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In Vitro Reconstitution of Chromaffin Granule-Cytoskeleton Interactions: Ionic Factors Influencing the Association of F-Actin With Purified Chromaffin Granule Membranes

Velia M. Fowler and Harvey B. Pollard

Section on Cell Biology and Biochemistry, Clinical Hematology Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

Chromaffin granules are the secretory vesicles directly involved in exocytosis of catecholamines, enkephalins, and other components from adrenal medullary cells. The granules occupy a large portion of the cytoplasmic volume and thus may interact extensively with cytoskeletal elements such as actin. Indeed, using both sedimentation techniques and falling ball viscometry [Fowler et al: J Cell Biol 88:388, 1981] to measure actin binding by membranes, we were able to show that chromaffin granules bind F-actin via a protein site on the membrane, and that these interactions are reversibly inhibited by raising the free calcium ion concentration to micromolar levels ($[Ca^{++}]_{free}$ for half-maximal inhibition approximately 2.6×10^{-7}M)[Fowler and Pollard: Nature 295:336, 1982]. Here, we show that F-actin-chromaffin granule interactions are unaffected by changes in pH between about pH 6.4 and 7.4 but are about 50% inhibited by raising the pH from 7.5 to 8.0. They are also 50% inhibited by increasing the KCl concentration to about 200 mM but are not significantly affected by increasing concentrations of K-glutamate up to 500 mM or by varying the $MgCl_2$ concentration between 0 and 6 mM. The interactions between chromaffin granule membranes and F-actin are also reduced in the presence of ATP, AMP-PNP, or free pyrophosphate; cAMP and AMP are without effect.

The ability of chromaffin granule membranes to interact with F-actin under conditions that may approximate the resting intracellular environment (neutral pH, low KCl, 1-2 mM $MgCl_2$, 1 mM ATP, $[Ca^{++}]_{free} < 10^{-7}$M, 30°C) suggests that these interactions may partially reconstitute naturally occurring associations between chromaffin granules and the cytoskeleton. Further, regula-

Velia M. Fowler is now at the Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

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tion of chromaffin granule membrane-actin interactions by ionic factors (pH, calcium, chloride ions, nucleotides) that could vary intracellularly leads us to propose that associations between actin and the chromaffin granule membrane could influence the location and dislocation of these organelles in the cytoplasm.

Key words: membrane association, actin binding sites, calcium-sensitive gelation, F-actin cross-linking, falling ball viscometer, membrane cytoskeleton, exocytosis, chromaffin granule, secretion

Actin-containing cytoskeletal and/or contractile elements have been proposed to be instrumental in the translocation of secretory vesicles to the plasma membrane during calcium-mediated exocytosis of neurotransmitters, hormones, and other secretory products from cells [1–5]. Biochemical evidence supporting a role for actin in exocytosis has come mainly from analysis of the structure and function of the secretory vesicles of the adrenal medulla (chromaffin granules) because they can be easily prepared in large quantities and high purity [6,7]. Actin has been reported by some [8–10], but not all [11], workers to be present on the chromaffin granule membrane, and chromaffin granules [12,13] as well as secretory vesicles from several other cell types [14,15] have also been reported to bind exogenous actin. Reports that an α-actinin-like polypeptide is associated with the chromaffin granule membrane [10,16] have further encouraged speculations that an actomyosin sliding filament mechanism could push or pull the secretory vesicle to the plasma membrane [1–4]. However, it is difficult to evaluate the significance of these observations due to lack of direct evidence concerning the molecular characteristics of actin's attachment to the membrane, lack of information about ionic factors regulating actin-membrane interactions, as well as questions about the relative purity of some of the granule and membrane preparations used.

Using both sedimentation techniques and a novel application [17,18] of falling ball viscometry [19,20] to measure F-actin binding to membranes, we show elsewhere that purified chromaffin granule membranes as well as intact chromaffin granules do, in fact, bind and cause large increases in the viscosity of F-actin [21]. Although other interpretations are possible [17,18,21], we have proposed that the increases in viscosity of F-actin caused by chromaffin granule membranes are a consequence of actin filament cross-linking by membranes containing multiple F-actin binding sites [17,18,21]. The actin-binding activity of the membranes is thermolabile and trypsin-sensitive, and interactions between membranes and F-actin are reversibly inhibited by raising the free calcium ion concentration to about 10^{-6} M. These observations led us to suggest that chromaffin granules could be attached via a protein site on the membrane to an actin-filament network in vivo and that calcium-induced solation of this network could be a prerequisite for organelle movement to the plasma membrane during calcium-mediated exocytosis [21].

Here, we further explore the effects on membrane-induced increases in viscosity of F-actin of ionic factors, in addition to calcium, that are potential candidates for regulation of exocytosis and other membrane and organelle movements in vivo. The results encourage us to believe that chromaffin granule membrane-F-actin interactions might indeed partially reconstitute naturally occurring associations between chromaffin granules and the cytoskeleton and that these interactions could potentially affect the cytoplasmic location and intracellular movements of these organelles.

MATERIALS AND METHODS

Preparation of Membranes

Chromaffin granules were prepared from bovine adrenal medullae by a modification of the procedure described by Bartlett and Smith [22]. Medullae dissected from 40–60 adrenal glands (100–150 gm medullae) were placed in 4 volumes of ice cold 330 mM sucrose, 40 mM HEPES, pH 7.4–7.6, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.87% ethanol (n = 1.3490, d = 1.045), disrupted with two 3-sec bursts in a Waring blender, and then homogenized using a motor driven homogenizer with a loose-fitting Teflon pestle (0.050–0.051 inch clearance) in a 15-ml glass homogenizer (Kontes). The homogenate was filtered through four layers of surgical gauze, its pH was checked and titrated to pH 7.4–7.6 with 1 N NaOH, and then it was centrifuged for 10 min at 2,000 rpm, 2–4°C, in the Sorvall SS-34 rotor to remove cellular debris, nuclei, and contaminating red blood cells. The supernatant was decanted into new tubes and centrifuged 30 min, 18,000 rpm, 2–4°C in the SS-34. The supernatants were discarded and the pellets resuspended to 90 ml by homogenization with a loose fitting glass homogenizer (Dounce) in the 330 mM sucrose-HEPES isolation medium. This was distributed between six Beckman type 35 ultracentrifuge tubes (15 ml in each) and underlaid with 35 ml each of ice cold 1.6 M sucrose, 40 mM HEPES, pH 7.4–7.6, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.87% ethanol (n = 1.4107, d = 1.205). Centrifugation for 1 h, 34,000 rpm, 2–4°C, in the Beckman Type 35 rotor separated the intact chromaffin granules from the bulk of the mitochondria as well as from other material that remained on top of the 1.6 M sucrose shelf. The supernatants were completely discarded, and the chromaffin granule pellets were lysed by homogenization in 60 ml of ice cold 5 mM HEPES, pH 7.8–8.0, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.87% ethanol. The volume of the lysate was brought to 250 ml and the membranes were collected by centrifugation for 30 min, 20,000 rpm, 2–4°C, in a Sorvall SS-34 rotor. Pellets were resuspended in 150–200 ml ice cold 5 mM HEPES lysis buffer and centrifuged again as above. The crude chromaffin granule membranes were resuspended up to 40 ml in the same buffer, and the pH of the suspension was checked with a pH electrode. If necessary, it was titrated to pH 8.0–8.2 by addition of a small volume of 0.1 N NaOH (it usually started at about pH 7.4–7.5). The membranes were distributed between two SW 27 cellulose nitrate tubes (Beckman) (20 ml in each tube) and underlaid with 17 ml ice cold 1.0 M sucrose, 40 mM HEPES, pH 7.6–7,8, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.87% ethanol (n = 1.3815, d = 1.126) and centrifuged 1 h, 26,000 rpm, 2–4°C in the SW 27 rotor. The chromaffin granule membranes accumulated as an intense salmon-pink band on top of the 1.0 M sucrose shelf [11,24,25] and were collected by Pasteur pipette, resuspended to about 150 ml in the 5 mM HEPES buffer, and centrifuged again for 30 min. The homogeneous pink pellets were resuspended in a small volume of the 5 mM HEPES buffer and stored on ice for up to ten days with little or no loss of actin binding activity. Protein concentrations of membranes were determined after trichloroacetic acid precipitation of samples by the method of Lowry et al [26], with bovine serum albumin as a standard.

For experiments in which the actin binding activity of intact chromaffin granules was studied, the granules were purified by centrifugation through 1.8 M sucrose (n = 1.4205, d = 1.230) and collected on top of a d = 1.28 sucrose cushion [11,23]. Five volumes of the 0.33 M sucrose-HEPES isolation medium were added

drop-wise to the granule suspension while stirring on ice. The granules were then collected by centrifugation for 40 min at 18,000 rpm, 2–4°C in the SS-34 rotor. The granules were washed once again in 0.33 M sucrose-HEPES and then resuspended in a small volume of the same solution. The protein loss from the chromaffin granules during the gradual dilution with 0.33 M sucrose represented about 50%. Membranes were not prepared routinely from granules isolated by this procedure because yields were substantially less than obtained using a 1.6 M sucrose shelf [22].

To optimize the yield, it was critical to buffer the solutions used to between pH 7.4–7.8, because as the pH was decreased from pH 8.0 to pH 6.0, an increasingly greater proportion of the intact granules remained on top of the 1.6 M sucrose shelf along with the mitochondria and other lighter components. Similarly, an increasing proportion of the chromaffin granule membranes sedimented through the 1.0 M sucrose shelf along with the contaminating denser components present in the crude membranes. These effects of pH have not been previously described in the literature, and were probably due to our inclusion of EDTA and PMSF in the solutions used for chromaffin granule and membrane isolation, in contrast to conventional published procedures. Membranes prepared without the addition of EDTA and PMSF were completely inactive in binding actin.

Purification of Actin

Actin was prepared from an acetone powder of rabbit skeletal muscle with one or two cycles of polymerization and sedimentation from 0.8 M KCl [27] and was depolymerized and stored as previously described [17]. The protein concentration of the monomeric actin (G-actin) was determined by the absorbance at 290 nm minus that at 320 nm, assuming the A_{290} of 1 mg/ml G-actin to be 0.62 [28]. Immediately before each experiment, the actin was polymerized at 5–8 mg/ml in 100 mM KCl, 2.0 mM MgATP, for 30–60 min at 30°C. Unless otherwise stated, F-actin was used in all of the viscosity experiments.

Viscosity Measurements

Viscosity was measured using a low-shear falling ball viscometer [19, 20] as previously described [17,20]. Purified chromaffin granule membranes were mixed at 0°C with F-actin in a buffer containing 20 mM KCl, 40 mM PIPES, pH 6.8, 1.0 mM $MgCl_2$, 5.0 mM EGTA, and 0.25 mM $CaCl_2$ ($[Ca^{++}]_{free} \sim 4 \times 10^{-8}$ M, calculated according to Caldwell [29] using a Kapp of 1.95×10^{-6} M). (The membranes and actin contributed approximately an additional 1–3 mM HEPES, 0.2–0.5 mM EDTA, 0.3–0.6 mM DTT, 0.2 mM Tris-Cl, 0.02 mM $CaCl_2$, 0.22 mM ATP, 0.2 mM $MgCl_2$, and 0.002% NaN_3; concentrations were held constant for each experiment.) To economize on the membranes and actin, sample volumes of only 150 μl were used. Thus, only 50 μl was drawn up into each of the three 100 μl micropipettes used for the viscosity measurements. Micropipettes were then placed in a horizontal position [17] and incubated for 1 h at 30°C before the falling times of the ball were measured. Due to the smaller sample volume used, the times were measured over a 2-cm rather than over a 6-cm distance as in the previous work [17,18,20]. These times were converted into apparent viscosities using standard curves generated empirically with a series of glycerol solutions of known viscosities [20]. The viscosities (in centipoise) of the glycerol solutions were determined using a Cannon-Manning viscometer, as previously stated [20]. In Table I the slopes and

TABLE I. Slopes and y-Intercepts of Standard Curves of the Form, y = mx + b, Describing the Relationship Between 1/Speed and Viscosity for Glycerol Solutions in the Falling Ball Viscometer*

Measuring angle	Slope (m)	y-intercept (b)
20°	0.377	0.179
30°	0.197	0.259
40°	0.136	0.197
50°	0.109	0.092
60°	0.094	0.042
70°	0.082	0.040
80°	0.073	−0.0024

*The slopes and y-intercepts of these lines were determined by linear regression analysis of the 1/speed vs viscosity data that was obtained empirically for each angle as described in the Methods and in reference [20]. The correlation coefficients for all of the lines were 0.998 or better. Seven to ten (1/speed, viscosity) points were obtained for each angle to determine these lines. The range of viscosities over which these standard curves were determined for each angle was approximately as follows: 20° (1–20 cp), 30° (1–30 cp), 40° (1–30 cp), 50° (1–200 cp), 60° (2–200 cp), 70° (2–200 cp), 80° (4–500 cp). The deviations of the y-intercepts from 0 cp [19] may be due to the fact that both the viscosities of the glycerol solutions used and the standard curves themselves were determined empirically. These are the standard curves that were used previously by Fowler and Taylor [20], Fowler et al [17], Luna et al [18] and Fowler and Pollard [21] to determine apparent viscosities of various experimental solutions.

y-intercepts of the standard curves determined for each angle are presented. Although the linear relationship between 1/speed and viscosity only holds for Newtonian solutions such as glycerol [19], conversion of falling times to (apparent) viscosities is useful as it enables measurements made at one angle to be compared with measurements made at another. This extends greatly the range and sensitivity of the technique. The apparent viscosities presented are averages of triplicate determinations for each sample.

Electron Microscopy

Membranes were prepared for electron microscopy by fixation at 0°C for 1 h in 2% glutaraldehyde, 100 mM HEPES, pH 7.4–7.6, and then pelleted by centrifugation for 45 min at 2–4°C at 20,000 rpm in the Sorvall SS-34 rotor. Pellets were postfixed in 1% osmium tetroxide before dehydration and embedding. Sections were stained with aqueous lead citrate and uranyl acetate and examined by conventional transmission electron microscopy.

SDS-Polyacrylamide Gel Electrophoresis

Membranes were prepared for electrophoresis by dilution 1:1 in distilled water, addition of one-quarter volume of 5×-concentrated SDS-electrophoresis sample buffer [18], and denaturation for 5 min at 60–70°C. Samples were electrophoresed on 7.5–15% linear gradient polyacrylamide slab gels using the discontinuous buffer system of Laemmli [30]. Boiling of the purified chromaffin granule membrane samples was avoided because this resulted in accumulation of Coomassie brilliant blue staining material that did not enter the gel, as well as in streaking and poor resolution of the stained bands. This may have been due to irreversible aggregation of the cytochrome component of the membrane (personal communication, Pat Fleming, Department of Biochemistry, Georgetown University,

Washington, DC). Increasing the SDS concentration in the sample buffer to 2% (from 1%) also seemed to improve the appearance of the stained bands somewhat. Molecular weight standards used were bands 1 and 2 of spectrin (240 and 220 $\times$ 10^3 daltons, respectively). Phosphorylase a (94K) albumin (67K), catalase (60K), actin (42K), lactate dehydrogenase (36K), soybean trypsin inhibitor (20.1K), and myoglobin (16.9K). Numbers in parentheses refer to apparent polypeptide molecular weights in kilodaltons.

RESULTS

Isolation and Characterization of Chromaffin Granule Membranes

Chromaffin granules come ready made with their cytoplasmic surface externally oriented and thus are particularly attractive candidates for studying interactions of membranes with filamentous cytoskeletal elements such as actin. However, we have chosen to study the interaction of F-actin with membranes prepared by hypotonic lysis of granules rather than with the intact chromaffin granules themselves for several reasons. First, other dense subcellular components which tend to co-isolate wih intact chromaffin granules can be readily separated from hypotonically lysed chromaffin granules membranes by centrifugation of the lysed membranes over a 1.0 M sucrose shelf (Fig. 1) [11, 24, 25]. The chromaffin granule membranes (Fig. 1b) are recovered as an intense salmon-pink-colored band (due to the presence of the characteristic chromaffin granule membrane cytochrome b_{561} [6,33] on top of the 1.0 M sucrose, while the contaminating material (Fig. 1c) forms a brownish pellet. Between 50 and 75% of the total protein from the crude chromaffin granule membranes is recovered in the pink band remaining on top of the 1.0 M sucrose. Second, ionic parameters of interest can be varied without concern for their effects on the osmotic stability of the intact chromaffin granule. Third, because isolated chromaffin granules are osmotically unstable, they can not be stored for more than a day or two without undergoing extensive lysis and breakdown.

The isolated chromaffin granule membranes constitute a fairly homogeneous population of closed, smooth-surfaced vesicles between about 0.1 and 0.6 μm in diameter (Fig. 1b). This is similar to the size range of chromaffin granules in situ [31]. Although a few vesicles are observed whose protein matrix has not been completely removed by the low ionic strength washes used in their preparation (Fig. 1b), the major intragranular proteins are greatly reduced in amount when examined by SDS-polyacrylamide gel electrophoresis (Fig. 2, lane b; compare [9]). Also, although there is evidently some vesiculation as well as entrapment of small vesicles within larger ones (Fig. 1b), membranes prepared from granules by hypotonic lysis apparently retain their native orientation since they are able to reseal and accumulate catecholamines [32]. Furthermore, the ability of intact chromaffin granules to bind and induce increases in the viscosity of F-actin is comparable to that of the isolated membranes (not shown), taking into account that the membrane protein represents $\sim$20% of the total granule protein.

The polypeptide composition of the isolated chromaffin granule membranes is illustrated in Figure 2b. Although it superficially resembles that of the total membranes (Fig. 2a), it is clearly different from that of the material which sediments through the 1.0 M sucrose (Fig. 2c). For example, the doublet at about

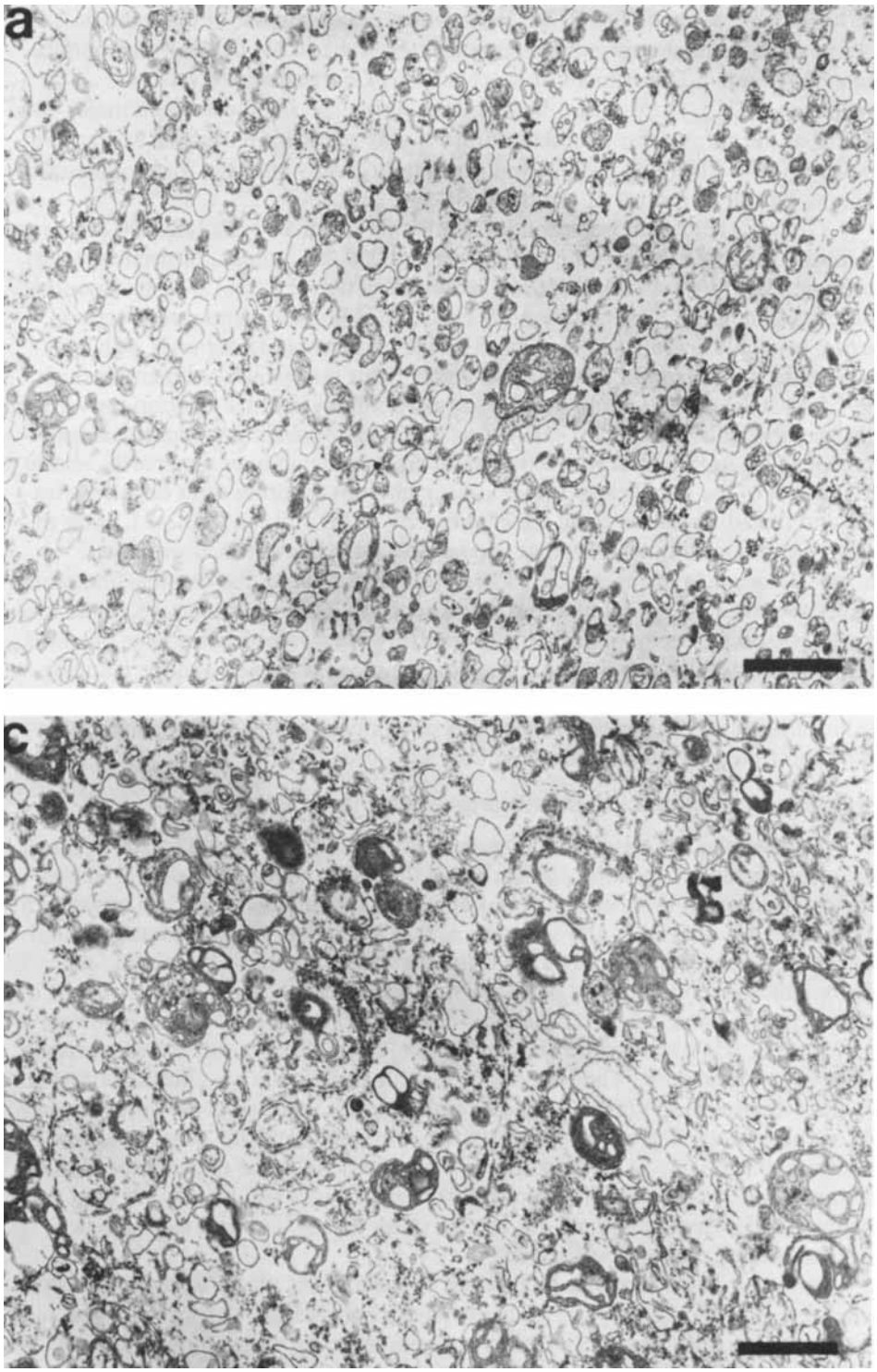
25,000–28,000 daltons that is greatly enriched in the material remaining on top of the 1.0 M sucrose shelf (Fig. 2) has been shown to contain the cytochrome b_{561}, a specific marker for the chromaffin granule membrane [6,33, personal communication, Pat Fleming, Department of Biochemistry, Georgetown University, Washington, DC]. Dopamine β-hydroxylase, also a specific marker and major component of the chromaffin granule membrane, has a subunit molecular weight of about 74,000–77,000 daltons [34] and is similarly greatly enriched in the material remaining on top of the 1.0 M sucrose shelf (Fig. 2).

On the other hand, polypeptides comigrating with actin and myosin (apparent molecular weights of approximately 42,000 and 200,000 daltons, respectively) are preferentially enriched in the material sedimenting through the 1.0 M sucrose shelf and correspondingly depleted from the chromaffin granule membrane fraction. Dense, insoluble actomyosin complexes would be expected to copurify with the chromaffin granules until this stage, at which point they would sediment through the 1.0 M sucrose. This could occur irrespective of any specific associations with the other components in the pellet. However, it is also possible that reduced associations of this putative actin with chromaffin granule membranes may partly be due to isolation of the membranes under ionic conditions favoring actin depolymerization and thus dissociation from the membrane [17,18, and see Methods]. Most of the studies purporting to show that endogenous actin is associated with the chromaffin granule membrane [8,10,13,16] did not separate the chromaffin granule membranes from the contaminating dense material. The two studies which did look for the presence of actin in purified chromaffin granule membranes have obtained conflicting results on this question using immunological [9] and peptide mapping [11] techniques. This question should be reinvestigated since SDS-polyacrylamide gel electrophoresis of the purified membranes does indeed reveal a band comigrating with rabbit skeletal muscle actin (Fig. 2b).

Ionic Factors Influencing Membrane–Actin Interactions

Intracellular pH has been increasingly implicated as a second messenger, analogous to calcium or to cAMP, in mediating the cell's response to external stimuli [35,36]. Changes in intracellular pH of about 0.3 to 0.5 units have been reported to occur upon cell activation and secretion in sea urchin eggs [37], platelets [38], and pancreatic β-cells [39], as well as upon stimulation of glycolysis by insulin [40]. Figure 3 illustrates that over a potentially physiological pH range changes in pH between about 6.4 and 7.4 have little effect on membrane-induced increases in viscosity of F-actin, while raising the pH from about 7.5 to 8.0 diminishes the increase in viscosity by about 50%. The viscosity of F-actin alone is not significantly affected by these changes in pH (Fig. 3). These results suggest that unless the intracellular pH of the resting cell were to be maintained at or above pH 7.5, small changes in pH would be unlikely to affect secretion via a direct influence on actin-membrane interactions. It may be significant that Baker and Knight [42] also found that changes in pH between 6.4 and 7.4 had no effect on calcium-activated secretion from permeabilized chromaffin cells.

Although large increases in viscosity of membranes plus F-actin are observed below pH 6.4 (Fig. 3), they are probably due to nonspecific, nonphysiological, interactions since the intracellular pH of a mammalian cell such as the chromaffin cell is not likely to be below pH 6.4 [35,36]. Moreover, the apparent viscosity of F-actin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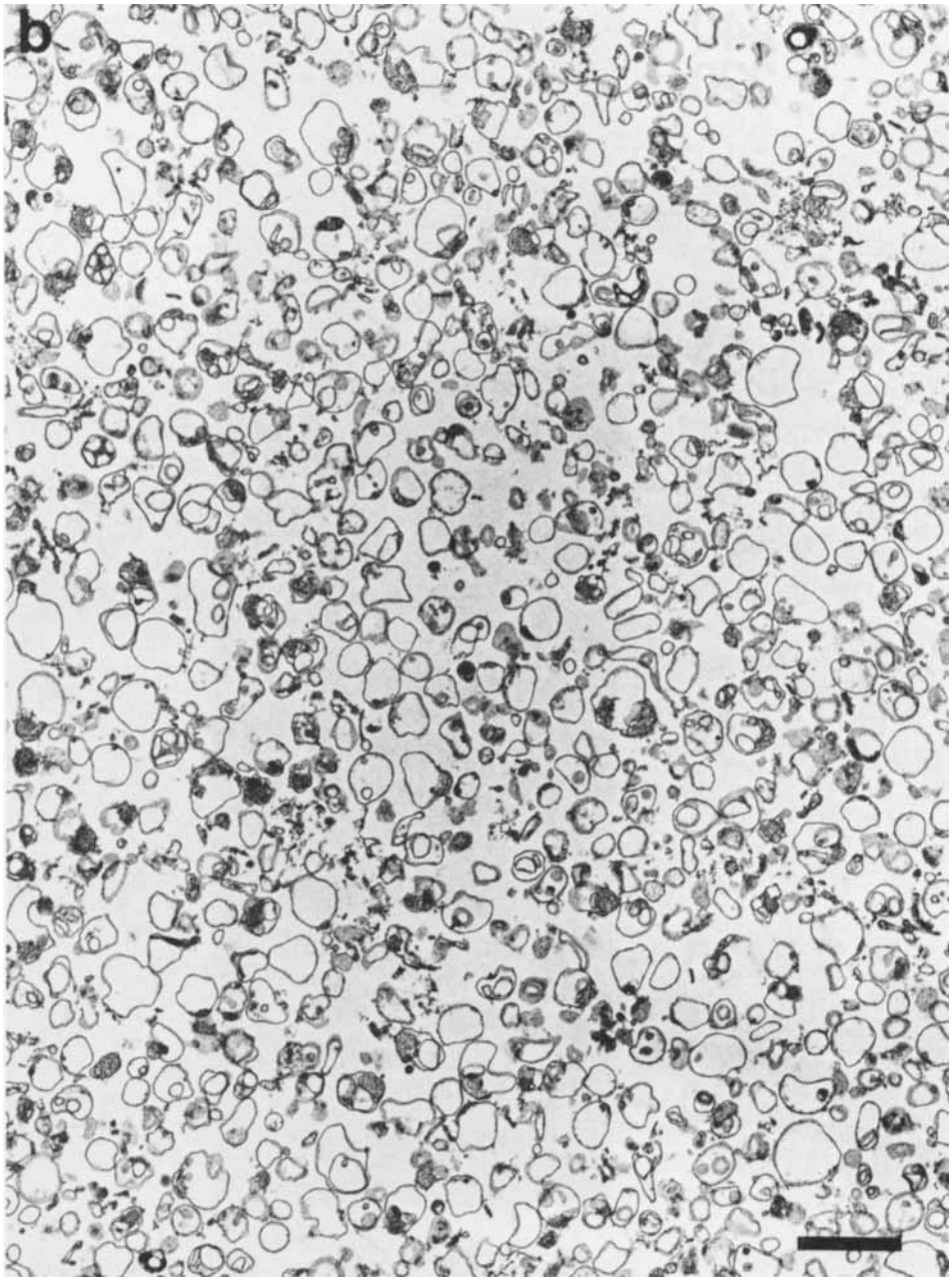

Fig. 1. Electron micrographs of (a) crude chromaffin granule membranes, (b) chromaffin granule membranes isolated over a 1.0 M sucrose shelf, and (c) material sedimenting through a 1.0 M sucrose shelf. Bars, 1 μm $\times$ 14,000 (approx).

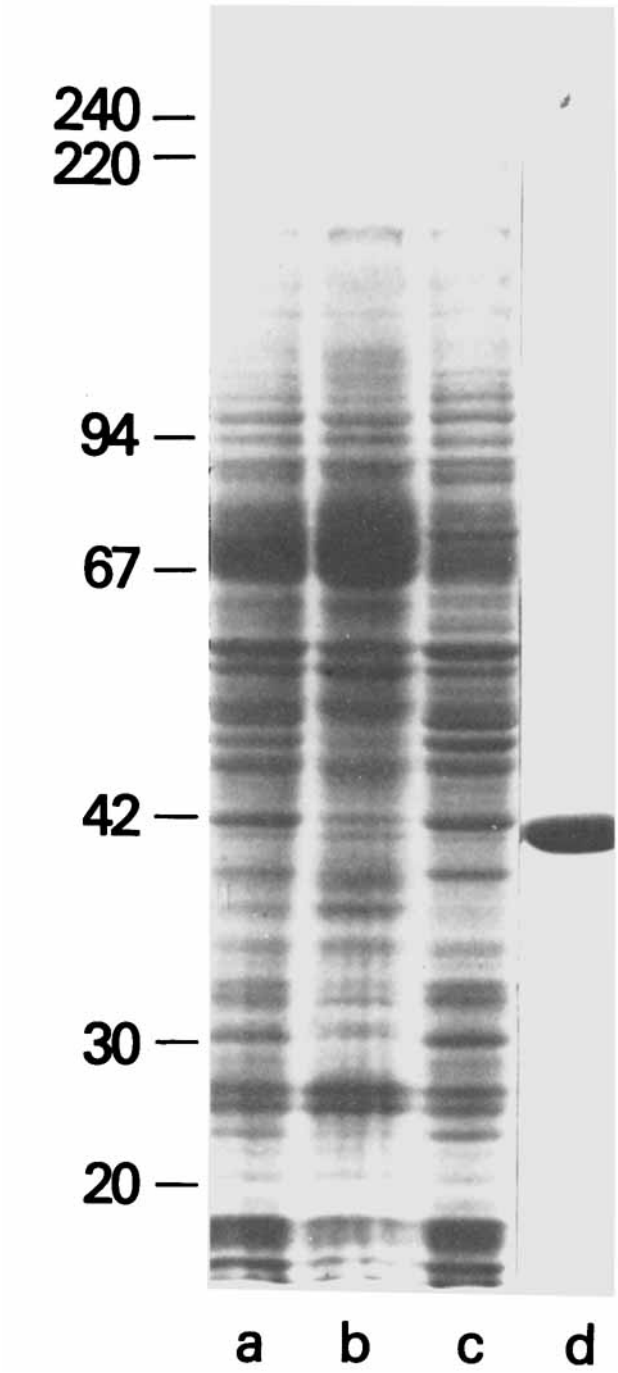

Fig. 2. 7.5 to 15% SDS-polyacrylamide gels of (a) crude chromaffin granule membranes, (b) chromaffin granule membranes isolated over a 1.0 M sucrose shelf, (c) material sedimenting through a 1.0 M sucrose shelf, and (d) purified rabbit skeletal muscle actin. Membranes were purified as described in Materials and Methods. Membrane fractions in (a,b,c) correspond to the electron micrographs in Figure 1 (a,b,c), respectively. 26 μg, 27 μg and 25 μg of membrane protein, and 5 μg of pure actin were electrophoresed in lanes a,b,c, and d, respectively.

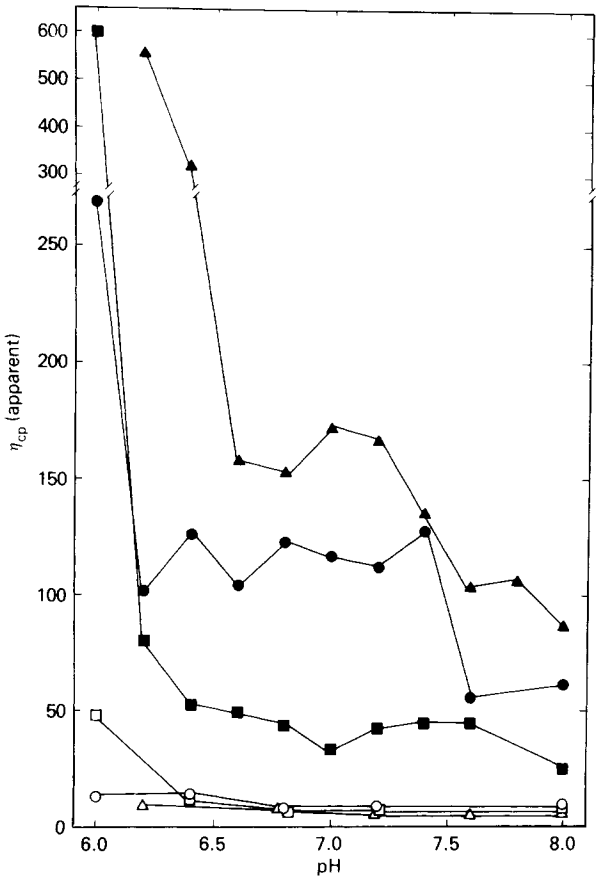

Fig. 3. Effect of pH on the apparent viscosity (centipoise) of (●, ■, ▲) chromaffin granule membranes plus F-actin and on the viscosity of (○, □, △) F-actin alone. The different symbols represent results from three different experiments using different preparations of membranes. Chromaffin granule membranes [final concentrations of (●) 1.64, (■) 1.60, and (▲) 2.26 mg/ml], or an equivalent volume of 5 mM HEPES lysis buffer (see methods) for the actin alone controls, were mixed with buffer and salts in a very small beaker (~ 1 ml capacity) and titrated at 0°C to the desired pH (± .05) with 0.1 N NaOH or HCl before addition of F-actin (final concentration 0.8 mg/ml). Samples were incubated 1 h, 30°C for viscosity measurements as described in Materials and Methods. Sodium phosphate stocks of the appropriate pH were used to give final buffer concentrations of (●, ■) 40 mM or (▲) 20 mM in the assay mixture. A different Ca^{++}/EGTA stock solution was prepared for each pH so that the free calcium ion concentration would be about 10^{-8} M for each pH [calculated as described by Caldwell [26] using the Kapps for each pH given by Amos [41]]. The final EGTA concentration in the assay mixture was 5 mM. Final concentrations of other components were as specified in Materials and Methods. Direct titration of membrane samples was necessary because the buffering capacity of the sodium phosphate varied somewhat over the pH range from 6.0 to 8.0 and was thus influenced by the addition of membranes more at some pHs than at others (addition of membranes tended to drop the pH). Addition of actin had no effect on the pH. A series of buffers was not used (eg, MES, PIPES, HEPES) because the buffers themselves influenced the viscosity of membranes plus actin. For example, at pH 7.0, 40 mM PIPES enhanced the viscosity of membranes plus actin with respect to 40 mM HEPES.

TABLE II. Effect of Magnesium, Nucleotides, and Phosphate on the Apparent Viscosity (η_{cp}) of Chromaffin Granule Membranes Plus F-Actin

Additions	Experiment 1 η_{cp}[a]		Experiment 2 η_{cp}	
	Membranes + F-actin	F-actin	Membranes + F-actin	F-actin
0 mM $MgCl_2$	–	–	208	8
1 mM $MgCl_2$	100	10	285	9
6 mM $MgCl_2$	139	9	231	11
1 mM $MgCl_2$ + 5 mM MgATP[b]	47	12	114	11
1 mM $MgCl_2$ + 5 mM $MgPP_i$[c]	60	–	85	11
1 mM $MgCl_2$ + 5 mM MgAMP-PNP[d]	51	–	90	11
1 mM $MgCl_2$ + 5 mM ATP	71	9	94	10
1 mM $MgCl_2$ + 5 mM AMP	105	–	256	12
1 mM $MgCl_2$ + 5 mM cAMP	120	–	337	12
1 mM $MgCl_2$ + 5 mM NaH_2PO_4 (pH 6.8)	–	–	221	12

[a]Apparent viscosity (η) expressed in centipoise (cp).
[b]MgATP made by mixing $MgCl_2$ with ATP before addition to the assay mixture.
[c]Magnesium pyrophosphate ($MgPP_i$) made as for MgATP.
[d]Magnesium 5'-adenylylimidodiphosphate (MgAMP-PNP) made as for MgATP.
Purified chromaffin granule membranes at final concentrations of 2.16 mg/ml (Experiment 1) or 2.35 mg/ml (Experiment 2) were mixed with 0.8 mg/ml F-actin and incubated for 1 h at 30°C in micropipettes before measuring the apparent viscosity as described in the Materials and Methods section. $MgCl_2$, nucleotide, or phosphate concentrations were as indicated in the Table. Other ionic conditions were specified in the Materials and Methods.

alone begins to rise in this region (Fig. 3), heat-treated membranes cause large increases in the viscosity of F-actin in this region (not shown), and as the pH is lowered further toward the isoelectric point of actin, the protein all precipitates and the viscosity drops to that of the buffer. Similar large, abrupt increases in viscosity near pH 6.0 and below have also been observed for the interaction of a variety of actin cross-linking proteins with actin [20,43,44], as well as for the self-association of actin filaments [43].

Cytoplasmic levels of MgATP might also be expected to influence actin-membrane interactions, since secretion from intact cells is energy dependent, and release from permeabilized chromaffin cells is specifically dependent on MgATP [42]. However, although 5 mM MgATP inhibits the increases in viscosity of membranes plus F-actin by about 50%, both free pyrophosphate and a nonhydrolyzable analogue of ATP (AMP-PNP) also inhibit the increases in viscosity of chromaffin granule membranes plus F-actin (Table II). The effects of ATP and pyrophosphate are not simply due to an effect on ionic strength, since the ionic strength of 200 mM potassium chloride that causes 50% inhibition of membrane-actin interactions is 0.2 (200 mM KCl, see Fig. 4), while that of 5 mM magnesium pyrophosphate is 0.05 (assuming the charge on the pyrophosphate to be -4). Also, changes in the $MgCl_2$ concentration between 0 and 6 mM have only slight but variable effects on membrane-induced increases in viscosity of F-actin, and AMP, cAMP, and sodium phosphate have no significant effects. Thus, the energy dependence of secretion must be sought elsewhere. Indeed, there is accumulating evidence that the MgATP-dependent chemiosmotic mechanism for release of contents from isolated chromaffin granules may also apply to secretion from intact cells [7].

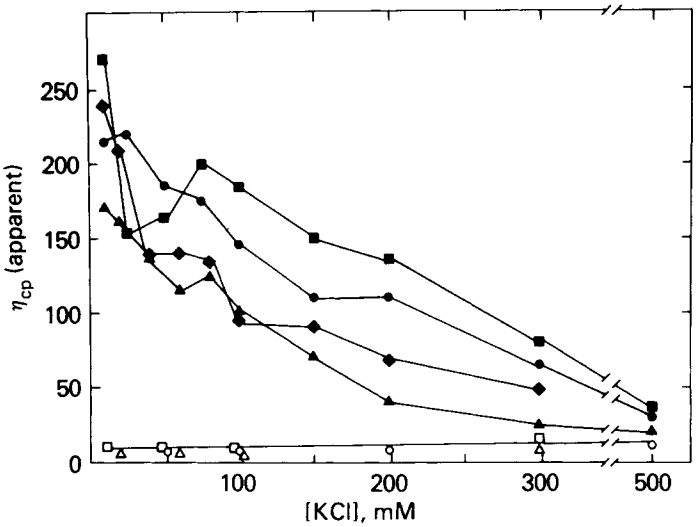

Fig. 4. Effect of increasing KCl concentration (mM) on the apparent viscosity (centipoise) of (●, ■, ▲, ◆) chromaffin granule membranes, plus F-actin and on the viscosity of (○, □, △) F-actin alone. The different symbols represent results from four separate experiments using different preparations of membranes. Chromaffin granule membranes at (●) 1.90, (■) 1.73, and (▲,◆) 1.80 mg/ml, or equivalent volumes of the 5 mM HEPES lysis buffer were mixed with 0.8 mg/ml F-actin and incubated as described in Materials and Methods before measuring the viscosities. To determine whether reducing the KCl concentration to 0 mM would have any adverse effect on the increases in viscosity, we added G-actin rather than preformed F-actin to the assay, and allowed the actin to polymerize in the presence of the membranes. This was necessary because the F-actin routinely used in our assays was polymerized before addition to the membranes in 100 mM KCl at about ten times its final concentration in the assay thus contributing 10 mM KCl to the assay mixture. We found that the increases in viscosity were the same in 0 mM KCl as in 10 or 20 mM KCl. (Under the conditions used in these assays (0.8 mg/ml actin, 40 mM PIPES, pH 6.8, 1 mM $MgCl_2$, $[Ca^{++}]_{free} \sim 4 \times 10^{-8}$M, 30°C) G-actin polymerizes spontaneously to F-actin [17,18,21]. Also, the final viscosity of membranes plus preformed F-actin is the same as that of membranes plus actin added as G-actin and allowed to polymerize in the presence of the membranes.)

Intracellular levels of free chloride ions in many cells are probably only a few millimolar [45,46]. High levels of chloride ions have been shown to inhibit gelation of actin-containing cytoplasmic extracts [19,20,47,48], to inhibit interactions between actin and microtubules in vitro [43,44], and to solubilize squid axoplasm [49,50]. On the other hand, organic anions such as glutamate do not have these effects [44,49]. We similarly find that increasing concentrations of KCl inhibit the increases in viscosity of F-actin induced by chromaffin granule membranes (Fig. 4), while increasing concentrations of K-glutamate do not (Fig. 5). A slight enhancement in the viscosity of F-actin alone is observed at the highest KCl and K-glutamate concentrations tested (500 mM) (Fig. 4,5). It will be interesting to see if KCl reduces membrane-actin interactions by changing the affinity of actin for its attachment site on the membrane, or alternatively, by eluting that component from the membrane, if it were a loosely bound component. Since the extracellular concentration of chloride ions in plasma is on the order of 150 mM, it is tempting to speculate that stimulus-triggered influx of chloride across the membrane could po-

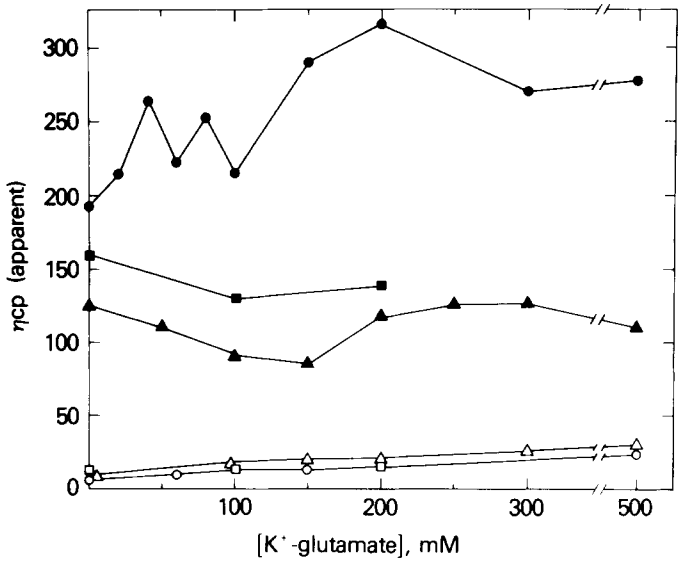

Fig. 5. Effect of increasing K-glutamate concentration (mM) on the apparent viscosity (centipoise) of (●, ■, ▲) chromaffin granule membranes plus F-actin and on the viscosity of (○, □, △) F-actin alone. The different symbols represent results from three separate experiments using different preparations of membranes. Chromaffin granule membranes at (●) 1.80, (■) 2.46, and (▲) 1.56 mg/ml, or (○, □, △) equivalent volumes of the 5 mM HEPES lysis buffer were mixed with 0.8 mg/ml F-actin (final concentration) and incubated as described in Materials and Methods before measuring the viscosities. Buffer and ionic conditions were specified in Materials and Methods except that (●) 8, (■) 10, or (▲) 16 mM KCl was present in the assays (contributed by the addition of the F-actin, which was polymerized in 100 mM KCl).

tentially provide another mechanism for controlling the association of the chromaffin granules with the actin cytoskeleton.

DISCUSSION

The three-dimensional cytoplasmic matrix of the chromaffin and other cells is pervaded by an anastomosing system of filamentous elements that consists of actin filaments, microtubules, and intermediate filaments, as well as other filaments of uncertain origin [47,51–55]. Subcellular organelles, including secretory vesicles such as chromaffin granules, appear to be embedded within and attached to these cytoskeletal structures [53–58]. Thus, it is not unreasonable to suppose that the placement and displacement of chromaffin granules in the cytoplasm in preparation for and during exocytosis might be controlled by changes in the structure and organization of these cytoskeletal structures. Indeed, preliminary biochemical [12,13] and morphological [10,12,13] evidence indicated that actin filaments could, in fact, bind to chromaffin granules in vitro. However, until recently [21], there have been no experiments that have addressed the question of whether interactions between granules and cytoskeletal components might be active, in the sense of specific and regulated attachments, or passive, in the sense of the granules simply being trapped in an actin-containing cytoskeletal meshwork.

We have approached this problem by determining whether the association of actin with chromaffin granules might be influenced by ionic factors that could control exocytosis and other membrane movements in the cell. We have used a low shear viscometric technique [19,20] partly because it is a sensitive and convenient method to measure F-actin binding to membranes [17,18,21] but also because it may allow us to determine whether regulation of F-actin-membrane interactions could influence cytoplasmic structure and consistency. This approach is thus an extension of that used previously to study gelation of actin-containing cytoplasmic extracts [19,20] and to study interactions of filamentous actin with proteins that cross-link [19,20,44,59,60], inhibit self-association [61,62], or depolymerize or otherwise act to shorten actin filaments [60,63,64].

However, the low shear viscometric assay does not directly address the molecular mechanism by which a given ionic factor (pH, chloride, calcium) inhibits the membrane-induced increases in viscosity of F-actin. Since falling ball viscometry is only an indirect measure of F-actin binding to membranes [17,18], different viscosities of membrane-F-actin solutions under different conditions could be due to differences in the length or amount of filamentous actin, changes in the mode of actin attachment to the membrane (lateral vs end-on) as well as to differences in the absolute amount of actin bound to the membranes [17–21]. Deciding among these possibilities will require correlation of viscosity measurements with direct measurements of actin binding to membranes, relative proportions of monomeric and filamentous actin, as well as ultrastructural observations to determine the mode of actin association with the membrane.

As described in an earlier publication [21], we found that associations between F-actin and chromaffin granule membranes were influenced by free calcium ion concentrations similar to those thought to activate secretion in vivo [1,42,65]. Here, we show that these associations are also affected by changes in pH over a potentially physiological pH range, as well as by changes in the concentrations of nucleotides or chloride ions. Our conclusion is that associations of chromaffin granules with the actin component of the cytoskeleton may indeed be active, since they are regulated by ionic factors that could vary intracellularly. Information about the actual changes in the intracellular ionic environment during exocytosis and other cellular processes will be essential to evaluate the exact significance of our observations concerning the effects of calcium, pH, nucleotides, and anions on chromaffin granule membrane-actin associations. In addition, it will be necessary to obtain morphological data concerning the structural nature of actin's attachment to the membrane, as well as biochemical data concerning the nature of the protein component to which actin binds. This may permit us to determine how actin attachment to the chromaffin granule membrane might control the placement and displacement of chromaffin granules in the cytoplasm.

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