Annexins V and XII Insert into Bilayers at Mildly Acidic pH and Form Ion Channels†

J. Mario Isas,‡ Jean-Philippe Cartailler,§ Yuri Sokolov,‡ Darshana R. Patel,‡ Ralf Langen,|| Hartmut Luecke,§ James E. Hall,‡ and Harry T. Haigler*,‡

Departments of Physiology and Biophysics, and Molecular Biology and Biochemistry, University of California, Irvine, California 92697, and Department of Biochemistry & Molecular Biology, University of Southern California, Los Angeles, California 90033

Received September 27, 1999; Revised Manuscript Received December 20, 1999

ABSTRACT: The functional hallmark of annexins is the ability to bind to the surface of phospholipid membranes in a reversible, Ca^{2+}-dependent manner. We now report that human annexin V and hydra annexin XII reversibly bound to phospholipid vesicles in the absence of Ca^{2+} at low pH; half-maximal vesicle association occurred at pH 5.3 and 5.8, respectively. The following biochemical data support the hypothesis that these annexins insert into bilayers at mildly acidic pH. First, a photoactivatable reagent (3-trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine) which selectively labels proteins exposed to the hydrophobic domain of bilayers reacted with these annexins at pH 5.0 and below but not at neutral pH. Second, in a Triton X-114 partitioning assay, annexins V and XII act as integral membrane proteins at low pH and as hydrophilic proteins at neutral pH; in the presence of phospholipids half-maximal partitioning into detergent occurred at pH ≈ 5.0. Finally, annexin V or XII formed single channels in phospholipid bilayers at low pH but not at neutral pH. A model is discussed in which the concentrations of H^+ and Ca^{2+} regulate the reversible conversion of three forms of annexins—soluble, peripheral membrane, and transmembrane.

Annexins form a large multigene family of proteins structurally defined by a conserved core domain consisting of four homologous repeats, each of which contains ∼70 amino acids (*1*). Eleven different vertebrate annexin gene products have been identified, and they share approximately 50% amino acid sequence identity in their core domains (*2*). Each annexin also has a shorter nonconserved amino terminal domain. The functional hallmark of annexins is the ability to bind to phospholipids in a reversible Ca^{2+}-dependent manner (*1*). Although the exact biological functions of annexins have not been clearly defined, they have been implicated in a number of membrane-related events including membrane fusion, vesicular trafficking, and ion-channel formation (*1*).

The X-ray crystal structures of the soluble form of several different annexins all reveal a common backbone fold in which each of the four repeats in the core domain contains five α-helices connected by short loops (*1*). Two of these loops come together to form each of the "type II" Ca^{2+}-binding sites (*3*). High-affinity membrane association of annexins is mediated by the joint coordination of Ca^{2+} by these loops and the polar headgroup of phosphatidylserine on the surface of membranes (*4*). Although these studies of the Ca^{2+}-dependent peripheral membrane-bound annexins undoubtedly describe the structure of a biologically important form of the protein, they do not, in our opinion, provide a structural framework that can explain the ion-channel activity observed for annexin V and other annexins (*5*). As discussed in more detail below, we proposed that annexin-dependent channel activity is mediated by another form of the protein, one that is inserted into the hydrophobic core of the bilayer in a Ca^{2+}-independent manner (*6*).

In addition to the extensive studies of Ca^{2+}-dependent binding, there are a few reports of Ca^{2+}-independent association of annexins with biological membranes (see the review by Moss in ref *1*). While the biological relevance is not yet clear, recent in vitro studies suggest that Ca^{2+}-independent binding of annexins to phospholipid membranes can be induced by mildly acidic pH (*6*−*8*). Kohler et al. presented clear evidence that annexin V associated with phosphatidylserine-containing vesicles at pH 4.0 in the absence of Ca^{2+} but required Ca^{2+} for association at neutral pH (*7*). In a separate study, Rosengarth et al. showed that, in the absence of Ca^{2+}, annexin I increased the surface pressure of phospholipid monolayers at pH 6.0 but not at pH 7.4, and concluded that annexin I penetration into the monolayers caused the pressure changes (*8*).

Recent studies in our laboratory have used site-directed spin labeling to investigate the interaction of annexin XII with phospholipid bilayers. At neutral pH annexin XII underwent Ca^{2+}-dependent binding to the surface of phospholipid bilayers and assembled into trimers (*9*). Under these conditions, EPR studies of numerous spin-labeled sites were

† This work was supported by the National Institutes of Health Grants GM55651 (to H.T.H.), GM57998 (to J.E.H.), and GM56445 (to H.L.).

* To whom correspondence should be addressed. Phone: (949) 824-6304. Fax: (949) 824-8540. E-mail: hhaigler@uci.edu.

‡ Department of Physiology and Biophysics, University of California.

§ Department of Molecular Biology and Biochemistry, University of California.

|| University of Southern California.

10.1021/bi9922401 CCC: $19.00
Published on Web 02/24/2000

consistent with the conclusion that the overall backbone fold of the soluble form of the protein that was observed by crystallography was retained when the protein underwent Ca^{2+}-dependent binding to membranes (ref *9* and unpublished results). However, major structural rearrangements clearly occurred at pH 4 (*6*). A nitroxide scan of amino acids at and around the putative Ca^{2+} coordination site between the D and E helices of the second repeat showed that, upon membrane binding in the absence of Ca^{2+} at pH 4, the helix−loop−helix motif observed in the crystal structure in this region was converted into a continuous α-helix that appeared to be transmembrane. The transmembrane helix was asymmetrically solvated with one side facing the hydrophobic core of the bilayer and the other side facing water. Inspection of the amino acid sequence around other Ca^{2+}-binding sites revealed several additional putative transmembrane helices. All helices had amphipathic character with Glu and Asp residues located at or near the hydrophobic face. Protonation of these acidic side chains would greatly reduce the unfavorable free energy of transfer from an aqueous environment to the hydrophobic core of the bilayer (*10*). A model was proposed in which protonation of acidic residues in several segments of the annexin XII conserved core domain triggers the formation of amphipathic transmembrane α-helices which assemble into a transmembrane structure with a hydrophilic central pore (*6*).

We now report a study in which we characterized the pH dependence of membrane association of both hydra annexin XII and human annexin V and tested the model of membrane insertion by two independent biochemical approaches. In addition, the proposal that several amphipathic transmembrane helices come together to form a water-filled pore (*6*) was tested by determining whether annexin V or XII could induce ion channels in planar lipid bilayers in a pH-dependent manner. Taken together these studies provide additional experimental support for the proposal that annexin XII undergoes H^+-induced membrane insertion. This study also provides the first direct evidence that annexin V inserts into membranes. Membrane insertion of both annexins V and XII was reversible, thereby implying that there is an equilibrium between three forms of these proteins—soluble, peripheral membrane, and transmembrane—that is modulated by the H^+ and Ca^{2+} concentration.

MATERIALS AND METHODS

Materials. Recombinant annexins V (*14*) and XII (*15*) were expressed and purified as previously described. Phosphatidylserine (bovine brain, catalog no. 840032) and phosphatidylcholine (egg yolk, catalog no. 840051) were obtained from Avanti Polar Lipids (Alabaster, AL). The photoactivatable probe 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-diazirine (abbreviated ^{125}I-TID)[1] was purchased from Amersham (Piscataway, NJ).

Measurement of Phospholipid Vesicle Binding. Large unilammelar vesicles composed of phosphatidylserine and phosphatidylcholine (2:1 molar ratio) were prepared according to the Reeves/Dowben protocol (*16*). The association of annexins with these vesicles was measured by a copelleting assay as described (*17*). The standard assay contained annexin V or XII (10 μg) and vesicles (1:1000 molar ratio of protein to phospholipid) in a solution (0.5 mL) containing either $CaCO_3$ (500 μM) or EGTA (500 μM). The following buffers (100 mM) were used: sodium acetate for pH 4.0−5.6, MES for pH 6.0−6.5, and Tris−HCl for pH 7.4. Repeating the experiments with other buffers showed that the results were dependent on pH and not the buffer used. After a 10 min incubation at room temperature, the solutions were centrifuged (20000*g*, 5 min) to separate vesicles along with associated protein (pellet) from soluble protein (supernatant). Control experiments at all pH values showed that neither annexin V nor annexin XII was found in the pellet following centrifugation in the absence of phospholipid vesicles. Samples were analyzed by SDS−PAGE followed by staining with Coomassie blue and densitometry as previously described (*18*).

Labeling with ^{125}I-TID. The topography of membrane-bound annexins was probed with ^{125}I-TID by published methods (*11*, *12*). Annexin V or XII (12 μg) was incubated with vesicles (1:1000 molar ratio of protein to phospholipid) in a solution (30 μL final volume) containing either $CaCO_3$ (500 μM) or EGTA (500 μM) at the indicated pH as described in the phospholipid vesicle binding assay (see above). The ^{125}I-TID solutions were diluted with ethanol to a concentration of 1 μCi/μL. After protein and phospholipid were incubated for 10 min, 1 μCi of ^{125}I-TID was added, and the solution was incubated for 20 min in the dark. The sample was then subjected to a 30 min irradiation from an ultraviolet light (Minerallight Lamp model UVSL-25, 366 nm) at a distance of 5 cm to generate the reactive carbene. All incubations were at room temperature. After the incubation, Laemmli SDS sample buffer was added, the samples were analyzed by SDS−PAGE, and the dried gels were exposed to Kodak XAR film with intensifying screens (−80 °C, 48 h).

Triton X-114 Phase Partitioning. The hydrophobicity of annexins at different pH values was investigated by a previously characterized method involving the partitioning of proteins during phase separation in solutions of Triton X-114 (*13*). Annexin V or XII (20 μg) was added to a solution (35 μL final volume) containing NaCl (150 mM) and buffer (10 mM) appropriate for the indicated pH (see above) and either $CaCO_3$ (500 μM) or EGTA (500 μM). The indicated solutions also contained vesicles (1:1000 molar ratio of protein to phospholipid). Following a 10 min incubation at 4 °C, each sample was added to a solution (175 μg) at the same pH as the sample containing Triton X-114 (0.5% w/v). The mixture was overlaid on a 300 μL cushion of ice-cold sucrose (6% w/v) and Triton X-114 (0.06% w/v) in the same buffer and at the same pH as the sample. The samples were then subjected to a 30 °C incubation (above the cloud point for Triton X-114) for 3 min, which caused the solution to phase separate. The hydrophobic detergent phase was separated from the aqueous phase by centrifugation and washing as described in the original description of the method (*13*). Aliquots of the detergent and aqueous phases were analyzed by SDS−PAGE and Coomassie blue staining, and the scanned gels were quantified using NIH Image.

Channel Activity in Lipid Bilayers. Bilayers were formed at room temperature by the union of two monolayers formed

[1] Abbreviations: EPR, electron paramagnetic resonance; ^{125}I-TID, probe 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine.

from a mixture (1:1 by weight) of phosphatidylcholine (PC) and phosphatidylserine (PS) as described elsewhere (*19*, *20*). Briefly, lipid monolayers were opposed over a hole approximately 200 μm in diameter in a 15−20 μm thick Teflon partition dividing the two aqueous phases. The hole, punched by electric spark, was precoated with about 5 μL of a 2.5 vol % solution of squalene in *n*-pentane. Salt solutions were 100 mM NaCl, buffered by either 10 mM MES−NaOH to pH 6.0 or 6.5 or 10 mM Tris−HCl to pH 7.0−7.4. Bilayer formation was monitored by measuring capacitance. Silver/silver chloride wires were used as electrodes to apply voltages across the bilayer. When asymmetric solutions were used, the Ag/AgCl electrodes were connected to the solutions using bridges made by fitting glass tubes packed with glass fibers into a short piece of solution-filled silicon tubing. The bridge-filling solution was 2 M KCl. The rear chamber potential was taken as ground, and the additions of annexins were made to the front chamber. Voltages were generated and currents digitized at a resolution of 12 bits by an AD Lab ADC/DAC board driven by software written in the lab. Currents were measured by an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) connected to the AD Lab board as described above.

RESULTS

pH-Dependent Association of Annexins with Phospholipid Vesicles. It is well-known that at neutral pH annexins V and XII bind with high affinity to phospholipid bilayers in the presence of Ca^{2+} but have no measurable binding affinity in the absence of Ca^{2+} (*1*). Using a fluorescence resonance energy transfer assay, it recently was shown that annexin V could bind to bilayers in the absence of Ca^{2+} under mildly acidic conditions (*7*). We confirmed this observation by showing that annexin V cosediments with large phospholipid vesicles in the absence of Ca^{2+} at low pH but not at neutral pH (Figure 1). Half-maximal vesicle association occurred at approximately pH 5.3. In the presence of Ca^{2+}, between 90% and 100% of the added annexin V associated with vesicles at all pH values tested (data not shown). Annexin XII also associated (90−100%) with vesicles at all pH values tested in the presence of Ca^{2+} (data not shown). In the absence of Ca^{2+} annexin XII associated with vesicles only at mildly acidic pH, with half-maximal vesicle association occurring at approximately pH 5.8 (Figure 1).

The following experiments were performed to determine whether association of annexins V and XII with vesicles at low pH was reversible. Annexin V was incubated with vesicles in the absence of Ca^{2+} at pH 4.5, and the solution then was centrifuged. The pellet containing vesicles and associated protein was resuspended in a solution buffered to pH 7.4 and again was centrifuged. Approximately 85% of the annexin V that was associated with vesicles at low pH (Figure 2A) was released following incubation at neutral pH (Figure 2B). The annexin V that was released by the neutral pH treatment still retained the ability to bind to vesicles at neutral pH in the presence of Ca^{2+} (Figure 2C). Annexin XII behaved in a manner very similar to that of annexin V in these assays (Figure 2). These experiments showed that annexins V and XII could be reversibly converted from a form that underwent Ca^{2+}-dependent association with vesicles to a form that underwent Ca^{2+}-independent association at low pH.

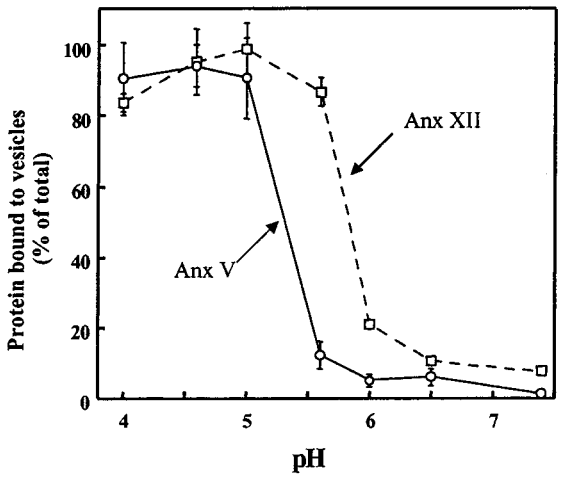

FIGURE 1: Association of annexins V and XII with phospholipid vesicles as a function of pH in the absence of Ca^{2+}. Annexin V or XII was incubated with phospholipid vesicles at the indicated pH in the presence of EGTA (500 μM). The solutions were centrifuged to separate the vesicles and associated protein from soluble protein. The pellets of triplicate samples were analyzed by SDS−PAGE followed by staining with Coomassie blue and densitometry as described in the Experimental Procedures. The results are expressed as a percentage of the total protein added. The data shown are typical of the results from four experiments, each of which gave the same pH response curve.

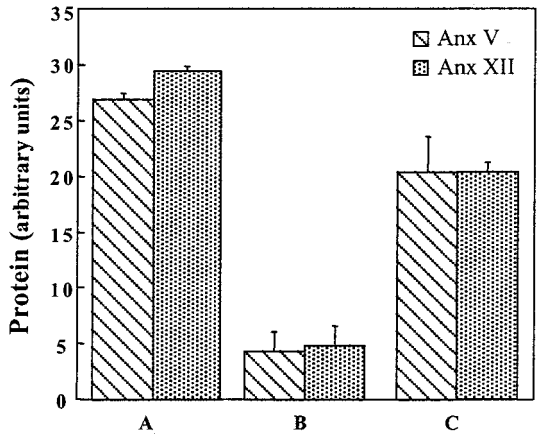

FIGURE 2: Reversibility of annexins V and XII binding to vesicles. Annexin V or XII was incubated with phospholipid vesicles at pH 4.5 in the presence of EGTA and then centrifuged as described in the Experimental Procedures. Protein in the pellet was analyzed by SDS−PAGE followed by Coomassie blue staining and densitometry and is represented by histogram A. Replicate pellets containing protein−vesicle complexes were suspended in pH 7.4 buffer, incubated for 10 min, and then centrifuged. The protein in the second pellet was analyzed (histogram B). The protein in the supernatant from B was incubated with vesicles in the presence of Ca^{2+} at pH 7.4 and then was centrifuged to form pellet C. The entire experiment was performed at room temperature. The data reported correspond to values from the scanned gels in arbitrary units.

Labeling Membrane-Associated Annexins with ^{125}I-TID. Previous studies have established that Ca^{2+}-dependent association of annexins V and XII with phospholipid bilayers at neutral pH occurs by peripheral binding of trimers of the proteins to the surface of bilayers (*9*, *21*−*23*). In contrast site-directed spin-labeling studies indicate that under mildly acidic conditions annexin XII inserts into the bilayer and does not form the trimer (*6*). As an independent test of the topography of annexins on bilayers, we investigated their

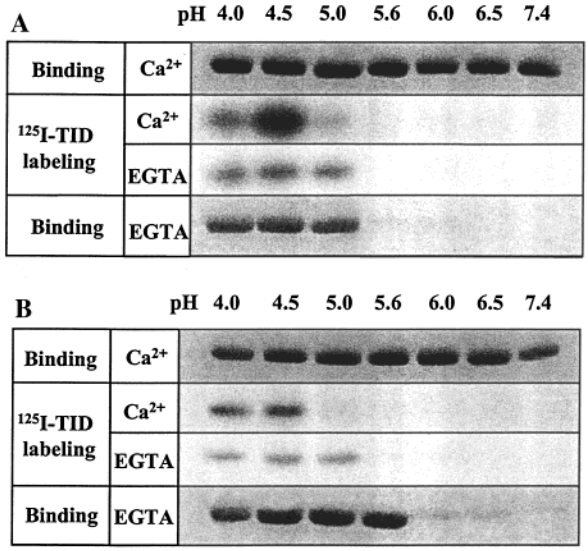

FIGURE 3: Labeling of annexins V and XII with ^{125}I-TID. Annexins V (panel A) and XII (panel B) were incubated with phospholipid vesicles at the indicated pH in the presence of either Ca^{2+} or EGTA and then labeled with ^{125}I-TID as described in the Experimental Procedures. The entire sample then was subjected to SDS−PAGE and autoradiography. The radioactive annexin bands on the autoradiogram are shown in the rows marked "^{125}I-TID labeling". No other radioactive bands were observed except for low molecular weight material at the dye front of the gel. The data shown are typical of five experiments, each of which gave the same pH-dependent response. The upper and lower rows show the results from parallel vesicle binding assays in which annexin association with vesicle pellets was analyzed by Coomassie blue staining as described in the legend of Figure 1 and the Experimental Procedures. In parallel control experiments under the above conditions at pH 4.0 and 7.4, no detectable label was incorporated into carbonic anhydrase, while the integral membrane protein, major intrinsic protein, reconstituted into liposomes (*37*) was labeled at a level comparable to that of the most heavily labeled annexin bands (data not shown). Other control experiments showed that the annexins were not labeled when incubated with ^{125}I-TID in the absence of vesicles (data not shown).

ability to react with ^{125}I-TID, a photoactivatable hydrophobic reagent which selectively partitions into the hydrophobic core of bilayers and labels proteins exposed to this region (*11*, *12*). After photoactivation this reagent labels proteins that are exposed to the hydrophobic core of bilayers but does not label peripheral membrane or soluble proteins (*12*). At neutral pH in the presence of Ca^{2+}, conditions at which annexins bind to the surface of bilayers (*9*, *21*−*23*), neither annexin V nor annexin XII was radiolabeled with ^{125}I-TID (Figure 3). In striking contrast, both annexins were labeled by ^{125}I-TID at pH $\approx$ 5 or below, indicating that they were exposed to the core of the bilayers (Figure 3). In the absence of Ca^{2+}, half-maximal labeling of both annexins V and XII occurred between pH 5.0 and pH 5.6. A slightly lower pH was required for maximal labeling if Ca^{2+} was present (Figure 3), thereby implying that Ca^{2+} stabilizes the peripherally bound form and keeps it from inserting into the hydrophobic core of the bilayer. It is also interesting to note that, in the absence of Ca^{2+}, annexin XII bound to vesicles at pH 5.6 yet was not labeled with ^{125}I-TID (Figure 3B). This raises the possibility that a Ca^{2+}-independent surface-bound intermediate exists before annexin XII inserts into the bilayer.

Partitioning of Annexins in Trition X-114. The response of annexins to low pH also was investigated by measuring

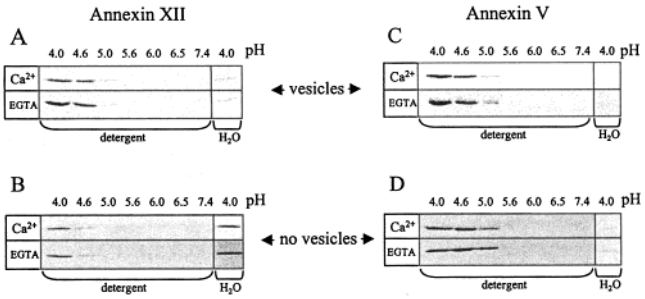

FIGURE 4: Triton X-114 phase separation of annexins V and XII. Annexin XII (panels A and B) and V (panels C and D) were incubated in the presence (panels A and C) or absence (panels B and D) of phospholipid vesicles in solutions containing either Ca^{2+} or EGTA and then mixed with Triton X-114 at 4 °C, a temperature below the detergent cloud point. After the temperature was raised to induce phase separation, the hydrophobic detergent phase was separated from the aqueous phase as described in the Experimental Procedures. Aliquots of the detergent and aqueous phases were analyzed by SDS−PAGE, and the Coomassie blue-stained annexin bands are shown in the figure. The data shown are typical of three experiments, each of which gave the same pH-dependent response. In parallel control experiments at either pH 7.4 or pH 4.0, bovine serum albumin and carbonic anhydrase were recovered in the aqueous phase, while the integral membrane protein, major intrinsic protein, was recovered in the detergent phase (data not shown).

their behavior in a Triton X-114 partitioning assay (*13*). In this assay, membrane proteins were solubilized in a solution of Triton X-114, and phase separation then was induced by increasing the temperature to 30 °C. Hydrophilic peripheral membrane proteins should partition into the aqueous phase, and amphiphilic integral membrane proteins should be soluble in the hydrophobic detergent phase (*13*). At neutral pH annexin V was not detected in the detergent phase following incubation in either the presence (Figure 4C) or absence (Figure 4D) of phospholipid vesicles. However, at pH $\approx$ 5 and below, annexin V was found in increasing amounts in the detergent phase. At pH 4.0 in the presence of vesicles, there was no detectable annexin V remaining in the aqueous phase, and in the absence of vesicles only a small amount (<10%) was in the aqueous phase (Figure 4C,D). Ca^{2+} had little effect on the behavior of annexin V in this assay. Thus, annexin V had the properties of a hydrophilic protein at neutral pH and of an amphiphilic integral membrane protein below pH 5.0. Annexin XII behaved very similarly to annexin V in the presence of phospholipid vesicles, with the amount of protein in the detergent phase increasing from <5% to >90% as the pH was dropped from 7.4 to 4.0 (Figure 4A). In contrast to that of annexin V, only partial (~35%) partitioning of annexin XII into the detergent phase occurred at pH 4.0 in the absence of phospholipid vesicles (Figure 4B).

Ion Channel Activity of Annexins V and XII. The effects of annexins V and XII on lipid bilayer conductance as a function of pH were also investigated. Although we observed bilayers with added annexin V or XII for many hours, no annexin-induced channels were ever observed at pH 7.4 in either the presence or absence of Ca^{2+}. Thus, our failure to observe channels at neutral pH was not due to lack of effort. In contrast, we found that both annexins V and XII induced ion channels in lipid bilayers when the pH was lowered to 6.0−6.5 even in the absence of added Ca^{2+}. Lowering the pH below 6.0 in the presence of either annexin XII or annexin V usually was followed by a rapid increase in bilayer

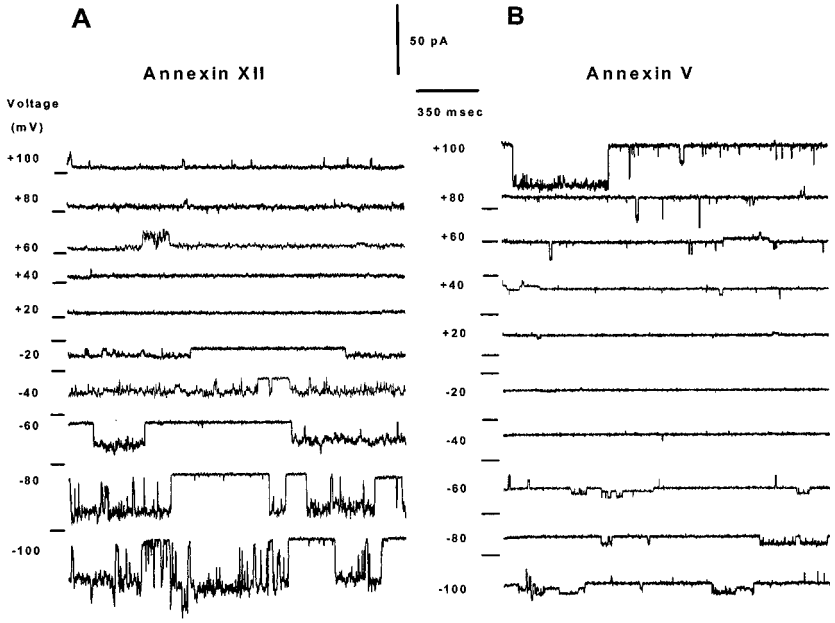

FIGURE 5: Channels produced by annexins V and XII in lipid bilayers. Bilayers were formed from the mixture of PC and PS (1:1 by weight). Annexins were added at the front chamber; both chambers contained 100 mM NaCl. The rear chamber was taken as ground. Traces are labeled by the applied voltage step in millivolts. For clarity, the point of zero current at zero voltage has been shifted to separate the traces. A short bar on the left indicates the point of zero current for each trace. The order of the zero current bars and the current traces is the same from top to bottom. (A) Channels produced by annexin XII (100 nM) at pH 6.5. (B) Channels produced by annexin V (200 nM) at pH 6.0.

conductance, typically resulting in the destruction of the bilayer. Although the electrophysiological changes occurred at a higher pH than the biochemical changes reported above (Figures 1, 3, and 4), it is important to remember that the association of annexins V and XII with bilayers at low pH was reversible (Figure 2), thereby indicating that the free and bound states are in equilibrium. We interpret the channel activity and membrane destruction in the bilayer chamber as being caused by a small fraction of the total annexin that is in the inserted state even at pH values too high for detection by the less sensitive biochemical methods. A number of site-directed mutants of annexins V and XII had different electrophysiological characteristics even though they were purified by the same procedures used to purify the wild-type proteins used herein (data not shown). These observations suggest that the annexins and not a contamination in the preparation mediated the observed channel activity because it is unlikely that each preparation would contain a different contamination that produced a distinctively different artifact.

Typical records of the channels formed by annexins V and XII in phosphatidylserine-containing bilayers are shown in Figure 5. On the basis of the number of simultaneous openings to the main conductance level seen, parts A and B of Figure 5 show one channel each. We do not have enough data to perform detailed statistical analysis based on the probability of one channel closing and another opening, but the simplest and most likely interpretation of the data is that there is only one channel present in each of the membranes from which the data in Figure 5 were recorded. The single-channel conductance amplitudes induced by both annexins are similar and show multiple single-channel levels with conductance values between ~80 and 320 pS in 100 mM NaCl. Both annexin V and annexin XII channels are voltage-dependent. Annexin XII channels tend to open at negative voltages (Figure 5A), and annexin V channels tend to open at higher voltages regardless of their sign (Figure 5B). These asymmetries may be a result of the direction of insertion of the channels, but we cannot yet say whether the direction of insertion is random. Thus, we do not know whether the difference in asymmetry in annexins V and XII arises from differences in the topography of insertion or from the amino acid sequences of the proteins.

Annexin XII showed (Figure 6A) a linear IV curve (generated by a voltage sweep) which (assuming the principal conductance level of Figure 5A corresponds to one channel) probably reflects one channel in the membrane. Annexin V showed (Figure 6B) a swept IV curve which (assuming the principal conductance level of Figure 5B corresponds to one channel) probably reflects about five channels in the membrane. Both annexins V and XII channels show weak cation selectivity in asymmetrical NaCl solutions (Figure 6). For annexin XII the reversal potential was 11 mV in a 1:2 ratio of NaCl concentration, giving a calculated Na^+:Cl^- permeability ratio of 4.7. For annexin V the reversal potential was 20 mV in a 1:3 ratio of NaCl concentration, giving a calculated Na^+:Cl^- permeability ratio of 5.0.

DISCUSSION

Annexins are soluble proteins that can bind with high affinity to the surface of phospholipid membranes in a Ca^{2+}-dependent manner (*1*). Although these membrane-surface forms of annexins surely are biologically important, they do not suggest plausible mechanisms to explain certain activities of annexins including their ability to cross membranes (*24*), cause membrane fusion (*25*), and mediate ion channel activity (*5*). Insights into the mechanisms underlying these activities may come from a small but growing body of literature indicating that Ca^{2+}-independent membrane association can occur at low pH in vitro. Annexin V has been shown to induce pH-dependent vesicle aggregation (*26*), vesicle fusion

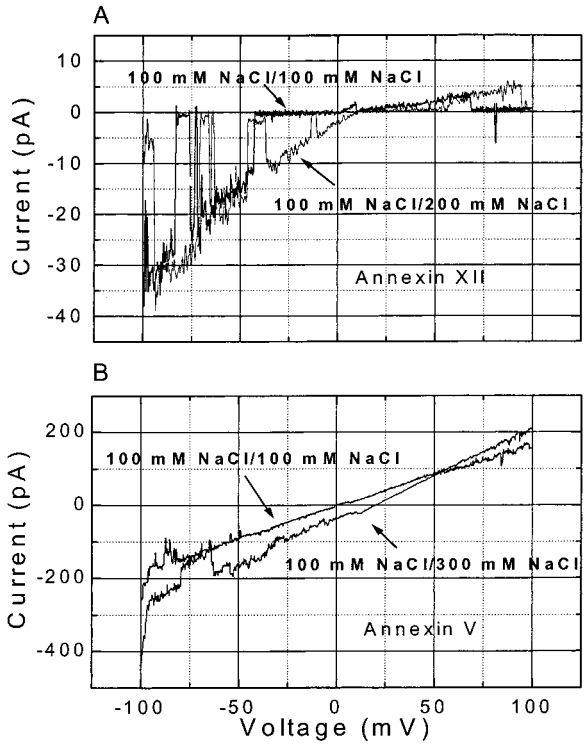

FIGURE 6: Current−voltage curves of bilayers containing annexins V and XII channels in symmetrical and asymmetrical ionic conditions. Using the same experimental conditions described in Figure 5, the voltage was ramped from −100 to +100 mV at a rate of 20 mV/s while the current was recorded in the presence of either annexin XII (panel A) or annexin V (panel B). KCl-filled agar bridges were used in these selectivity experiments. After the trace in symmetrical ionic conditions was recorded for either annexin XII (panel A) or annexin V (panel B), an appropriate amount of 4 M NaCl was added to the rear chamber to increase the concentration to either 200 mM (panel A) or 300 mM (panel B). The reversal potential shifted from 0 to 11 mV for annexin XII and from 0 to 20 mV for annexin V, thereby indicating that both were cation selective.

(*7*), and exposure of hydrophobic residues to solvent in the absence of vesicles (*27*). Site-directed spin-labeling studies of annexin XII (*6*) and measurement of the surface pressure of phospholipid monolayers in the presence of annexin I (*8*) indicated that these proteins penetrated into the hydrophobic core of the membrane. Studies reported herein investigated annexins V and XII and showed that they can reversibly insert into membranes and form ion channels at mildly acidic pH.

Both annexins V and XII reversibly associated with phosphatidylserine-containing vesicles in the absence of Ca^{2+} with half-maximal association occurring at pH 5.3 and 5.8, respectively (Figures 1 and 2). Following association with vesicles at low pH, annexins V and XII could be labeled with the hydrophobic, photoactivatable reagent ^{125}I-TID (Figure 3). In contrast, these proteins were not labeled with ^{125}I-TID when they were bound to the surface of bilayers at neutral pH in the presence of Ca^{2+} (Figure 3). Thus, they appear to undergo pH-dependent insertion into the hydrophobic core of the bilayer. Although numerous reports have shown that ^{125}I-TID reacted with proteins that were exposed to the hydrophobic core of bilayers, there was at least one report in which this reagent labeled a hydrophobic pocket in a water-soluble protein (*28*). However, labeling artifacts does not appear to be likely for annexins V and XII because they were not labeled when bound to the surface of the bilayer. Furthermore, labeling was dependent on the phospholipid composition of the vesicles. ^{125}I-TID labeling of annexins XII and V was either undetected or reduced ~10-fold, respectively, at low pH when phosphatidylserine-containing vesicles were replaced with vesicles containing only phosphatidylcholine (data not shown).

The membrane topography of annexins also was probed using a phase separation assay in which integral membrane proteins partition into the Triton X-114 detergent phase while hydrophilic proteins partition into the aqueous phase (*13*). In the presence of phosphatidylserine-containing vesicles, annexins V and XII partitioned into the aqueous phase at neutral pH and into the hydrophobic detergent phase (Figure 4) with a pH dependence very similar to that observed for ^{125}I-TID labeling (Figure 3). Annexin XII but not annexin V required phosphatidylserine-containing vesicles for pH-induced association with the detergent phase (Figure 4). Previous site-directed spin-labeling studies of annexin XII also showed that pH-induced structural changes were minimal until vesicles were added (*6*). Vesicles containing only phosphatidylcholine showed little effect on the partitioning of either annexin V or annexin XII (data not shown).

Our site-directed spin-labeling studies of annexin XII showed that, in the presence of membranes at low pH, there were major changes in the tertiary structure of annexin XII (*6*). Detailed analysis of a helical hairpin that is involved in forming one of the type II Ca^{2+}-binding sites showed that the region refolded at low pH and formed a continuous transmembrane α-helix (*6*). The transmembrane helix had a hydrophobic face that was exposed to the acyl chains of the phospholipid bilayer and a hydrophilic face that was solvated by water. We proposed that several transmembrane amphipathic helices in annexin XII assembled to form a structure in which the hydrophilic faces of the helices formed a central transmembrane aqueous pore. We proposed that the trigger for membrane insertion was protonation of Glu or Asp residues on the hydrophobic face of the transmembrane helices. Many of the putative switch residues are likely to participate in Ca^{2+} coordination in the membrane-surface form of the protein at neutral pH. Data presented in the current study are entirely consistent with the spin-label studies and provide independent support for the proposal that annexin XII inserts into bilayers. In addition, the current study provides the first direct experimental evidence that annexin V inserts into bilayers. Inspection of the amino acid sequence of human annexin V reveals several putative transmembrane amphipathic helices containing ~20 amino acids. Like annexin XII, most of these sequences in annexin V are around Ca^{2+}-binding A−B and D−E loops and contain putative Glu or Asp switch residues on the hydrophobic faces.

To our knowledge, annexins V and XII are the only proteins known to be capable of reversibly adopting a soluble, a peripherally membrane-bound, and a transmembrane state. The model presented in Figure 7 illustrates these three different forms and shows how H^+ and Ca^{2+} regulate their interconversion. The soluble monomer and the surface trimer have similar backbone folds, while the structure of the transmembrane form is unknown but clearly is radically different. A single amphipathic transmembrane helix of annexin XII has been characterized (*6*), and the other

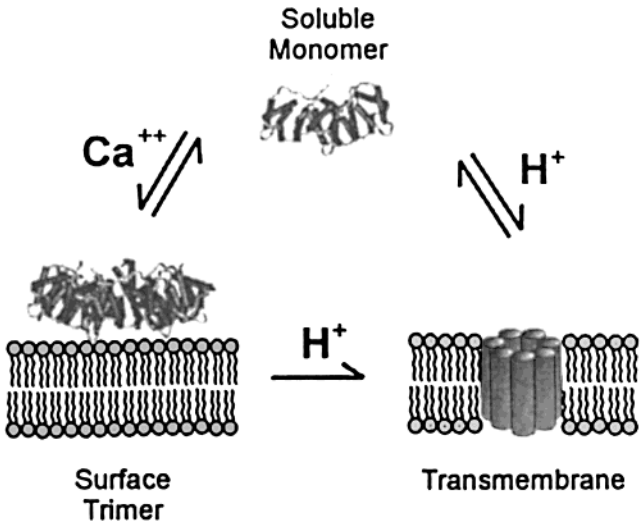

FIGURE 7: Model of Ca^{2+} and H^+ Switching of annexin XII membrane association. Annexin XII is a monomer in solution and assembles into trimers on the surface of membranes in the presence of Ca^{2+} (*9*). The backbone folds of both the monomer and trimer are similar to the fold observed in the crystal structure of the protein (*9*). At low pH, major structural reorganization occurs due to protonation of carboxylate switch residues, and the protein inserts into bilayers. The transmembrane form is depicted as a monomer with seven transmembrane helices, but there are no experimental data concerning the quaternary structure or the number of transmembrane helices. Annexin XII can be reversibly converted between the three states, with the equilibrium being regulated by the concentration of H^+ and Ca^{2+}. It is not yet known whether the transmembrane form can be directly converted to the surface trimer by raising the pH and Ca^{2+} concentration. The transition between the different forms is not necessarily a single-step process as illustrated. In fact, at pH 5.6, annexin XII associated with bilayers but was not labeled with ^{125}I-TID (Figure 3B), thereby implying that an intermediate between the soluble and transmembrane forms exists on the surface of the bilayer. This model also appears to apply to annexin V in addition to annexin XII. However, the structures of other annexins on bilayers have not been determined, so it is not known whether the model applies to them.

transmembrane helices are speculative on the basis of amino acid sequence analysis of annexins V and XII. This model predicts a central water-filled pore that could function as a channel.

To test the transmembrane model of annexin structure, annexins V and XII channel activities were investigated in planar bilayers, a method that is not as sensitive as patch pipets but is less prone to artifacts (*29*, *30*). Using this method, single-channel currents were reproducibly detected when annexins V and XII were incubated with phosphatidylserine-containing bilayers at pH 6.0−6.5 (Figure 5) but never at neutral pH in either the presence or absence of Ca^{2+}. This is the first time annexin XII ion channel activity has been investigated, but several studies of annexin V have been reported (*31*−*33*). In general the previous studies of annexin V were performed in the presence of high concentrations of Ca^{2+} and at neutral pH—conditions under which we did not detect channel activity. The previously reported single-channel conductance values of annexin V studied by patch pipet methods at neutral pH in the presence of high Ca^{2+} were of a single level and had significantly lower values (~30 pS) than the ones observed herein (Figures 5 and 6). The reason for the differences between our planar bilayer channel data and these studies is not yet known. In one previous study, annexin V was investigated in planar bilayers (*33*). In that study annexin V solutions buffered to pH 5.6 were incorporated into bilayers, and they produced a range of conductance levels that overlapped the range observed herein (Figures 5 and 6). Thus, methods that use planar bilayers and methods that use patch pipets may observe different characteristics of annexin channels.

Although additional studies are required to reconcile all reported annexin channel data, our model of annexin insertion into bilayers (Figure 7) provides a structural framework for explaining annexin-mediated channel activity in vitro. The important question of whether annexin channels are physiologically significant remains unanswered. Several reports have shown that different annexins, including annexin XII (*17*), can associate with biological membranes in a Ca^{2+}-independent manner (*1*). Furthermore, two studies reported membrane association of annexins that may have involved insertion; annexin V from bone matrix vesicles (*34*) and annexin XIIIb from epithelial transport vesicles (*35*, *36*) had the properties of integral membrane proteins in lipophilic partitioning assays. Annexins clearly are primarily cytosolic proteins, and the pH of the cytosol is significantly higher than the pH required to induce membrane association and insertion of a large enough fraction of protein to be detected by biochemical methods (Figures 1, 3, and 4). However, membrane insertion could be induced by transient local decreases in pH, by specialized local phospholipid environments that alter the pK_a values of key annexin switch residues, or by unidentified accessory proteins. Furthermore, since the different forms of annexins are in equilibrium (Figures 2 and 7), a very small but still physiologically significant fraction of the cellular annexin could be in the inserted form.

Recent studies identified annexin 31, a human annexin with novel features in its amino acid sequence at key positions in the core domain. It lacks the Glu or Asp residues that coordinate Ca^{2+} in the type II Ca^{2+} sites in all four of its D−E loops (*6*). It will be interesting to determine whether this unusual annexin which lacks these putative carboxylate switch residues has lost the ability to undergo Ca^{2+}-dependent binding to membranes and/or gained the ability to insert into membranes at neutral pH. A remarkable range of versatility is possible within the annexin family, because, in addition to having multiple gene products with different amino acid sequences, their tertiary/quaternary structure and function can be modulated by environmental factors including the concentration of H^+ and Ca^{2+} and the lipid composition of membranes.

In summary, this study showed that annexins V and XII associated with membranes in the absence of Ca^{2+} at low pH. Evidence was presented that these forms of the proteins were exposed to the hydrophobic core of the bilayer and formed ion channels. On the basis of the observation that several putative transmembrane amphipathic helices containing carboxylate switch residues could be recognized within the amino acid sequence of annexins V and XII, a model for annexin membrane insertion and ion channel formation was proposed. These studies form a structural framework for evaluating possible physiological functions of annexin channels.

ACKNOWLEDGMENT

We gratefully thank Elizabeth M. Baer and Christine C. Jao for their excellent technical support.

REFERENCES

1. Seaton, B. A. (1996) in *Annexins: Molecular Structure to Cellular Function* (Seaton, B. A., Ed.) R. G. Landes Co., Austin, TX.
2. Morgan, R. O., Bell, D. W., Testa, J. R., and Fernandez, M. P. (1999) *Gene 227*, 33−8.
3. Huber, R., Schneider, M., Mayr, I., Romisch, J., and Paques, E. P. (1990) *FEBS Lett. 275*, 15−21.
4. Swairjo, M. A., Concha, N. O., Kaetzel, M. A., Dedman, J. R., and Seaton, B. A. (1995) *Nat. Struct. Biol. 2*, 968−74.
5. Luecke, H., Chang, B. T., Mailliard, W. S., Schlaepfer, D. D., and Haigler, H. T. (1995) *Nature 378*, 512−515.
6. Langen, R., Isas, J., Hubbell, W., and Haigler, H. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 14060−14065.
7. Kohler, G., Hering, U., Zschoring, O., and Arnold, K. (1997) *Biochemistry 36*, 8189−8194.
8. Rosengarth, A., Wintergalen, A., Galla, H. J., Hinz, H. J., and Gerke, V. (1998) *FEBS Lett. 438*, 279−284.
9. Langen, R., Isas, J. M., Lueke, H., Haigler, T. H., and Hubbell, W. L. (1998) *J. Biol. Chem. 273*, 22453−22457.
10. Wimley, W. C., Creamer, T. P., and White, S. H. (1996) *Biochemistry 35*, 5109−5124.
11. Brunner, J., and Semenza, G. (1981) *Biochemistry 20*, 7174−7182.
12. Hoppe, J., Brunner, J., and Jørgensen, B. B. (1984) *Biochemistry 23*, 5610−5616.
13. Bordier, C. (1981) *J. Biol. Chem. 256*, 1604−7.
14. Schlaepfer, D. D., Jones, J., and Haigler, H. T. (1992) *Biochemistry 31*, 1886−91.
15. Mailliard, W. S., Leucke, H., and Haigler, H. T. (1997) *Biochemistry 36*, 9045−9050.
16. Reeves, J. P., and Dowben, R. M. (1969) *J. Cell. Physiol. 73*, 49−60.
17. Schlaepfer, D. D., Fisher, D. A., Brandt, M. E., Bode, H. R., Jones, J. M., and Haigler, H. T. (1992) *J. Biol. Chem. 267*, 9529−39.
18. Schlaepfer, D. D., Mehlman, T., Burgess, W. H., and Haigler, H. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6078−82.
19. Zampighi, G. A., Hall, J. E., and Kreman, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8468−72.
20. Ehring, G. R., Lagos, N., Zampighi, G. A., and Hall, J. E. (1992) *J. Membr. Biol. 126*, 75−88.
21. Olofsson, A., Mallouh, V., and Brisson, A. (1994) *J. Struct. Biol. 113*, 199−205.
22. Voges, D. B., R. Burger, A., Demange, P., Baumeister, W., Huber, R. (1994) *J. Mol. Biol. 238*, 199−213.
23. Reviakine, I. B.-S., W., Brisson, A. (1998) *J. Struct. Biol. 121*, 356−361.
24. Christmas, P., Callaway, J., Fallon, J., Jones, J., and Haigler, H. T. (1991) *J. Biol. Chem. 266*, 2499−507.
25. Creutz, C. E. (1992) *Science 258*, 924−31.
26. Hoekstra, D., Buist-Arkema, R., Klappe, K., and Reutelingsperger, C. P. (1993) *Biochemistry 32*, 14194−202.
27. Sopkova, J., Vincent, M., Takahashi, M., Lewit-Bentley, A., and Gallay, J. (1998) *Biochemistry 37*, 11962−70.
28. Krebs, J., Buerkler, J., Guerini, D., Brunner, J., and Carafoli, E. (1984) *Biochemistry 23*, 400−3.
29. Coronado, R., and Latorre, R. (1983) *Biophys. J. 43*, 231−6.
30. Coronado, R. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 259−77.
31. Rojas, E., Pollard, H. B., Haigler, H. T., Parra, C., and Burns, A. L. (1990) *J. Biol. Chem. 265*, 21207−15.
32. Demange, P., Voges, D., Benz, J., Liemann, S., Gottig, P., Berendes, R., Burger, A., and Huber, R. (1994) *Trends Biochem. Sci. 19*, 272−6.
33. Arispe, N., Rojas, E., Genge, B. R., Wu, L. N., and Wuthier, R. E. (1996) *Biophys. J. 71*, 1764−75.
34. Genge, B. R., Wu, L. N., Adkisson, H. D. t., and Wuthier, R. E. (1991) *J. Biol. Chem. 266*, 10678−85.
35. Fiedler, K., Lafont, F., Parton, R. G., and Simons, K. (1995) *J. Cell Biol. 128*, 1043−53.
36. Wandinger-Ness, A., Bennett, M. K., Antony, C., and Simons, K. (1990) *J. Cell Biol. 111*, 987−1000.
37. Ehring, G. R., Zampighi, G., Horwitz, J., Bok, D., and Hall, J. E. (1990) *J. Gen. Physiol. 96*, 631−64.

BI9922401