps were repeated three times to remove mitochondria completely. Zymogen granules were stored in a solution containing 280 mM sucrose, 5 mM MES (pH 6.5), and 4 mM EGTA, and stored at 0°C until use. Osmotic swelling experiments were carried out at 10°C for granules within 48 h after preparation. The protein concentration was determined by the method of Lowry et al. (1951) and modified by Bensadoun and Weinstein (1976), with bovine serum albumin as standard.

Osmotic swelling

Granules with an initial diameter d_o are prepared in an aqueous solution containing membrane-impermeable solute near isoosmotic concentration C_o (mol/liter). Note $C_o = \gamma C$, where γ (≤ 1) is the osmotic activity coefficient and C is the concentration of amylase and other osmotically active molecules inside the granule. When a dilution buffer is added to reduce the solute concentration to C_e, water flows into the granule and the osmotic stress T_s increases the membrane area by $\Delta A = (d_f^2 - d_o^2)/d_o^2$, where d_f is the final diameter. If γ is assumed to be constant during dilution, a stress-strain relation $T_s = M\Delta A$ gives

$$d_f - d_o = (d_o d_f K_o/8)(1/M)[(d_o/d_f)^3 C_o - C_e]. \quad (1)$$

where K_o is the osmotic coefficient and M (dynes/centimeter) is the membrane elastic modulus (Miyamoto et al., 1988). Although the concentration of amylase inside the granule is very high, the osmotic pressure does not break the granule membrane. This is due to an internal organization of amylase molecules (Ermak and Rothman, 1978). Regardless of the mechanism of this organization, we can equivalently assume a very low osmotic activity coefficient γ of amylase molecules.

Dynamic light scattering

The granule diameter was determined by dynamic light scattering (DLS). The DLS apparatus and method of analysis are detailed elsewhere (Miyamoto et al., 1988). The decay rate Γ of the DLS spectrum for suspension of spheres is related to the particle diffusion coefficient D and diameter d by $D = \Gamma/K^2 = k_B T/(3\pi\eta d)$, where $K = (4\pi n/\lambda)\sin(\theta/2)$ (n: the refractive index of the medium; λ: the wavelength of incident light in vacuum; and θ: the scattering angle), k_B is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity. The tabulated values were used for n and η of sucrose solutions (Weast, 1976). The most probable d value for a given condition was determined by averaging d values of 10 successive measurements at $\theta = 90°$.

Characterization of zymogen granules

Characterization by DLS of our zymogen granules has been detailed elsewhere (Fujime et al., 1988*b*), which can be summarized as follows. Over the accessible range of K, the D vs. K^2 relationship reasonably agreed with a DLS theory for a slightly polydisperse suspension of spheres, showing no appreciable contamination of particles larger and/or smaller than zymogen granules. The number-average diameter d_n was ~800 nm and the relative dispersion in size distribution σ/d_n was 0.18. The number of granules with diameter in a range $d_n \pm 2\sigma$ was >96% of the total. Our granule suspension was substantially monodisperse, and the measured diameter was little dependent on the scattering angle around 90°. These are important for a quantitative study of osmotic swelling at only one angle of 90° (Fujime et al., 1988*a*).

RESULTS AND DISCUSSION

Dependence on pH of elastic modulus of granule membranes

Fig. 1 *a* shows some examples of the osmotic swelling curves at pCa 8 and various pH. On lowering the sucrose concentration C_e, a good linear relationship between $(d_f - d_o)$ and $(C_o - C_e)$ was observed, except a few points, in this concentration range. The slope of the straight line became slightly larger with the increase of pH. Fig. 1 *b* shows the diameter vs. pH relationship of the granule at 280 mM sucrose. The reasons why the diameter of the granule strongly depends on pH may be as follows. (*a*) The molecular structure of the granule membrane (bilayer membrane decorated with various kinds of proteins and cytoskeleton) changes depending on pH and (*b*) the transport system of the granule membrane is regulated by pH (Hellmessen et al., 1985; Carter et al., 1987). From *a* we infer that the granule assumes an invaginated form in a certain circumference. Even in a small invagination, the granule diameter may reduce to a great extent. Many invaginated granules can be observed on electron micrographs (Ermak and Rothman, 1978). At pH $\leq$ 5.5, the aggregation of granules took place, and the mean diameter of the granules became extremely large. This was easily checked visually under a phase contrast microscope. (However, neither mitochondria nor other organelles were observed, suggesting again a high purity of our samples). As shown in Fig. 1 *c*, the elastic modulus of the granule membrane decreased from 230 dyn/cm at pH 6.0 to almost zero at pH 7.5. The value of zero for the elastic modulus reflects that the granule membrane

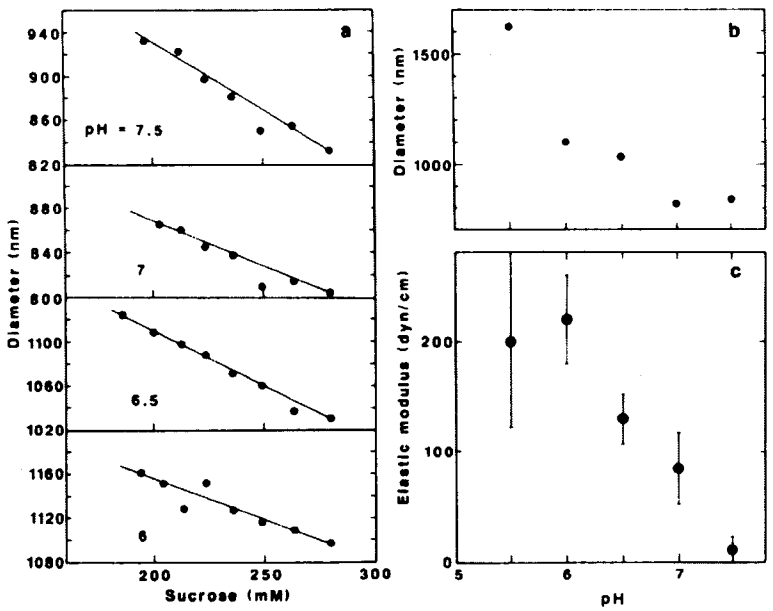

FIGURE 1 Membrane properties of zymogen granules at various pH. (*a*) Osmotic swelling curves. The granule suspension (100 μg/ml protein) was prepared in 280 mM sucrose, 1 mM EGTA, 5 mM MES (at each pH), and incubated for 20 min at 20°C. The dilution buffer containing 1 mM EGTA and 5 mM MES (at each pH) was added in steps of 50 μl with a speed of 10 μl/min. The circles without error bars have the standard deviation within the double size of the symbol. (*b*) The diameter at 280 mM sucrose vs. pH relationship. (*c*) The elastic modulus vs. pH relationship.

undergoes hydrolytic lysis, and that the granule content is solubilized at pH > 7.5. The elastic modulus of the granule membrane changes sensitively with pH around the granule. This suggests a possibility that fusion and exocytosis of zymogen granules can be triggered also by small pH changes around the granule.

For the rat pancreas acinar cell, pH of the cytosol of an unstimulated cell is generally ~pH 6.8 (Hellmessen et al., 1985). For the mouse pancreas acinar cell, Carter et al. (1987) reported an increase of pH inside the cell by 0.1–0.3 pH units and secretion of amylase by 5 to 10% of the total, after stimulation by such secretagogues as carbachol, caerulein, and Br-A23187. An increase of pH by 0.1–0.3 U may also occur in response to a change in external salt concentration as a stimulation. A change of pH in this range will result in a reduction in the elastic modulus of the granule membrane by 20–30 dyn/cm. This suggests that the state of the granule membrane switches to a more flexible one enough to osmotically swell just before exocytosis.

Dependence on pCa of elastic modulus of granule membranes

Fig. 2 *a* shows the osmotic swelling curves of the granules at pH 6.5 and various pCa. At high pCa, the diameter of the granule increased linearly with the dilution. But, the range of this linear relation became narrower at low pCa, suggesting occurrence of lysis. Fig. 2 *b* shows the diameter vs. pCa relationship at 280 mM sucrose. In the physiological [Ca^{2+}] range from 100 nM to 10 μM, the granule diameter slightly decreased or stayed almost constant. This fact suggests that in this [Ca^{2+}] range, neither aggregation nor fusion of granules took place. In a higher [Ca^{2+}] range, 1 and 10 mM, an abrupt increase of the granule diameter was observed. This is compatible with the results at millimolar [Ca^{2+}]s by Warashina (1981) and Rogers et al. (1987). This abrupt increase in the diameter probably corresponds to aggregation and/or fusion of granules. However, this aggregation seems not to be a physiological one, simply because [Ca^{2+}] is too high. Fig. 2 *c* shows the elastic modulus vs. pCa relationship of the granule membrane at pH 6.5. The elastic modulus of the granule membrane decreased from 300 dyn/cm at 100 nM Ca^{2+} to 50 dyn/cm at 100 μM Ca^{2+}. Additional experiments suggested no irreversible effect of low pCa on the granule membrane at 280 mM sucrose. After samples at pCa 5.0 and pH 6.5 were left standing at 10°C, they also gave high values (~300 dyn/cm) of elastic modulus at pCa > 7.5.

From quin-2 fluorescence measurements for the rat acinar cell, Ochs et al. (1983) reported that the cytosolic [Ca^{2+}] at an unstimulated state was (180 ± 4) nM, and at a stimulated state by such secretagogues as carbachol and cholecystokinin, the cytosolic [Ca^{2+}] rose to a maximal value of (860 ± 41) nM. As shown in Fig. 2 *c*, the

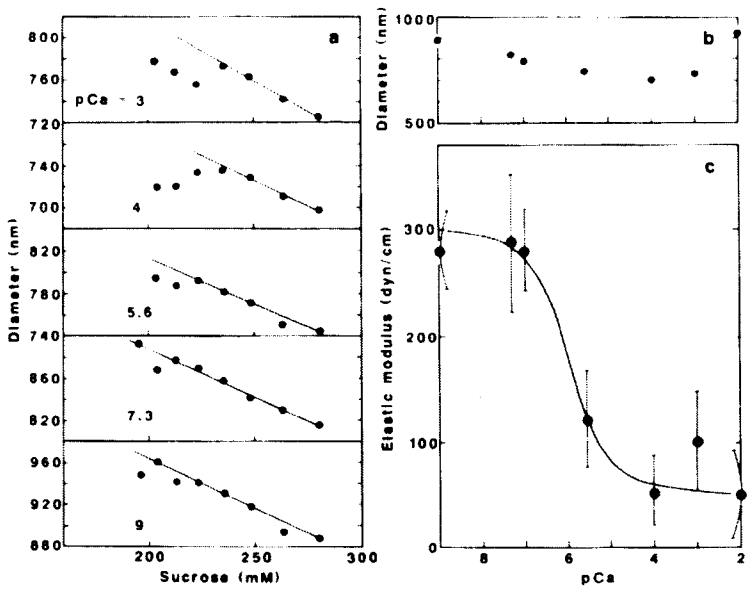

FIGURE 2 Membrane properties of zymogen granules at various pCa. (*a*) Osmotic swelling curves. The granule suspension (100 μg/ml protein) was prepared in 280 mM sucrose, 5 mM MES (pH 6.5), and each $[Ca^{2+}]$ adjusted by Ca^{2+}-EGTA buffer. The pCa values were determined by a multiequilibrium formulation, where the EGTA-calcium binding constant at our condition was assumed to be $10^{5.5}$. The dilution buffer contained 5 mM MES (pH 6.5) and the same amount of Ca^{2+}-EGTA buffer as that of the suspension. For others, see legend to Fig. 1. (*b*) The diameter at 280 mM sucrose vs. pCa relationship. (*c*) The elastic modulus vs. pCa relationship.

elasticity of the granule membrane steeply reduced at ~1 μM Ca^{2+}, corresponding to the rise of cytosolic $[Ca^{2+}]$. As in the case of the pH change, the state of the granule membrane switches to a more flexible one where osmotic swelling is easily activated as a prelude just before exocytosis. From the present results, we can expect a synergistic effect of pH and pCa on the change in elastic modulus of the granule membrane.

Some remarks

Absolute sizes of the granule diameter and elastic modulus for a given condition were different from preparation to preparation of suspensions of zymogen granules. For example, the diameter and elastic modulus at pH 6.5 and 1 mM EGTA in Fig. 1 are different from those at pCa > 7 and pH 6.5 in Fig. 2. This is inferred to be due to slight differences in the state, mean size, and/or size distribution of granules depending on preparation. However, the general trends of many other observations were all the same as those given above.

From this study as well as that for chromaffin granules (Miyamoto and Fujime, 1988), it is suggested that the membrane elastic modulus of the granule in the cell reduces to a great extent after a signal transduction to activate exocytosis. This indicates that the granule becomes easy to appose to the cell membrane with significant area, and to swell as a prelude to exocytosis. It remains to be elucidated, however, which biochemical event(s) is most responsible for the great reduction of membrane elasticity with pCa and/or pH.

This study as well as the previous ones (Li et al., 1986; Miyamoto et al., 1988; Miyamoto and Fujime, 1988) have proved that combination of osmotic swelling and DLS methods is very powerful in the study of the elastic behavior of the membranes of various granules and vesicles with submicroscopic sizes under controlled conditions.

CONCLUSIONS

In this study on exocrine zymogen granules, we observed a clear correlation between an increase in structural flexibility and activation of physiological function. This kind of correlation has also been observed not only for endocrine chromaffin granules and brush-border membrane vesicles (Miyamoto and Fujime, 1988; Miyamoto et al., 1988), but also for reconstituted F-actin and intact thin filaments of skeletal muscle (Ishiwata and Fujime, 1972; Yoshino et al., 1978; see also Fujime, 1987). As a result, it is generally speculated that the structure of biomolecular machinery becomes more flexible when its physiological function is activated.

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REFERENCES

Bensadoun, A., and D. Weinstein. 1976. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* 70:241–250.

Breckenridge, L. J., and W. Almers. 1987. Final steps in exocytosis observed in a cell with giant secretory granules. *Proc. Natl. Acad. Sci. USA.* 84:1945–1949.

Brocklehurst, K. W., and H. B. Pollard. 1988. Osmotic effects in membrane fusion during exocytosis. *Curr. Top. Membr. Trans.* 32:203–225.

Carter, K. J., P. L. Rutledge, M. L. Steer, and W. Silen. 1987. Secretagogue-induced changes in intracellular pH and amylase release in mouse pancreatic acini. *Am. J. Physiol.* 253:G690–G696.

Cohen, F. S., M. H. Akabas, and A. Finkelstein. 1982. Osmotic swelling of phospholipid vesicles causes them to fuse with a planar phospholipid bilayer membrane. *Science (Wash. DC.).* 217:458–460.

De Lisle, R. C., and J. A. Williams. 1986. Regulation of membrane fusion in secretory exocytosis. *Annu. Rev. Physiol.* 48:225–238.

Ermak, T. H., and S. S. Rothman. 1978. Internal organization of the zymogen granule: formation of reticular structure in vitro. *J. Ultrastruc. Res. 64:98–113.*

Fujime, S. 1987. Sarcomeric element flexibility. *In* Optical Studies of Muscle Cross-Bridges. R. J. Baskin and Y. Yeh, editors. CRC Press, Inc., Boca Raton, FL. 149–188.

Fujime, S., M. Takasaki-Ohsita, and S. Miyamoto. 1988*a*. Dynamic light scattering from polydisperse suspensions of thin ellipsoidal shells of revolution with submicron diameters. *Biophys. J.* 53:497–503.

Fujime, S., M. Takasaki-Ohsita, and S. Miyamoto. 1988*b*. Dynamic light scattering from polydisperse suspensions of large spheres: characterization of isolated secretory granules. *Biophys. J.* 54:1179–1184.

Hellmessen, W., A. L. Christian, H. Fasold, and I. Schultz. 1985. Coupled Na^+-H^+ exchange in isolated acinar cells from rat exocrine pancreas. *Am. J. Physiol.* 249:G125–G136.

Holz, R. W. 1986. The role of osmotic forces in exocytosis from adrenal chromaffin cells. *Annu. Rev. Physiol.* 48:175–189.

Ishiwata, S., and S. Fujime. 1972. Effect of calcium ions on the flexibility of reconstituted thin filaments of muscle studied by quasi-elastic scattering of laser light. *J. Mol. Biol.* 68:511–522.

Li, W., T. S. Aurora, T. H. Haines, and H. Z. Cummins. 1986. Elasticity of synthetic phospholipid vesicles and submitochondrial particles during osmotic swelling. *Biochemistry.* 25:8220–8229.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Meldolesi, J. 1983. Membranes of pancreatic zymogen granules. *Methods Enzymol.* 98:67–75.

Miyamoto, S., and S. Fujime. 1988. Regulation by Ca^{2+} of membrane elasticity of bovine chromaffin granules. *FEBS* (Fed. Eur. Biochem. Soc.) *Lett.* 238:67–70.

Miyamoto, S., T. Maeda, and S. Fujime. 1988. Change in membrane elastic modulus on activation of glucose transport system of brush border membrane vesicles studied by osmotic swelling and dynamic light scattering. *Biophys. J.* 53:505–512.

Ochs, D. L., J. I. Korenbrot, and J. A. Williams. 1983. Intracellular free calcium concentrations in isolated pancreatic acini: effects of secretagogues. *Biochem. Biophys. Res. Commun.* 117:122–128.

Rogers, J., E. K. Matthews, and D. B. McKay. 1987. Effects of a cytosolic protein on the interaction of rat pancreatic zymogen granules in vitro. *Biochim. Biophys. Acta.* 897:217–228.

Warashina, A. 1981. Properties of pancreatic zymogen granules studied by quasi-elastic light scattering. *Biochim. Biophys. Acta.* 672:158–164.

Weast, R. C. 1976. CRC Handbook of Chemistry and Physics. 57th ed. CRC Press, Cleveland, OH. 2506 pp.

Yoshino, S., Y. Umazume, Rb. Natori, S. Fujime, and S. Chiba. 1978. Optical diffraction study of muscle fibers. II. Electro-optical properties of muscle fibers. *Biophys. Chem.* 8:317–326.

Zimmerberg, J., M. Curran, F. S. Cohen, and M. Brodwick. 1987. Simultaneous electrical and optical measurements show that membrane fusion precedes secretory granule swelling during exocytosis of beige mouse mast cells. *Proc. Natl. Acad. Sci. USA.* 84:1585–1589.