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Lipid organization regulates Annexin A2 Ca²⁺-sensitivity for membrane bridging and its modulator effects on membrane fluidity

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Abstract

Annexin A2 (AnxA2) is a phospholipid binding protein that has been implicated in many membrane-related cellular functions. AnxA2 is able to bind different acidic phospholipids such as phosphatidylserine (PS) and phosphatidylinositol-4,5-bisphosphate (PI2P). This binding is mediated by Ca²⁺-dependent and Ca²⁺-independent mechanisms. The specific functions of annexin A2 related to these two phospholipids and the molecular mechanisms involved in their interaction remain obscure. Herein we studied the influence of lipid composition on the Ca²⁺-dependency of AnxA2-mediated membrane bridging and on membrane fluidity. Membrane models of ten different lipid compositions and detergent-resistant membranes from two cellular sources were investigated. The results show that the AnxA2-mediated membrane bridging requires 3 to 50 times less calcium for PS-membranes than for PI2P-membranes. Membrane fluidity was measured by the ratiometric fluorescence parameter generalized polarization method with two fluorescent probes. Compared to controls containing low phospholipid ligand, AnxA2 was found to reduce the membrane fluidity of

PI2P-membranes twice as much as the PS-membranes in the presence of calcium. On the contrary, at mild acidic pH in the absence of calcium AnxA2 reduces the fluidity of the PS-membranes more than the PI2P-membranes. The presence of cholesterol on the bilayer reduced the AnxA2 capacity to reduce membrane fluidity. The presented data shed light on the specific roles of PI2P, PS and cholesterol present on membranes related to the action of Annexin A2 as a membrane bridging molecule during exocytosis and endocytosis events and as a plasma membrane domain phospholipid packing regulator.

Keywords: Anionic phospholipids; Annexin A2; Calcium; Cholesterol; Membrane domains; Membrane fluidity.

1. Introduction

The characterization of lipid diversity and the distribution of specific lipids in cell organelles and plasma membrane domains is becoming essential for the fine understanding of membrane cell biology (for a review see [1]). Annexins (Anxs) ^(footnote) are a family of calcium and phospholipid binding proteins involved in different membrane-related cellular functions [2, 3]. Anxs are found in different organelles and specific regions of the plasma membrane and show different affinities for cellular membranes with specific calcium requirements [4, 5]. Their Ca²⁺-sensitivity for binding to different phospholipids has been studied in vitro revealing that each Anx possesses specific preferences for different phospholipids. For example, AnxA5 Ca²⁺-sensitivity was reported to be in the range of 0.16 to 0.75 mM for the following phospholipids in the order dioleoylphosphatidylglycerol < dioleoylphosphatidylserine < phosphatidylinositol (PI) < phosphatidic acid (PA) [6]. For AnxA4, the Ca²⁺-sensitivity was in the range of 40 to 120 μM for PA < phosphatidylserine (PS) < phosphatidylglycerol (PG) < phosphatidylinositol-4,5-bisphosphate (PI2P) < PI in this order [7]. Moreover, the individual calcium binding sites of the four annexin repeats can show characteristic affinities for the different phospholipids. For example, the fourth repeat of AnxA4 was reported to be involved in PS and PI binding but not in PG binding [8].

Phospholipid organization and distribution can be modified by protein binding. Phospholipid clustering by Anxs has been reported in vitro and in vivo. AnxA2 is able to induce PS and PI2P segregation and clustering in vitro [9, 10]. Anxs A4 and A6 were also shown to induce

clustering of their binding phospholipids PG and PA respectively [11, 12]. Moreover, the speed of PA clustering depended on the presence of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [13] indicating that lipids not directly bound to the protein are able to modulate protein-membrane interactions. Several experiments with cells suggested that the annexin family of proteins is responsible for Ca^{2+} -dependent lipid segregation in muscle cells [14] and that AnxA2 is involved in microdomain regulation of exocytic sites [15]. Cholesterol plays also an important role in Annexins functions by modulating the binding to negatively charged phospholipids [16-18] (for a review see [19]), and recently it was shown that ceramide increases AnxA1 binding to biological and artificial membranes [20]. Finally, the binding and cellular distribution of AnxA6 in Ca^{2+} -dependent and independent pools was shown to be modulated by cholesterol [21].

Several experiments demonstrated that cholesterol regulates the Ca^{2+} -dependent and independent binding of AnxA2 to membranes *in vitro* [22] and *in vivo* [17, 23]. Moreover, this Ca^{2+} -independent binding to membranes seemed to be induced by acidic pH as demonstrated *in vitro* [24, 25] and *in vivo* [26]. The role of PS in AnxA2 membrane binding is well known, but the role of PI2P in actin cytoskeleton membrane-related processes [27-29], in macropinocytosis [30] and exocytosis [31] has also been reported leading to the possibility that AnxA2 plays different cellular roles in membranes depending on their enrichment in PS or PI2P.

In spite of the fact that many studies on lipid-AnxA2 interaction have been performed, a simple explanation of the annexin A2 effects on the membranes including different parameters such as calcium concentration, pH, lipid composition and the physicochemical properties of the membrane is lacking. In this study we performed comparative experiments with large unilamellar vesicles (LUV) of different composition focusing on the relative importance of PS, PI2P and cholesterol on AnxA2-membrane interactions. The results show that AnxA2 interaction with membranes and its effects depend on lipid organization. The Ca^{2+} -sensitivity of AnxA2 is higher for PS-rich than for PI2P-rich membranes but it exerts a stronger reduction of membrane fluidity in PI2P-rich membranes. The membrane fluidity changes were also observed in cellular detergent-resistant membranes.

2. Material and Methods

2.1. Reagents. Egg yolk L- α -phosphatidylcholine (PC), egg yolk L- α -phosphatidylethanolamine (PE), brain L- α -glycerophosphatidyl-L-serine (PS) and cholesterol were purchased from Sigma-Aldrich. Brain L- α -phosphatidylinositol-4,5-bisphosphate (PI2P) was from Avanti polar lipids. Laurdan was from Molecular Probes. Di-4-ANNEPDHQ was obtained from Dr Leslie M. Loew (Connecticut, USA). Recombinant human AnxA2 and human S100A10 were purified as previously described [32]. The tetrameric complex (AnxA2T; (AnxA2-S100A10)₂) was obtained by mixing equimolar quantities of AnxA2 and S100A10. The rabbit polyclonal anti-AnxA2 was produced in our laboratory. The rabbit polyclonal anti-Caveolin1 was from Santa Cruz Biotechnology. The anti-S100A10 and the anti-E-cadherin were from Transduction Laboratories. The goat anti-rabbit IgG-peroxidase and the secondary fluorescent (FITC, CY3 and CY5) conjugated anti-rabbit or anti-mouse antibodies were from Jackson ImmunoResearch.

2.2. Large Unilamellar Vesicles. Multilamellar vesicles (MLVs) were obtained by dissolving the appropriate amounts of lipids (see table 1) in a mixture of chloroform and methanol, 2/1 (v/v), followed by solvent evaporation under nitrogen. For fluorescence experiments, lauridan was added at 0.1% weight. Lipid films were hydrated with buffer A (40 mM HEPES pH 7, 30 mM KCl, 1 mM EGTA) and vortexed extensively. Large unilamellar vesicles (LUV) were prepared by extrusion of MLVs through a polycarbonate filter (pore diameter 100 nm) as previously described [33].

2.3. Membrane bridging. LUVs aggregation by annexin A2-mediated membrane bridging was monitored by turbidimetry at 340 nm with a Cary spectrophotometer (Varian) as described [34]. The temperature was regulated with a Peltier device and controlled by a thermocouple. Free-Ca²⁺ concentration was controlled in buffer A as described [35]. The Ca²⁺ concentrations are expressed as pCa = -Log[Ca²⁺]. 5 μ g of monomeric or tetrameric AnxA2 were added to a final 250 μ l buffer containing 5 μ g of LUVs (20 μ g ml⁻¹ for both protein and phospholipids). In these conditions, the lipid/protein molar ratio for interaction is 25. Considering that an annexin protein can cover between 26 to 31 nm² and a phospholipid a surface of 0.6 nm² [6, 36, 37], there is enough protein to cover the entire surface of the vesicles. Moreover, in our pCa 3.3 and pH 4.5 experimental conditions there is 100% protein binding and vesicle aggregation [16, 22, 24, 32, 34]. In conclusion, the experiments are near saturation of bound

protein and therefore comparable. After protein addition the absorbance was followed for 7 minutes. At this time membrane aggregation is very close to the plateau. Graphics and curves fitting were performed with Graphpad Prism software.

2.4. Cell culture and immunofluorescence. Madin Darby Canine kidney (MDCK) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, France) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), penicillin (0.1 i.u./ml) and streptomycin (100 mg/ml). Cells were grown at 37°C in a 5% CO₂/air atmosphere. To obtain differentiated epithelial MDCK cells, the cultures were recovered 2 days after confluence. To obtain undifferentiated cells we used the Ca²⁺ switch strategy [38]. Briefly confluent MDCK cultures were incubated in a Ca²⁺-free medium for two days. This medium was prepared with Ca²⁺-free DMEM and serum dialyzed against PBS. For immunofluorescence the cells were cultured on glass cover-slips in 24 well plates. The cells were washed twice with PBS, and fixed with 3.7% paraformaldehyde in PBS for 15 minutes at room temperature. Then they were washed three times with PBS, NH₄Cl 50 mM and post-fixed and permeabilized with cold methanol (-20°C) for 1 minute and washed three times as before. Fixed cells were saturated with PBS, NH₄Cl 50 mM, BSA 1% for 20 minutes at room temperature. The first antibody was added at the right dilution during 1.5 hours at room temperature followed by three washes in saturation solution. The secondary antibody was incubated and washed as the primary antibody. Finally, the cover-slips were washed in water, dried and mounted in Mowiol. All the fluorescence confocal images were acquired with a TCS SP2 laser-scanning spectral system (Leica, Wetzlar, Germany) attached to a Leica DMR inverted microscope (Platform Imagerie cellulaire et tissulaire, IFR65) at the specific excitation wavelengths and appropriate emission channels for each fluorophore.

2.5. Detergent-resistant membranes from non differentiated and differentiated epithelial cells. The Triton X-100 detergent-resistant membranes (DRM) preparation was performed at 4°C. Cell cultures (from 75 mm flasks) were washed with PBS and lysed in buffer A containing Triton X-100 1% (w/v) and a protease inhibitor cocktail. The cell suspension was passed 10 times through G23 and G26 needles. The cell extract was diluted with 80% sucrose solution in buffer A to obtain a final 40% sucrose suspension (4 ml) that was loaded in a SW41 Beckman tube. 4 ml of 35% sucrose solution and 3.5 ml of 5% sucrose solution were sequentially added to the tube to obtain the discontinuous gradient. The tubes were centrifuged at 38 000 rpm for 18 hours at 4°C. After centrifugation, twelve 1 ml fractions

were recovered. Caveolin1 and AnxA2 contents were analyzed by Western blot on PVDF membranes by standard procedures using the ECL method (Amersham).

2.6. Fluorescence recording. Fluorescence measurements were performed with a Cary fluorimeter (Varian) as previously described [39]. The protein and LUV concentration and the temperature regulation were as described in section 2.3. The excitation and emission band-pass were set at 5 nm. For laurdan fluorescence, the emission spectra were recorded from 400 to 650 nm using 365 nm excitation. Sequential spectra were recorded as follows: first, free LUVs in buffer A, then the LUVs in the presence of calcium (0.5 mM), and finally 10 minutes after AnxA2 addition. For experiments at pH 4.5, the free-Ca²⁺ buffer B (40 mM acetate pH 4.5, 30 mM KCl, 1mM EGTA) was used. The excitation generalized polarization (GP) for laurdan was calculated as $GP = (I_{440} - I_{490}) / (I_{440} + I_{490})$, where I_{440} and I_{490} are the fluorescence intensities at the maximum emission wavelength in the ordered (440 nm) and disordered (490 nm) phases [40]. For DRMs, the probe di-4-ANNEPDHQ was used at 1 μ M. This probe such as laurdan, is able to monitor membrane fluidity [41]. Ca²⁺ was added to 200 μ l of DRMs for a final 500 μ M concentration. Then, 10 μ g of AnxA2 were added and incubated for 15 minutes. Finally, to measure reversibility, EGTA was added (2 mM) and incubated for 30 min. before spectrum recording. The sequential emission spectra were recorded from 500 to 750 nm using 485 nm excitation and the wavelengths used for GP calculation were 570 and 630 nm for the ordered and disordered phases respectively. The statistical significance of the GP differences in figure 3 was assessed by ANOVA with GraphPad Prism. * P<0.05, ** P<0.01, *** P<0.001.

3. Results

3.1. Ca²⁺-dependency for membrane bridging by AnxA2 depends on lipid composition. In order to study the role of lipid composition on the Ca²⁺-dependency of AnxA2 for membrane bridging, we compared large unilamellar vesicles (LUV) containing phosphatidylserine (PS) as the AnxA2 ligand phospholipid and different quantities of cholesterol, phosphatidylcholine and phosphatidylethanolamine (PE) (Table 1). Briefly, PS-LUV1 are rich in PS, PS-LUV2 are rich in PS and cholesterol, PS-LUV3 contain PS, PE and cholesterol in similar quantities, PS-LUV4 are rich in PE mimicking the inner leaflet of the plasma membrane without cholesterol and PS-LUV5 mimic the inner leaflet with cholesterol. Figure 1 shows that the Ca²⁺-

dependency for membrane bridging by AnxA2 in its monomeric and tetrameric (complexed to the S100A10 dimer) forms at 23 and 37°C depends on the phospholipid composition.

For the monomeric AnxA2, the higher Ca²⁺-sensitivity was observed for the PS-LUV3 at 23 and 37°C (see table 2). The half maximal aggregation was observed at pCa close to 5.8 (1.6 μM free-Ca²⁺). PS-LUV1 presented the lower Ca²⁺-sensitivity with a pCa close to 4.4 (40 μM free-Ca²⁺) at both temperatures. For the other PS-LUVs, the values were placed in between these calcium concentrations range. The Ca²⁺-sensitivity did not correlate with the quantity of any phospholipid in the membranes. PS-LUV3 contains less PS than PS-LUV1 but more than PS-LUV4 or 5 (see table 1). Inversely, PS-LUV3 contains more PE than PS-LUV1 and 2 but less than PS-LUV4 or 5. At 37°C, the cholesterol content does not explain the difference in calcium sensitivity because PS-LUV1 and 4 with no cholesterol showed a pCa for half maximal aggregation very different at 37°C (4 and 5.4 respectively), and PS-LUV2 and 3 with the same cholesterol content showed also a quite different pCa for half maximal aggregation (5 and 5.7 respectively). However, at 23°C, the Ca²⁺-sensitivity seemed to correlate with the cholesterol content showing lower Ca²⁺ concentration to bridge the PS-LUVs with cholesterol (tables 1 and 2).

For the tetrameric AnxA2, the Ca²⁺-sensitivity for membrane bridging at both temperatures was in the order: PS-LUV3>PS-LUV2>PS-LUV1>PS-LUV4>PS-LUV5 (Figure 1 and table 3). As for the monomer, the Ca²⁺-sensitivity does not correlate with any particular lipid. PS-LUV3, 4 and 5 contain PE, PS-LUV3, 2 and 5 contain cholesterol and the strongest quantity of the ligand phospholipid (PS) is in PS-LUV1 and 2. Overall, these results show that for both forms of the protein, there was no simple correlation of membrane bridging efficiency (Ca²⁺ concentration for half maximal effect) with the phospholipid composition.

Considering that the most important differences in Ca²⁺-dependency for membrane bridging were observed with the monomeric AnxA2, in the next experiments we compared only the monomeric AnxA2 bridging capacity on LUVs containing phosphatidylinositol-4,5-bisphosphate (PI2P-LUVs), the second phospholipid described to play an important role in AnxA2 binding to cell membranes. Figure 2 shows the Ca²⁺-dependency for aggregation of PI2P-LUVs at 23 and 37°C. The analysis of the Ca²⁺-sensitivity for aggregation revealed that, contrary to the PS-LUVs, the PI2P-LUVs 2 and 3 with high cholesterol content need more calcium to aggregate the membranes. The stronger Ca²⁺-sensitivity was observed for the

PI2P-LUVs with higher PE content and paradoxically with the lower PI2P content (table 2 and figure 2) suggesting that PE facilitates AnxA2 binding to PI2P.

The comparison of the AnxA2 Ca^{2+} -dependency for membrane bridging of PS versus PI2P-LUVs revealed different behavior for each phospholipid. The pCa for half maximal aggregation for PS-LUV5 and PI2P-LUV5 at both temperatures were about 5.2 and 5.4 respectively. This difference can be considered as not significant. However, the pCa for PS-LUV3 was about 5.8, a value completely different than that for PI2P-LUV3 (4.1 at both temperatures). The pCa values for LUV2 at 23°C showed also higher Ca^{2+} -sensitivity for PS than for PI2P containing LUVs (5.2 and 4.5 respectively).

These results show that the Ca^{2+} -sensitivity for AnxA2-mediated membrane bridging does not follow a simple rule based in the ligand phospholipid quantity, and suggest strongly that the determinant factor of Ca^{2+} -sensitivity for AnxA2-mediated membrane bridging is the organization of lipids in different membrane domains.

3.2. Annexin A2 modulates membrane fluidity. The binding of AnxA2 to specific phospholipids would be expected to induce changes in membrane organization and properties. Therefore, we investigated whether AnxA2 modified membrane fluidity by using the fluorescent probe laurdan. This probe shows a spectrum centered at 440 nm in membranes in the gel phase (rigid) and centered at 490 nm in liquid disordered (fluid) phase. A spectral shift towards the blue indicates a decrease in membrane fluidity, a shift towards the red an increase in membrane fluidity. The state of a membrane can be quantitatively characterized by the ratiometric function GP (generalized polarization) (see methods). Therefore protein-induced membrane changes can be estimated by the ΔGP ($\text{GP}_{\text{final}} - \text{GP}_{\text{initial}}$). A positive ΔGP indicates a decrease in fluidity and a negative ΔGP an increase in fluidity.

The ΔGP ($\text{GP}_{\text{AnxA2-Ca}^{2+}\text{-LUVs}}$ minus $\text{GP}_{\text{Ca}^{2+}\text{-LUVs}}$) was measured for the five different PS-LUVs. As a control we used LUVs with a small quantity of ligand phospholipid (2.5 % of PI2P). With these LUVs in the absence of presence of cholesterol, no membrane aggregation was observed even at 1 mM free Ca^{2+} , and laurdan spectra were not modified in the presence of the protein and Ca^{2+} . Several spectra showing the AnxA2 effects in different conditions are illustrated in the supplementary figure S1 (additional file), and figure 3 shows the measured AnxA2-induced ΔGP . We used a calcium concentration of 500 μM because in these conditions all membrane binding sites will be occupied by the protein. Therefore, we were

sure that the differences observed were not due to differences in calcium dependent protein binding efficiency (this is the same for H^+ concentration in the case of pH4.5 experiments). For the PS-LUVs in the presence of calcium (pCa 3.3), we observed a small decrease in membrane fluidity after AnxA2 addition at two temperatures (figure 3a). The PS-LUV4 (plasma membrane mimicking without cholesterol) showed the stronger decrease in fluidity with a ΔGP value of about 0.03. The controls showed small ΔGP changes (from -0.004 to 0.003). Because AnxA2 presents also a Ca^{2+} -independent H^+ -dependent phospholipid binding, we performed the same experiments at pH 4.5. Figure 3b shows variable small decrease in fluidity after AnxA2 addition. However, with the PS-LUV1 (rich in PS without cholesterol), the effect was stronger at both temperatures (ΔGP values about 0.04). AnxA2 induced less effect in PS-LUVs containing cholesterol compared to the cholesterol-free membranes. In a second series of experiments, we studied the AnxA2 effect on PI2P-LUVs in the presence of calcium (pCa 3.3) and at pH 4.5. The results in figure 3c show that AnxA2 induced a decrease in fluidity of all PI2P-LUVs tested. At 23°C the tendencies of four PI2P-LUVs were similar to those observed with the PS-LUVs but they showed higher ΔGP values. At 37°C, with the exception of PI2P-LUV5, the effect was stronger for PI2P-LUVs compared to PS-LUVs with ΔGP values ranging from 0.04 to 0.07 indicating that the protein reduces the fluidity of PI2P-membranes more than in PS-membranes. At pH 4.5 and 37°C, AnxA2 also induced high decrease in fluidity on PI2P-LUV4 compared to PS-LUVs (Fig. 3d). Overall, these results show that AnxA2-mediated reduction in fluidity is stronger in PI2P-LUVs than in PS-LUVs especially in the presence of calcium.

To test whether AnxA2 was able to modify the fluidity of biological membranes we prepared Triton X-100 resistant membranes (DRMs) from undifferentiated and differentiated (epithelial polarized) MDCK cells. Figure 4a shows that in epithelial polarized cells, AnxA2 is localized on the lateral membranes where cell contacts are established and on the apical membrane as previously described [42, 43]. In undifferentiated cells AnxA2 is mainly cytosolic but a fraction remains associated with the plasma membrane (Figure 4b). The DRMs were separated by flotation in twelve fractions that were characterized. AnxA2 and caveolin1 were found in fractions 4 and 5 of differentiated cells (Fig. 4c). Therefore we used these fractions for the next experiments.

The fluorescent probe used to monitor the fluidity changes of DRMs was di-4-ANEPPDHQ. Like laurdan, this probe is sensitive to the membrane fluidity and the cholesterol contents. It is

water soluble and fluoresces only when incorporated into the lipid bilayer. AnxA2 alone did not induce fluorescence indicating that the protein does not associate to the probe (supplementary Fig. S2 of additional file). Figure 4d shows the di-4-ANEPPDHQ spectra of DRM fraction 5 from differentiated (D5) and undifferentiated (I5) MDCK cells indicating that DRMs from differentiated cells are less fluid (blue shifted) than those from undifferentiated cells (red shifted). This difference, also observed for fractions 4, is due to the fact that the plasma membrane of differentiated cells and especially the apical membrane is rich in cholesterol and sphingomyelin. The GPs were measured before and after AnxA2 addition to DRMs in the presence of 500 μM free calcium and the ΔGP was calculated. Figure 5a shows that Ca^{2+} does not change significantly the spectra of the DRMs. The ΔGP values in Figure 5b, demonstrate that AnxA2 induces a decrease in fluidity of the DRMs at 23 and 37°C. The effect was smaller for the DRMs from differentiated MDCK cells (ΔGP values between 0.02-0.03) than for DRMs from undifferentiated cells with ΔGP values about 0.04 at 37°C and 0.06-0.07 at 23°C. The effect was reversible as shown by the addition of EGTA which induces AnxA2 membrane dissociation (Fig. 5a). These experiments demonstrate that AnxA2 is able to reduce the fluidity of membranes from biological origin the effect being stronger at low cholesterol content.

4. Discussion

The annexins specificity for different phospholipids has been studied in several conditions [6, 7, 10]. Phospholipid preferences are not exclusive of annexins and are shared by other domains such as the plekstrin domain of evt-2 protein which prefers PS to PIs [44]. Herein, we show that the Ca^{2+} -sensitivity for annexin A2 mediated membrane bridging does not correlate with the concentration of the anionic phospholipid involved in binding (PS or PI2P). However, some interesting tendencies were observed (supplementary figure S3). First, the presence of cholesterol results in a decrease in the calcium concentration necessary for membrane bridging of PS-LUVs. This fact is consistent with the previously published enhancement of AnxA2 membrane binding by cholesterol [16, 17, 22, 23]. However, for the PI2P-containing membranes the Ca^{2+} -sensitivity enhancement by cholesterol was not clear. The second tendency was that the PI2P-LUVs rich in PE showed lower calcium requirements.

These data demonstrate that the annexin A2-mediated membrane bridging depends more on the organization of phospholipids (membrane domains) than on the global concentration of a specific ligand and that each ligand phospholipid has specific environmental conditions for efficient Ca^{2+} -dependent binding of AnxA2. For example, with PS-LUV1, 2 and 3 AnxA2 required lower calcium concentrations compared to PI2P-LUVs, but AnxA2 required similar calcium concentration for PS- and PI2P-LUV5. However, in most cases, we observed higher calcium requirement for PI2P than for PS containing LUVs. The strong negative charge of PI2P would require higher Ca^{2+} concentration for charge titration (decrease in charge mediated repulsion) to allow AnxA2 bridging. These data suggest that the spontaneous distribution of membrane lipids create domains rich or poor in ligand phospholipids allowing the protein to bind at low or high calcium concentrations.

Considering the capacity of AnxA2 to change the membrane fluidity, we observed that the protein was able to reduce membrane fluidity. The strength of this effect was also dependent on the lipid composition. In the presence of calcium, PS-LUVs showed weak decrease in membrane fluidity after AnxA2 binding. PS-LUV4 which contains no cholesterol and low PS content showed moderate effect (supplementary figure S3). In the absence of calcium and at mild acidic pH, the decrease in fluidity of the PS-LUVs without cholesterol was stronger than in the presence of calcium. Interestingly, the capacity of AnxA2 to reduce the fluidity of PI2P-LUVs was stronger compared to the PS-LUVs. This effect was also enhanced in cholesterol-free PI2P-LUVs. This tendency was also observed at acidic pH. However, contrary to the PS-LUVs, the effect was stronger with calcium than at acidic pH demonstrating again phospholipid-dependent behavior.

To test the AnxA2 effects on membranes from biological origin, we prepared TX-100 resistant membrane fractions from differentiated and undifferentiated cells. These fractions can be considered as DRMs from different cell types. The differentiated MDCK cells present an apical membrane rich in cholesterol and sphingolipids which are characteristic of the raft-DRM domains and present higher order compared to the basolateral membrane (see the MDCK lipidomic characterization in [45]) The undifferentiated cells do not present this apical membrane. Therefore it was expected that the DRMs from these two cell types were different. Those from differentiated cells are rich in cholesterol and thus more rigid. The DRMs from undifferentiated cells have less cholesterol and are more fluid. The fact that DRMs can be of quite different composition was previously discussed in [46]. In spite of the fact that the TX-

100 treatment can induce the redistribution of cholesterol creating membrane "domains" [47], the idea in this work was to test the effect of Anx2 in two DRMs of different composition with different rigidity and cholesterol content and containing natural lipid species and membrane proteins. As shown by the ANNEPDHQ spectra, the differentiated DRMs rich in apical membrane show higher rigidity due in part to their high cholesterol content [41]. The results we observed with DRMs are consistent with the results from LUVs indicating that Anx2 induction of rigidity is stronger in fluid membranes than in rigid membranes. The presented data is also consistent with reports showing that some annexins are able to redistribute phospholipids [9, 11, 12] and change the membrane fluidity [48] depending on the phospholipid composition [10, 13].

The explanation for the AnxA2-mediated membrane fluidity reduction must be based on the capacity of annexins to bind specific anionic phospholipids. After phospholipid binding, the different affinities between all the lipids may determine their redistribution. This redistribution will depend on two facts: 1) the creation of new spacing between the anionic protein-bound phospholipid and 2) the change of the head group charge interactions. In other words, the binding of four anionic phospholipids to the four AnxA2 repeats, would change first the original distances between them restraining their movement, and second, the charges on the head groups would be differently oriented with the consequent change of repulsion-attraction forces between the bound phospholipids and the unbound lipids. This spacing and charge changes will allow lipid redistribution in different domains (Fig 6a). Considering that the overall lipid volume, the head group size and the net charge of PI2P are higher than PS, it is expected that AnxA2 bound to PS or PI2P will induce different effects on the membrane lipid redistribution with the consequent differences in the physicochemical properties of the membranes.

How do these data help to understand the role of lipids on AnxA2 functions? Based on our results we suggest that in the cell, the Ca^{2+} or H^+ requirements for membrane binding and the functions and effects of AnxA2 will be different depending on the target membrane. In the absence of Ca^{2+} at low H^+ concentration, AnxA2 would bind preferentially to PS-cholesterol-rich membranes and/or PI2P-cholesterol-poor membranes with a strong effect on the PI2P-membranes. This situation would be implicated for the endosomal functions of annexin A2 in which the protein seems to bind the membrane in a Ca^{2+} -independent manner [17, 23], but also to the reported H^+ dependent binding to the plasma membrane [26]. In the presence of

calcium, AnxA2 will be able to bind to the PIP2-cholesterol-rich membranes and/or to the PS-cholesterol-poor membranes with a strong decrease in fluidity of membranes poor in cholesterol and rich in PE. This situation would represent the annexin A2 role in Ca^{2+} -mediated processes such as the stimulated exocytosis in endothelial and chromaffin cells [15, 49, 50] in which AnxA2 was found to localize in PI2P-enriched exocytic domains [31]. Therefore, AnxA2 will induce specific effects for the reported functions such as Ca^{2+} -mediated secretion, endosomal regulation and plasma membrane domains organization.

5. Conclusions

The data schematically represented in figure 6b (see also supplementary figure S3), show that; 1) the PS membranes require less calcium than the PI2P membranes for AnxA2 membrane bridging. 2) Cholesterol reduces the calcium requirements for AnxA2 on PS membranes. 3) AnxA2 decreases the fluidity of PI2P membranes more than the PS membranes. 4) AnxA2 diminishes the fluidity of the PS membranes more in the presence of H^+ than in the presence of Ca^{2+} , but the effect on PI2P membranes is stronger in the presence of Ca^{2+} than at acidic pH, and 5). Cholesterol reduces AnxA2 capacity to reduce membrane fluidity. These data shed light on the molecular mechanisms necessary to understand the specific effects of Annexin A2 in membranes rich in PI2P, PS or cholesterol and also in membrane domains regulation [14, 15, 27-29]. However, for the fine understanding of the AnxA2 functions on membrane regulation, the future lipidomic analysis of cellular AnxA2 target membranes is the next step to take.

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Footnote

Abbreviations: Anx; Annexin, DRM; detergent-resistant membranes, LUV; large unilamellar vesicles, MDCK; Madin Darby canine kidney, PE; phosphatidylethanolamine, PI2P; phosphatidylinositol-4,5-bisphosphate, PS; phosphatidylserine

Figures

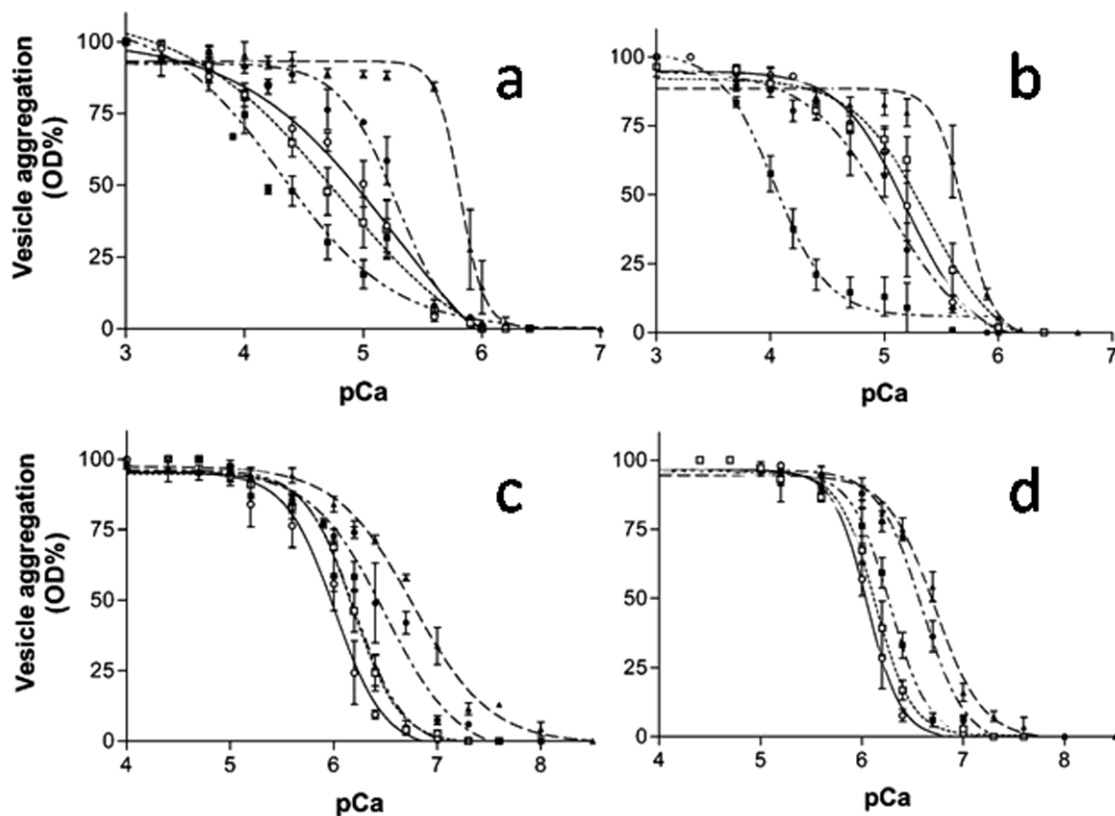


Figure 1. PS-LUVs bridging by Annexin A2 as a function of calcium. Vesicle aggregation in percent of maximal OD for monomeric AnxA2 at 23°C (a) and 37°C (b), and for tetrameric AnxA2 at 23°C (c) and 37°C (d). LUVs composition is shown in table 1. PS-LUV1 (■ dash double dotted line), PS-LUV2 (● dash dotted line), PS-LUV3 (▲ dashed line), PS-LUV4 (□ dotted line) and PS-LUV5 (○ continuous line). The calcium concentration for half maximal aggregation (EC_{50} as pCa) and the number of experiments performed are shown in tables 2 and 3.

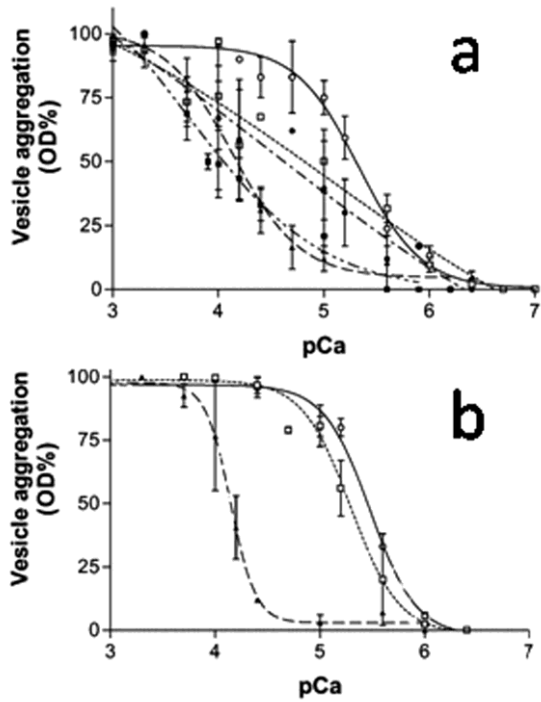


Figure 2. PI2P-LUVs bridging by Annexin A2 as a function of calcium. Vesicle aggregation in percent of maximal OD for AnxA2 at 23°C (a) and 37°C (b). The LUVs composition is in table 1. PI2P-LUV1 (■ dash double dotted line), PI2P-LUV2 (● dash dotted line), PI2P-LUV3 (▲ dashed line), PI2P-LUV4 (□ dotted line) and PI2P-LUV5 (○ continuous line). The calcium concentration for half maximal aggregation (EC50) and the number of experiments performed are in table 2.

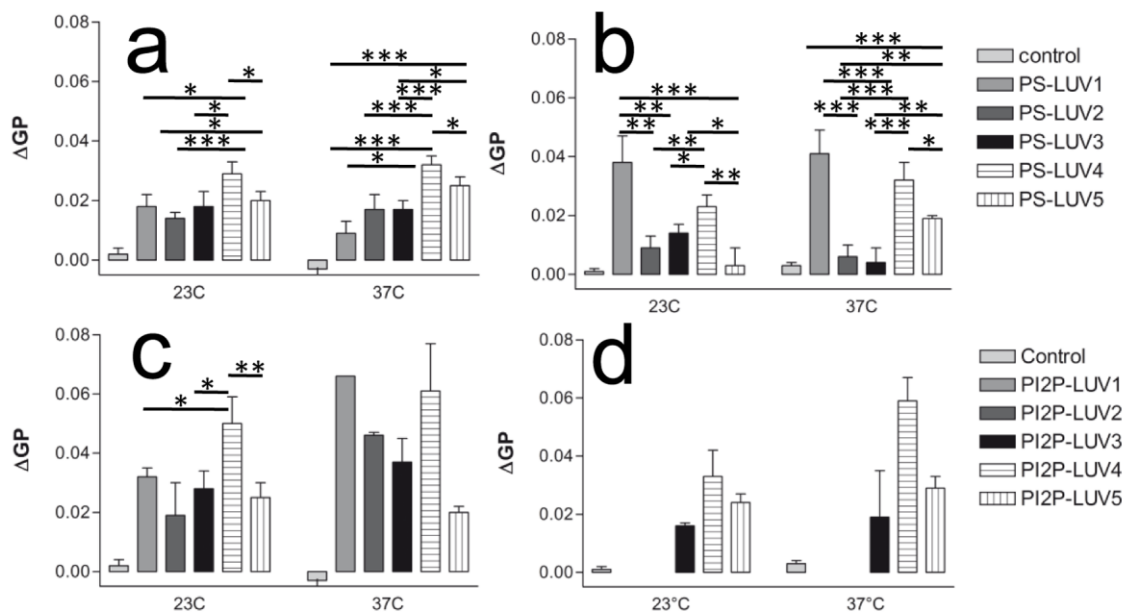


Figure 3. Changes in membrane fluidity induced by AnxA2. Changes in membrane fluidity expressed as ΔGP ($GP_{AnxA2-LUVs} - GP_{free-LUVs}$). PS-LUVs at 23 and 37°C in the presence of 500 μM calcium (a). PS-LUVs at 23 and 37°C at pH 4.5 (b). PI2P-LUVs at 23 and 37°C in the presence of 500 μM calcium (c). PI2P-LUVs at 23 and 37°C at pH 4.5 (d). Bars are the mean \pm Std. error from 2 to 8 experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

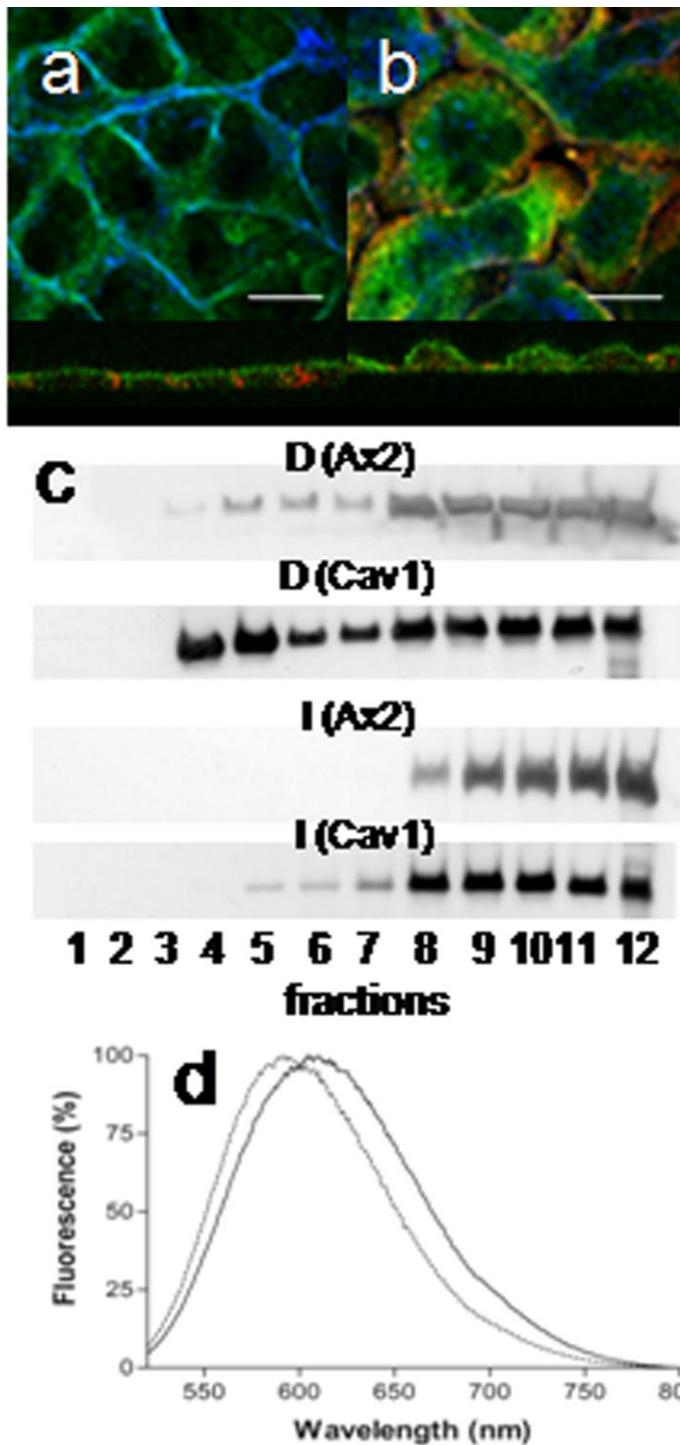


Figure 4. Cellular distribution of Annexin A2 and characterization of the DRM fractions of differentiated and undifferentiated MDCK cells. AnxA2 labeling in confocal slices of differentiated epithelial (a) and undifferentiated MDCK cells (b). AnxA2 (green), S100A10 (red) and E-cadherin (blue). Confocal images in the XY plane on top and in Z plane (AnxA2 (green) and E-cadherin (red)) at bottom. Bars; 10 μ m. (c); Western blots of AnxA2 and Caveolin1 in discontinuous sucrose gradients from differentiated (D) and undifferentiated (I) cells. DRMs are found in fractions 4 and 5. (d); Di-4-ANEPPDHQ spectra of fractions 5 from undifferentiated (continuous line), and differentiated cells (dotted line). DRMs from differentiated cells are less fluid (blue shifted) than those from undifferentiated cells.

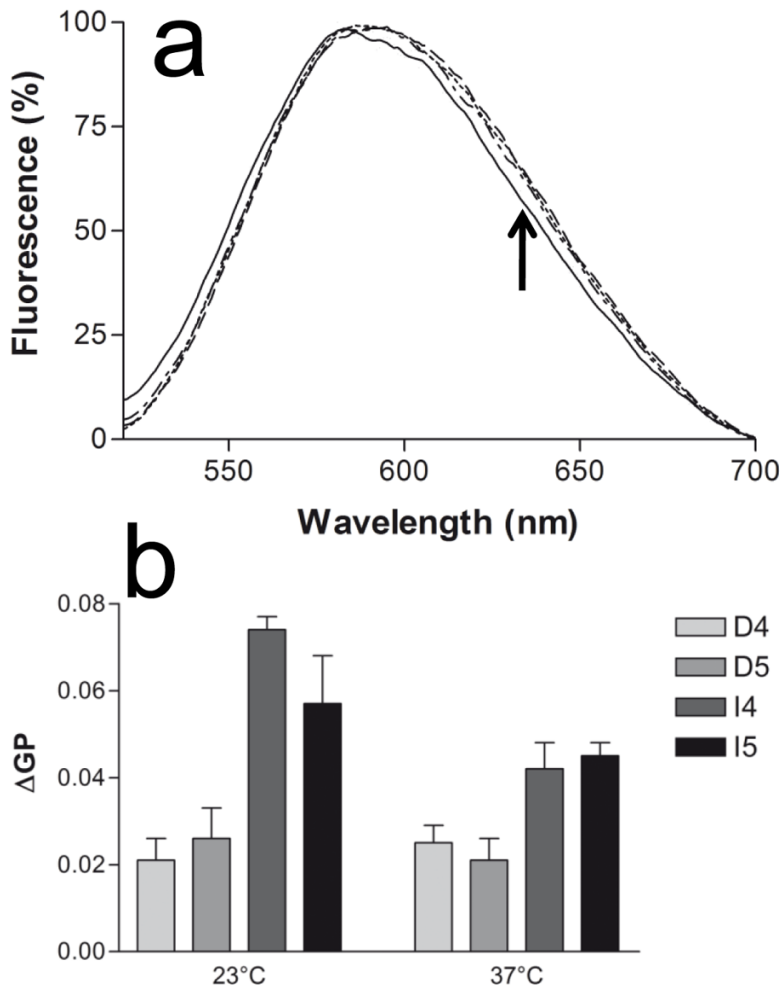


Figure 5. Annexin A2 reduces the fluidity of detergent resistant membranes (DRMs). (a); representative di-4-ANEPPDHQ spectra of DRM fraction 4 from undifferentiated cells (dotted line), after sequential addition of 500 μM free- Ca^{2+} (dashed line), AnxA2 (continuous line, arrow), and addition of EGTA 1 mM to test the reversibility of the AnxA2 effect (dash-dotted line). Spectra were obtained at 23°C. (b); ΔGP after AnxA2 addition on DRM fractions 4 and 5 from epithelial differentiated (D4, D5) and undifferentiated (I4, I5) MDCK cells at 23 and 37°C. Mean of two experiments in triplicate. Notice the stronger effect induced by AnxA2 on DRMs from undifferentiated cells (I).

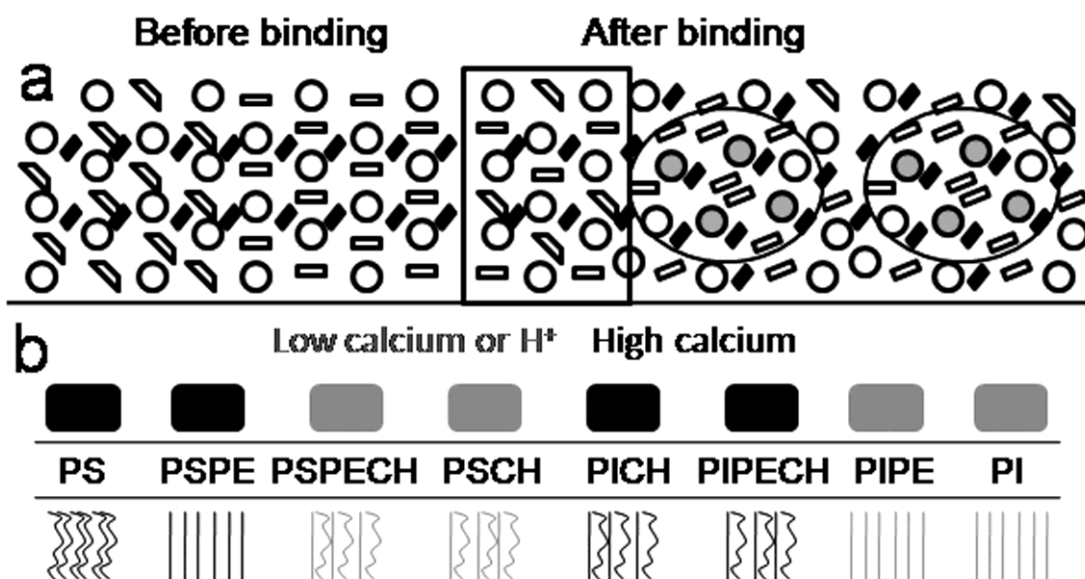


Figure 6. Schematic representation of AnxA2 effects on membranes. In left part of (a) the membrane is composed of different lipids (geometric figures) distributed in different domains. After AnxA2 (oval) binding, the ligand phospholipid associates strongly to the protein (gray circles). This interaction (right of a) provokes the redistribution of lipids with the consequent creation of new domains with different properties. The zone of the membrane that is not in contact with the protein might be modified by the protein-induced redistribution of lipids (box). (b) Illustrates the preferences of AnxA2 for the different phospholipids in the presence or the absence of calcium and the associated effect on fluidity. The Ca^{2+} -independent action is schematized in gray, the Ca^{2+} -dependent process in black. Phospholipids are abbreviated as in the text and cholesterol by CH. Membrane fluidity is schematized at the bottom. Strong reduction in membrane fluidity is coded by the straight bars, fluidity by rippled bars and moderate AnxA2 effect by straight-rippled bars. See details in the text.

Tables

Table 1. Lipid composition of large unilamellar vesicles (LUV).

	Lipid (weight %)			
LUV	PC ^a	PS or PI2P ^b	PE	Cholesterol
LUV1	75	25	0	0
LUV2	50	25	0	25
LUV3	20	20	35	25
LUV4	25	15	60	0
LUV5	17	12	52	19

a; Phosphatidylcholine

b; Depending on the experiment, the anionic phospholipid ligand for AnxA2 was phosphatidylserine (PS) or phosphatidylinositol-4,5-bisphosphate (PI2P).

Table 2. Calcium concentration for half maximal membrane bridging (EC50) by monomeric AnxA2 (pCa)^a.

	PS containing LUV				
	PS-LUV1	PS-LUV2	PS-LUV3	PS-LUV4	PS-LUV5
AnxA2 23°C	4.37±0.09 (8)	5.28±0.05 (6)	5.81±0.03 (8)	4.78±0.14 (9)	5.26±0.28 (9)
AnxA2 37°C	4.04±0.04 (5)	5.04±0.07 (6)	5.70±0.04 (5)	5.38±0.08 (7)	5.19±0.06 (6)
	PI2P containing LUV				
	PI2P-LUV1	PI2P-LUV2	PI2P-LUV3	PI2P-LUV4	PI2P-LUV5
AnxA2 23°C	3.72±0.53 (5)	4.57 (2)	4.14±0.11 (4)	5.07±1.12 (5)	5.33±0.07 (4)
AnxA2 37°C	nd	nd	4.15 (2)	5.30±0.07 (3)	5.48±0.03 (3)

a: The values are expressed in pCa ± Std. error (N).

nd: not determined.

Table 3. Calcium concentration for half maximal membrane bridging (EC50) by tetrameric AnxA2 (pCa)^a.

	PS containing LUV				
	PS-LUV1	PS-LUV2	PS-LUV3	PS-LUV4	PS-LUV5
AnxA2T 23°C	6.20±0.04 (6)	6.50±0.07 (4)	6.77±0.04 (4)	6.19±0.03 (4)	6.01±0.06 (5)
AnxA2T 37°C	6.28±0.03 (4)	6.59±0.03 (4)	6.72±0.04 (4)	6.14±0.03 (5)	6.05±0.03 (6)

a: The values are expressed in pCa ± Std. error (N).

nd: not determined.