Articles

Chromaffin Granules Release Calcium on Contact with Annexin VI: Implications for Exocytosis†

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ABSTRACT: Chromaffin granules represent a substantial and exchangeable intracellular calcium pool which is thought to be regulated by a sodium/calcium exchange protein and also by a putative inositol trisphosphate-activated calcium channel. A family of calcium-binding proteins, called the annexins, has been shown to bind to chromaffin granules. We have therefore investigated the possible involvement of these proteins in the regulation of chromaffin granule sequestered calcium. Annexin VI (A-VI) produced a concentration-dependent release of $^{45}Ca^{2+}$ from chromaffin granules; half-maximal release occurred at 1 μM A-VI, with near-maximum release being at 20 μM A-VI. The A-VI-induced release of $^{45}Ca^{2+}$ was rapid, being essentially complete by our first time point of 7 s, and corresponded to 40% of the total sequestered $^{45}Ca^{2+}$. A-VI-induced release occurred at extravesicular Ca^{2+} concentrations ranging from a pCa^{2+} of 4.12 to 6.86 and also appeared specific to this protein since neither annexin I nor annexin II (tetramer) could evoke any $^{45}Ca^{2+}$ release. Given the predominant localization of A-VI to the apical plasmalemma, these results suggest that this protein could participate in the secretory event by mediating the localized release of Ca^{2+} at sites of contact between the chromaffin granule and plasma membrane.

Chromaffin cells of the adrenal medulla have been extensively used for studying stimulus–secretion coupling *in vivo* (Phillips & Pryde, 1987), and isolated secretory granules from these cells have been used widely used for studying membrane–membrane interactions *in vitro* (Drust & Creutz, 1988). Further, because of the dependence of most cells on an elevation of intracellular calcium concentration for secretion [e.g., see Zimmerman (1990)], these studies have primarily focused on calcium-dependent events which seemingly couple the calcium signal to the exocytotic event. In this context, there are two features of adrenal medullary secretory granules which have been independently described, but not fully exploited, in terms of developing an understanding of the role of chromaffin granules in stimulus–secretion coupling. The ability of chromaffin granules to sequester (Kostron et al., 1977) and also release calcium (Yoo & Albanesi, 1990a) has presented the possibility that the secretory granules might contribute to the Ca^{2+} signal necessary for secretion. This potential contribution of intragranular Ca^{2+} to the Ca^{2+} signal for stimulus–secretion coupling could indeed be very substantial, especially when the following properties of chromaffin granules are considered.

The 25 000 secretory granules *per* medullary cell typically occupy about 10% of the cell volume (Nordmann, 1984), possess a membrane Na^+/Ca^{2+} exchanger (Krieger-Brauer & Gratzl, 1982; Jones et al., 1994), and contain a high-capacity calcium-binding protein within their lumen (Winkler et al., 1986). These characteristics taken together allow chromaffin granules to accumulate up to 30 mM Ca^{2+} (Phillips & Allison, 1977) with granule matrix-free [Ca^{2+}] being in the range of 4–24 μM (Bulenda & Gratzl, 1985). It was originally thought that the purpose of this sequestration was to enable cellular Ca^{2+} to be eliminated from the cell during exocytosis (von Grafstein & Powis, 1989). Although recent studies described inositol trisphosphate (IP_3)-induced release of Ca^{2+} from chromaffin granules (Yoo & Albanesi, 1990a; Yoo, 1991), it is unlikely that this mechanism plays a major role in stimulus–secretion coupling in these cells, because muscarinic agents which stimulate IP_3 production result in minor activation of secretion (O'Sullivan et al., 1989; Cheek et al., 1989, 1990). Conversely, as discussed below, certain annexin proteins have been shown to be involved in secretion (Ali et al., 1989), but their mechanisms of action have been modeled in terms of their Ca^{2+}-dependent ability to bind to and aggregate secretory granules to the plasma membrane.

The annexins (Klee, 1988) are Ca^{2+}-dependent, phospholipid-binding proteins, two of which, the tetrameric form of annexin II and also annexin VI, have been predominantly localized to the apical surface of the plasmalemma (Drust & Creutz, 1991). Aside from this localization implying a role for these proteins in secretion, both monomeric and tetrameric annexins II have been shown to reconstitute secretion in permeabilized adrenal medullary cells (Ali et al., 1989), but annexin VI (A-VI)[1] seemingly does not (Ali & Burgoyne, 1990).

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[1] Abbreviations: A-I, annexin I (lipocortin I, calpactin II, chromobindin 6, p35); A-IIt, annexin II tetramer (L-85, p86); A-V, annexin V (endonexin II, lipocortin V); A-VI, annexin VI (p68, 67k calelectrin, lipocortin VI); A-VII, annexin VII (synexin); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,-*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; buffer A, 300 mM sucrose, 25 mM Hepes (pH 7.5), and 5 mM EGTA; buffer B, 300 mM sucrose, 25 mM Hepes (pH 7.5), and 1 mM EGTA; buffer C, 300 mM sucrose/25 mM Hepes (pH 7.5); buffer D, 300 mM sucrose, 25 mM Hepes (pH 7.5), 0.5 mM EGTA, and 0.65 mM $CaCl_2$; buffer E, 300 mM sucrose, 25 mM Hepes (pH 7.5), 0.25 mM EGTA, and 0.325 mM $CaCl_2$.

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The inability of A-VI to reconstitute secretion is rather surprising, since this protein has been demonstrated to be ubiquitously distributed throughout many secretory tissue types (Kaetzel et al., 1990), and in ameloblasts and odontoblasts has been detected exclusively at the plasma membrane undercoat of specialized secretory regions (Goldberg et al., 1991). Additionally, one of the fundamental properties of the annexins is their ability to bind to chromaffin secretory granules *in vitro* (Zaks & Creutz, 1990), and furthermore, annexins I (Pollard et al., 1992), V (Rojas et al., 1990), VI (Pollard et al., 1992), and VII (Pollard & Rojas, 1988) have been reported to produce ion channel activity in artificial lipid bilayers. The annexin localization studies, when considered with the observation of ion channel activity associated with annexins I, V, VI, and VII, led us to ask if the annexin proteins might also play a role in chromaffin granule Ca^{2+} homeostasis. As a part of our recent study of the Na^+-dependent Ca^{2+} uptake and release pathway of chromaffin granules, we examined the ability of annexin proteins I–VI inclusive to inhibit the uptake of Ca^{2+} by these granules (Jones et al., 1994). We found that of those annexins examined, only A-V and A-VI were able to reduce the amount of Ca^{2+} sequestered by chromaffin granules. However, if these two proteins formed Ca^{2+} channels in the chromaffin granule membrane, or inhibited the Na^+/Ca^{2+} exchange protein, inhibition of uptake would be expected. Given the previously mentioned association between A-VI and the specialized sites of secretion (Goldberg et al., 1991), we concentrated on examining the possibility that A-VI might display the ability to release previously sequestered Ca^{2+} from chromaffin granules. We report here that A-VI caused a rapid and substantial release of stored $^{45}Ca^{2+}$ from these secretory vesicles and speculate that this Ca^{2+} release observed *in vitro* may represent an *in vivo* process which could contribute to the intracellular Ca^{2+} signal for stimulus–secretion coupling.

EXPERIMENTAL PROCEDURES

Chromaffin Granule Purification. Chromaffin granules were purified according to Morris and Schovanka (1977) with modification. Bovine adrenal glands were obtained from a local slaughterhouse and placed on ice within 5 min of animal death. All isolation procedures were performed at 4 °C. Adrenal medullas were minced and homogenized in buffer A [300 mM sucrose/25 mM Hepes (pH 7.5) containing 5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] and centrifuged at 1500*g* for 10 min, and the supernatant was centrifuged for 40 min at 10000*g*. The upper layer of the pellet was washed away with buffer B [300 mM sucrose, 25 mM Hepes (pH 7.5), and 1 mM EGTA], and the resulting pellet was resuspended in buffer B and then top-loaded onto a continuous 300 mosM sucrose/metrizamide density gradient consisting of 100% metrizamide [36 g/100 mL, containing 1 mM EGTA/25 mM Hepes (pH 7.5)] at the bottom and 40% metrizamide (diluted with buffer B) at the top. After centrifugation in a Beckman SW41Ti rotor at 32500*g* for 60 min, the middle band of three was removed and washed twice by centrifugation at 1000*g* for 40 min in buffer B. The pellet was resuspended in the same buffer, and the purified granules (between 4 and 8 mg/mL) were stored at 4 °C until used.

$^{45}Ca^{2+}$ Sequestration by Chromaffin Granules. Purified chromaffin granules (diluted to 2 mg/mL with buffer B) were loaded with $^{45}Ca^{2+}$ by incubation in a final reaction mixture of 300 mM sucrose, 25 mM Hepes (pH 7.5), and 0.5 mM EGTA at 37 °C for 45 min. The reaction was initiated by addition of 0.65 mM $CaCl_2$ containing $^{45}Ca^{2+}$ (10 cpm/pmol). The Ca^{2+} sequestration buffer, buffer D, therefore contained a final $CaCl_2$/EGTA ratio of 0.65/0.50 ($[Ca^{2+}]_f$ of 150 μM). All incubations in buffer D, unless otherwise stated, were at 37 °C, and in all cases, the reaction was terminated by filtration (nitrocellulose, pore size 0.45 μm; Metricel, Gelman Sciences). The filters were washed with 10 mL of buffer A at 4 °C and subjected to liquid scintillation spectrometry. The values obtained by this procedure represent the chromaffin granule-sequestered Ca^{2+}.

$^{45}Ca^{2+}$ Release from Chromaffin Granules. Chromaffin granules were loaded with $^{45}Ca^{2+}$ in buffer D [containing 300 mM sucrose, 25 mM Hepes (pH 7.5), 0.5 mM EGTA, and 0.65 mM $CaCl_2$] as detailed above. To determine the effects of various proteins on $^{45}Ca^{2+}$ release, 100 μL of the incubation mixture (for each sample point) was diluted with an equal volume of buffer C [300 mM sucrose/25 mM Hepes (pH 7.5)], which was used as the control value representing the total granule-sequestered Ca^{2+}, or an equal volume of buffer C containing various amounts of annexin proteins (dialyzed against buffer C) and incubated at 37 or 4 °C as specified. Therefore, the final Ca^{2+}/EGTA ratio in the Ca^{2+} release buffer, buffer E, was 0.325 mM $CaCl_2$/0.25 mM EGTA (i.e., $[Ca^{2+}]_f$ = 75 μM). $^{45}Ca^{2+}$ remaining in granules was determined by nitrocellulose filtration with 10 mL of buffer A at 4 °C. $^{45}Ca^{2+}$ release from chromaffin granules was calculated according to the equation: % Ca^{2+} released = [1 − ($[^{45}Ca^{2+}]$ retained in granules in the presence of test substance)/($[^{45}Ca^{2+}]$ retained in granules)] × 100.

A-VI-Induced Release of $^{45}Ca^{2+}$ into Various Extravesicular Ca^{2+} Concentrations. In order to determine the Ca^{2+} dependence of A-VI-dependent Ca^{2+} release from Ca^{2+}-loaded chromaffin granules, granules were allowed to sequester $^{45}Ca^{2+}$ by incubation in buffer D [containing 300 mM sucrose, 25 mM Hepes (pH 7.5), 0.5 mM EGTA, and 0.65 mM $CaCl_2$] at 37 °C for 45 min, as detailed above. Aliquots were then diluted in an equal volume of buffer C containing either no A-VI (control for determination of total granule-sequestered Ca^{2+}) or 20 μM A-VI and appropriate amounts of $CaCl_2$ or EGTA (in buffer C) to yield the desired Ca^{2+} concentration. After a 10-min incubation, aliquots were filtered on nitrocellulose membranes and washed with 10 mL of buffer A at 4 °C. Alternatively, the Ca^{2+} concentration of the wash buffer was adjusted to match that of the incubation buffer such that granules were incubated with A-VI and washed on the nitrocellulose filters at the same Ca^{2+} concentration. Parallel control experiments were performed in which $^{45}Ca^{2+}$ release from granules was initiated by the addition of buffer C instead of A-VI. The Ca^{2+} concentration of the incubation buffer was determined by substitution centrifugation of the granules (15600*g* for 2 min in an Eppendorf 5414) for filtration of the granules. The Ca^{2+} concentration of the supernatant obtained from the centrifugation was estimated by the method of Fabiato and Fabiato (1979) and verified by Ca^{2+} electrode and FURA-2 measurement, following the procedure of Grynkiewaiz et al. (1985). The Ca^{2+} released from chromaffin granules was determined from a comparison of the $^{45}Ca^{2+}$ retained in the granules in the presence and absence of A-VI as detailed above.

Na^+-Dependent Release of $^{45}Ca^{2+}$ from Chromaffin Granules. Chromaffin granules were loaded by incubation at 37 °C for 45 min in buffer D (as described above). An equal volume of a buffer containing 300 mM NaCl/25 mM Hepes (pH 7.5) (giving a final NaCl concentration of 150 mM NaCl) or an equal volume of 529 mM sucrose/25 mM Hepes (pH 7.5) (the osmotic control) was added at the start of a 10-min

incubation at 37 °C. The reaction was then terminated by filtration in buffer A, as above.

Determination of Catecholamine Content of Isolated Chromaffin Granules. Purified chromaffin granules were incubated in buffer D for 45 min at 37 °C. The incubation mixture was diluted with an equal volume of buffer C [300 mM sucrose/25 mM Hepes (pH 7.5)] or an equal volume of buffer C containing 20 μM A-VI (dialyzed against buffer C) and incubated at 37 °C for 10 min. Aliquots were removed and centrifuged (15600*g* for 2 min in an Eppendorf 5414), and the pellet content of catecholamines was determined by the trihydroxyindole spectrophotometric assay (von Euler & Lishajko, 1961) with modifications (Sharman, 1971). Samples were read at 412-nm excitation, 523-nm emission on a Perkin Elmer 650-10S fluorescence spectrophotometer.

Determination of Na^+ by Flame Emission Spectrophotometry. Extravesicular Na^+ was determined by flame emission spectrophotometry (Haigh et al., 1989) using a Phillips Pye Unicam SP9.

Ca^{2+} Dependence of A-VI Binding to Isolated Chromaffin Granules. The Ca^{2+} dependence of A-VI binding to isolated chromaffin granules was determined in parallel experiments to those which examined the dependence of A-VI-induced $^{45}Ca^{2+}$ release on extravesicular Ca^{2+}. Chromaffin granules were incubated in buffer D for 45 min at 37 °C, and then aliquots were diluted in an equal volume of buffer C containing 20 μM A-VI and appropriate amounts of $CaCl_2$ or EGTA to yield the desired incubation buffer Ca^{2+} concentration. The incubation was continued for 10 min at 37 °C. Aliquots were removed and centrifuged (15600*g* for 2 min 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 A-VI protein bands thus obtained were extracted overnight by pyridine extraction (25%) as *per* the method of Fenner et al. (1975) and then quantified spectrophotometrically by measuring the absorbance at 605 nm.

Dose Dependence of A-VI Binding to Isolated Chromaffin Granules. The binding of A-VI to chromaffin granules was examined under identical conditions to those for the A-VI-dependent Ca^{2+} release experiments except that to terminate the experiment, granules were not filtered but centrifuged. Essentially, chromaffin granules were incubated in buffer D for 45 min at 37 °C, and aliquots were removed and then adjusted with an equal volume of buffer C containing various concentrations of A-VI. The incubation was continued at 37 °C for 10 min, and aliquots were removed and centrifuged (15600*g* for 2 min 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 those A-VI protein bands obtained were extracted overnight by the pyridine method (25% pyridine) as *per* Fenner et al. (1975) and then quantified spectrophotometrically by measuring the absorbance at 605 nm.

Preparation of Proteins. Annexin I, A-II, and A-VI were prepared from bovine lung (Khanna et al., 1990) and were stored in 50 mM KCl at −70 °C. Proteins were essentially homogeneous as determined by SDS–PAGE. Prior to use, proteins were thawed and equilibrated by chromatography on a PD 10 column (Pharmacia) with buffer C. Where stated, A-VI was denatured by incubation at 95 °C for 10 min.

Miscellaneous Techniques. Protein concentrations were determined according to Bradford (1976), using BSA as a standard.

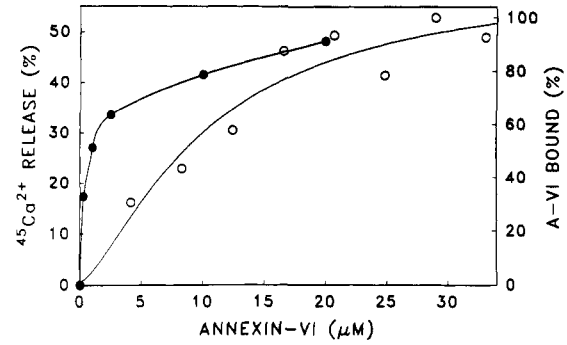

FIGURE 1: Annexin VI-induced $^{45}Ca^{2+}$ release and annexin VI binding to chromaffin granules. Chromaffin granules were $^{45}Ca^{2+}$-loaded by incubation in a buffer containing 300 mM sucrose, 25 mM Hepes (pH 7.5), 0.5 mM EGTA, and 0.65 mM $CaCl_2$ containing $^{45}Ca^{2+}$ (10 cpm/pmol) at 37 °C for 45 min. In order to examine A-VI-dependent release of Ca^{2+}, the mixture was then diluted with an equal volume of buffer C containing 300 mM sucrose/25 mM Hepes (pH 7.5) or buffer C containing various concentrations of A-VI and then incubated at 37 °C for 10 min. Aliquots were removed, and either the granule content of $^{45}Ca^{2+}$ was determined by filtration with 10 mL of 300 mM sucrose, 25 mM Hepes (pH 7.5), and 5 mM EGTA (Experimental Procedures) (●), or the binding of A-VI to chromaffin granules (○) was determined by centrifugation (2 min at 15600*g* in an Eppendorf 5414) and resuspension of the pellet in SDS disruption buffer. Subsequent to SDS–PAGE, protein bands were visualized with Coomassie Brilliant Blue (R-250), and A-VI protein bands thus obtained were cut from the gel and extracted overnight by pyridine extraction (25%) as *per* the method of Fenner et al. (1975) and quantified spectrophotometrically by measuring the absorbance at 605 nm. Binding is expressed as percent of maximum bound.

Materials. All chemicals were of analytical grade: potassium ferricyanide from Aldrich; BSA, Hepes, and EGTA (acid form) from Sigma Chemical Co.; sucrose from BDH Chemicals; metrizamide from Accurate Chemicals and Scientific Corp.; $^{45}Ca^{2+}$ from Amersham; Metricel filters (0.45 μM) from Gelman Sciences; PD 10 column from Pharmacia, Bovine adrenal glands were obtained from XL Beef Co., Calgary, Alberta. Coomassie blue dye (Brilliant Blue R-250), bis-(acrylamide), and SDS were from Calbiochem Corp. All solutions were made in Milli-Q deionized water (Millipore).

RESULTS

Concentration Dependence of A-VI-Induced $^{45}Ca^{2+}$ Release from Chromaffin Granules. The previously observed A-VI-dependent inhibition of Ca^{2+} uptake by chromaffin granules (Jones et al., 1994) suggested that A-VI might either block the Na^+/Ca^{2+} exchange protein of the granules or alternatively promote the release of Ca^{2+} from granules concurrently with uptake. It has been reported (Pollard et al., 1992) that A-VI can form Ca^{2+} channels in artifical phospholipid membranes; however, the ability of A-VI to do so in biological membranes has not been tested. In order to investigate the possibility that A-VI promoted release of Ca^{2+} from the granules, the granules were $^{45}Ca^{2+}$-loaded and then incubated with A-VI, and the $^{45}Ca^{2+}$ content of the granules was measured (see Experimental Procedures). A-VI produced a concentration-dependent decrease in chromaffin granule-sequestered $^{45}Ca^{2+}$ (Figure 1). Half-maximal decrease in granule-sequestered $^{45}Ca^{2+}$ occurred at a total A-VI concentration of 1 μM A-VI, with a near-maximal decrease (45% of total sequestered $^{45}Ca^{2+}$) being seen at 20 μM total A-VI. These results therefore suggest that A-VI promoted the release of $^{45}Ca^{2+}$ from the $^{45}Ca^{2+}$-loaded granules.

In parallel experiments, the amount of A-VI associated with chromaffin granules during Ca^{2+} release was also determined (Figure 1). Half-maximal binding occurred at a

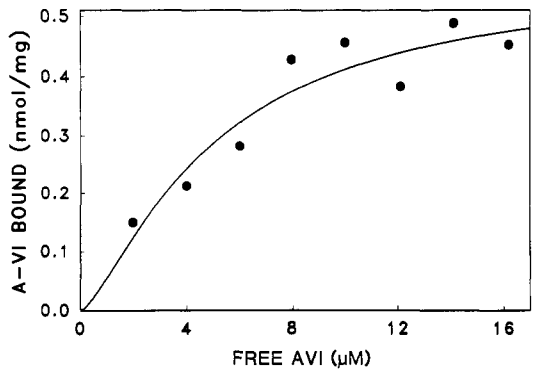

FIGURE 2: Isotherm of annexin VI binding to purified chromaffin granules. Binding of A-VI to chromaffin granules was determined by incubating granules at 37 °C in 300 mM sucrose, 25 mM Hepes (pH 7.5), 0.5 mM EGTA, and 0.65 mM $CaCl_2$ for 45 min. Aliquots were removed and adjusted with equivalent volumes of buffer C containing various concentrations of A-VI. The incubation was continued at 37 °C for 10 min, and aliquots were removed and centrifuged (15600*g* for 2 min 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 A-VI protein bands obtained were cut from the gel and extracted overnight by pyridine extraction (25%) as *per* the method of Fenner et al. (1975) and quantified by measuring the absorbance at 605 nm. Data were analyzed according to the equation $y = m/[1 + (k/x)^n]$ where m = maximal binding, k = annexin VI concentration at half-maximal binding, n = Hill coefficient, y = bound annexin VI concentration, and x = free annexin VI concentration. Values determined from the curve fit are $m = 0.563 \pm 0.16$, $k = 4.86 \pm 2.3$, and $n = 1.40 \pm 0.7$.

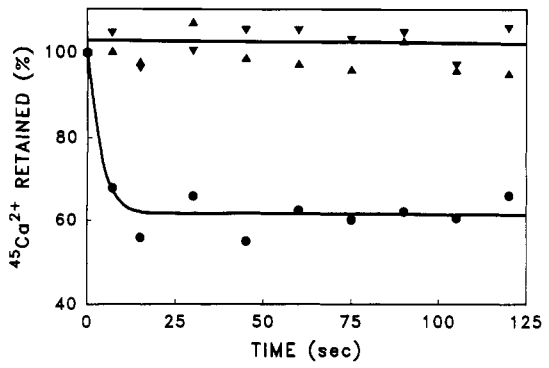

FIGURE 3: Time course of annexin VI-induced $^{45}Ca^{2+}$ release from isolated chromaffin granules. Chromaffin granules were loaded with $^{45}Ca^{2+}$ as described in the Figure 1 legend and adjusted with an equal volume of buffer C [300 mM sucrose/25 mM Hepes (pH 7.5)] (▲) or buffer C containing either 20 μM A-VI (●) or 20 μM heat-denatured A-VI (▼). Aliquots were removed at the times stated and filtered with 10 mL of buffer containing 300 mM sucrose, 25 mM Hepes (pH 7.5), and 5 mM EGTA (Experimental Procedures). A-VI was denatured by incubation at 95 °C for 10 min. The results are representative of five independent experiments.

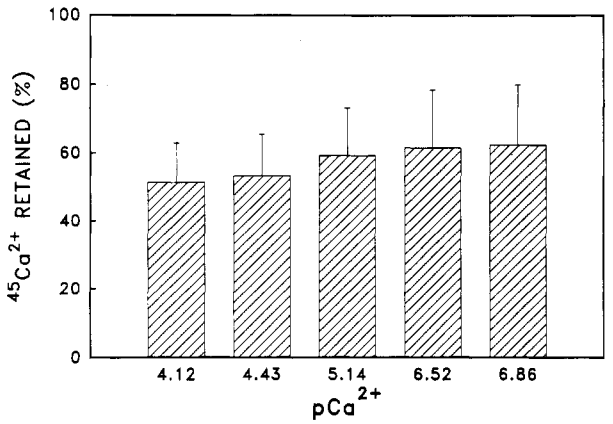

FIGURE 4: Effect of extravesicular $[Ca^{2+}]$ on annexin VI-induced release of sequestered $^{45}Ca^{2+}$ from isolated chromaffin granules. Purified chromaffin granules which had been loaded with $^{45}Ca^{2+}$ (as *per* Figure 1 legend) were adjusted with equivalent volumes of 300 mM sucrose/25 mM Hepes (pH 7.5) containing 20 μM A-VI, 0.325 mM $CaCl_2$, 0.250 mM EGTA, and various concentrations of EGTA to give the final Ca^{2+} concentration shown. $^{45}Ca^{2+}$ retained in the granules was determined as *per* Experimental Procedures.

total concentration of 9 μM A-VI whereas the corresponding half-maximal point of Ca^{2+} release was 1 μM A-VI, demonstrating that maximal release of Ca^{2+} occurs well before saturation of the A-VI-binding sites on the chromaffin granules. These data raise the possibility that A-VI initiates release of Ca^{2+} either by binding to and activating existing release mechanisms or by the formation of *de novo* ion channels by A-VI in the chromaffin granule membrane. Furthermore, it may be that higher concentrations of A-VI do not cause release of Ca^{2+} in excess of 40% of sequestered $^{45}Ca^{2+}$ because all of the $^{45}Ca^{2+}$ sequestred by the chromaffin granules may not be exchangeable.

We also examined the affinity and stoichiometry of binding A-VI to chromaffin granules. These experiments were performed at isoosmotic conditions to avoid lysis of the granules, and the binding isotherm is presented in Figure 2. Analysis of the binding curve by nonlinear regression suggests that half-maximal binding occurred at a free A-VI concentration of 5 μM, with binding essentially saturated at 12 μM A-VI_f. The number of binding sites on the chromaffin granules was determined to be about 563 pmol of A-VI *per* milligram of granules. Assuming that 1 mg of granule protein is equivalent to 4×10^{12} granules (Creutz et al., 1978), this corresponds to about 40 molecules of A-VI per granule at half-maximal binding.

Time Course of A-VI-Induced Release. In order to gain some insight into the possible mechanisms of release of Ca^{2+} from chromaffin granules by A-VI, we examined the time course of that release. Saturating A-VI (20 μM) elicited a 40% decrease in granule-sequestered $^{45}Ca^{2+}$ within our first time point of 7 s (Figure 3). The kinetics of this release were very similar to glutathione-induced Ca^{2+} release from bovine pituitary granules, i.e., occurring within 7 s (Lorensen et al., 1990), but appreciably faster than IP_3-induced release of Ca^{2+} from chromaffin granules, which takes greater than 2 min to reach its maximum (Yoo, 1991). Additionally, the A-VI-mediated release appeared capable of overwhelming any Ca^{2+} reuptake process within the 2-min duration of the experiment, and when extended to 5 min (data not shown), there was still no indication of Ca^{2+} reuptake. Conversely, neither heat-treated A-VI nor its carrier buffer was capable of eliciting any observable release of sequestered Ca^{2+}.

Dependence of A-VI-Induced $^{45}Ca^{2+}$ Release on Extravesicular $[Ca^{2+}]$. Inevitably, if the documented release of $^{45}Ca^{2+}$ from chromaffin granules were to have any physiological relevance, the binding of A-VI to chromaffin granules, and also the A-VI-induced of release of $^{45}Ca^{2+}$ from them, should be observed at physiological extravesicular Ca^{2+} concentrations. The A-VI-mediated release of $^{45}Ca^{2+}$ from granules occurred at extravesicular Ca^{2+} concentrations from pCa^{2+} 4.12 to 6.86 (Figure 4), suggesting that relese could indeed occur under physiological (i.e., cytosolic) Ca^{2+} concentrations. Given that the A-VI-mediated release of Ca^{2+} from chromaffin granules occurred within this range of extravesicular pCa^{2+}, we considered it necessary to investigate the ability of that protein to bind to granules within and also outside of this range of pCa^{2+}. A-VI displayed essentially maximal binding between pCa^{2+} 4.12 and 6.86 (data not

Table 1: Temperature Dependence of Calcium Release[a]

temp (°C)	$^{45}Ca^{2+}$ release (%)
37	37.5 ± 8.0
4	40.2 ± 7.3

[a] Purified chromaffin granules (see Experimental Procedures) were loaded with $^{45}Ca^{2+}$ by incubation in a final reaction mixture of 300 mM sucrose, 25 mM Hepes (pH 7.5), and 0.5 mM EGTA at 37 °C for 45 min. The reaction was initiated by addition of 0.65 mM $CaCl_2$ containing $^{45}Ca^{2+}$ (10 cpm/pmol). The reaction mixture was subsequently incubated in the presence or absence of 20 μM A-VI at either 37 or 4 °C for 10 min and the granule content of $^{45}Ca^{2+}$ determined by filtration (see Experimental Procedures). Results are expressed as mean ± SD (n = 4).

shown). Since $[Ca^{2+}]_f$ values (obtained by mixing Ca^{2+} and EGTA) below 6.86 could not be accurately verified, binding and release analyses were not conducted below this value. However, in the presence of 5 mM EGTA ($pCa^{2+} > 8.0$), only 18% of A-VI bound to granules, and there was no concomitant release of $^{45}Ca^{2+}$ from granules (data not shown).

Temperature Dependence of $^{45}Ca^{2+}$ Release from Chromaffin Granules. The rapidity of the $^{45}Ca^{2+}$ release (Figure 3) suggests that the release is unlikely to be occurring *via* the Na^+/Ca^{2+} exchanger. To examine the possibility of Ca^{2+} efflux *via* this route, we investigated the temperature dependence of the release, as it very well established that Na^+/Ca^{2+} exchange proteins operate maximally within a very limited temperature range (e.g., Bersohn et al., 1991). Whereas the uptake of Ca^{2+} into chromaffin granules displays a clear temperature dependence between 4 and 37 °C (Jones et al., 1994), in contrast the A-VI-induced release of Ca^{2+} from them is temperature-independent within the same range of temperatures (Table 1). Granues which were incubated in the presence of saturating (20 μM) A-VI at 4 and 37 °C showed no statistical difference in their release of Ca^{2+}, when compared against paired controls at the corresponding temperature (37.5 ± 8.0% at 37 °C as opposed to 40.2 ± 7.3% at 4 °C).

Catecholamine Content of A-VI-Treated Chromaffin Granules. It is conceivable that the observed A-VI-induced release of Ca^{2+} from chromaffin granules could result from lysis of the chromaffin granule membrane, rather than by activation of a specific Ca^{2+} release mechanism. In order to allow for this possibility, we assayed the catecholamine content of chromaffin granules which had been treated with A-VI or identical volumes of buffer C using two different experimental paradigms. By the use of centrifugation, we found that A-VI treatment did not induce any significant release of catecholamines (Table 2). Chromaffin granule pellets which had been incubated with saturating (20 μM) A-VI contained 2.53 ± 0.40 μmol of catecholamines/mg of granule protein (mean ± SD, n = 5), as compared to control pellets which had been incubated with an equivalent volume of buffer C, and contained 2.29 ± 0.15 μmol of catecholamines/mg of granule protein (mean ± SD, n = 5). Similarly, using an identical filtration technique to that used for the determination of $^{45}Ca^{2+}$ release from chromaffin granules (see Experimental Procedures), we observed 8.0 ± 1.0% (mean ± SD) of catecholamines released from granules incubted with buffer C as opposed to 7.7 ± 0.8% (mean ± SD) from those incubated with saturating (20 μM) A-VI.

Effect of Various Ca^{2+} Wash Conditions on $^{45}Ca^{2+}$ Levels in Chromaffin Granules. Experiments designed to test the extravesicular Ca^{2+} dependency of $^{45}Ca^{2+}$ release from chromaffin granules has been performed by the incubation of $^{45}Ca^{2+}$–loaded chromaffin granules at various extravesicular

Table 2: A-VI-Dependent Release of Catecholamines from Chromaffin Granules

conditions	chromaffin granule catecholamine content (μmol/mg)
1 (centrifugation)[a]	2.29 ± 0.1
2 (filtration)[b]	2.53 ● 0.40

[a] Purified chromaffin granules were incubated at 37 °C in buffer containing 300 mM sucrose, 25 mM Hepes (pH 7.5), 0.5 mM EGTA, and 0.65 mM $CaCl_2$ for 45 min. Subsequently, the reaction mixture was incubated in the presence or absence of 20 μM A-VI for 10 minutes at 37 °C, and the granule content of catecholamines was determined spectrophotometrically (see Experimental Procedures). [b] Purified chromaffin granules were incubated at 37 °C in buffer containing 300 mM sucrose, 25 mM Hepes (pH 7.5), 0.5 mM EGTA, and 0.65 mM $CaCl_2$ for 45 min. Subsequently, the reaction mixture was incubated in the presence or absence of 20 μM A-VI for 10 min at 37 °C, the granules were filtered, and the content of the filtrate was determined spectrophotometrically (see Experimental Procedures).

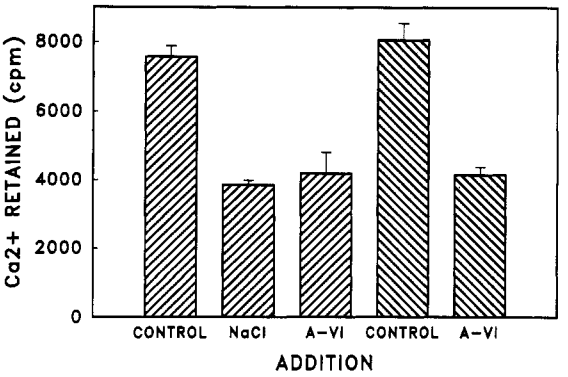

FIGURE 5: Comparison of wash conditions on $^{45}Ca^{2+}$ release from isolated chromaffin granules by annexin VI. Chromaffin granules were loaded with $^{45}Ca^{2+}$ as described in the Figure 1 legend and then incubated with equivalent volumes of 529 mM sucrose/25 mM Hepes (pH 7.5) (*CONTROL*, hatch left to right, up), 300 mM sucrose/25 mM Hepes (pH 7.5) (*CONTROL*, hatch left to right, down), 300 mM sucrose/25 mM Hepes (pH 7.5) containing 20 μM A-VI (*A-VI*), or 300 mM NaCl/25 mM Hepes (pH 7.5) (*NaCl*). After a 10-min incubation at 37 °C, $[^{45}Ca^{2+}]$ remaining in the granules was determined by filtration (see Experimental Procedures), hatched bars (left to right, up) represent samples washed with 300 mM sucrose, 25 mM Hepes (pH 7.5), and 5 mM EGTA, and hatched bars (left to right, down) represent samples washed with 300 mM sucrose/25 mM Hepes (pH 7.5) containing 0.325 mM $CaCl_2$ and 0.250 mM EGTA. Data shown are mean ± SD of three independent experiments.

Ca^{2+} concentrations, followed by addition of A-VI to initiate release and subsequent washing of these granules on nitrocellulose filters with buffer A (which contains 5 mM EGTA). The chromaffin granule $^{45}Ca^{2+}$ content was then determined by liquid scintillation spectrometry of the washed nitrocellulose filters. These experiments inherently assumed that washing granules with buffer A would not influence the A-VI-dependent release of $^{45}Ca^{2+}$ from the granules. In order to test the effect of the washing conditions of chromaffin granules on A-VI-dependent $^{45}Ca^{2+}$ release directly, we modified the experimental procedure so that chromaffin granules were incubated in, and washed with, identical Ca^{2+} concentrations. As shown in Figure 5, when chromaffin granules were incubated with A-VI in a buffer containing 0.325 mM $CaCl_2$ and 0.250 mM EGTA, followed by washing and filtration of the reaction mixture at this same $CaCl_2$/EGTA ratio, $^{45}Ca^{2+}$ release from the chromaffin granules was seen to occur to the same extent as when granules were incubated with A-VI in a buffer containing 0.325 mM $CaCl_2$ and 0.250 mM EGTA and subsequently washed with buffer A. Therefore, the Ca^{2+}

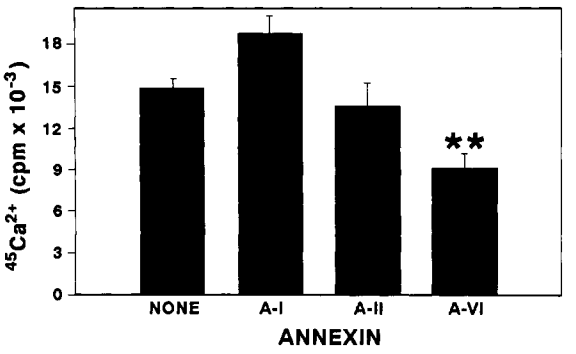

FIGURE 6: Comparison of $^{45}Ca^{2+}$ release from isolated chromaffin granules by annexin VI and other annexing proteins. Chromaffin granules were loaded with $^{45}Ca^{2+}$ as described in the Figure 1 legend. Various annexins were tested for $^{45}Ca^{2+}$ release activity by incubating the specified proteins at 20 μM for 10 min at 37 °C with $^{45}Ca^{2+}$-loaded chromaffin granules as detailed in the Figure 2 legend. The release reaction was terminated by filtration with buffer A. Granule-sequestered $^{45}Ca^{2+}$ was determined as indicated under Experimental Procedures. None = control-incubated granules; A-I, annexin I; A-II, annexin II tetramer; A-VI, annexin VI. Data are shown as mean ± SD of four independent experiments. Two asterisks, $p < 0.01$.

content of the wash conditions does not appear to artifically induce A-VI-dependent $^{45}Ca^{2+}$ release during the wash step. Furthermore, when the Na^+-dependent release of Ca^{2+} was examined (Figure 5), it was seen that there was no statistical difference between $^{45}Ca^{2+}$ retained by granules in this condition when compared to those incubated with A-VI. On the basis of the assumption that a strong extravesicular to intravesicular NaCl gradient would completely deplete the exchangeable intravesicular Ca^{2+} pool, *via* activation of the Na^+/Ca^{2+} exchanger, these data suggest that the incubation of chromaffin granules with A-VI leads to a similar depletion of the exchangeable internal Ca^{2+} pool of the granules. This also suggests that the Na^+/Ca^{2+} exchanger and A-VI access a similar (exchangeable) pool of intragranular Ca^{2+}.

Specificity of A-VI-Mediated $^{45}Ca^{2+}$ Release. Given that the annexin proteins share some common structural homology (Klee, 1988), and as a result of these similarities might possess a common ability to release sequestered Ca^{2+} from chromaffin granules, the effects of two other annexins, A-I and A-IIt, were examined in this context. Neither of these annexins at 20 μM produced any effect on the release of sequestered $^{45}Ca^{2+}$ from granules, whereas A-VI at the same concentration reduced the level of acquired $^{45}Ca^{2+}$ to 60% of the control value (Figure 6). This therefore suggests that the ability of A-VI to release $^{45}Ca^{2+}$ from chromaffin granules is not a property shared by all of the annexins.

DISCUSSION

Our results clearly demonstrate that the $^{45}Ca^{2+}$ content of purified chromaffin granules is decreased by incubation with A-VI. Similarly, we have found that A-V also decreases the $^{45}Ca^{2+}$ content of purified chromaffin granules (data not shown). On the basis of current knowledge of the mechanisms of ion translocation across membranes in other systems, this release might therefore be mediated either by an A-VI-activated, ATP-dependent process, the A-VI activation of the chromaffin granule Na^+/Ca^{2+} exchanger, or activation by A-VI of an existing ion channel, or by the formation of a novel ion channel by A-VI, in the chromaffin granule membrane. It is unlikely that an A-VI-activated, ATP-dependent Ca^{2+} extrusion mechanism is present in the membrane of chromaffin granules because it has already been reported that chromaffin granules do not display any immunoreactivity against the Ca^{2+}/ATPase present in the adrenal medulla endoplasmic reticulum membrane (Burgoyne et al., 1989). Similarly, we have also been unable to demonstrate the presence of a Ca^{2+}/ATPase in chromaffin granule membranes (Jones et al., unpublished observation). Therefore, it appears that the A-VI-mediated release of Ca^{2+} from chromaffin granules of the adrenal medulla is not enzymatic.

Consequently, we considered alternative mechanisms by which A-VI might mediate the relase of Ca^{2+} from chromaffin granules. Since the release of $^{45}Ca^{2+}$ occurred in the absence of extravesicular Na^+, it is unlikely that A-VI acted to reverse the Na^+/Ca^{2+} exchanger. Furthermore, the Na^+/Ca^{2+} exchange rates of chromaffin granules (Jones et al., 1994) and the exchange proteins of other cellular membranes (e.g., Bersohn et al., 1991) have been seen to be substantially inhibited at 4 °C. However, as shown in Table 1, there is no statistical difference between the extent of release of $^{45}Ca^{2+}$ at 4 and 37 °C (i.e., the granule-sequestered $^{45}Ca^{2+}$ had decreased by 40% by 30 s at either temperature, Table 1). This demonstration of the temperature independence of $^{45}Ca^{2+}$ release within the range of 4–37 °C also serves as further evidence against this release being due to the activation of a Ca^{2+}–ATPase. Additionally, our observation that A-VI treatment of chromaffin granules does not cause concomitant release of both catecholamines and Ca^{2+} suggests that A-VI-dependent release of $^{45}Ca^{2+}$ (within the range of pCa^{2+} 4.12–6.86, Figure 4) does not occur by a disruption of granule membrane integrity (Table 2).

Therefore, within the current knowledge of Ca^{2+} release mechanisms, there are two remaining possibilities which could explain the observed A-VI-induced Ca^{2+} release. First, A-VI has been shown to be able to form a *de novo* ion channel in phospholipd membranes (Pollard et al., 1992) and may be doing so in chromaffin granules. Alternatively, A-VI may activate an existing receptor-operated Ca^{2+} channel in the chromaffin granule membrane, as has been shown for the calcium release channel of sarcoplasmic reticulum (Diaz-Munoz et al., 1990). Indeed, there is evidence for a voltage-dependent K^+ channel in *Torpedo* synaptic vesicles (Rahamimoff et al., 1988, 1989), a Ca^{2+}-dependent cation channel in the membrane of rat neurohypophysis secretory granules (Lemos et al., 1989), and also a voltage- and Ca^{2+}-dependent cation channel in the secretory granule membrane of mast cells (Hirashima & Kirino, 1992). It is therefore conceivable, therefore, that a preexisting cation channel in chromaffin granule membranes could be modulated by annexin VI. Further support for our assertion that A-VI activates, or itself forms, an ion channel comes from the observation that this protein is able to inhibit Ca^{2+} sequestration (Jones et al., 1994) in addition to initiating Ca^{2+} release from $^{45}Ca^{2+}$-loaded granules. The formation of Ca^{2+}-permeable ion channels in the granule membrane by A-VI during Ca^{2+} uptake would presumably result in a concurrent loss, from the exchangeable Ca^{2+} pool, of some of the $^{45}Ca^{2+}$ being sequestered *via* the Na^+/Ca^{2+} exchanger. The net effect of this concurrent uptake and release of Ca^{2+} would be an apparent inhibition of uptake. Inevitably, the methodolgoy utilized in this study cannot, by virture of its intrinsic limitations, tease apart the fairly complex Ca^{2+}-dependent events of the Ca^{2+} release process described here, and we are presently conducting other studies which will help elucidate the mechanism (or mechanisms) by which A-VI mediates the release of intravesicular Ca^{2+}.

On the basis of the previously discussed absence of an active Ca^{2+} transport mechanism in chromaffin granule membranes,

it would be reasonable to predict that any movement of Ca^{2+} from intravesicular to extravesicular space would be dependent upon the Ca^{2+} concentration gradient. Our results demonstrate that A-VI mediates the movement of Ca^{2+} across the chromaffin granule membrane at extravesicular Ca^{2+} levels from pCa^{2+} 4.12 to 6.86. This observation therefore suggests that the intravesicular Ca^{2+}, concentration in granules which have been loaded with $^{45}Ca^{2+}$ is higher than pCa^{2+} 4.12 (75 μM Ca^{2+}). On the assumption that under physiological conditions intravesicular $[Ca^{2+}]_f$ is between 4 and 20 μM (Bulenda & Gratzl, 1985) and cytosolic $[Ca^{2+}]_f$ ranges between 0.1 and 1 μM, it is reasonable to propose that A-VI-dependent $^{45}Ca^{2+}$ release from the granules would be favored within a physiological range of $[Ca^{2+}]$. However, if release of Ca^{2+} from granules by A-VI does occur *in vivo*, it would presumably be coupled to cellular stimulation, in that chromaffin granules are entrapped within an actin barrier in the resting cell (Kondo et al., 1982) and therefore are prevented from contacting A-VI which is present in the plasma membrane undercoat. Conversely, when the cell is stimulated to secrete, the actin barrier is reorganized (Cheek & Burgoyne, 1986, 1987; Burgoyne & Cheek, 1987), and secretory granules are free to translocate to the inner surface of the plasma membrane where A-VI is localized at secretory sites (Goldberg et al., 1991) and contact between this protein and chromaffin granules is possible. Thus, A-VI-induced release of Ca^{2+} might only then be triggered.

The Ca^{2+} dependence of A-VI-induced Ca^{2+} release from chromaffin granules was seen to occur within a range of pCa^{2+} 4.12 to pCa^{2+} 6.8 (Figure 4) and was mirrored by our observation that A-VI binding to intact chromaffin granules was maximal within the same range of extravesicular $[Ca^{2+}]$ (data not shown). In contrast, an existing study of the A-VI Ca^{2+} dependence of binding to chromaffin granules showed a $K_d(Ca^{2+})$ of 60 μM (Zaks & Creutz, 1991), although it should be noted that this observation was made on A-VI binding to isolated chromaffin granule membranes. Furthermore, we determined the affinity and stoichiometry of A-VI binding to chromaffin granules, at a Ca^{2+} concentration of 150 μM, to be 5 μM and 563 pmol of A-VI/mg of chromaffin granules (about 110 pmol of A-VI/mg of granule protien), respectively, compared to a half-maximal binding value, determined at 100 μM Ca^{2+}, of 50 nM and a capacity of about 500 pmol of A-VI/mg of chromaffin granules (Zaks & Creutz, 1990). The discrepancy between these existing data and our present observations is not surprising given that the previous study used disrupted chromaffin granule membranes whereas our present study used intact granules (Table 2). The higher concentrations of A-VI used in our study, compared to that reported by Zaks and Creutz (1990), could also cause the apparent shift in Ca^{2+} sensitivity. It is also interesting to note that the total $^{45}Ca^{2+}$ content of granules was not released by their incubation with A-VI. There are three immediately obvious possible explanations for this phenomenon: (i) that the sequestered $^{45}Ca^{2+}$ mixes with already incorporated granule Ca^{2+} and is rendered nonreleasable by its binding to, and precipitation of, intragranular chromogranin (Yoo & Albanesi, 1990b); (ii) that the putative ion channel formed by A-VI may display Ca^{2+}-dependent Ca^{2+} release and therefore may not permit the release of more than the 45% of $^{45}Ca^{2+}$ acquired; (iii) that a physically distinct Ca^{2+} pool, possibly represented by the previously observed intragranular granules (Ornberg et al., 1986), may irreversibly sequester 55% of the $^{45}Ca^{2+}$ sequestered by the chromaffin granule.

The ability of A-VI to bind to chromaffin granules at a wide range of extravesicular pCa^{2+}, when considered with its ability to release Ca^{2+} from intact granules at physiologically relevant extravesicular pCa^{2+} *in vitro*, raises the possibility that this system may be capable of sequestering Ca^{2+} and then releasing that Ca^{2+} upon contact with A-VI *in vivo*. Furthermore, the kinetics of A-VI-induced Ca^{2+} release demonstrated here, when considered with the localization of this protein to specialized secretory sites of the plasmalemma, suggest that should this release occur with similar characteristics *in vivo* it would display appropriate spatial and temporal characteristics to allow its participation in the production of the Ca^{2+} signal prior to exocytosis. The existing model of exocytosis postulates that the Ca^{2+}-dependent binding of secretory granules with the plasma membrane is one of the final stages of secretion and considering our present observations it is interesting to speculate whether this interaction also involves contact between A-VI and secretory granules at the plasmalemma. The formation of a complex between secretory granules, plasma membrane, and A-VI could be a necessary part of the exocytotic process, causing a release of granular Ca^{2+} which augments the transmembrane Ca^{2+} signal and assists in the induction of membrane fusion. We are currently investigating this possibility and also examining whether the A-VI modulation of $^{45}Ca^{2+}$ release is a widely distributed property in endocrine tissues.

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