Salt Dependency of Chromaffin Granule Aggregation by Annexin II Tetramer†

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ABSTRACT: Annexin II tetramer (AIIt) is a Ca^{2+} and phospholipid binding protein that has been shown to reconstitute secretion in permeabilized adrenal medulla cells. In the present study, we have characterized the interactions of AIIt with biological membranes using isolated adrenal medulla secretory granules as a model system. Without added salt, maximal binding of AIIt to chromaffin granules occurred in the absence of AIIt-dependent chromaffin granule aggregation, whereas increasing the osmolality of the reaction mixture with sucrose did not activate AIIt-dependent chromaffin granule aggregation. As the KCl or potassium glutamate concentration of the reaction mixture was increased to between 30 and 50 mM salt, AIIt-dependent chromaffin granule aggregation increased to a maximum, while AIIt binding to chromaffin granules decreased. As the salt concentration was increased from 50 to 150 mM, both AIIt-dependent chromaffin granule aggregation and the binding of AIIt to chromaffin granules were decreased. Furthermore, at optimal salt concentration, KCl and potassium glutamate activated AIIt-dependent aggregation of chromaffin granules to maximum values of about 210% and 195% of control, respectively, whereas potassium phosphate supported AIIt-dependent aggregation of chromaffin granules to only 120% of control. The concentration of AIIt for half-maximal binding to chromaffin granules without added salt or at 50 mM KCl was 0.163 ± 0.007 (mean ± SD, $n = 3$) or 0.173 ± 0.034 μM AIIt (mean ± SD, $n = 3$), respectively, and binding of AIIt to chromaffin granules was not measurable at 150 mM KCl. In contrast, at 50 mM KCl, half-maximal AIIt-dependent chromaffin granule aggregation required 0.171 ± 0.001 μM AIIt (mean ± SD, $n = 3$) and was not measurable without added salt or in the presence of 150 mM KCl. Without added salt, at 50 mM KCl, or at 150 mM KCl, the Ca^{2+} concentrations for half-maximal aggregation of chromaffin granules and the maximal extent of chromaffin granule aggregation (A_{max}) were $pCa^{2+} = 3.79 \pm 0.062$ (mean ± SD, $n = 3$) and $A_{max} = 127\%$ of control, $pCa^{2+} = 6.07 \pm 0.021$ (mean ± SD, $n = 3$) and $A_{max} = 185\%$ of control, or pCa^{2+} 4.41 ± 0.07 (mean ± SD, $n = 3$) and $A_{max} = 156\%$ of control, respectively. The stimulation of chromaffin granule aggregation activity and the chromaffin granule binding activity of AIIt was reversible by removal of Ca^{2+}. These results suggest that both ionic strength and salt composition modulate both AIIt-dependent chromaffin granule aggregation and binding to the membranes of these secretory granules.

The annexins [e.g., see Klee (1988)] are a family of Ca^{2+}-dependent proteins that bind to acidic phospholipids, and they are further identifiable as members of this family by the presence of a region of amino acid homology in each of the proteins called the "annexin fold" (Geisow *et al.*, 1986). To date, 12 annexins have been purified and characterized. The ability of the annexins to bind to phospholipids in a Ca^{2+}-dependent manner has led to speculation that these proteins might be involved in a number of membrane trafficking events, such as endocytosis or exocytosis.

Annexin II can exist as a monomer (AIIm[1]) or as a tetramer comprising two subunits of AIIm and two molecules of an 11 kDa protein (AIIt) (Gerke & Weber, 1984; Erikson *et al.*, 1984), and the formation of the tetramer appears to drive association of the complex with the plasma membrane (Harder *et al.*, 1993). In line with its localization to the plasma membrane implying its role in exocytosis (Goldberg *et al.*, 1990; Nakata *et al.*, 1990), AIIt has also been seen in many secretory tissues (Gould *et al.*, 1984; Erickson *et al.*, 1984; Rhoads *et al.*, 1985; Regnouf *et al.*, 1991) and secretory granules of the adrenal medulla. Additionally, this protein has been shown to be localized to regions of the cytosolic face of the plasma membrane that are enriched with actin (Goldberg *et al.*, 1990; Nakata & Hirokawa, 1992).

The possible involvement of AIIt in exocytosis was first postulated on the basis of both the localization of AIIt to the plasma membrane and the ability of AIIt to aggregate isolated chromaffin granules. It was suggested that the *in vitro* chromaffin granule aggregation activity of AIIt might model the *in vivo* docking of chromaffin granules with the

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[1] Abbreviations: AI, annexin I (lipocortin I, calpactin II, chromobindin 6, p35); AIIm, annexin II monomer (lipocortin II, calpactin I, p36); AIIt, annexin II tetramer (L-85, p86); AIII, annexin III (lipocortin III, PAP-III); AIV, annexin IV (lipocortin IV, endonexin, chromobindin 4); AV, annexin V (lipocortin V, endonexin II); AVI, annexin VI (lipocortin VI, p68, 67k calelectrin); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; OD, optical density; BSA, bovine serum albumin; TEA, triethanolamine; buffer A, 340 mosm/kg sucrose, 5 mM EGTA, and 25 mM Hepes (pH 7.5); buffer B, 340 mosm/kg sucrose and 25 mM Hepes (pH 7.5); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; A_{max}, maximum change in optical density; $A_{50\%}$, half-maximal change in optical density.

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apical plasma membrane prior to secretion (Drust & Creutz, 1988). In this study of AIIt-dependent aggregation of highly purified adrenal chromaffin secretory granules, it was reported that AIIt caused half-maximal aggregation of these secretory granules at physiological, i.e., micromolar, levels of Ca^{2+}. In contrast, the heavy chain of AIIt, AIIm, could not aggregate granules at levels of Ca^{2+} as high as 1 mM (Drust & Creutz, 1988). AIIt has also been suggested to be involved in the endocytotic pathway [Emans *et al.*, 1993; reviewed in Gruenberg and Emans (1993)]. Furthermore, both AIIt and AIIm have been reported to reconstitute secretion in annexin-depleted, permeabilized chromaffin cells at micromolar Ca^{2+} levels (Ali *et al.*, 1989), although AIIm may be less potent than AIIt (Sarafian *et al.*, 1991).

The apparent inconsistencies in the AIIm data obtained from the two *in vitro* model systems could be attributable to the different buffer systems used by Drust and Creutz (1988) and by Ali *et al.* (1989) in that Drust and Creutz (1988) used 30 mM KCl plus 240 mM sucrose while Ali *et al.* (1989) used 139 mM potassium glutamate. Given that the existing data were derived from two different *in vitro* systems that used different ionic strength buffer systems, we examined the salt dependence of chromaffin granule aggregation by AIIt. We found that the salt concentration affects the relationship between the chromaffin granule binding activity of AIIt and the chromaffin granule aggregation activity of that protein. We also found that the salt concentration affected the Ca^{2+} dependency of both AIIt-dependent aggregation and binding to chromaffin granules. Further, considering the different salt requirements for the chromaffin granule aggregation activity of AIIt and AIIm with the high degree of sequence homology within the annexin family and their shared ability to bind to phospholipids, it was possible that all of these proteins could aggregate chromaffin granules under appropriate conditions of ionic strength. However, of the six annexins tested (annexins I–VI), only AIIt caused a significant aggregation of chromaffin granules at low micromolar Ca^{2+}. These data are discussed within the context of the existing *in vitro* models of secretion.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were of analytical grade, and all solutions were buffered with triethanolamine: Hepes (acid form) and EGTA, Sigma Chemical Co.; triethanolamine, Fisher; sucrose, BDH Chemicals; Percoll, Pharmacia; Coomassie Blue dye reagent, Bio-Rad; SDS, bisacrylamide, and acrylamide, Boehringer Mannheim. Bovine adrenal glands were from XL Beef Co., Calgary, Alberta.

Chromaffin Granule Purification. Bovine adrenal glands were obtained from a local slaughterhouse and placed on ice within 5 min of the death of the animal, chromaffin granules were purified essentially *per* the method of Meyer and Burger (1979). All isolation procedures were performed at 4 °C, and all solutions were buffered with triethanolamine. Medullae were coarsely homogenized in 10 vol of buffer A [340 mosm/kg sucrose, 5 mM EGTA, and 25 mM Hepes (pH 7.5)], in a Waring commercial food blender at the highest setting (3 × 2 s bursts, 1 min apart). This coarse tissue suspension was further disrupted by several strokes in a Dounce homogenizer and then centrifuged at 1000*g* for 10 min. The supernatant was filtered through two layers of cheesecloth and centrifuged further (40 min, 10000*g*). The upper layer of the pellet was washed away with buffer A to partially remove mitochondrial contamination, and the pellet was resuspended in 42 mL of buffer A in readiness for loading onto a Percoll gradient. The Percoll gradient consisted of 3 × 12 mL each of 20%, 40%, and 60% Percoll that contained 1 mM EGTA (pH 7.5) and were each adjusted to a final osmolality of 340 mosm/kg by the addition of sucrose. Each gradient was loaded with 7 mL of the crude granule suspension and centrifuged for 60 min at 25000*g*. Purified granules, which collected at the 40%/60% interface, were washed twice (10000*g* for 40 min) in 100 mL of buffer B [340 mosm/kg sucrose and 25 mM Hepes (pH 7.5)] and stored at between 4 and 8 mg/mL, in buffer B, at 4 °C.

Table 1: Contaminating Na^+ and K^+ in the Reaction Mixture[a]

Hepes (hemi-sodium) and triethanolamine	Hepes (free acid) and triethanolamine
$Na^+ = 2$ mM	$Na^+ = 16.80 \pm 0.72$ μM
$K^+ = 0.5$ mM	$K^+ = 14.72 \pm 0.45$ μM

[a] Purified chromaffin granules (Experimental Procedures) were incubated in a buffer containing 300 mM sucrose and 25 mM Hepes, either the free acid or hemi-sodium form, adjusted to pH 7.5 with triethanolamine. After centrifugation (1200*g*, 30 min), the buffer was removed from the pellet and assayed for Na^+ and K^+ content by flame spectrophotometry (see Experimental Procedures).

The Na^+ and K^+ content of sucrose/Hepes buffer, adjusted to pH 7.5 with NaOH, was determined to be 2 mM Na^+ and 0.5 mM K^+ by atomic absorption (Table 1). Therefore, the sucrose/Hepes buffer used in all experiments was prepared from the acid form of Hepes, adjusted to pH 7.5 with triethanolamine. This protocol reduced contaminating Na^+ and K^+ in the sucrose/Hepes buffer to 16.80 ± 0.72 and 14.72 ± 0.45 μM ($n = 6$), respectively.

Determination of AIIt-Induced Aggregation of Chromaffin Granules. The reaction mixture contained, in a final volume of 0.6 mL, purified chromaffin granules at an OD of 0.30, in 25 mM Hepes/TEA (pH 7.5), and 20 μM $CaCl_2$, and sucrose to a final osmolality of 340 mosm/kg with sucrose. When the granules were incubated with various concentrations of salts, the final osmolality of the reaction buffer was maintained at 460 mosm/kg by the addition of sucrose. The maintenance of the osmolality of the reaction mixture at 460 mosm/kg by the addition of sucrose therefore allowed the manipulation of the salt concentration to as high as 200 mM salt, without changing the osmolality of the reaction mixture. The optical density of the aliquot was read prior to protein addition, and further absorbance readings were taken at the different time intervals specified in a Pye Unicam PU8800 spectrophotometer at a wavelength of 540 nm and 20 °C. Unless otherwise stated, the addition of 1 μM AIIt initiated the reaction.

Determination of AIIt Binding to Chromaffin Granules. After measurement of chromaffin granule aggregation (preceding section), the reaction mixture was centrifuged at 20 °C (5 min at 15600*g* in an Eppendorf 5414). The pellet was resuspended in SDS disruption buffer and run on SDS–PAGE. Protein bands were visualized with Coomassie Brilliant Blue (R-250), and both the protein band at 67–70 kDa and the AIIm protein bands were cut from each lane of the polyacrylamide gel, extracted overnight with pyridine (25%) *per* the method of Fenner *et al.* (1975), and then quantified spectrophotometrically by measuring absorbance at 605 nm. A small variation in the amount of pellet loaded

onto each lane of the SDS-polyacrylamide gel was observed. This possibly was due to the slight variation in the degree of solubilization of the pellet. We therefore corrected for this variation in the amount of SDS-disrupted pellet that was applied to the gel by extracting, from each lane of the SDS gel, both the AII band and the band at 67−70 kDa. The 67−70 kDa region of the gel was therefore used as an internal granule marker to normalize data. The values (A_{605}) for the 67−70 kDa band, extracted from each lane of the SDS gel, were averaged, and the 67−70 kDa band value of each lane of the SDS gel was used to normalize the amount of AIIt present in that lane according to the formula; A_{605} (normalized AIIt present in lane) = A_{605}(average 67−70 kDa value in all lanes)/A_{605} (67−70 kDa region in lane) × [A_{605}(AIIt in lane)]. Where necessary, the quantity of AIIt present in the lane was calculated after determination of the A_{605} values of AIIt standards. When chromaffin granules were pelleted, disrupted in SDS disruption buffer, and separated by SDS−PAGE, chromaffin granule protein bands with mobilities corresponding to that of AII were not visualized.

Determination of Ca^{2+} in Buffers. Ca^{2+} concentrations were estimated by the method of Fabiato and Fabiato (1979) and verified by Ca^{2+} electrode and FURA-2 measurement, following the procedure of Grynkiewicz *et al.* (1985).

Scanning Electron Microscopy of Chromaffin Granules. Chromaffin granules were fixed in a solution containing 2.5% glutaraldehyde, 0.2 M sodium cacodylate (pH 7.2), and sucrose to 340 mosm/kg at 4 °C, overnight. After rinsing in buffer B, the granules were further fixed for 12 h at 4 °C in a solution containing 1% osmium tetraoxide, 0.3 M sucrose, and 0.2 M sodium cacodylate (pH 7.2). The granules were washed with buffer B and partially dehydrated through a series of graded alcohols. The samples were then critical point dried onto 25 mm Nucleopore polycarbonate filters with CO_2 and then sputter coated with gold−palladium prior to examination (Hitachi S450, SEM).

Miscellaneous Techniques. Annexins I−VI were prepared from bovine lung (Khanna *et al.*, 1990) and were stored in 50 mM KCl at −70 °C. Annexin II tetramer contained less than 1% phosphate. Prior to use, proteins were thawed and equilibrated by chromatography on a PD 10 column (Pharmacia) with buffer B. Protein concentration was measured using the Bradford (1976) Coomassie Blue dye binding assay using BSA as a standard. Concentrations were further determined spectrophotometrically with an extinction coefficient for AIIt of A_{280} = 0.65 for 1 mg/mL (Gerke & Weber, 1985). SDS−PAGE was performed in a slab-gel apparatus using the methods described by Laemmli (1970). Na^+ and K^+ in reaction buffers were determined by flame emission spectrometry (Haigh *et al.*, 1989) using a Phillips Pye Unicam SP9.

RESULTS

KCl Dependence of AIIt-Induced Aggregation of Chromaffin Granules. The *in vitro* aggregation of chromaffin granules by AIIt had been previously examined in a buffer that contained 30 mM KCl and 240 mM sucrose (Drust & Creutz, 1988). Figure 1 compares the rate of AIIt-dependent aggregation of chromaffin granules incubated in an isotonic reaction mixture containing buffer B [340 mosm/kg sucrose and 25 mM Hepes (pH 7.5)] with that of a reaction mixture

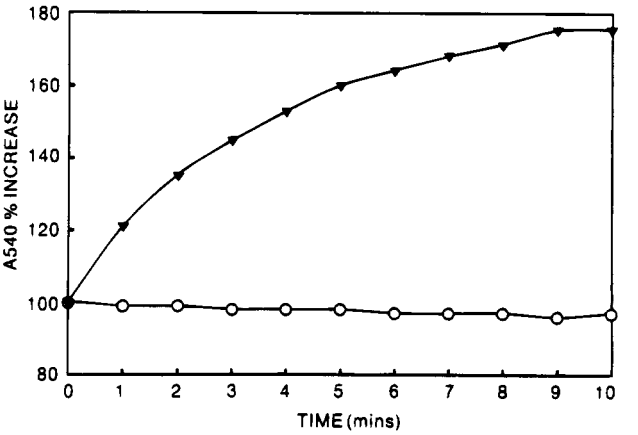

FIGURE 1: Time course of KCl dependence of AIIt-induced aggregation of purified chromaffin granules. Chromaffin granules were purified *per* the Experimental Procedures and then incubated in a final volume of 0.6 mL with sucrose/Hepes buffer [0.3 M sucrose and 25 mM Hepes/TEA (pH 7.5)] containing 20 μM $CaCl_2$, in the presence (▼) or absence of 50 mM KCl (○). The reaction was initiated by the addition of 0.46 μM AIIt. Immediately prior to AIIt addition, the optical absorbance (A_{540}, nm) of the aliquot was determined, and changes in optical absorbance were expressed as a percentage of the starting optical density (no added AIIt) determined spectrophotometrically at a wavelength of 540 nm (see Experimental Procedures). Data shown are representative of five independent experiments.

containing buffer B and 50 mM KCl. Incubation of chromaffin granules in a reaction mixture containing buffer B did not activate AIIt-dependent aggregation of the chromaffin granules, whereas when the reaction mixture contained buffer B plus 50 mM KCl, time-dependent aggregation of chromaffin granules by AIIt was observed. The chromaffin granule aggregation activity of AIIt, assayed in the presence of buffer B and 50 mM KCl, was maximal at 10 min. In contrast, in the absence of AIIt or in the presence of AIIt but the absence of Ca^{2+}, aggregation of chromaffin granules did not occur (data not shown).

Osmolality Dependence of Chromaffin Granule Aggregation. The data presented in Figure 1 show that the addition of 50 mM KCl to the reaction mixture activated AIIt-dependent aggregation of chromaffin granules. Since the addition of KCl to the reaction medium would increase both the osmolality and the ionic strength of the medium, we tested the effect of increased osmolality on the AIIt-dependent aggregation of chromaffin granules. As shown in Figure 2, increasing the osmolality of the reaction mixture from 340 (buffer B) to 640 mosm/kg, by the addition of sucrose, did not activate the AIIt-dependent aggregation of granules. In contrast, when the osmolality of the reaction medium was increased by the addition of KCl to the reaction mixture, a dose-dependent effect of KCl addition on AIIt-dependent chromaffin granule aggregation was observed. The KCl-dependent activation of AIIt-dependent chromaffin granule aggregation reached a maximum between 370 and 390 mosm/kg KCl. Increasing the osmolality of the reaction mixture above 390 mosm/kg, by the addition of KCl resulted in progressive inhibition of AIIt-dependent chromaffin granule aggregation until about 640 mosm/kg, where AIIt-dependent chromaffin granule aggregation was almost totally inhibited.

Salt Dependence of AIIt-Induced Chromaffin Granule Aggregation. In order to examine whether the salt composition or the ionic strength of the reaction mixture was

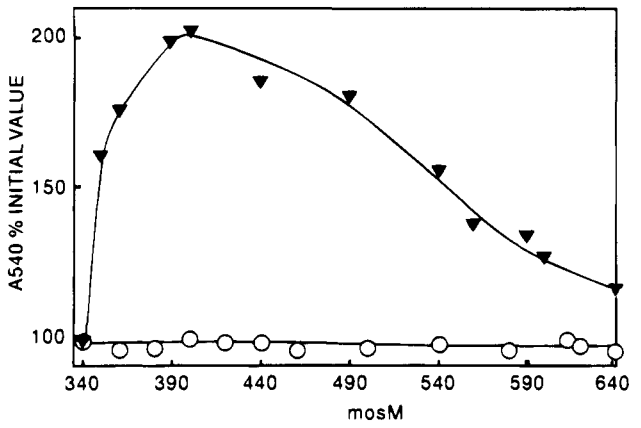

FIGURE 2: Osmolality dependence of AIIt-induced aggregation of purified chromaffin granules. Chromaffin granules were purified *per* the Experimental Procedures and then incubated with 25 mM Hepes (pH 7.5), 0.3 M sucrose, 20 μM $CaCl_2$, and various concentrations of KCl (▼) and sufficient added sucrose to produce the osmolalities shown (○). The reaction was initiated by the addition of 0.46 μM AIIt and analyzed after 10 min. Changes in optical absorbance are expressed as a percentage of the starting optical density (no added AIIt) determined spectrophotometrically at a wavelength of 540 nm (see Experimental Procedures). Data shown are representative of three independent experiments.

necessary to activate the chromaffin granule aggregation activity of AIIt, we examined several different salts for their ability to support AIIt-dependent chromaffin granule aggregation. In these experiments, the final osmolality of the reaction mixture was adjusted to 460 mosm/kg by the addition of sucrose. Therefore, under these experimental conditions, the addition of salt to the reaction mixture results in changes in the ionic strength but not in the osmolality of the reaction mixture. Consistent with the results presented in Figure 2, AIIt-dependent aggregation of chromaffin granules was not activated in the absence of added salt and at an osmolality of 460 mosm/kg. Figure 3A demonstrates that at concentrations between 30 and 50 mM salt, both KCl and potassium glutamate support chromaffin granule aggregation to maximum values of 210% and 195% of the starting values, respectively, whereas potassium phosphate at its optimum concentration of 10 mM supported the aggregation of granules to a maximum value of 120% of the starting value. Similar results were observed when sodium salts were substituted for potassium salts. We also investigated the possibility that the incubation of chromaffin granules with high salt concentrations might inhibit the ability of the granules to aggregate by damaging them. Therefore, chromaffin granules were incubated with 150 mM NaCl at 20 °C for 30 min, washed with buffer B, and incubated with AIIt under standard conditions for the aggregation reaction. The AIIt-dependent aggregation of the NaCl-incubated granules was decreased slightly by 14% compared with granules that had been incubated with buffer B. This suggests that the decrease in AIIt-dependent chromaffin granule aggregation, observed at 150 mM NaCl, was not due to salt-induced damaging of the chromaffin granules.

We also examined the possibility that the low levels of AIIt-dependent aggregation of chromaffin granules observed in the presence of potassium phosphate could be due to chelation of Ca^{2+} by phosphate. However, when chromaffin granules were incubated at 20 mM potassium phosphate, increasing the Ca^{2+} concentration from 10 to 200 μM increased the AIIt-dependent aggregation from 116 ± 4.8%

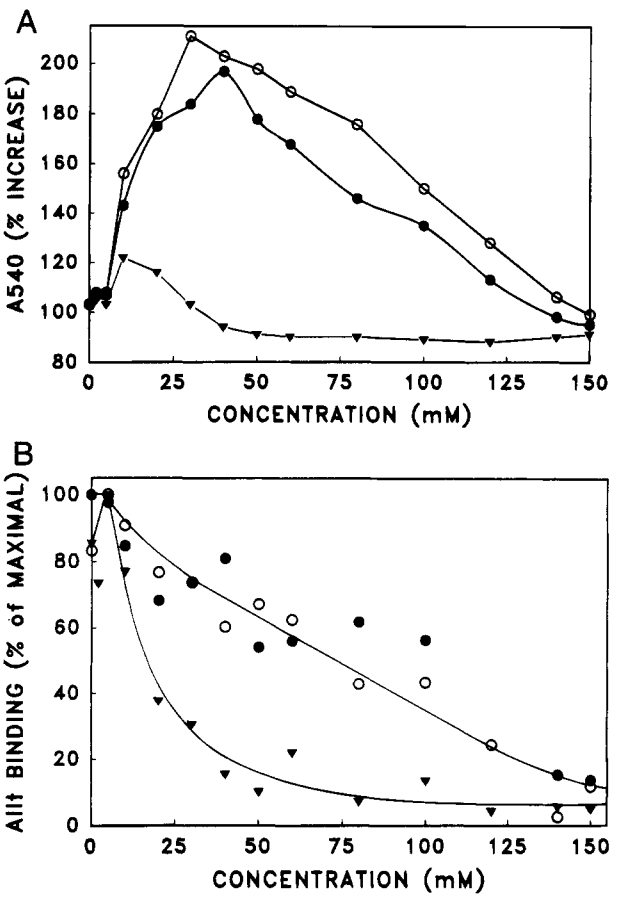

FIGURE 3: (A) Salt dependence of AIIt-induced aggregation of purified chromaffin granules. Chromaffin granules were purified *per* the Experimental Procedures and then incubated in a reaction mixture containing 25 mM Hepes (pH 7.5), 20 μM $CaCl_2$, and increasing concentrations of KCl (●), potassium glutamate (○), or potassium phosphate (▼). Sucrose was added to the reaction mixture to maintain an osmolality of 460 mosm/kg. The reaction was initiated by the addition of 1.0 μM AIIt and analyzed after 10 min. Changes in optical absorbance are expressed as a percentage of the starting optical density (no added AIIt) determined spectrophotometrically at a wavelength of 540 nm (see Experimental Procedures). Data shown are representative of three independent experiments. (B) Salt-dependence of AIIt binding to chromaffin granules. Chromaffin granules were purified *per* the Experimental Procedures and incubated with 25 mM Hepes (pH 7.5), 20 μM $CaCl_2$, and increasing concentrations of KCl (●), potassium glutamate (○), or potassium phosphate (▼) . Sucrose was added to the reaction mixture to maintain an osmolality of 460 mosm/kg. The reaction was initiated by the addition of 1.0 μM AIIt. The reaction was allowed to proceed for 10 min, and the binding of AIIt to granules was quantitated by centrifugation of the sample, disruption of the pellet in SDS buffer with subsequent visualization by SDS electrophoresis, and pyridine extraction of the protein bands (see Experimental Procedures). Data are representative of three independent experiments.

(mean ± SD, $n = 4$) to 140 ± 7.3% (mean ± SD, $n = 4$) of control. In contrast, when chromaffin granules were incubated at 150 mM potassium phosphate, increasing the Ca^{2+} concentration from 10 to 200 μM did not activate the AIIt-dependent aggregation of the chromaffin granules. Therefore, the inability of potassium phosphate to stimulate AIIt-dependent chromaffin granule aggregation to the levels observed with KCl is not due to chelation of Ca^{2+} by phosphate. The inability of potassium phosphate to support chromaffin granule aggregation to levels approaching those produced by KCl and potassium glutamate suggests that ionic strength is not the only factor that activates AIIt-dependent

chromaffin granule aggregation. The differential effect of salts on chromaffin granule aggregation raised the possibility that these salts might allosterically interast with AIIt and that the chromaffin granule aggregation activity of AIIt might be affected by both the salt composition of the reaction mixture as well as the ionic strength of the reaction mixture. The inhibition of aggregation of chromaffin granules by AIIt at high salt concentrations was not caused by a salt-dependent dissociation of the p11 light chain, since the final step in the purification of AIIt involves gel permeation chromatography in the presence of 150 mM KCl. Furthermore, the dissociation of the p11 light chain from the tetramer requires the presence of high concentrations of urea (Johnsson *et al.*, 1988).

The high salt concentrations used in the experiment reported in Figure 3A could lead to granule lability and time-dependent lysis during the aggregation reaction. This could therefore result in a decline in turbidity and a masking of the aggregation reaction. Therefore, chromaffin granules were incubated in several concentrations of KCl, potassium glutumate, and potassium phosphate in the absence of AIIt, and the turbidity was measured after 10 min *per* the Experimental Procedures. In the presence of 0, 50, 100, and 150 mM KCl, the A_{540} (% initial value) expressed as mean ± SD, ($n = 3$) was 93.2 ± 2.6, 94.6 ± 3.1, 94.3 ± 0.8, and 93.7 ± 2.0. At 50, 100, and 150 mM potassium phosphate, these values were 98.3 ± 0.1, 98.5 ± 1.3, and 98.8 ± 2.9, and at similar concentrations of potassium glutumate these values were 98.7 ± 1.4, 97.4 ± 2.3, and 98.2 ± 1.7. Clearly, the loss in the chromaffin granule aggregation activity of AIIt, at high KCl or potassium glutamate concentrations or at moderate concentrations of potassium phosphate, could not be due to a salt-dependent decline in the turbidity of the chromaffin granules masking the AIIt aggregation activity.

Salt Dependency of AIIt Binding to Chromaffin Granules. The data presented in Figure 3A suggested that the chromaffin granule aggregation activity of AIIt is influenced by both the ionic strength and the salt composition of the reaction mixture. Since the binding of AIIt to the chromaffin granules was an important step in the aggregation reaction, it was possible that the effects of ionic strength on the chromaffin granule aggregation activity of AIIt could be due to an effect of ionic strength on the binding of AIIt to the chromaffin granules. Therefore, the effects of increasing salt concentration on the binding of AIIt to chromaffin granules were examined. As shown in Figure 3B, maximal binding of AIIt was observed in the absence of added salt, whereas under these experimental conditions, AIIt-dependent aggregation was not activated (Figure 3A). As the salt concentration was increased, and the final osmolality of the reaction mixture was maintained at 460 mosm/kg, AIIt binding to chromaffin granules decreased in a dose-dependent manner to about 15% of maximal binding at 150 mM KCl or 150 mM potassium glutamate and about 4% of maximal binding at 150 mM potassium phosphate. Surprisingly, as the KCl or potassium glutamate concentration was increased to between 25 and 50 mM salt, AIIt-dependent aggregation increased (Figure 3A) whereas AIIt-dependent binding decreased (Figure 3B). Conversely, as the potassium phosphate concentration was increased, AIIt-dependent aggregation was not activated at levels of potassium phosphate above 25 mM. The binding of AIIt to chromaffin granules was half-maximally inhibited at about 15 mM potassium phosphate whereas half-maximal binding of AIIt to chromaffin granules required about 75 mM KCl or potassium phosphate. These data therefore suggest that the addition of salt to the reaction mixture results in a decrease in the binding of AIIt to chromaffin granules, whereas AIIt-dependent aggregation of chromaffin granules shows a biphasic response: activation at low salt concentrations and inhibition at high salt concentrations.

AIIt Dose Dependence of Chromaffin Granule Aggregation at 0, 50, and 150 mM KCl. From the data presented in Figure 3A, it may be concluded that AIIt-dependent aggregation of granules is not activated in the absence of added salt, is maximally potentiated between 25 and 50 mM KCl, and is almost completely inhibited at 150 mM KCl. In order to determine whether increasing the salt concentration could affect the affinity of the interaction of AIIt with chromaffin granules, the dose dependence of AIIt aggregation of chromaffin granules at 50 mM KCl, 150 mM KCl, and without added salt was examined (Figure 4A). For these experiments, the final osmolality of the reaction mixture, after addition of KCl, was adjusted to 460 mosm/kg by the addition of sucrose. With no added salt, little aggregation was observed over the range of AIIt concentrations tested. In contrast, at 50 mM KCl, half-maximal aggregation was seen at 0.171 ± 0.001 μM AIIt (mean ± SD, $n = 3$), maximum aggregation in this case being 168% of control. Conversely, at 150 mM KCl, increasing the AIIt concentration could not, over the concentration range examined, compensate for the inhibitory effects of this concentration of KCl.

Dependence of AIIt Binding to Chromaffin Granules on KCl. In order to determine whether the salt concentration effects on the chromaffin granule activity of AIIt were due to a KCl-dependent change in the binding of AIIt to chromaffin granules, the binding of AIIt to granules was examined under conditions identical to those used for the experiments detailed in Figure 4A. Chromaffin granules that were incubated in a reaction mixture containing 50 mM KCl that was adjusted to 460 mosm/kg showed half-maximal binding of AIIt at 0.163 ± 0.007 μM AIIt (mean ± SD, $n = 3$), with maximal binding of AIIt to the chromaffin granules occurring at about 0.6 μM (Figure 4B). Similarly, in the absence of added KCl, half-maximal binding of AIIt to chromaffin granules occurred at 0.173 ± 0.034 μM AIIt (mean ± SD, $n = 3$). In contrast, minimal binding of AIIt to granules incubated in 150 mM KCl was observed (Figure 4B). Therefore, these results suggest that increasing the KCl concentration to 50 mM minimally affects the dose dependency of the binding of AIIt to chromaffin granules; however at KCl concentrations higher than 50 mM, the binding of AIIt to chromaffin granules is inhibited (Figure 4B).

Ca^{2+} Dependence of AIIt-Induced Aggregation of Chromaffin Granules at Various Concentrations of KCl. In order to further clarify the effects of salt concentration on AIIt-dependent chromaffin granule aggregation, the Ca^{2+} dependence of AIIt-induced aggregation at several concentrations of KCl was examined. Without added salt, half-maximal aggregation occurred at $pCa^{2+} = 3.79 \pm 0.062$ (mean ± SD, $n = 3$), and the maximal extent of aggregation was 127% of control (Figure 5A). We also examined the possibility that, in the absence of added salt, increasing the $CaCl_2$ concentration to 1 mM $CaCl_2$ might activate AIIt-dependent chromaffin granule aggregation due to the ionic strength of the $CaCl_2$

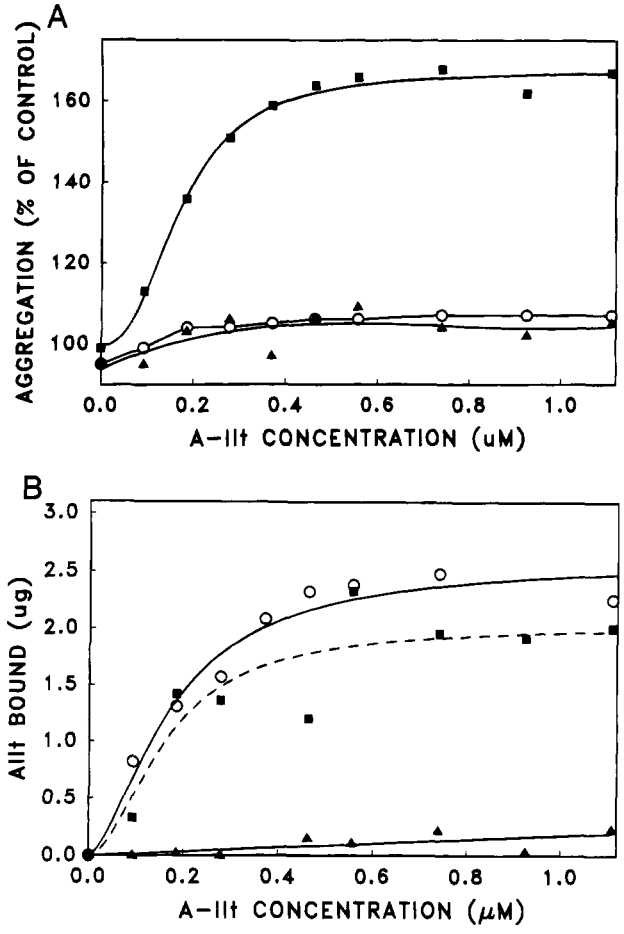

FIGURE 4: (A) Dependence of the AIIt-dependent aggregation of purified chromaffin granules on KCl. Chromaffin granules were purified *per* the Experimental Procedures and incubated in a reaction mixture containing 25 mM Hepes (pH 7.5) and various concentrations of AIIt, in the absence of added salt (○), the presence of 50 mM KCl (■) or the presence of 150 mM KCl (▲). Sucrose was added to adjust the final osmolality to 460 mosm/kg. The reaction was initiated by the addition of 20 μM $CaCl_2$. Optical absorbancies were read at the point immediately prior to the addition of $CaCl_2$ and again at 10 min and are expressed as a percentage of the starting optical density determined spectrophotometrically at a wavelength of 540 nm (see Experimental Procedures). Data shown are representative of three independent experiments. (B) Dependence of the AIIt binding to purified chromaffin granules on KCl. Chromaffin granules were purified *per* the Experimental Procedures and incubated in a reaction mixture containing 25 mM Hepes (pH 7.5) and various concentrations of AIIt, in the absence of added salt (○), the presence of 50 mM KCl (■) or the presence of 150 mM KCl (▲). Sucrose was added to adjust the final osmolality to 460 mosm/kg. The reaction was initiated by the addition of 20 μM $CaCl_2$. After 10 min, the binding of AII to granules was quantitated by centrifugation of the sample, disruption of the pellet in SDS buffer with subsequent visualization by SDS electrophoresis, and pyridine extraction of the protein bands (see Experimental Procedures). Data are representative of three independent experiments.

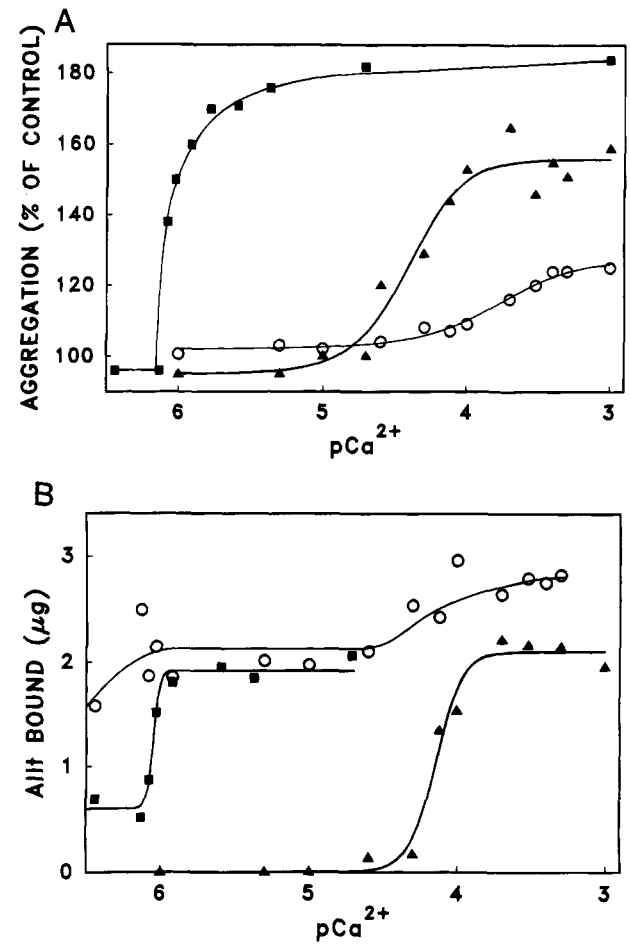

FIGURE 5: (A) Dependence of AIIt-induced aggregation of purified chromaffin granules on Ca^{2+} at various concentrations of KCl. Chromaffin granules were purified *per* the Experimental Procedures and then incubated in a reaction mixture containing 25 mM Hepes (pH 7.5) and various amounts of $CaCl_2$ to give the final concentrations of Ca^{2+} shown, in the absence of added salt (○), the presence of 50 mM KCl (■), or the presence of 150 mM KCl (▲). Sucrose was added to adjust the final osmolality to 460 mosm/kg, and the reaction was initiated by the addition of 1 μM AIIt. Optical absorbancies were read at the point immediately prior to AIIt addition and again at 10 min and are expressed as a percentage of the starting optical density determined spectrophotometrically at a wavelength of 540 nm (see Experimental Procedures). Data shown are representative of three independent experiments. (B) Dependence of AIIt binding to chromaffin granules on Ca^{2+}. Chromaffin granules were purified *per* the Experimental Procedureds and then incubated in a reaction mixture containing 25 mM Hepes (pH 7.5) and various amounts of $CaCl_2$ to give the final concentrations of Ca^{2+} shown, in the absence of added salt (○), the presence of 50 mM KCl (■), or the presence of 150 mM KCl (▲). Sucrose was added to adjust the final osmolality to 460 mosm/kg, and the reaction was initiated by the addition of 1 μM AIIt. The binding of AIIt to granules was quantitated by centrifugation of the sample, disruption of the pellet in SDS buffer with subsequent visualization by SDS electrophoresis, and pyridine extraction of the protein bands (see Experimental Procedures). Data shown are representative of three independent experiments.

addition. Since 2 mM KCl did not activate AIIt-dependent aggregation in the presence of 20 μM Ca^{2+} (Figure 2), it is unlikely that in, the absence of added salt, AIIt activation of AIIt-dependent chromaffin granule aggregation at pCa^{2+} 3.0 (1 mM $CaCl_2$) was due to an ionic strength effect of the $CaCl_2$ addition (data not shown). In the presence of 50 mM KCl, half-maximal AIIt-dependent aggregation of chromaffin granules occurred at $pCa^{2+} = 6.07 \pm 0.018$ (mean ± SD, $n = 3$), and the maximal extent of aggregation of the granules was 185% of control. In the presence of 150 mM KCl, half-maximal aggregation of granules occurred at $pCa^{2+} = 4.41 \pm 0.07$ (mean ± SD, $n = 3$), and the maximal extent of granule aggregation was 156% of control.

Dependence of AIIt Binding to Chromaffin Granule on Ca^{2+} at Various Concentrations of KCl. In order to determine whether the shift in Ca^{2+} dependence of the chromaffin granule aggregation activity of AIIt at the differing KCl concentrations was reflective of a change in

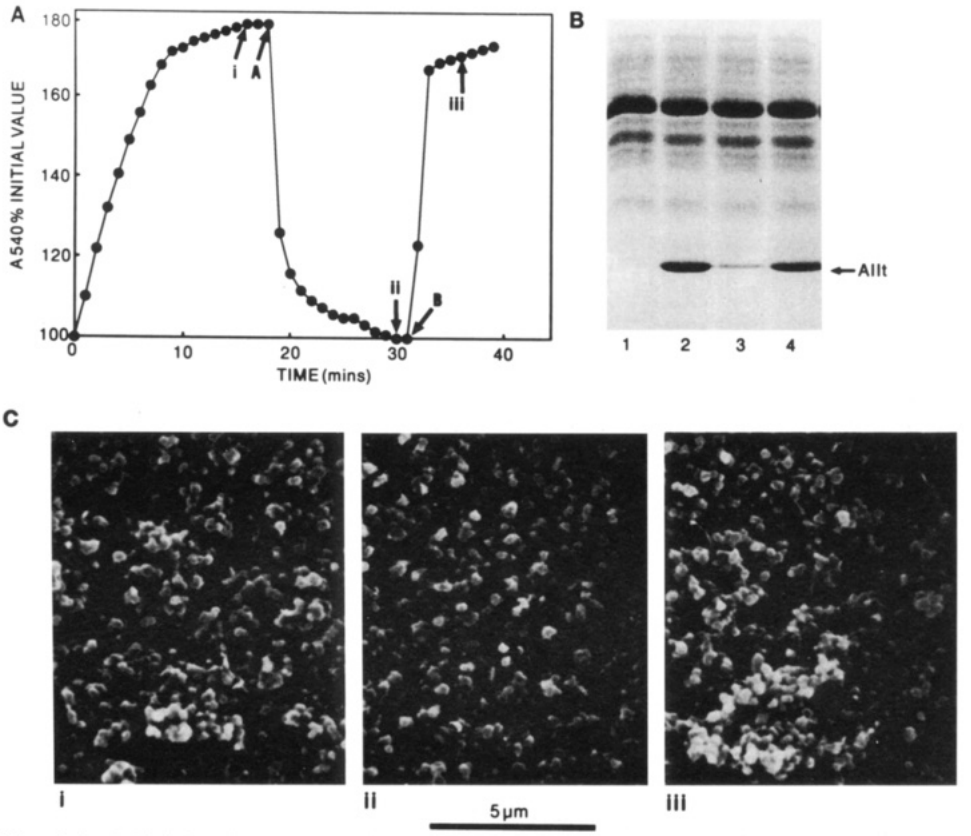

FIGURE 6: Reversibility of the $Ca2^{+}$-induced aggregation of chromaffin granules. (A) Chromaffin granules were prepared *per* the Experimental Procedures, and then incubated with 50 μM $CaCl_2$, 50 mM KCl, and sucrose to a final concentration of 340 mos in 25 mM Hepes/TEA (pH 7.5). At 0 min, 1 μM AIIt was added to initiate the reaction. Immediately prior to the addition of AIIt, the optical absorbance of the aliquot was determined, and changes in optical absorbance, at the times shown, are expressed as a percentage increase over the starting optical density determined spectrophotometrically at a wavelength of 540 nm (see the Experimental Procedures). At point A, EGTA was added to a final concentration of 1 mM, and at point B, a total of 2 mM Ca^{2+} was added. Data shown are representative of five independent experiments. (B) SDS−PAGE of AIIt binding to chromaffin granules. Aliquots (0.1 mL) of the reaction mixture were removed before the addition of AIIt (lane 1), at point i (lane 2), and at point ii (lane 3) (part A). 100 μL aliquots of reaction mixture were taken at points i,ii, and iii. The binding of AIIt to granules was quantitated by SDS analysis of centrifuged chromaffin granules (see Experimental Procedures). Data are representative of three independent experiments. (C) Scanning electron microscopy of chromaffin granules during Ca^{2+}-induced aggregation (i), EGTA reversal of aggregation (ii), and Ca^{2+} reinitiation of aggregation (iii). 100 μL aliquots of the reaction mixture were taken at points i, ii, and iii of part A and processed for scanning electron microscopy *per* the Experimental Procedures. Scale bar = 5 μm.

the ability of AIIt to bind to granules, the binding of AIIt to chromaffin granules was examined under similar conditions. The Ca^{2+} dependence of binding of AIIt to chromaffin granules was measured in a reaction mixture adjusted to a final osmolality of 460 mosm/kg and containing 50, 150, or no added KCl. As shown in Figure 5B, half-maximal binding of AIIt to granules occurred at pCa = 6.04 ± 0.08 (mean ± SD, n = 3) in 50 mM KCl. In the absence of added salt, AIIt binding was essentially maximal over the range of Ca^{2+} examined. Furthermore, in the presence of 150 mM KCl, half-maximal binding occurred at pCa^{2+} = 4.14 (mean ± SD, n = 3) (Figure 4B).

Reversibility of AIIt Aggregation of Chromaffin Granules by Chelation of Ca^{2+}. Electron microscopic evidence has suggested that AIIt promotes the Ca^{2+}-dependent aggregation but not the fusion of chromaffin granules (Drust & Creutz, 1988). However, it has not been established whether the AIIt-dependent aggregation of granules is reversible by the removal of Ca^{2+}. As shown in Figure 6A, the addition of 20 μM Ca^{2+} to the reaction mixture containing AIIt and chromaffin granules, results in the rapid aggregation of the granules. When an aliquot of the reaction mixture was removed after 16 min and centrifuged, SDS-PAGE analysis of the pelleted granules suggested that binding of AIIt to the chromaffin granules had occurred (Figure 6B, lane 2). Analysis of an additional 16 min aliquot, by scanning electron microscopy, revealed the presence of large aggregates of chromaffin granules (Figure 6C, panel i). The aggregation reaction was allowed to proceed for 18 min, at which time the Ca^{2+} in the reaction mixture was reduced to less than 1.0 nM Ca^{2+} by the addition of EGTA. As shown in Figure 6A, the addition of EGTA results in a rapid reversal of the aggregation reaction. After about 30 min, the aggregation was completely reversed and aliquots of the reaction were removed and analyzed by centrifugation and scanning electron microscopy. The addition of EGTA resulted in the loss of binding of AIIt to the chromaffin granules (Figure 6B, lane 3). Furthermore, the presence of aggregated chromaffin granules was not observed by electron microscopy (Figure 6C, panel ii). These results confirmed that the reduction in Ca^{2+} in the reaction mixture, by the addition of EGTA, completely reversed the aggregation of the chromaffin granules by AIIt. In order to examine whether the aggregation reaction, which had been reversed by the addition of EGTA, could be reinitiated by the readdition of Ca^{2+} , the reaction mixture was adjusted to 1 mM Ca^{2+} at 31 min. As seen in Figure 6A, the AIIt-dependent aggregation of the chromaffin granules was reinitiated rapidly by the addition of Ca^{2+}. Additional aliquots of the reaction mixture were analyzed at 36 min.

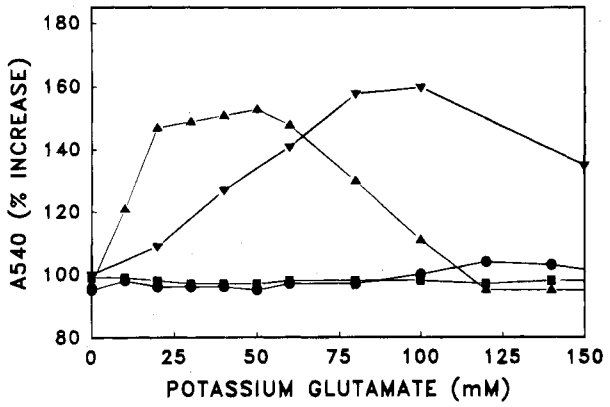

FIGURE 7: Effect of potassium glutamate on the ability of various annexin proteins to induce the aggregation of purified chromaffin granules. Chromaffin granules were purified *per* methods and then incubated with 50 μM $CaCl_2$ (▲, ●, ■) or 200 μM $CaCl_2$ (▼) and the various concentrations of potassium glutamate shown, in a buffer containing 0.3 M sucrose and 25 mM Hepes/TEA (pH 7.5) to a final concentration of 340 mosm. At 0 min, 2 μM AI (▲), AIIm (▼), AIV (●), or AVI (■), was added to the reaction mixture, bringing the final volume to 1 mL and initiating the reaction. Changes in optical absorbance are expressed as a percentage of the starting optical density (no added protein) determined spectrophotometrically at a wavelength of 540 nm (see Experimental Procedures). Data shown are representative of three independent experiments.

Centrifugation of the mixture and analysis of the pelleted granules by SDS−PAGE revealed the reassociation of AIIt with the chromaffin granules (Figure 6B, lane 4). Furthermore, scanning electron microscopy revealed the reformation of large aggregates of chromaffin granules (Figure 6C, panel iii).

Potassium Glutamate Dependence of Chromaffin Granule Aggregation by Various Annexins. Of the many annexins previously tested for chromaffin granule aggregation activity at micromolar Ca^{2+}, only AIIt and AI have been shown to possess this activity (Drust & Creutz, 1988). However, the data presented in Figure 3 suggest that the optimal chromaffin granule aggregation activity of AIIt occurs over a narrow range of salt concentration. This result presented the possibility that other annexins might possess chromaffin granule aggregation activity if assayed at their optimal salt concentration. Therefore, the aggregation of chromaffin granules by annexins I−VI was examined over a range of potassium glutamate concentrations. This paradigm revealed that only AIIt and AI could aggregate chromaffin granules to a significant level at low micromolar Ca^{2+}. Whereas when Ca^{2+} was increased to 200 μM Ca^{2+}, significant aggregation of chromaffin granules was observed by the annexin II monomer (Figure 7), in sharp contrast, annexins III−VI did not aggregate chromaffin granules above baseline (data for annexins III and V not shown). Furthermore, the optimum chromaffin granule aggregation activity of AIIm required 100 mM potassium glutamate, compared with the 30 mM potassium glutamate required for optimal chromaffin granule aggregation activity of AIIt (Figure 3A).

DISCUSSION

Previous reports that partially characterized the kinetics and mechanism of annexin-dependent aggregation of phospholipid liposomes or chromaffin granules did not consider tonicity or ionic strength as factors that might affect the fundamental properties of these proteins.

The relationship between AIIt-dependent aggregation of chromaffin granules, and AIIt binding to chromaffin granules may be summarized as follows. First, without added salt, maximal binding of AIIt to chromaffin granules is observed; however, under these conditions AIIt-dependent chromaffin granule aggregation is not measurable (Figure 3). Second, as potassium glutamate or KCl is added to the reaction mixture to a salt concentration of between 30 and 50 mM, AIIt-dependent chromaffin granule aggregation increased to a maximum while AIIt binding to chromaffin granules decreased. Third, as the salt concentration is increased from 30−50 to 150 mM salt, both AIIt-dependent chromaffin granule aggregation and AIIt binding to granules are decreased. Although AIIt-dependent chromaffin granule aggregation is only marginally stimulated by potassium phosphate, a similar biphasic dependence of AIIt-dependent chromaffin granule aggregation on potassium phosphate was measured. Over the range of potassium phosphate tested, AIIt binding to the chromaffin granules decreased compared to the amount of AIIt bound in the absence of added salt. Surprisingly, half-maximal inhibition of AIIt binding to the chromaffin granules occurred at about 15 mM potassium phosphate, whereas half-maximal inhibition of AIIt binding to chromaffin granules occurred in the presence of about 75 mM KCl or 75 mM potassium glutamate (Figure 3B). These results clearly demonstrate that the extent of AIIt binding to chromaffin granules is not tightly coupled to AIIt-dependent aggregation of the chromaffin granules. Furthermore, the effect of the salt concentration on both AIIt-dependent chromaffin granule aggregation and AIIt binding to chromaffin granules is not entirely dependent on the ionic strength of the salt but may also be due to the salt species.

We have also examined whether the effects of salt concentration on AIIt binding to chromaffin granules and AIIt-dependent chromaffin granule aggregation are due to an effect of salt on the dose dependency or Ca^{2+} dependence of AIIt-dependent aggregation or binding to chromaffin granules. When we examined the effects of 50 mM KCl, 150 mM KCl, and no salt addition on AIIt-dependent chromaffin granule aggregation and binding, we observed that the concentration of AIIt for half-maximal binding to chromaffin granules is only slightly reduced at 50 mM KCl compared to no added salt (Figure 4B). In contrast, at 150 mM KCl, AIIt-dependent binding to chromaffin granules is barely measurable over the concentrations of AIIt examined. Furthermore, over the range of AIIt examined, AIIt-dependent aggregation of chromaffin granules is only measurable at 50 mM KCl (Figure 4B). At 50 mM KCl, significant AIIt-dependent aggregation of granules was measured at 0.1 μM AIIt, whereas at concentrations as high as 1.1 μM AIIt, AIIt-dependent aggregation of chromaffin granules could not be detected at 150 mM KCl or in the absence of added salt.

Given that AIIt is a protein that is dependent upon Ca^{2+} for its binding to either phospholipids or natural membranes, it might be expected that the difference in the ability of this protein to aggregate chromaffin granules, at different salt concentrations, might be reflected in a change in the Ca^{2+} dependency of that process. As shown in Figure 5A, the pCa^{2+} for half-maximal activation of the AIIt-dependent aggregation of granules varies with the salt concentration. In the absence of added salt, half-maximal aggregation of

chromaffin granules occurs at $pCa^{2+} = 3.79$, compared to $pCa^{2+} = 4.41$ at 150 mM KCl and $pCa^{2+} = 6.07$ in the presence of 50 mM KCl. These results therefore suggest that the Ca^{2+} dependency of AIIt-dependent chromaffin granule aggregation is modulated by the salt concentration of the incubation mixture. Similarly, the pCa^{2+} for half-maximal binding of AIIt to chromaffin granules is also modulated by the salt concentration of the reaction mixture (Figure 5B). Creutz *et al.* (1978) reported that the chromaffin granule aggregation activity of annexin VII was stimulated by 2−3-fold by the salts of monovalent ions. The reaction was stimulated beween 0 and 30 mM salt, after which the effect was saturated.

Although speculative, it is reasonable to propose that the AIIt-dependent aggregation of granules requires two distinct conformations of AIIt. The first conformation (conformation A) requires the presence of Ca^{2+}, and the generation of conformation A allows the binding of AIIt to the chromaffin granules. However, aggregation of the granules is not supported by conformation A. The observed inhibition of AIIt binding to chromaffin granules upon the addition of salt could be explained, within this hypothesis, as an inhibitory effect of salt concentrations on the generation of conformation A. Mechanistically, it appears that increasing salt concentrations increase the Ca^{2+} requirement for the generation of conformation A. When AIIt changes to conformation B, chromaffin granule binding activity is retained and the chromaffin granule aggregation activity of the protein is promoted. The second conformation, conformation B, requires the presence of an optimal concentration of salt. The Ca^{2+} dependency of the generation of conformation B on the salt concentration appears to be biphasic and varies from about 160 μM in the absence of added salt to about 0.9 μM in the presence of the optimal salt concentration (50 mM KCl) to 40 μM at 150 mM KCl. The simplest explanation for the chromaffin granule aggregation activity of conformation B is that the protein, when in conformation B, possesses two membrane binding sites, whereas conformation A possesses only a single binding site. The observation that the chromaffin granule aggregation and binding activity of AIIt can be both activated and inhibited by cycles of Ca^{2+} and EGTA (Figure 6) suggests that the generation of conformations A and B is fully reversible. Recent studies from our laboratory have shown that the phosphorylation of AIIt results in the inhibition of liposomal vesicle aggregation (Johnstone et al., 1992) and chromaffin granule aggregation (data in preparation) without affecting the binding of AIIt to these structures. The phosphorylation data therefore support the hypotheses that distinct conformations of AIIt are required for AIIt binding to chromaffin granules and for AIIt-dependent aggregation of chromaffin granules and that conformation B may be regulated by protein phosphorylation.

The involvement of AIIt in exocytosis has been suggested, on the basis of the observed low micromolar Ca^{2+} dependency of aggregation of chromaffin granules (Drust & Creutz, 1988), by the localization of AIIt to the cytosolic face of the plasma membrane and also as a result of the ability of AIIt to reconstitute secretion in permeabilized cells of the adrenal medulla (Ali *et al.*, 1989). Both the aggregation of chromaffin granules and the permeabilized adrenal medullary cells have been proposed as model systems for studies of the role of Ca^{2+} binding proteins in exocytosis. Wilson and Kirschner (1983) reported that incubation of permeabilized cells with sucrose-containing medium resulted in only a 130% increase in the Ca^{2+}-dependent release of catecholamines, compared with a 238% increase in the Ca^{2+}-dependent release of catecholamines in the presence of 150 mM potassium glutamate. These results are consistent with our observation of the inability of sucrose to stimulate the AIIt-dependent aggregation of chromaffin granules (Figure 1). Also, Wilson and Kirschner (1983) reported that, in the presence of 150 mM salt, the release of catecholamines displayed a biphasic dependency on Ca^{2+}, with limited catecholamine release at $A_{50\%}$ ($Ca^{2+} = 3-4$ μM) and a large catecholamine release at $A_{50\%}$ ($Ca^{2+} = 0.4$ mM). Ali *et al.* (1989) reported that the reconstitution of secretion by AIIt, assayed in the presence of 139 mM potassium glutamate, required about 5 μM Ca^{2+}. Clearly, the $A_{50\%}$ ($Ca^{2+} = 40$ μM) for the AIIt-dependent aggregation of chromaffin granules in the presence of 150 mM potassium glutamate (Figure 5) is within the same order of magnitude as the results obtained with permeabilized cells. Furthermore, Drust and Creutz (1988) reported that when assayed at 30 mM KCl and 1 mM $CaCl_2$, AIIm did not aggregate chromaffin granules. However, the results presented in Figure 7 show that when assayed at 200 μM Ca^{2+} in the presence of 150 mM potassium glutamate, AIIm displayed significant aggregation of chromaffin granules, which is consistent with the observation that AIIm can reconstitute exocytosis in permeabilized cells (Ali *et al.*, 1989).

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