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Interactions of annexin V with phospholipid monolayers

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Abstract

To understand the mechanism of annexin V–membrane interactions, we measured the interaction of human recombinant annexin V with phospholipid monolayers with differing head group and acyl group structures. Annexin V interacted with anionic phospholipid monolayers via non-specific electrostatic interactions, which was highly dependent on the surface pressure of monolayer with a sharp maximum. The unique surface pressure dependence of the annexin V-monolayer binding is strikingly similar to that observed for the binding of Ca^{2+} to anionic phospholipid monolayers, which indicates that the annexin V-bound Ca^{2+} binds two phospholipids at the membrane surface and that factors governing the Ca^{2+}-phospholipid complex formation regulate the overall annexin V-Ca^{2+}–membrane interactions.

Keywords: Annexin V; Calcium; Phospholipid monolayer; Phospholipid headgroup spacing

1. Introduction

Annexins are a family of cytosolic proteins which interact with the membrane containing anionic phospholipids in a Ca^{2+}-dependent manner (for recent reviews, see [1,2]). Although these proteins have been found in many tissues and show a wide variety of in vitro activities, their exact physiological roles remain unknown. Since biological activities of annexins depend mainly on their ability to interact with biological membranes, annexin–membrane interactions have been extensively studied by a wide variety of physico-chemical techniques. In particular, annexin V has been most thoroughly characterized thanks to its well-defined tertiary structure and availability. Several lines of evidence have indicated that annexin V interacts with membranes primarily electrostatically [3,4] and forms an aggregate on the membrane surface [5,6]. Also, it has been generally believed that calcium ions mediate electrostatic interactions between annexin and phospholipids [4,7,8]. Indeed, X-ray crystallographic analyses of annexin V have revealed that this predominantly α-helical protein has symmetrical arrangement of four subdomains with multiple Ca^{2+} binding sites in the putative membrane-binding surface [9–11]. However, the mechanism of Ca^{2+}-dependent annexin V–membrane interactions, including the stoichiometry of annexin-Ca^{2+}-phospholipid complex, is not fully understood yet. To better understand the mechanism, we have measured the interaction of human annexin V with various phospholipid monolayers. Results described herein indicate that each annexin V-bound Ca^{2+} binds two phospholipids at the membrane surface and that factors affecting Ca^{2+}-phospholipid complex formation, including surface charge and phospholipid head group spacing, govern the overall annexin V-Ca^{2+}–membrane interactions.

2. Materials and methods

2.1. Materials

The annexin V gene was a generous gift from Dr. Michael Jaye (Rorer Biotechnology). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), -glycerol (POPG) and -serine (POPS), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and -serine (DOPS) were all pur-

Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; EGTA, ethyleneglycol bis(β-aminoethyl ether)tetraacetic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine.

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chased from Avanti Polar Lipids. Phospholipid concentrations were determined by phosphate analysis [12].

2.2. Expression and purification of annexin V

The annexin V gene was subcloned into the pET-21-d(+) vector (Novagen) and then transformed into BL21(DE3)pLysS cells for protein expression. Typically, cells were grown in 2 × 1 l of Luria broth containing 50 μg/ml of ampicillin at 37°C. When the absorbance at 600 nm reached ca. 0.4, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM. The cells were allowed to grow at 37°C for 3 h and then placed on ice for 30 min before being harvested by centrifugation (5000 × *g*, 10 min, 4°C). The cells were re-suspended in 20 mM Hepes, pH 7.4 containing 1 mM EGTA and 0.16 M NaCl and then were lysed by a freeze/thaw method. The cell lysate was centrifuged for 3 h at 100 000 × *g* at 4°C. To the supernatant were added 1 mM POPS/POPC (7:3) multilamellar liposomes and 5 mM $CaCl_2$ and the mixture was shaken on ice for 20 min. The solution was centrifuged at 100 000 × *g* at 4°C for 2 h. The pellet was re-suspended in 20 mM Tris-HCl, pH 8.0 containing 10 mM EGTA, 0.16 M NaCl, and the solution was centrifuged at 150 000 × *g* at 4°C for 2 h. The supernatant was concentrated and desalted in an ultrafiltration chamber using a YM-10 membrane (Amicon). The sample was applied to a Mono-Q column (Pharmacia), connected to the fast protein liquid chromatography system (Pharmacia) and equilibrated with 20 mM Tris-HCl, pH 8.0. The protein was eluted with a linear gradient from 0 to 1 M NaCl in the same buffer. The annexin peak emerged at 0.25 M NaCl and showed a single band with an expected molecular mass (35 kDa) on a SDS-polyacrylamide electrophoresis gel. Protein concentration was determined by the bicinchoninic acid method [13].

2.3. Monolayer measurements

Surface pressure (π) of solution in a circular Teflon trough was measured using a du Nouy ring attached to a computer-controlled Cahn electrobalance (Model C-32) as described previously [14]. The trough (5 cm diameter × 1 cm deep) has a 0.5 cm deep well for magnetic stir bar and a small hole drilled at an angle through the wall to allow an addition of protein solution. 10 to 20 μl of phospholipid solution in ethanol/hexane (1:9 (v/v)) was spread onto the subphase containing 10 mM Hepes, pH 7.4, with 0.16 M NaCl, 1 mM $CaCl_2$ to form a monolayer with a given initial surface pressure (π_0). It was shown that Na^+ or K^+ significantly displaced Ca^{2+} bound to the phosphatidyl serine monolayer and thereby prevented the condensation of monolayers [15]. We found that due to the presence of 0.16 M NaCl in the subphase Ca^{2+} did not cause any detectable condensation of anionic phospholipid monolayers under our experimental condition. Once the surface pressure reading of monolayer became stable (after ca. 1 min), the protein solution was added to the subphase and the change in surface pressure ($\Delta\pi$) was measured as a function of time at 23°C. Typically, the $\Delta\pi$ value reached a maximum after 5 min. The maximal $\Delta\pi$ value depended on the protein concentration at the low concentration range and reached a saturation when the protein concentration was higher than 0.1 μM. Protein concentrations were therefore maintained above 0.1 μM to ensure that the observed $\Delta\pi$ represented a maximal value and that a loss of protein by the adsorption to the trough wall was negligible. Surface pressure-area (π–A) isotherms were measured by a Lauda Surface Film Balance FW2 (Brinkmann) using the same subphase buffer.

3. Results

Phospholipid monolayers are an excellent model membrane in which the penetration of protein into phospholipids can be sensitively monitored in terms of the change in surface pressure ($\Delta\pi$) [16]. It was previously reported that annexin VI did not significantly penetrate monolayers of various phospholipids (i.e., $\Delta\pi = 2$ to 6 dyne/cm) but instead formed an array of two-dimensional crystal on the monolayer surface [17]. To systematically analyze the interactions of annexin V with monolayers, we measured the interaction of annexin V with a wide variety of phospholipid monolayers with differing head group and acyl group structures. We first measured the Ca^{2+}-dependent penetration of annexin V into POPS, POPG and POPC phospholipid monolayers as a function of their initial surface pressure (π_0); a higher π_0 value indicates higher phospholipid packing density. POPC, POPG and POPS showed essentially identical surface pressure–area isotherms (data not shown) and thus have the identical head group spacing at a given surface pressure despite some potential differences in acyl group conformations [18]. In the absence of Ca^{2+}, annexin V resulted in no detectable increase in surface pressure toward any monolayer with π_0 up to 24 dyne/cm (data not shown). As shown in Fig. 1, annexin V injected into the subphase in the absence of phospholipid monolayers or in the presence of zwitterionic POPC monolayer did not show any detectable change in surface pressure, indicating that annexin V per se is not a surface-active protein. Annexin V penetrated into anionic POPS and POPG monolayers but the degree of penetration was, in general, much smaller than that caused by other surface-active proteins, which normally reaches up to 15 dyne/cm [16]. Also, annexin V showed no detectable penetration to any phospholipid monolayers in the presence of 1 M NaCl in the subphase (Fig. 1). Taken together, these results are consistent with the notion that the binding of annexin V to phospholipid monolayers is largely driven by non-specific electrostatic interactions without substantial membrane penetration [3].

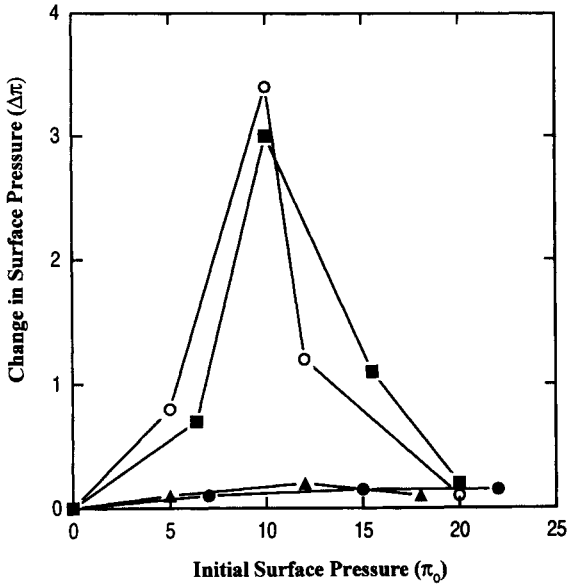

Fig. 1. Effect of initial surface pressure of monolayers on the penetration of annexin V. The monolayers used were POPS (○), POPG (■), and POPC (▲). The protein concentration in the subphase (10 mM Hepes, pH 7.4, with 0.16 M NaCl and 1 mM $CaCl_2$) was 0.15 μM. The penetration of annexin V into the POPS monolayer was also measured in the presence of 1 M NaCl in the subphase (10 mM Hepes, pH 7.4, with 1 M NaCl and 1 mM $CaCl_2$) (●). Each data point represents the average of three measurements.

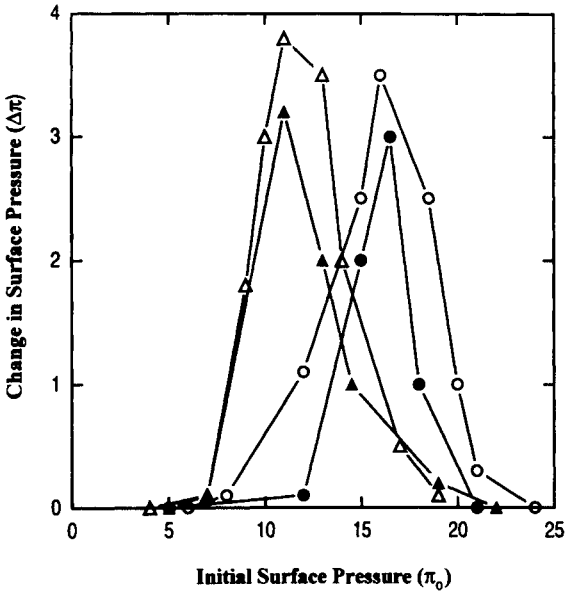

Fig. 2. Effect of initial surface pressure of mixed monolayers on the penetration of annexin V. The monolayers used were POPS/POPC (7:3) (△), POPS/POPC (3:7) (○), POPG/POPC (7:3) (▲) and POPG/POPC (3:7) (●). The protein concentration in the subphase (10 mM Hepes, pH 7.4, with 0.16 M NaCl and 1 mM $CaCl_2$) was 0.15 μM. Each data point represents the average of three measurements.

Most importantly, the $\Delta\pi$ vs. π_0 plot for POPS and POPG monolayers showed a sharp maximum, indicating the presence of an optimal phospholipid packing density (or head group spacing) for the annexin V–monolayer interactions. To better understand the origin of the unique surface pressure dependence of the annexin V–anionic phospholipid interactions, we measured the Ca^{2+}-dependent penetration of annexin V into the mixed monolayers of POPS/POPC and POPG/POPC, respectively, with varying compositions; i.e., POPS/POPC (7:3), POPS/POPC (3:7), POPG/POPC (7:3) and POPG/POPC (3:7). As shown in Fig. 2, $\Delta\pi$ vs. π_0 plots for these mixed monolayers also showed sharp maximums. Toward the POPS/POPC (3:7) mixed monolayer, for instance, the $\Delta\pi$ value increased with an increase in π_0, reaching a maximal value of 3.5 dyne/cm at $\pi_0 = 16 \pm 1$ dyne/cm, then sharply decreased as π_0 further increased. For the POPS/POPC (7:3) mixed monolayer, the same pattern was observed but the maximal $\Delta\pi$ value occurred at $\pi_0 = 11 \pm 1$ dyne/cm. As shown in Fig. 2, POPG/POPC mixed monolayers showed essentially the same behaviors. For both types of mixed monolayers, the optimal π_0 shifted to a lower value with the increase in the proportion of anionic phospholipid in the mixed monolayer, which again underscores the importance of the head group spacing between anionic phospholipids. Finally, we measured the interaction of annexin V with phospholipid monolayers with differing acyl chain compositions, i.e., mixed monolayers of DOPS/DOPC (3:7), DOPS/DOPC (7:3). As shown in their force–area curves (Fig. 3), DOPC (or DOPS) molecules with two *cis*-unsaturations occupy a higher surface area than POPC (or POPS) molecules with

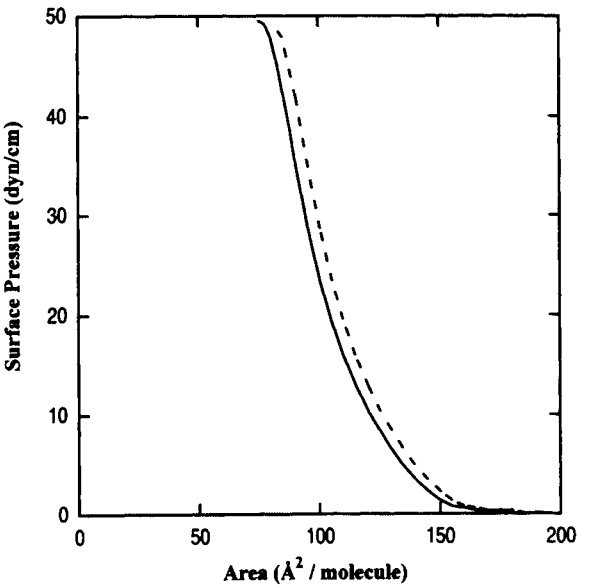

Fig. 3. Surface pressure–area curves for POPC (solid line) and DOPC (broken line). Curves for POPS monolayer and POPS/POPC mixed monolayers were identical to that for POPS monolayer while DOPS monolayer and DOPS/DOPC mixed monolayers all showed the same curve as DOPS monolayer. Each curve was drawn from a minimum of five independent measurements.

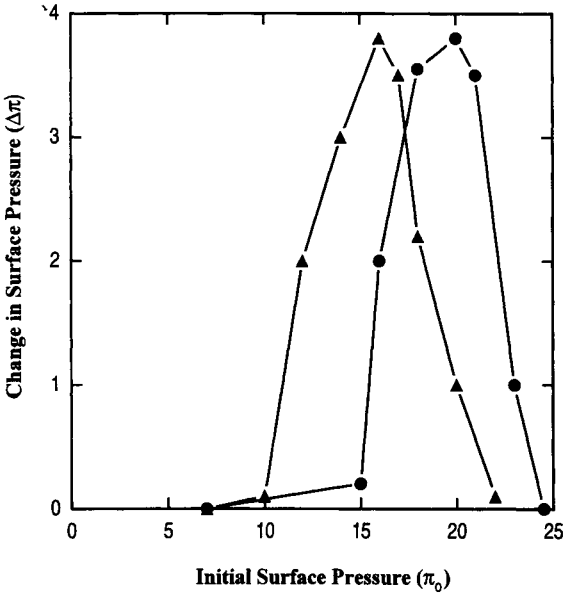

Fig. 4. Effect of initial surface pressure on the penetration of annexin V into DOPS/DOPC (7:3) (▲) and DOPS/DOPC (3:7) (○) mixed monolayers. The protein concentration in the subphase (10 mM Hepes, pH 7.4, with 0.16 M NaCl and 1 mM $CaCl_2$) was 0.15 μM. Each data point represents the average of three measurements.

only one *cis*-unsaturation, thereby having a larger head group spacing. Compared to POPS/POPC mixed monolayers, DOPS/DOPC mixed monolayers should thus be compressed to a higher surface pressure to attain the same surface area per molecule that is a direct indicative of phospholipid head group spacing. In agreement with this notion, $\Delta\pi$ vs. π_0 plots for the DOPS/DOPC monolayers (Fig. 4) showed a pattern similar to that observed for the POPS/POPC monolayers but with maximums shifted to higher values (Table 1). Most importantly, different optimal π_0 values for DOPS/DOPC (7:3) and POPS/POPC (7:3) monolayers correspond to essentially the same surface area per molecule value (Table 1) and the same is true for DOPS/DOPC (3:7) and POPS/POPC (3:7) mixed monolayers.

Table 1
Parameters for surface pressure dependence of the annexin V penetration into various phospholipid monolayers

Phospholipids	Optimal surface pressure (dyne/cm)	Optimal surface area (Å^2/molecule)
POPS	10±1	122±5
POPS/POPC (7:3)	11±1	120±5
POPS/POPC (3:7)	16±1	110±5
DOPS/DOPC (7:3)	16±1	115±5
DOPS/DOPC (3:7)	21±1	108±5

Optimal surface pressure values were determined from Figs. 1, 2 and 4. Optimal surface area values were calculated from corresponding optimal surface pressure values using surface pressure–area curves in Fig. 3.

4. Discussion

The monolayer penetration studies described herein show that the annexin-membrane binding is largely driven by non-specific electrostatic interactions. It was previously reported that Ca^{2+} bound to anionic phospholipid monolayers but not to phosphatidyl choline monolayers [15]. Also, the binding of Ca^{2+} to the anionic phosphatidyl inositol monolayer showed a sharp maximum as a function of phospholipid packing density, which was interpreted as the result of preferential formation of a ternary complex between one metal ion and two phospholipids [19]. Interestingly, the sharp maximum in the $\Delta\pi$ vs. π_0 plot for the binding of annexin V to monolayers is strikingly similar to that observed for the Ca^{2+}-monolayer binding. Also, the optimal surface area per molecule for the annexin-POPS monolayer binding (122 ± 10 Å^2/molecule) is reasonably consistent with the optimal surface area per molecule for the Ca^{2+} binding to phosphatidyl inositol monolayer (ca. 100 Å^2/molecule) [19]. The modest difference could be accounted for in terms of the difference between free Ca^{2+} and protein-bound Ca^{2+}. Taken together, these similarities point to the importance of the Ca^{2+}-phospholipids binding in the overall annexin V-Ca^{2+}-membrane binding and also indicate that annexin V-bound Ca^{2+} might bind two anionic phospholipid molecules on the membrane surface in the course of the annexin–membrane interactions. Indeed, the tertiary structures of annexin V show that high affinity Ca^{2+} binding sites contain two water molecules as a ligand, which could be replaced by two phospholipids in the putative annexin V-Ca^{2+}-phospholipids [2,10,11]. Reported Ca^{2+}-binding stoichiometries of annexin V vary from 5 to 12 mol of Ca^{2+}/mol of protein [10,20]. Based on these values, an annexin V molecule is expected to bind 10 to 24 anionic phospholipid molecules, which is consistent with reported values [9,21]. Finally, results from the binding of annexin V to mixed monolayers with different compositions indicate the formation of anionic phospholipid domains in the process of annexin V-monolayer binding. This notion is based on the finding that the optimal surface area per molecule value for the POPS/POPC (7:3) mixed monolayer (see Table 1) is essentially the same as that for pure POPS monolayer. Also, the difference in optimal surface area per molecule between POPS/POPC (7:3) and POPS/POPC (3:7) mixed monolayers is not as large as expected from their significantly different compositions. Thus, it appears that the regions of monolayer interacting with annexin V contain mostly anionic phospholipids. It is less likely, however, that annexin V binds the anionic phospholipid domain pre-formed by Ca^{2+} because Ca^{2+} does not induce the formation of anionic phospholipid domains in the absence of annexin V under our experimental condition [15] (also see Section 2). Presumably, the phospholipid domain formation is caused by the annexin V molecule containing multiple binding sites for the calcium ions which in turn

bind a large number of anionic phospholipids. The mechanism of the anionic phospholipid domain formation and the interplay between phospholipid and protein aggregation at the membrane surface are currently under investigation.

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References

[1] Creutz, C.E. (1992) Science 258, 924–931.
[2] Swairo, M.A. and Seaton, B.A. (1994) Annu. Rev. Biophys. Biomol. Struct. 23, 193–213.
[3] Voges, D., Berendes, R., Berger, A., Demange, P., Baumeister, W. and Huber, R. (1994) J. Mol. Biol. 238, 199–213.
[4] Meers, P., Daleke, D., Hong, K. and Papahadjopoulos, D. (1991) Biochemistry 30, 2903–2908.
[5] Mosser, G., Ravanat, C., Freyssinet, J.M. and Bisson, A. (1991) J. Mol. Biol. 217, 241–245.
[6] Concha, N.O., Head, J.F., Kaetzel, M.A., Dedman, J.R. and Seaton, B.A. (1992) FEBS Lett. 314, 159–162.
[7] Anree, H.A., Reutelingsperger, C.P.M., Hauptman, R., Hemker, H.C., Hermers, W.Th. and Willems, G.M. (1990) J. Biol. Chem. 265, 4923–4928.
[8] Meers, P. (1990) Biochemistry 29, 3325–3330.
[9] Huber, R., Romisch, J. and Paques, E.P. (1990) EMBO J. 9, 3867–3874.
[10] Huber, R., Schneider, M., Mayr, I., Romisch, J. and Paques, E.P. (1990) FEBS Lett. 275, 15–21.
[11] Concha, N.O., Head, J.F., Kaetzel, M.A., Dedman, J.R. and Seaton, B.A. (1993) Science 261, 1321–1324.
[12] Kates, M. (1986) Techniques of Lipidology, 2nd Edn., pp. 113–116, Elsevier, Amsterdam.
[13] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[14] Shen, Z., Wu, S.-K. and Cho, W. (1994) Biochemistry 33, 11598–11607.
[15] Rojas, E. and Tobias, J.M. (1965) Biochim. Biophys. Acta 94, 394–404.
[16] Verger, R. and Pattus, F. (1982) Chem. Phys. Lipids 30, 189–227.
[17] Newman, R., Tucker, A., Ferguson, C., Tsemolou, D., Leonard, K. and Crumpton, M.J. (1989) J. Mol. Biol. 206, 213–219.
[18] Pascher, I., Lundmark, M., Nyholm, P.-G. and Sundell, S. (1992) Biochim. Biophys. Acta 1113, 339–373.
[19] Hauser, H. and Dawson, R.M.C. (1968) Biochem. J. 109, 909–916.
[20] Evans, T.C. and Nelsestuen, G.L. (1994) Biochemistry 33, 13231–13238.
[21] Meers, P. and Mealy, T.R. (1993) Biochemistry 32, 5411–5418.