Effect of divalent metal ions on annexin-mediated aggregation of asolectin liposomes✿

Vladimir I. Mel'gunov✉, Elena I. Akimova and Kirill S. Krasavchenko

Department of Molecular Biology, School of Biology, M.V. Lomonosov Moscow State University, Moscow 119899, Russia

Annexins belong to a family of Ca^{2+}- and phospholipid-binding proteins that can mediate the aggregation of granules and vesicles in the presence of Ca^{2+}. We have studied the effects of different divalent metal ions on annexin-mediated aggregation of liposomes using annexins isolated from rabbit liver and large unilamellar vesicles prepared from soybean asolectin II-S. In the course of these studies, we have found that annexin-mediated aggregation of liposomes can be driven by various earth and transition metal ions other than Ca^{2+}. The ability of metal ions to induce annexin-mediated aggregation decreases in the order: $Cd^{2+}>Ba^{2+}$, $Sr^{2+}> Ca^{2+}>>Mn^{2+}>Ni^{2+}>>Co^{2+}$. Annexin-mediated aggregation of vesicles is more selective to metal ions than the binding of annexins to membranes. We speculate that not every type of divalent metal ion can induce conformational change sufficient to promote the interaction of annexins either with two opposing membranes or with opposing protein molecules. Relative concentration ratios of metal ions in the intimate environment may be crucial for the functioning of annexins within specialized tissues and after treatment with toxic metal ions.

Annexins are a family of structurally related proteins capable of binding to phospholipid membranes in a Ca^{2+}-dependent manner [1–3]. Annexins are amphipathic proteins, different from soluble and integral membrane proteins. The biological roles of annexins are presumably intimately connected with their lipid-binding properties. Most experiments with Ca^{2+}-dependent binding of annexins to membranes and aggregation of membranes driven by annexins were performed using artificial membranes containing a high percentage of phosphatidylserine [4]. However, the situation within the cell is rather different: the lipid composition of membrane bilayers is very complex; in addition, not a single

---

annexin but several members of the annexin family are present in nearly every plant and animal cell. The ionic composition within the cell and in its environment is also complex but surprisingly there are only a few reports concerning the interaction of annexins with divalent metal ions other than Ca^{2+} [5–10].

To gain further insights into the mechanisms by which annexins bind to membranes in the presence of different metal ions, we have studied the interaction of a mixture of annexins isolated from rabbit liver with liposomes prepared from soybean phospholipids, asolectin [11]. The binding depended on the type of the ion (Ca^{2+}, Ba^{2+}, Sr^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}) present in the assay medium. In addition, it has been shown that the isolation of skeletal muscle cell membranes in the presence of Ba^{2+} leads to the formation of tightly-bound forms of annexins V and VI [12].

Originally, the annexin was defined as a protein that potentiates Ca^{2+}-dependent aggregation of phospholipid vesicles [13]. Later on, the ability of annexins to promote Ca^{2+}-dependent aggregation of secretion granules and liposomes turned out to be one of the general features of annexins [14]. With respect to the interaction of annexins with phospholipid membranes in the presence of Ca^{2+}, two groups within the annexin family of proteins are distinguished: (i) annexins enhancing the aggregation of vesicles (I, II, IV, VI, VII) [14, 15] , and (ii) annexins inhibiting vesicle aggregation (III, V) [15].

Site-directed spin labeling demonstrated that different annexins, such as human annexin V and hydra annexin XII, can form hetero-multimers [16]. Since all mammalian cells express different annexin genes, the interaction between various annexins can be one possible regulatory elements. In this paper we have tested the properties of a broad range of divalent metal ions in substituting Ca^{2+} for the induction of liposome aggregation mediated by annexins prepared from rabbit liver.

MATERIALS AND METHODS

Isolation of annexins. Annexins were isolated from rabbit liver (tissue stored at −70°C), as described previously [17]. Briefly, the procedure involves successive extraction of the tissue in a buffer containing 2 mM EDTA, precipitation of proteins in the presence of 2 mM Ca^{2+} solubilization of the pellet in a buffer containing 10 mM EGTA, binding of annexins into liposomes (at lipid:protein weight ratio of 3:1) in the presence of 2 mM Ca^{2+}, and final elution of the bound protein by 10 mM EGTA. The annexin preparation constituted mainly of annexins VI, V, and IV; other annexins (I–III) were found in trace amounts [17].

Preparation of metal ion stock solutions. Stock solutions containing different concentrations of metal ions were prepared in buffer A (50 mM Hepes, 100 mM KCl, 1 mM EGTA, pH 7.0), as in [15], with precautions described in [18]. The buffer concentration was chosen to avoid changes in pH after dilution; indeed, the pH of metal ion stock solutions after 1:4 (v/v) dilution with buffer A changed by less than 0.03 pH unit throughout the range of metal ion concentrations used in the experiments.

Preparation of liposomes. Liposomes were prepared by hydrating 250 mg of asolectin II-S (from Sigma, previously dried from chloroform/methanol mixture, 2:1 v/v, under nitrogen) with 25 ml of buffer A containing 240 mM sucrose [11]. Following ultrasonic treatment for 30 min at 22 kHz, the liposome suspension was diluted 3-fold with buffer A and centrifuged at $20000 \times g$ for 20 min in a Beckman JA-20 rotor. The pellet was washed twice by resuspending the liposomes in buffer A and centrifugation under the same conditions. The final pellet was suspended in 10 ml of buffer A.

Aggregation of liposomes. The annexin-mediated aggregation of liposomes in the presence of metal ions was determined as follows. Before assay, the mixture of rabbit liver

annexins was dialyzed against buffer A. On the day of the determination, liposome preparation was diluted by buffer A to reach an absorbance value at 540 nm of 0.8 and then mixed with buffer A containing the appropriate cations (0.5 ml of liposome suspension and 1.5 ml of buffer A in the presence or absence of various cations). After equilibration for 1 min in 1-cm plastic cuvettes, the reaction was started by the addition of 100 μl of annexin preparation (from 1 mg/ml stock solution). Absorbance was measured in a Hitachi 200-20 spectrophotometer equipped with a Cell Programmer unit that allowed simultaneous (time of cell repositioning was 3 s) registration of absorbance at 540 nm in four cells within programmed time intervals.

Miscellaneous. The ionized metal concentrations were assayed either directly with an electrode (Orion Research, U.S.A.) selective to the divalent metal ions, or were calculated using a self-written program. The program served to compute the concentrations of free ions and ligands in mixtures prepared from stock solutions with known concentrations of metal ion and buffer ligand. The rationale of the program was the same, as described by the algorithm of Fabiato & Fabiato [19]. The program used a modified Newton method [20] for calculations. Protein concentrations were determined by the Bradford method [21] using bovine serum albumin as a standard.

RESULTS

It is well established [15, 22] that the binding efficacy of annexins correlates with the relative content of the anionic phospholipids in the membrane. When we have studied the effect of various divalent metal ions on the competition of annexins for binding sites on phospholipid vesicles, we have used liposomes prepared from soybean asolectin containing 23.5% of phosphatidylcholine, 20% of phosphatidylethanolamine, 14% of inositol phosphatides, 39.5% of other phospholipids, lipids,

and carbohydrates, 2% of neutral lipids (triglycerides, tocopherols, sterols), and 0.3% of hexane insolubles. The approximate fatty acid composition was the following: 20% of palmitic, 4% of stearic, 10% of oleic, 59% of linoleic and 7% of linolenic; the polyunsaturated/saturated fatty acids ratio amounted to 2.8 (Dr. Rainer Dittrich, Sigma-Aldrich Chemie GmbH, personal communication). Under the conditions described in [11] we have found that the number of binding sites accessible for annexins on liposome membranes is limited and is sufficient only for the binding of no more than 45% of the protein present in the incubation medium. Therefore, we determined the aggregation of liposomes by annexins as a function of protein concentration. In the presence of 1.09 mM Ca^{2+}, the rate and extent of liposome aggregation is augmented with increasing amounts of protein in the assay medium (Fig. 1). The concentration of annexins required for maximal
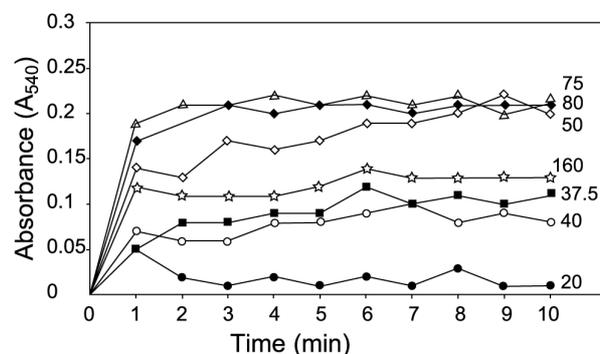

Figure 1. Effects of protein concentration on annexin-driven aggregation of asolectin liposomes.

The aggregation of liposomes as a function of annexin concentration was determined by measuring the change in turbidity (absorbance at 540 nm) induced in a suspension of liposomes in a buffer (50 mM Hepes, 100 mM KCl, 1 mM EGTA, pH 7.0) containing 1.09 mM Ca^{2+} by the addition of annexins (100 μl). Numbers at the end of experimental curves denote the concentration of annexins in μg/ml. Each point on the experimental curves represents a mean value obtained from at least four separate determinations for each protein concentration; values varied by 2–6%. Error bars are omitted for clarity.

change in the absorbance of liposome suspension at 540 nm amounted to 50 μg/ml. Further increases in the annexin concentration resulted in a decrease, rather than in an increase, of the extent of liposome aggregation. This may arise from the depletion of the number of available binding sites at the membrane and/or by membrane distortion by the annexins already bound [23].

According to the results of our previous experiments [11], the free concentration of Ca^{2+}, Ba^{2+}, Sr^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} for maximal binding of annexins to liposomes, were 480.8, 1623, 1622, 125, 130, and 6.5×10^{-6} M, respectively. The concentration of Cd^{2+} for maximal binding amounted to 4.3×10^{-9} M (unpublished data). Therefore, in the experiments presented in this report we have used the following range of ion concentrations: 2×10^{-4}–1.5×10^{-3} M for Ca^{2+}, 4×10^{-4}–2×10^{-3} M for Ba^{2+}, Sr^{2+} and Mg^{2+}, 3×10^{-5}–2×10^{-4} M for Mn^{2+} and Co^{2+}, 1.8×10^{-6}–1.5×10^{-5} M for Ni^{2+}, and 10^{-9}–10^{-8} M for Cd^{2+}.

Annexin-mediated liposome aggregation includes at least three primary interactions between three constituents of ternary complex, namely: (i) interaction of liposome with metal ion; (ii) interaction of liposome with protein, and (iii) interaction of protein with metal ion. Therefore, in every experiment we evaluated the role of such interactions and made respective controls. A typical result of one of such experiment is shown in Fig. 2. Without annexins and metal ions, no aggregation of liposome occurs (*curve 5*). *Curve 3* reflects only the slight sedimentation of liposomes with time, in the absence of both metal ions and annexins. The self-association of annexins in the presence of a metal ion (in the absence of liposomes) is rather small (*curve 5*). A related phenomenon has been observed for annexins in solution, using light scattering and fluorescence measurements [24] and during the preparation of annexin crystals [25]. The addition of annexins to liposomes suspended in metal-free medium (*curve 4*) always induces a slight in-

crease of liposome sedimentation rather than their aggregation. The same conclusion is true for the interaction of liposomes with metal ions in the absence of annexins (*curve 2*). The latter observation indicates that the ion concentrations used by us are lower than threshold concentrations of divalent metal ions necessary to induce the aggregation of large and
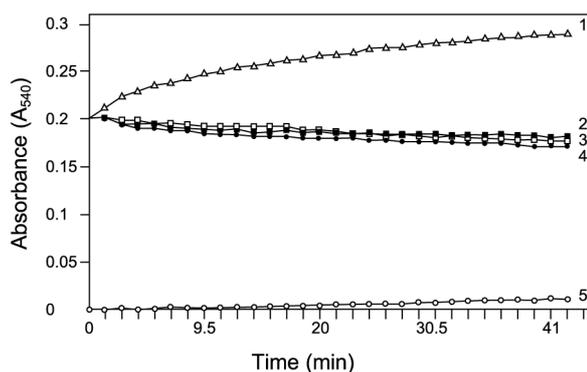

Figure 2. Time-dependent effect of 1.09 mM Ca^{2+} on annexin-mediated liposome aggregation.

In all experiments with liposomes, the initial value of absorbance at 540 nm was 0.2; the concentration of protein was 50 μg/ml. Absorbance was measured at 540 nm and data collected every 1.5 min. The curves from a single representative experiment demonstrate: *1*, aggregation of liposomes in a complete system consisting of liposomes, metal ions, and annexins; *2*, interaction of liposomes with metal ions in an annexin-free medium; *3*, sedimentation of vesicles with time in a metal- and annexin-free medium; *4*, interaction of liposomes with annexins in a metal-free medium; *5*, self-association of annexins in the presence of metal ions in liposome-free medium.

small unilamellar vesicles [26]. The aggregation of liposomes is observed only after the addition of annexins to a binary system consisting of liposomes and metal ions (Fig. 2, *curve 1*). The formation of liposome aggregates was also examined by electron microscopy in negatively stained specimens (not shown). The pattern of aggregated vesicles is very similar to that observed by other investigators [27].

Annexin-driven aggregation of liposomes in the presence of metal ions was assessed by subtracting respective control values from the

values of absorbance at 540 nm obtained in experiments where all interacting constituents of the ternary complex, i.e. annexin-lipid-ion, were present. As an example, data illustrating the effect of Ba^{2+} concentration on liposome aggregation by annexins is shown in Fig. 3. As indicated by the absorbance change at 540
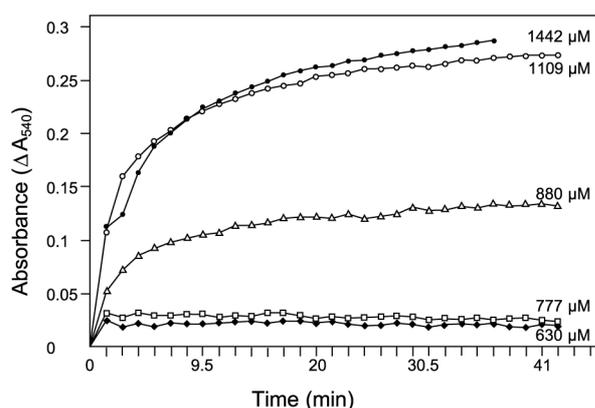

Figure 3. Effect of various concentrations of Ba^{2+} on annexin-driven liposome aggregation.

Aggregation of liposomes was determined as described under Material and Methods. The curves reflecting annexin-driven aggregation of asolectin liposomes in the presence of Ba^{2+} at concentrations indicated in the abscissa were obtained by subtracting, from determinations made in the presence of annexins, control values of absorbance obtained withouth the protein. Numbers at the end of experimental curves denote concentrations of Ba^{2+}. Mean values of four experiments are shown; values varied by 2–6%. Error bars are omitted for clarity.

nm, annexin-mediated aggregation of phospholipid vesicles reached a steady state within 30 min. Therefore, the absorbance change (ΔA_{540}) after 30 min of incubation was taken as a measure (index) of aggregation in the presence of other cations. Final values are depicted in Fig. 4, showing the effects of various cation concentrations on the aggregation of asolectin liposomes driven by annexins. The extent of liposome aggregation is dramatically affected by the metal ions investigated. Maximum aggregation is observed at 6.9×10^{-9} M Cd^{2+} and at millimolar concentrations of Ba^{2+}, Sr^{2+}, and Ca^{2+}. Other divalent ions, Mn^{2+}, Ni^{2+}, and to a lesser extent Co^{2+}, except

Mg^{2+}, also support annexin- driven aggregation of liposomes but with an apparently lower efficacy.

DISCUSSION

The results described in this study show that annexin-mediated aggregation of liposomes can be driven by ions of various earth and transition metals other than Ca^{2+}. The ability to mediate annexin-driven aggregation of liposomes decreases in the order $Cd^{2+}>Ba^{2+}$, $Sr^{2+}>Ca^{2+}>>Mn^{2+}>Ni^{2+}>>Co^{2+}$. Observations made by other investigators, concerning the interactions of annexins with divalent ions other than Ca^{2+} can be divided into two categories.

The first set of data describes the ability of various ions to mediate annexin binding to phospholipid membranes. Among the divalent cations studied, Zn^{2+} increased the binding of annexin V to phospholipid bilayers [8]. At free-ion concentrations below 10^{-6} M, Zn^{2+} was as effective as Ca^{2+} in promoting the binding of annexins to phosphatidylserine liposomes [7]. The capacity of Me^{2+} to stimulate the binding depends on the ionic radius in the folowing order: $Ca^{2+}>Zn^{2+}> Mn^{2+}> Co^{2+}> Ba^{2+}>Mg^{2+}$ [8]. Some transition metals, such as Cd^{2+}, Cu^{2+} and $Ni^{2+,}$ were also capable of forming ternary complexes with liposomes and annexins [7]. Annexin V seems to have the same ability to bind phospholipid vesicles in the presence of 10 μM of La^{3+}, Ce^{3+}, Tb^{3+} and Gd^{3+}, as it does in the presence of 1 mM Ca^{2+} [28]. With the limited number of accessible binding sites, when no more than 45% of annexins present in the incubation medium could bind to liposomes, the binding was stimulated in the presence of Ni^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}, Sr^{2+}, and Co^{2+} [11].

The second set of data on interactions between annexins and lipid membranes mediated by cations concerns the ion channel properties of annexins. Ion-replacement studies showed that the ion channel formed by

annexin VII molecules exhibits higher specificity to Ca^{2+} than to Ba^{2+} and Mg^{2+} [5]. The voltage-gated channels formed by annexin V molecules were selective for divalent cations in the folowing order of preference: Ca^{2+} $>Ba^{2+}>Sr^{2+}>>Mg^{2+}$. The annexin V channels also transported Li^+, Cs^+, Na^+ and to a lesser

tively [30]. It appears, therefore, that the effectiveness of divalent cations is closely related to the ionic radius and one can expect that the most potent mediators of annexin-driven liposome aggregation must be divalent cations of an ionic radius similar to that of Ca^{2+}. Indeed, our results show that Cd^{2+}
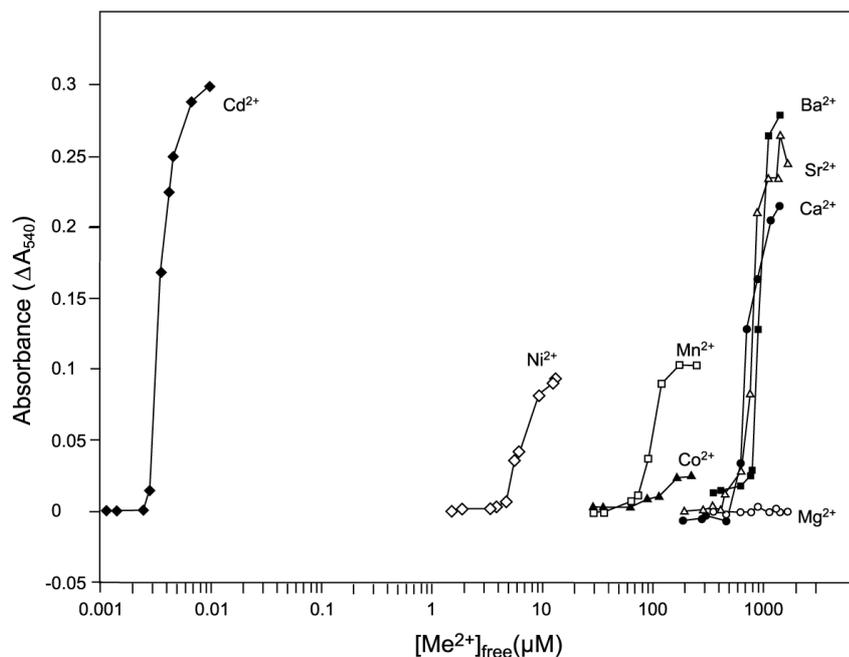

Figure 4. Effects of various divalent cations on annexin-driven liposome aggregation.

The absorbance changes (ΔA_{540}) during 30 min incubations were taken as an index of aggregation. EC_{50} values for Cd^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}, Sr^{2+}, and Ca^{2+} are 0.0035, 6.8, 100, 122, 730, 800 and 880 μM, respectively. The final values are plotted as a function of metal ion concentration shown in the abscissa to the figure. Each point represents a mean value of four experiments; values varied by 2–6%. Error bars are omitted for clarity.

extent K^+ while La^{3+} blocked these channels [9]. In addition, Zn^{2+} and Cd^{2+}, upon binding to annexin, inhibited the calcium channel activity of annexin V reconstituted into phosphatidylserine membranes [6, 9, 29], while Mn^{2+} or Mg^{2+} had no effect [29]. Cd^{2+}, a potent blocker of other voltage-gated Ca^{2+} channels at 100 μM, blocked the annexin VII channels only at very high concentrations (≥ 10 mM) [5]. Unfortunately, all of the data cited above were obtained with patch-clamp systems using very high concentration gradients of a metal ion (50 mM : 1 mM) and a difference of pH (6.5 *versus* 7.4) between the chamber and the pipette, raising the question of the significance of these results at the level of biological membranes.

It is known that the effective ionic radius of Ca^{2+} varies with its coordination numbers, namely from 0.1 to 0.107 and to 0.112 nm, for six, seven and eight-fold coordination, respec-

(ionic radius of 0.097 nm) is the most potent ion that promotes annexin-driven aggregation of liposomes at extremely low concentrations (6.9×10^{-9} M). This observation is in a good agreement with data on ion specificity of the fusion of mixed lipid vesicles [31] and cation-induced aggregation of membrane vesicles isolated from vascular smooth muscles [32]. In both cases, Cd^{2+} exhibited the same or more pronounced effects than Ca^{2+}, while other ions showed lower efficiencies.

The maximum aggregation of liposomes driven by annexins is also observed in the presence of Ba^{2+} (ionic radius of 0.135 nm), Sr^{2+} (ionic radius of 0.113 nm) and Ca^{2+} (ionic radius of 0.099 nm) but at millimolar concentrations of these ions. Other divalent ions such as Mn^{2+} (ionic radius of 0.081 nm), Ni^{2+} (ionic radius of 0.069–0.072 nm) and Co^{2+} (ionic radius of 0.070 nm) are less efficient. Mg^{2+} neither promotes the binding of anne-

xins to liposomes nor annexin-driven aggregation at all. This finding corroborates well with the lack of annexin-membrane binding in the presence of Mg^{2+} [33] and inability of Mg^{2+} to be transported by the channels formed by annexin V [9]. No activity of Mg^{2+} in supporting annexin-membrane interactions can be related to the properties of magnesium ions such as high charge density, propensity for solvatation and the formation of "covalent" bonds with ligands, constant coordination number equal to 6, and strict geometry of internal sphere.

Summarizing, the data presented in this work and by other investigators indicate that annexins interact with membranes in the presence of various divalent and trivalent metal ions. Crystallographic analyses of annexins in complex with cations revealed that molecules of all annexins have an overall flat, slightly curved shape, with a convex surface containing exposed conserved acidic residues that bind Ca^{2+} and a concave surface where the variable N-terminus is localized [34]. All calcium sites found so far in annexins are located on the convex surface and this surface plays a role in binding of annexins to membranes [35]. However, the dynamic nature of the bilayer constitutes a serious problem for detailed structural analysis [36].

The capacity of various metal ions to stimulate the binding of annexins to membranes seems to be of particular importance for the functioning of annexins within specialized tissues, and/or due to the pollution of the environment with toxic metal ions. On the other hand, the annexin-mediated aggregation of vesicles is more selective to a metal ion. Two topological models have been proposed to account for vesicle aggregation by annexins. The first model is that a single annexin molecule with two-phospholipid-binding sites can contact two membranes simultaneously [37, 38]. The second mechanism requires the self-association of membrane-bound annexin to form a protein–protein bridge [24, 39]. One can assume that not every divalent metal ion can induce conformational change within annexin molecule sufficient to promote lipid–protein or protein–protein interactions.

REFERENCES

1. Geisow, M.J., Walker, J.H., Boustead, C. & Taylor, W. (1987) Annexins — new family of Ca^{2+}-regulated phospholipid binding proteins. *Biosci. Rep.* **7**, 289–298.

2. Mel'gunov, V.I. (1990) *Advances in Science and Technology: Biophysics;* vol. **34**, pp. 1–196, VINITI, Moscow (in Russian).

3. Mel'gunov, V.I. (1991) Annexins — a new family of Ca^{2+} binding proteins. *Biochemistry (Moscow)* **56**, 107–122.

4. Raynal, P. & Pollard, H.B. (1994) Annexins: The problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochim. Biophys. Acta* **1197**, 63–93.

5. Pollard, H.B. & Rojas, E. (1988) Ca^{2+}-activated synexin forms highly selective, voltage-gated Ca^{2+} channels in phosphatidylserine bilayer membranes. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 2974–2978.

6. Sauer, G.R., Adkisson, H.D., Genge, B.R. & Wuthier, R.E. (1989) Regulating effect of endogenous zinc and inhibitory action of toxic metal ions on calcium accumulation by matrix vesicles in vitro. *Bone Miner.* **13**, 214–218.

7. Genge, R., Wu, L.N.Y. & Wuthier, R.E. (1989) Identification of phospholipid-dependent calcium-binding proteins as constituents of matrix vesicles. *J. Biol. Chem.* **264**, 10917–10921.

8. Andree, H.A., Reutelingsperger, C.P., Hauptmann, R., Hemker, H.C., Hermens, W.T. & Willems, G.M. (1990) Binding of vascular anticoagulant α (VAC α) to planar phospholipid bilayers. *J. Biol. Chem.* **265**, 4923–4928.

9. Rojas, E., Pollard, H.B., Haigler, H.T., Parra, C. & Burns, A.L. (1990) Calcium-activated endonexin II forms calcium channels across acidic phospholipid bilayer membranes. *J. Biol. Chem.* **265**, 21207–21215.

10. Zaks, W.J. & Creutz, C.E. (1990) Annexin — chromaffin granule membrane interactions: A comparative study of synexin, p32 and p67. *Biochim. Biophys. Acta* **1029,** 149–160.

11. Mel'gunov, V.I. & Nabokina, S.M. (1994) Interaction of rabbit liver annexins with various divalent metal ions: Competition of proteins with each other and change in the order of annexin binding with phospholipid vesicles. *Biochemistry (Moscow)* **59,** 411–415.

12. Krasavchenko, K.S., Akimova, E.I. & Mel'gunov, V.I. (1999) Formation of tightly bound forms of annexins V and VI in rabbit skeletal muscle membranes isolated in the presence of barium ions. *Biochemistry (Moscow)* **64,** 1169–1175.

13. Geisow, M.J., Fritsche, U., Hexham, J.M., Dash, B. & Johnson, T. (1986) A consensus amino acid sequence repeat in *Torpedo* and mammalian Ca^{2+}-dependent membrane binding protein. *Nature* **320**, 636–638.

14. Creutz, C.E. (1992) The annexins and exocytosis. *Science* **258**, 924–931.

15. Blackwood, R.A. & Ernst, J.D. (1990) Characterization of Ca^{2+}-dependent phospholipid binding, vesicle aggregation and membrane fusion by annexins. *Biochem. J.* **266**, 195–200.

16. Langen, R., Isas, J.M., Luecke, H., Haigler, H. & Hubbell, W.L. (1998) Membrane-mediated assembly of annexins studied by site-directed spin labeling. *J. Biol. Chem.* **273**, 22453–22457.

17. Mel'gunov, V.I. & Nabokina, S.M. (1992) Rabbit annexins: Comparison of proteins in cell-free homogenates and membranes of various tissues and fast preliminary identification of individual components after two-dimensional electrophoresis. *Biochemistry (Moscow)* **57**, 1194–1204.

18. Dinjus, U., Klinger, R. & Wetzker, R. (1984) Ca^{2+}/EGTA solutions: Comparison between measured and calculated free calcium ion concentrations in the micromolar range. *Biomed. Biochim. Acta* **43**, 1067–1072.

19. Fabiato, A. & Fabiato, F. (1979) Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol. (Paris)* **75**, 463–505.

20. D'yakonov, V.P. (1987) *Handbook of Algorithms and Programs in BASIC for Personal Computers.* Nauka, pp. 86–93, Moscow (in Russian).

21. Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–255.

22. Mel'gunov, V.I., Mamedova, N.A., Akimova, E.I. & Adzhimolaev, T.A. (1990) Calcium-dependent phospholipid-binding proteins associated with the membranes of rabbit skeletal muscle. *FEBS Lett.* **26**, 79–82.

23. Bazzi, M.D. & Nelsestuen, G.L. (1991) Highly sequential binding of protein kinase C and related proteins to membranes. *Biochemistry* **30**, 7970–7977.

24. Zaks, J. & Creutz, C.E. (1991) Ca^{2+}-dependent annexin self-association on membrane surfaces. *Biochemistry* **30**, 9607–9615.

25. Lewit-Bentley, A., Morera, S., Huber, R. & Bodo, G. (1992) The effect of metal binding on the structure of annexin V and implications for membrane binding. *Eur. J. Biochem.* **210**, 73–77.

26. Ohki, S., Düzgünes, N. & Leonards, K. (1982) Phospholipid vesicle aggregation: Effect of monovalent and divalent ions. *Biochemistry* **21**, 2127–2137.

27. Bandorowicz-Pikula, J. & Pikula, S. (1998) Modulation of annexin VI-driven aggregation of phosphatidylserine liposomes by ATP. *Biochimie* **80**, 613–620.

28. Funakoshi, T., Furushima, K., Mizokami, H. & Kojima, S. (1996) Effects of lanthanide ions on the binding ability of annexin V to phospholipid vesicle. *Biochem. Mol. Biol. Int.* **38**, 965–972.

29. Kirsch, T., Nah, H.D., Demuth, D.R., Harrison, G., Golub, E.E., Adams, S.L. & Pacifici, M. (1997) Annexin V-mediated calcium flux across membranes is dependent on the lipid composition: Implications for cartilage mineralization. *Biochemistry* **36**, 3359–3367.

30. Shannon, R.D. & Prewitt, C.T. (1969) Effective ionic radii in oxides and fluorides. *Acta Crystallogr. (Sect. B)* **25**, 925–945.

31. Liao, M.J. & Prestegard, J.H. (1980) Ion specificity in fusion of phosphatidic acid-phosphatidylcholine mixed lipid vesicles. *Biochim. Biophys. Acta* **601**, 453–461.

32. Kwan, C.Y. (1986) Cation-induced aggregation of membrane vesicles isolated from vascular smooth muscle. *J. Bioenerg. Biomembr.* **18**, 487–505.

33. Tsao, F.H.C. (1990) Purification and characterization of two rabbit lung Ca^{2+}-dependent phospholipid-binding proteins. *Biochim. Biophys. Acta* **1045**, 29–39.

34. Swairjo, M.A., Concha, N.O., Kaetzel, M.A., Dedman, J.R. & Seaton, B.A. (1995) Ca^{2+}-bridging mechanism and phospholipid head group recognition in the membrane binding protein annexin V. *Nature Struct. Biol.* **2**, 968–974.

35. Liemann, S. & Huber, R. (1997) Three-dimensional structure of annexins. *Cell. Mol. Life Sci.* **53**, 516–521.

36. Nelsestuen, G.L. & Ostrowski, B.G. (1999) Membrane association with multiple calcium ions: Vitamin-K-dependent proteins, annexins and pentraxins. *Curr. Opin. Struct. Biol.* **9**, 433–437.

37. Andree, H.A., Willems, G.M., Hauptmann, R., Maurer-Fogy, I.X., Stuart, M.C., Hermens, W.T., Frederik, P.M. & Reutelingsperger, C.P. (1993) Aggregation of phospholipid vesicles by a chimeric protein with the N-terminus of annexin I and the core of annexin V. *Biochemistry* **32**, 4634–4640.

38. Meers, P., Mealy, T., Pavlotsky, N. & Tauber, A.I. (1992) Annexin-mediated vesicular aggregation: Mechanism and role in human neutrophils. *Biochemistry* **31**, 6372–6382.

39. Liu, L. (1999) Calcium-dependent self-association of annexin II: A possible implication in exocytosis. *Cell. Signal.* **11**, 317–324.