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Regulation of the Sarcoplasmic Reticulum Ca^{2+}-Release Channel Requires Intact Annexin VI

P. Hazarika, A. Sheldon, M.A. Kaetzel, M. Díaz-Muñoz, S.L. Hamilton, and J.R. Dedman

Department of Physiology and Cell Biology, University of Texas Medical School, Houston, Texas 77225 (P.H., A.S., M.A.K., J.R.D.) and Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas 77030 (M.D.-M., S.L.H.)

Abstract Annexin VI has eight highly conserved repeated domains; all other annexins have four. Díaz-Muñoz et al. (*J Biol Chem* 265:15894, 1990) reported that annexin VI alters the gating properties of the ryanodine-sensitive Ca^{2+}-release channel isolated from sarcoplasmic reticulum. To investigate the domain structure of rat annexin VI (67 kDa calcimedin) required for this channel regulation, various proteolytic digestions were performed. In each case, protease-resistant core polypeptides were produced. Annexin VI was digested with V8 protease and two core polypeptides were purified by Ca^{2+}-dependent phospholipid binding followed by HPLC. The purified fragments were shown to be derived from the N- and C-terminal halves of annexin VI, and demonstrated differential immunoreactivity with monoclonal antibodies to rat annexin VI. While both core polypeptides retained their ability to bind phospholipids in a Ca^{2+}-dependent manner, they did not regulate the sarcoplasmic reticulum Ca^{2+}-release channel as did intact annexin VI.

Key words: Ca^{2+}-dependent, phospholipid-binding, proteolysis, purification, repeats, immunoreactivity

During the course of independent investigations, a family of Ca^{2+}- and phospholipid-binding proteins (annexins) is emerging as a potentially important component in cellular regulation [for review, see 1,2]. Members of this family of Ca^{2+}/phospholipid-binding proteins have been isolated from a variety of tissues, and all have been cloned and sequenced [1,3–6]. These data show that the annexins do not contain EF-hand structures found in the troponin-C/calmodulin/S-100 superfamily or demonstrate sequence similarity with other Ca^{2+}-regulated proteins, such as protein kinase C and synaptophysin. Annexins contain repeat sequences of approximately 70 amino acids in length. The data are consistent with two successive gene duplications of a single repeat-containing precursor leading to the formation of the 4-repeat proteins, which in turn were duplicated to generate annexin VI [7]. It was originally reported by Geisow et al. [8] that each repeat contained a more highly conserved sequence of 17 amino acids which was termed the "endonexin fold" by Kretsinger and Creutz [9]. This fold has been proposed to be involved in Ca^{2+}-dependent phospholipid binding [10]. Marriott et al. [11] have spectroscopic data supporting this hypothesis.

Defining the cellular functions of the annexins has proved difficult. Each member is capable of inhibiting phospholipase A_2 and blood coagulation in vitro, due to its depletion of substrate and co-factors. Functions for specific members of the annexin family have been proposed. Ali and Burgoyne [12] have shown, using permeabilized adrenal chromaffin cells, that annexin II stimulates Ca^{2+}-dependent exocytosis. Ross et al. [13] have suggested annexin III to be an inositol 1,2-cyclic phosphate 2-phosphohydrolase. Annexin VI has been reported by Díaz-Muñoz et al. [14] to dramatically alter the gating properties of the ryanodine-sensitive Ca^{2+}-release channel from sarcoplasmic reticulum. This structurally complex channel is critical in excita-

Abbreviations used: HPLC, high pressure liquid chromatography; FPLC, fast protein liquid chromatography (Pharmacia); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; EDTA, ethylenediamine tetraacetic acid; EGTA, [ethylene bis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; 2-D, two-dimensional; MOPS, 3-(N-morpholino)propanesulfonic acid.

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Address reprint requests to Dr. John R. Dedman, Department of Physiology and Cell Biology, University of Texas Medical School, P.O. Box 20708, Houston, Texas 77030.

tion-contraction coupling; its regulation is under intense investigation. In order to define the specificity of the Ca^{2+}-release channel regulation by annexin VI, proteolytically-produced 37 kDa four-domain core polypeptides were purified and tested in the reconstituted artificial planar lipid bilayer system.

MATERIALS AND METHODS

Materials

Proteases were purchased either from Boehringer Mannheim (Staphylococcus aureus protease V8 and Bacillus thermoproteolyticus thermolysin) or from Sigma Chemical Co. [TPCK-treated bovine pancreas trypsin (type XIII), TLCK-treated bovine pancreas alpha-chymotrypsin (type VII), and Streptomyces griseus pronase E (type XXV)]. $^{45}CaCl_2$ was obtained from ICN Radiochemicals.

Purification of Rat Annexins I–V, and Annexin VI and Its Core Polypeptides

Annexins I–V were purified using methods described in Kaetzel et al. [15]. Annexin VI was purified from rat tissues as described in Mathew et al. [16], with minor modifications. Rat annexin VI was further purified by the method of HPLC[1] using a Pharmacia Mono Q HR5/5 FPLC column with a 0–500 mM NaCl gradient in 10 mM Tris-HCl, 0.5 mM EDTA, pH 7.2. Aliquots from protein-containing fractions were subjected to electrophoresis on SDS polyacrylamide gels [17] to assess purity. The identity of each annexin was confirmed using monospecific antibodies.

Core polypeptides were produced by incubation of annexin VI with either V8 protease or thermolysin, and this mixture was applied to a phenyl-Sepharose column (2 ml bed volume), pre-equilibrated with 20 mM Tris-HCl, 2 mM $CaCl_2$, 0.02% NaN_3, 1M NaCl, pH 7.4 (buffer P). Prior to application of sample, this column was treated with 1 mg/ml brain extract type III phospholipids (phosphatidylserine-enriched; Sigma Chemical Co.) dissolved in buffer P. The column was washed with buffer P to remove unbound protein (i.e., protease), and the core polypeptides were then eluted with buffer P containing 3 mM EGTA instead of Ca^{2+}. The eluted protein was dialyzed against 10 mM Tris-HCl, 0.5 mM EDTA, pH 7.2, applied to a Pharmacia Mono Q FPLC column pre-equilibrated in the same buffer, and eluted with a 0–400 mM NaCl gradient. Fractions containing purified individual core polypeptides were either dialyzed against deionized water for amino acid sequencing, or reapplied to and eluted from a phosphatidylserine-treated phenyl-Sepharose column, as described above, in order to demonstrate that the individual core polypeptides are capable of binding in a Ca^{2+}-dependent manner.

Amino Acid Composition and Sequence Analysis

Lyophilized protein samples were prepared for amino acid composition determinations by hydrolysis under vacuum in constant boiling point HCl at 110°C for 24 h, including 0.1% phenol. L-norleucine (10 nmol) was added as an internal standard to monitor residue recovery. Analyses were performed with an LKB 4151 Alpha Plus amino acid analyzer, using an LKB buffer system. N-terminal sequence determination of annexin VI polypeptides was performed using an Applied Biosystems 447A automated sequencer.

Calcium-Binding Assay

Samples containing 10 μg of intact annexin VI or core polypeptides were applied to nitrocellulose paper using a dot-blot apparatus. $^{45}Ca^{2+}$-overlay experiments were performed according to the procedure of Maruyama et al. [18]. Autoradiography was performed using Kodak X-Omat film. Protein concentrations were determined by the method of Lowry [19], as modified by Peterson [20], and standardized by amino acid analysis for native rat annexin VI and its proteolytic cores.

Measurement of Ca^{2+}-Release Channel Activity

The activity of the Ca^{2+}-release channel was studied in a planar lipid bilayer system according to the technique described by Díaz-Muñoz et al. [14]. The episodes were recorded with a pulse protocol. Microsomal membranes from skeletal muscle, prepared according to Hamilton et al. [21], were added in the *cis* chamber. Cs_2SO_4 was used in a gradient (*cis:* 125 mM; *trans:* 25 mM); the buffer used was 10 mM MOPS, 8 μM $CaCl_2$, pH 7.4.

RESULTS AND DISCUSSION

Díaz-Muñoz et al. [14] recently reported that annexin VI modified the gating behavior of the sarcoplasmic reticulum Ca^{2+}-release channel by increasing both the probability of opening (2.7

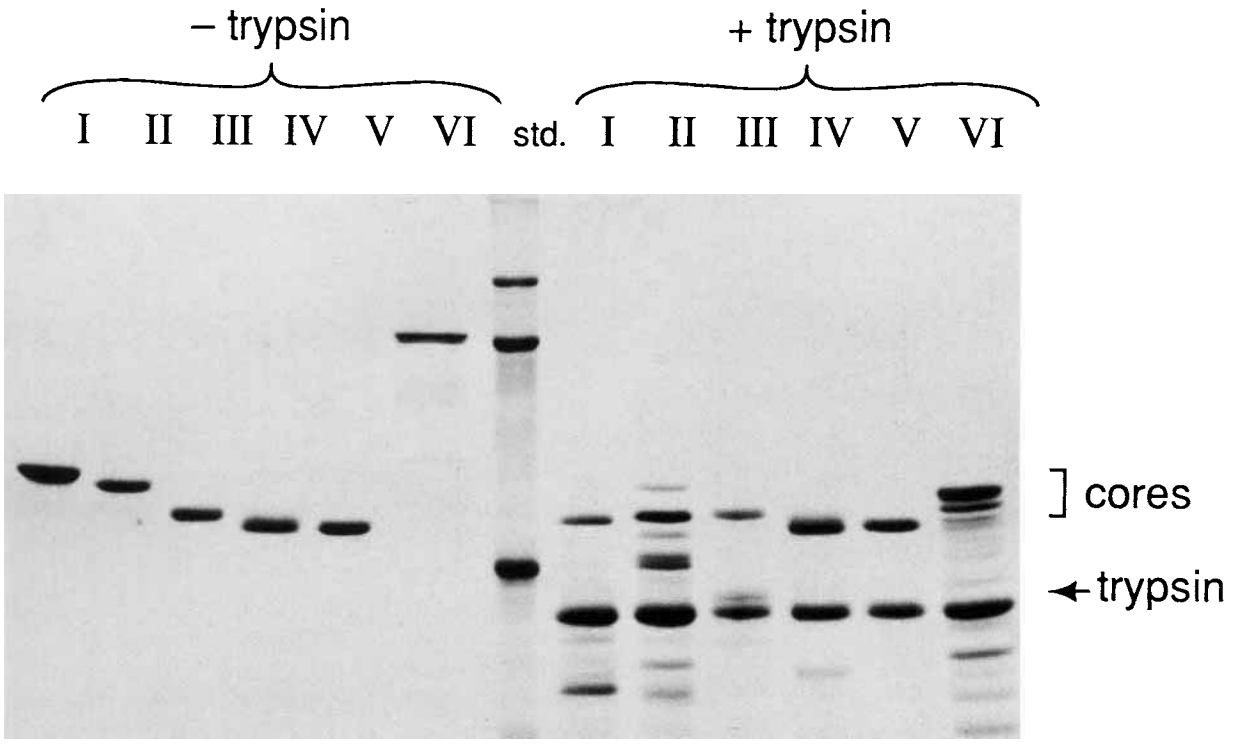

Fig. 1. Trypsin digestion of rat annexins I–VI. Approximately 2 μg annexins I–VI were either untreated or digested with 2–4 μg trypsin in 20 mM Tris-HCl, 200 mM NaCl, 5 mM Ca^{2+}, 1 mM DTT, pH 7.4. Digestions were performed at room temperature for 0.5–2 h. Samples were then electrophoresed in a 12% SDS polyacrylamide gel and stained with Coomassie blue. Molecular weight standards were carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), and phosphorylase b (97 kDa).

fold) and the mean open time (82-fold). Hazarika et al. [22] have shown by indirect immunofluorescence that annexin VI in situ is associated with the sarcoplasmic reticulum.

Purified annexin VI, isolated from various rat tissues, appears as a closely-spaced doublet on SDS-PAGE (see Fig. 1, lane 6), as does p68 (annexin VI) from murine and human tissues [23,24]. cDNAs isolated for both murine and human p68 suggest that alternatively-spliced mRNAs may encode for two p68 protein species, one lacking a six amino acid fragment [23], thereby explaining the presence of a doublet on SDS gels. The isoform mixture was used in the present studies. The existence of protease-resistant "core" polypeptides derived from 4-repeat annexins has been described for annexins I, II, and IV [25–31]. Several laboratories have produced proteolytic fragments (doublets of approximately 33 kDa and 34 kDa) from the 67 kDa protein using V8 protease or chymotrypsin [32] or thermolysin [33,34]. The properties of these proteolytic fragments, however, were not analyzed in detail. As seen in Figure 1, proteolysis of annexins I–VI was found to produce protease-resistant fragments of similar molecular weight, demonstrating that the core structures are a common characteristic of the family. In the case of annexin VI, these two 37 kDa core polypeptides, once formed, were resistant to further proteolysis for over 24 h under the conditions described. The stability of annexin VI-derived core polypeptides is further evidenced by the approximately 1:1 protein:trypsin ratio used for the digestion. As seen in Figure 2, proteolysis of the 67 kDa protein by chymotrypsin, V8, and pronase E also produced 37 kDa core polypeptides.

In order to analyze the properties of the individual annexin VI cores, those polypeptides were produced and purified. Annexin VI was digested with V8 protease, and the resulting core polypeptides were separated from the enzyme by means of Ca^{2+}-dependent phospholipid-treated phenyl-Sepharose chromatography. Under the conditions used, neither annexin VI nor its core polypeptides bind to phenyl-Sepharose that has not been treated with phospholipids. The core polypeptides were then separated from each other by ion-exchange HPLC, using a gradient of increas-

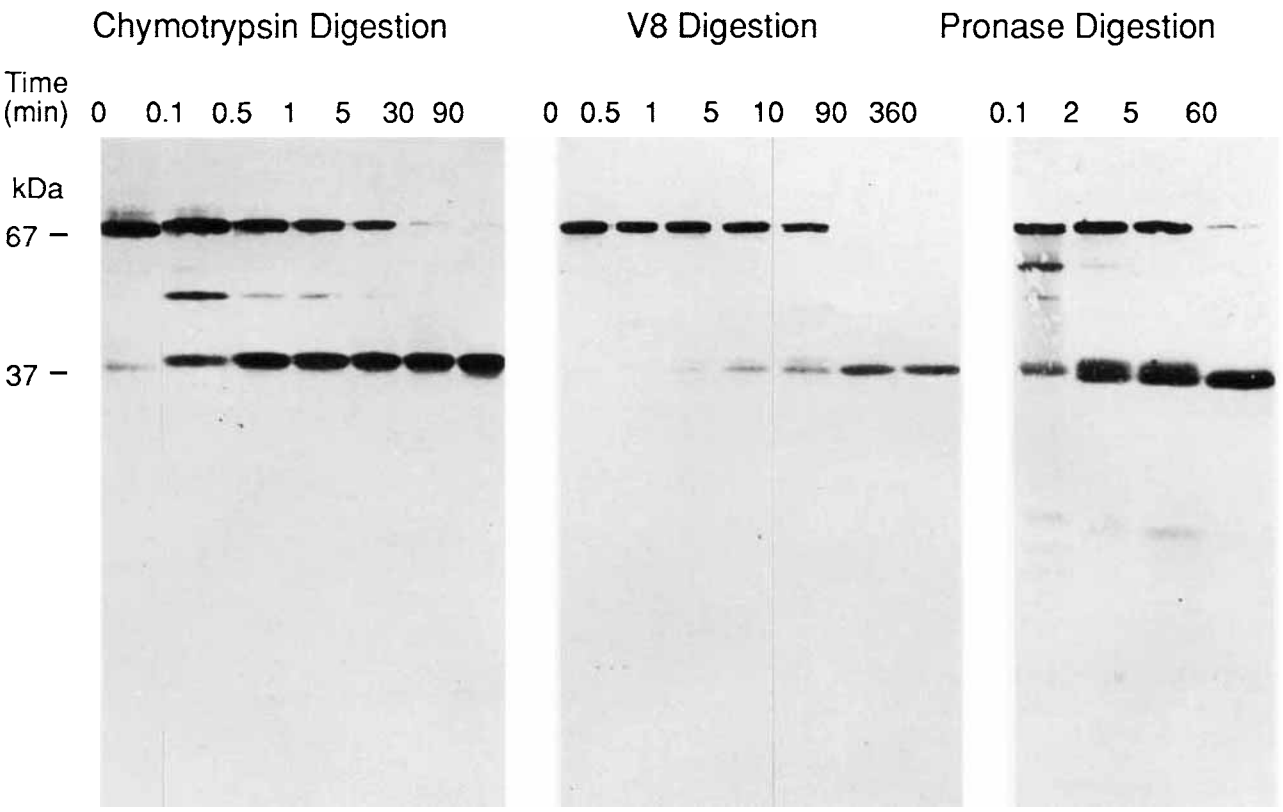

Fig. 2. Proteolytic digestion of rat annexin VI. Approximately 20 μg annexin VI was incubated at 37°C in 20 mM Tris-HCl, 200 mM NaCl, 5 mM Ca^{2+}, 1 mM DTT, pH 7.4, with either 0.1 μg chymotrypsin, 4 μg V8 protease, or 0.01 μg pronase E. 2 mM $MgCl_2$ was included in the pronase E digestion. At the times indicated, an aliquot was removed and added to 90°C SDS sample buffer to arrest proteolysis. Samples were subjected to 12% SDS-PAGE, transferred to nitrocellulose, and detected by immunoblotting with 1:2000 anti-annexin VI monoclonal antibody [35] and 1:1000 peroxidase-conjugated goat anti-mouse IgG.

ing salt concentration. The polypeptides eluted in two peaks at approximately 100 mM and 225 mM NaCl under these conditions (termed peaks "A" and "B," respectively). Both purified polypeptides individually retain Ca^{2+}-dependent binding activity to phospholipid-treated phenyl-Sepharose (Fig. 3), demonstrating that the intact 67 kDa protein is not the minimal Ca^{2+}/phospholipid-binding unit. The methods used, however, are only qualitative; the effects of cleavage on binding affinity have not been determined.

Peaks A and B were shown to be distinct protein fragments using anti-rat annexin VI monoclonal antibodies having different specificities [35]. These monoclonal antibodies were utilized as a tool to characterize the peak A and B polypeptides. Differential immunoreactivity was observed; monoclonal antibody #2 reacted only with peak B core polypeptide, while monoclonal antibody #7 detected only peak A core polypeptide (Fig. 4). These data suggested that the polypeptides contained in the two HPLC peaks

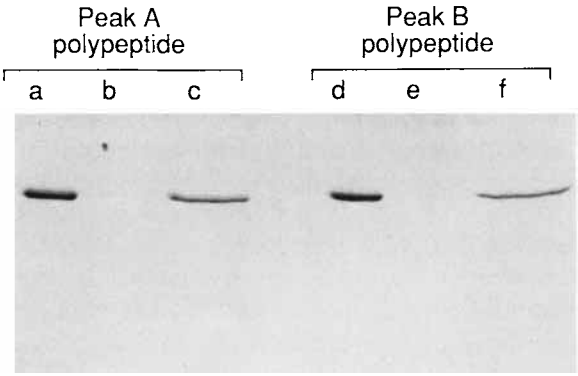

Fig. 3. Interaction of purified core polypeptides with phospholipids. Individual core polypeptides purified by HPLC (peaks A and B) were applied to phospholipid-treated phenyl-Sepharose in the presence of 1 mM Ca^{2+} (**lanes a, d;** unbound material is in **lanes b, e**) and eluted with 3 mM EGTA (**lanes c, f**). Samples from each fraction were subjected to 12% SDS-PAGE and stained with Coomassie blue.

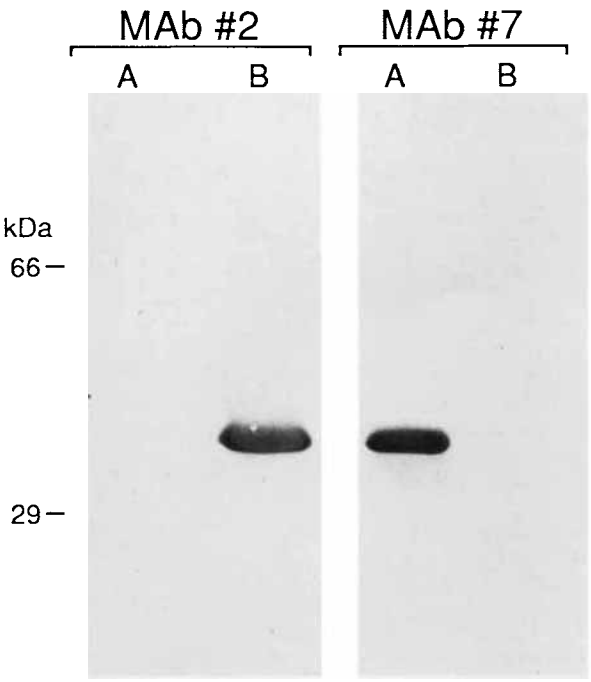

Fig. 4. Immunoreactivity of purified core polypeptides with anti-annexin VI monoclonal antibodies. Core polypeptides purified by HPLC were electrophoresed in a 12% SDS polyacrylamide gel, transferred to nitrocellulose, and probed with 1:2000 anti-annexin VI monoclonal antibodies (designated MAb #2 and MAb #7) and 1:1000 peroxidase-conjugated goat anti-mouse IgG.

are structurally dissimilar and that each is a different half of the parent protein.

In order to further define the identity of these two fragments, both peaks were analyzed by amino acid sequencing. Both intact annexin VI and peak B were not sequenceable, suggesting that the N-termini were blocked to Edman degradation and that peak B comprised the N-terminal half. Peak A produced the amino-terminal sequence, ELSAVSRVELKGTVCA; this starts at amino acid position 344 [24]. The V8 cleavage occurs, therefore, in the linkage region between domains 4 and 5, producing halves of almost equal molecular weight. Crystallographic studies of human p68 (annexin VI) support our biochemical findings. Newman et al. [36] demonstrated protein densities with a two-domain structure by image analysis of electron micrographs of 2-D crystalline arrays.

Since the core polypeptides retained their phospholipid-binding properties, studies were undertaken to determine whether or not they also retained their ability to bind Ca^{2+} directly. Samples containing equal microgram amounts of annexin VI and peak A and peak B core

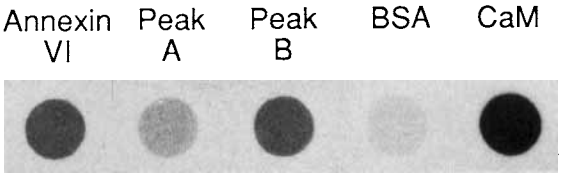

Fig. 5. Ca^{2+}-binding by annexin VI and core polypeptides. 10 μg samples of annexin VI, peak A and peak B polypeptides, and bovine serum albumin (BSA) and rat testis calmodulin (CaM; 1 μg), as controls, were subjected to $^{45}Ca^{2+}$-overlay as described in Methods. Autoradiography was performed for 2 days at room temperature.

polypeptides were applied to nitrocellulose. $^{45}Ca^{2+}$-overlay experiments demonstrated that intact annexin VI and its core polypeptides all were able to bind Ca^{2+} in the absence of phospholipid (Fig. 5). The autoradiographic intensity of the annexin VI and peak B polypeptide dots is intermediate between those corresponding to peak A polypeptide and calmodulin; these differences may reflect differences in the number and affinities of Ca^{2+}-binding sites.

Since the core polypeptides retained the ability to bind phospholipid in a Ca^{2+}-dependent manner, studies were undertaken to determine if the cores not only retained physicochemical properties demonstrated by the parent molecule, but also functional properties. Díaz-Muñoz et al. [14] found that annexin VI, in a range of 5–40 nM, modified the gating behavior of the Ca^{2+}-release channel; the probability of channel opening and the mean open time were increased by 2.7- and 82-fold, respectively. The effect of annexin VI was observed only when the protein was added to the *trans* chamber, corresponding to the luminal side of sarcoplasmic reticulum. The individual core polypeptides (peak A or peak B; 60 nM final concentration) were added sequentially to the *trans* chamber and then to the *cis* chamber, individually or in combination, and Ca^{2+}-release channel activity was monitored (Fig. 6). Neither the amino- nor carboxyl-terminal halves of annexin VI had a significant effect on the gating properties. The probability of opening for the control, amino terminal core- and carboxyl terminal core-treated channel were, respectively: 14.7 ± 3.1, 15.7 ± 3.6 and 15.0 ± 2.9 ($n = 3$). Subsequent addition of 6 nM intact annexin VI to the *trans* chamber caused the Ca^{2+}-release channel to respond by increasing the probability of opening by 3.3-fold. The mean open time (msec) for the control, amino terminal core- and carboxyl terminal core-treated channel were, respectively: 0.6 ± 0.1, 0.7 ± 0.2

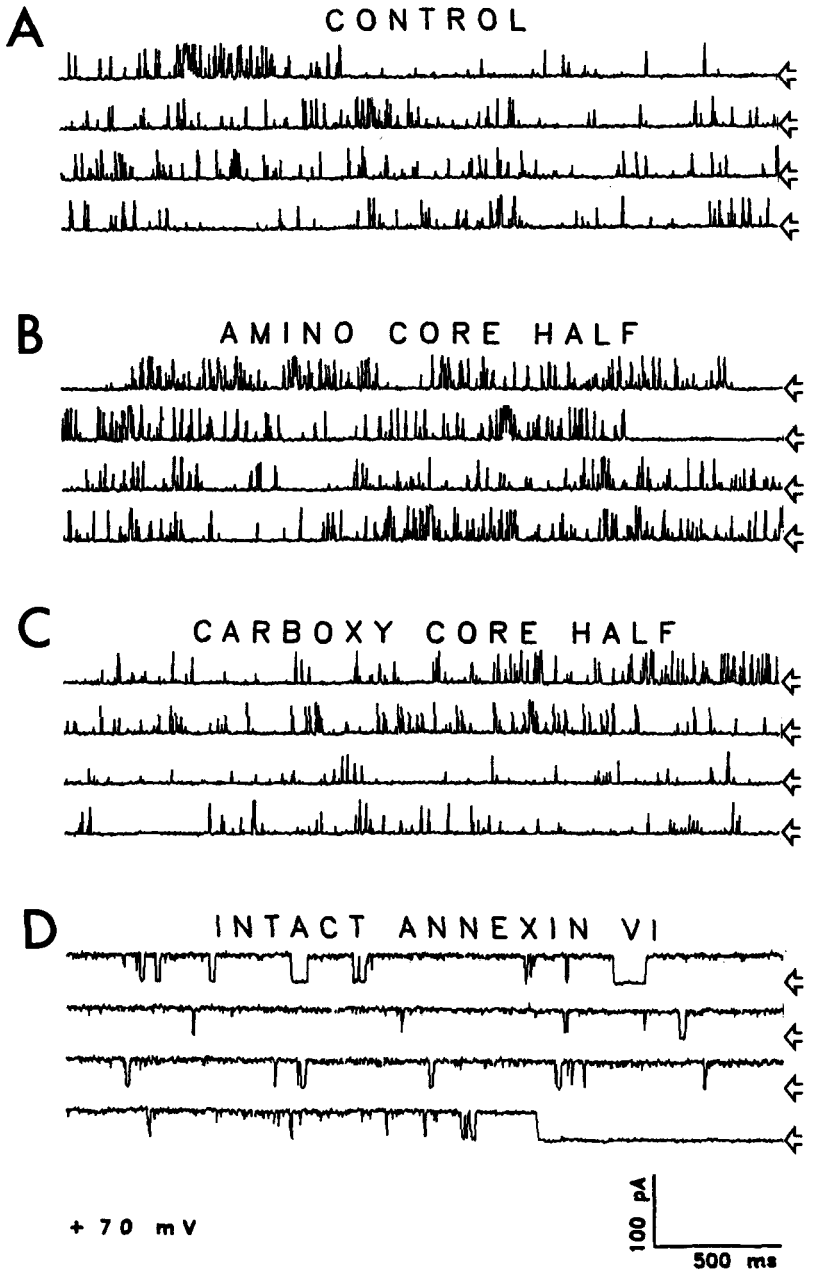

Fig. 6. Effects of intact annexin VI and core polypeptides on Ca^{2+}-release channel activity. Recordings were made as described in Methods; the activities shown were monitored during the pulse. (**A**) Control; (**B**) 60 nM amino core polypeptide (peak B) added to the *trans* chamber followed by addition to the *cis* chamber; (**C**) the same preparation had 60 nM carboxyl core polypeptide (peak A) added to the *trans* chamber followed by addition to the *cis* chamber; (**D**) the same preparation had 6 nM intact annexin VI added to the *trans* chamber. Arrows indicate the closed state of the channel. The tracings are representative of three independent experiments.

and 0.6 ± 0.1. Addition of 6 nM intact annexin VI increased the mean open time by 71-fold. These changes are in agreement with our previously published results [14]. Preliminary studies using each annexin, I through V, at 60 nM concentration showed no change in channel activity. Thus, the intact 67 kDa molecule is required for functional properties.

Members of the annexin family, although they share many similarities in sequence and physicochemical properties, require an intact structure for full function. For example, a 33 kDa proteolytic core fragment of calpactin I (annexin II) can bind Ca^{2+} and phospholipids, but the intact 36 kDa molecule containing the amino-terminus is required for interaction with p10 in order to form the native heterotetramer complex [25,37], which in turn is necessary for actin filament bundling [38]. In addition, the complex is required for the promotion of granule aggregation [39] and for stimulation of exocytosis from permeabilized chromaffin cells [12]. Analogously, we have shown that an intact annexin VI molecule is capable of modulating the gating properties of the Ca^{2+}-release channel, while neither four-domain half of the protein, in 10-fold excess, affects this function. Since the four-domain cores retain Ca^{2+}-dependent phospholipid binding, these data suggest that channel modulation by intact annexin VI reflects more than alteration of the phospholipid environment. These results indicate a biological specificity of annexin VI regulation of the Ca^{2+}-release channel.

Based on dissociation constants of calsequestrin, the free Ca^{2+} concentration within the lumen of the sarcoplasmic reticulum would keep annexin VI in a permanently activated state. This chronic effect on the Ca^{2+}-release channel is consistent with the patch-clamping measurements of intact SR [40]; gating properties are not rapidly "flickering," but are more similar to the long mean open and closed times we have demonstrated in the presence of annexin VI. Acute regulation of the channel during each contraction/relaxation cycle may originate from the myoplasmic side. For example, Smith et al. [41] found calmodulin to reduce the mean duration of single channel open events from the myoplasmic side, thereby acting as a feedback mechanism following a Ca^{2+}-release event. Thus, annexin VI may play a unique role in the complex process of intracellular Ca^{2+} homeostasis.

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REFERENCES

1. Crompton MR, Moss SE, Crumpton MJ: Cell 55:1, 1988.
2. Smith VL, Kaetzel MA, Dedman JR: Cell Regul 1:165, 1990.
3. Pepinsky RB, Tizard R, Mattaliano RJ, Sinclair LK, Miller GT, Browning JL, Chow EP, Burne C, Huang K-S, Pratt D, Wachter L, Hession C, Frey AZ, Wallner BP: J Biol Chem 263:10799, 1988.
4. Burns AL, Magendzo K, Shirvan A, Srivastava M, Rojas E, Alijani MR, Pollard HB: Proc Natl Acad Sci 86:3798, 1989.
5. Hauptmann R, Maurer-Fogy I, Krystek E, Bodo G, Andree H, Reutelingsperger CPM: Eur J Biochem 185: 63, 1989.
6. Johnston PA, Perin MS, Reynolds GA, Wasserman SA, Sudhof TC: J Biol Chem 265:11382, 1990.
7. Sudhof TC, Slaughter CA, Leznicki I, Barjon P, Reynolds GA: Proc Natl Acad Sci 85:664, 1988.
8. Geisow MJ, Fritsche U, Hexham JM, Dash B, Johnson T: Nature 320:636, 1986.
9. Kretsinger RH, Creutz CE: Nature 320:573, 1986.
10. Taylor WR, Geisow MJ: Prot Eng 1:183, 1987.
11. Marriott G, Kirk WR, Johnsson N, Weber K: Biochemistry 29:7004, 1990.
12. Ali SM, Burgoyne RD: Cell Signalling 2:265, 1990.
13. Ross TS, Tait JF, Majerus PW: Science 248:605, 1990.
14. Díaz-Muñoz M, Hamilton SL, Kaetzel MA, Hazarika P, Dedman JR: J Biol Chem 265:15894, 1990.
15. Kaetzel MA, Hazarika P, Dedman JR: J Biol Chem 264:14463, 1989.
16. Mathew JK, Krolak JM, Dedman JR: J Cell Biochem 32:223, 1986.
17. Laemmli UK: Nature 227:680, 1970.
18. Maruyama K, Mikawa T, Ebashi S: J Biochem 95:511, 1984.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
20. Peterson GL: Methods Enzymol 91:95, 1983.
21. Hamilton SL, Mejia-Alvarez R, Fill M, Hawkes MJ,

Brush KL, Schilling WP, Stefani E: Anal Biochem 183: 31, 1989.
22. Hazarika P, Kaetzel MA, Sheldon A, Karin NJ, Fleischer S, Nelson TE, Dedman JR: J Cell Biochem 46:78–85, 1991.
23. Crompton MR, Owens RJ, Totty NF, Moss SE, Waterfield MD, Crumpton MJ: EMBO J 7:21, 1988.
24. Moss SE, Crompton MR, Crumpton MJ: Eur J Biochem 177:21, 1988.
25. Glenney JR: J Biol Chem 261:7247, 1986.
26. Haigler HT, Schlaepfer DD, Burgess WH: J Biol Chem 262:6921, 1987.
27. Johnsson N, Vandekerckhove J, Van Damme J, Weber K: FEBS Lett 198:361, 1986.
28. Johnsson N, Marriott G, Weber K: EMBO J 7:2435, 1988.
29. Chuah SY, Pallen CJ: J Biol Chem 264:21160, 1989.
30. Liu Y, Brew K, Carraway KL, Carraway CAC: J Cell Biochem 35:185, 1987.
31. Weber K, Johnsson N, Plessmann U, Van PN, Soling H-D, Ampe C, Vandekerckhove, J: EMBO J 6:1599, 1987.
32. Geisow MJ: FEBS Lett 203:99, 1986.
33. Kobayashi R, Tashima Y: Biochem J 262:993, 1989.
34. Martin F, Derancourt J, Capony J-P, Colote S, Cavadore J-C: Biochem Biophys Res Comm 145:961, 1987.
35. Kaetzel MA, Hazarika P, Dedman JR: In Dedman JR, Smith VL (eds): "Stimulus-Response Coupling: The Role of Ca^{2+}-Binding Proteins." Boca Raton, FL: CRC Press, 1990.
36. Newman R, Tucker A, Ferguson C, Tsernoglou D, Leonard K, Crumpton MJ: J Mol Biol 206:213, 1989.
37. Glenney JR, Boudreau M, Galyeau R, Hunter T, Tack B: J Biol Chem 261:10485, 1986.
38. Ikebuchi NW, Waisman DM: J Biol Chem 265:3392, 1990.
39. Drust DS, Creutz CE: Nature 331:88, 1988.
40. Kwok WM, Best PM: Biophys J 57:168 (abstr), 1990.
41. Smith JS, Rousseau E, Meissner G: Circ Res 64:352, 1989.