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Cholesterol regulates membrane binding and aggregation by annexin 2 at submicromolar Ca^{2+} concentration

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Abstract

Annexin 2 is a member of the annexin family which has been implicated in calcium-regulated exocytosis. This contention is largely based on Ca^{2+}-dependent binding of the protein to anionic phospholipids. However, annexin 2 was shown to be associated with chromaffin granules in the presence of EGTA. A fraction of this bound annexin 2 was released by methyl-β-cyclodextrin, a reagent which depletes cholesterol from membranes. Restoration of the cholesterol content of chromaffin granule membranes with cholesterol/methyl-β-cyclodextrin complexes restored the Ca^{2+}-independent binding of annexin 2. The binding of both, monomeric and tetrameric forms of annexin 2 was also tested on liposomes of different composition. In the absence of Ca^{2+}, annexin 2, especially in its tetrameric form, bound to liposomes containing phosphatidylserine, and the addition of cholesterol to these liposomes increased the binding. Consistent with this observation, liposomes containing phosphatidylserine and cholesterol were aggregated by the tetrameric form of annexin 2 at submicromolar Ca^{2+} concentrations. These results indicate that the lipid composition of membranes, and especially their cholesterol content, is important in the control of the subcellular localization of annexin 2 in resting cells, at low Ca^{2+} concentration. Annexin 2 might be associated with membrane domains enriched in phosphatidylserine and cholesterol.

Keywords: Annexin 2; Cholesterol; Membrane binding; Aggregation; Chromaffin granule

1. Introduction

Annexins are soluble Ca^{2+}-regulated proteins, which bind to phospholipids. In this family, annexin 2 has been implicated in several steps of membrane traffic, such as endocytosis [1–4] and exocytosis [5–10] in several cell types. Annexin 2 exists as monomers, p36 (human protein: 338 residues), and tetramers where two p36 heavy chains, and two p11 light chains are associated. Both forms bind to membranes and induce their aggregation in a Ca^{2+}-dependent manner, this association being mediated by the C-terminal core of p36 (residues 30–338) [11–13] and requiring the presence of anionic phospholipids such as phosphatidylserine (PS) or phosphatidylinositol. The calcium sensitivity of these reactions is regulated, in part, by the N-terminal domain of the p36 heavy chain (residues 1–29), which also contains the binding site for the p11 light chain (residues

Abbreviations: MBCD, methyl-β-cyclodextrin; PC, phosphatidylcholine; PS, phosphatidylserine; DBH, dopamine β-hydroxylase

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1–14) [14,15]. However, annexin 2, especially in its tetrameric form, has also been shown to bind to PS liposomes in the absence of calcium [16]. More recently, the association of annexin 2 with early endosome membranes of BHK cells has been shown to be independent of Ca^{2+} and an N-terminal domain of the protein (residues 15–24) was proposed to be involved in this Ca^{2+}-independent association [17]. Moreover, annexin 2 was quantitatively released by the cholesterol sequestering agents filipin and digitonin from the membranes of early endosomes of BHK cells [18], together with a set of cortical cytoskeletal proteins, including actin.

In resting chromaffin cells at low intracellular Ca^{2+} concentration, cell fractionation experiments indicated that a large fraction (70%) of annexin 2 was soluble, but that the protein was also present in a Triton X-100-insoluble fraction. Under nicotine stimulation, at 10 μM intracellular Ca^{2+} concentration, confocal microscopy revealed a large translocation of the protein from the cytosol to the subplasmalemmal region, which was accompanied by an increase of the protein in the Triton X-100-insoluble fraction [5,7]. This fraction was partly soluble in the detergent octylglucoside, and the octylglucoside-soluble fraction contained plasma membrane and granule membrane proteins, together with some actin whereas the octylglucoside-insoluble one contained several cytoskeletal proteins, such as myosin, spectrin and actin. Recent data suggest that microdomains of the plasma membrane result from lateral assembly of insoluble sphingolipids and cholesterol that are able to form a liquid ordered phase in the membrane (detergent-insoluble glycolipid-enriched domains (DIG), detergent-resistant membranes (DRM), or rafts), which are thought to be involved in numerous cellular events including membrane traffic, sorting and retention of GPI-anchored proteins in the endocytic pathway, exocytosis, signal transduction and organization of microdomains of the plasma membrane [19–25].

We have previously suggested that the Triton X-100-insoluble, octylglucoside-soluble fraction in chromaffin cells might represent such a specialized lipidic membrane compartment where annexin 2 would be concentrated upon stimulation of secretion [5]. In this regard, we investigated the relationship of annexin 2 binding to membranes with the cholesterol content in chromaffin granule membrane by using a selective cholesterol sequestering agent, methyl-β-cyclodextrin (MBCD). Since these experiments indicated a role for cholesterol in the Ca^{2+}-independent binding of annexin 2 to chromaffin granule membranes, we studied the binding and aggregation properties of monomeric and tetrameric annexin 2 with liposomes of varying phospholipid composition, with or without cholesterol.

2. Materials and methods

2.1. Materials

Methyl-β-cyclodextrin, cholesterol, bovine brain phosphatidylcholine (PC) and phosphatidylserine (PS) were obtained from Sigma. [1α,2α(*n*)-^{3}H]-cholesterol (specific activity 35–50 Ci/mmol) was purchased from Amersham. The monoclonal antibody against annexin 2 was from Transduction laboratories (Lexington, USA) and the affinity-purified rabbit anti-bovine annexin 2 antibody was obtained as previously described [6].

2.2. Preparation of proteins

Human annexin 2 heavy chain cDNA (gift of Dr E. Solito and F. Russo-Marie, INSERM U332, Paris, France) was subcloned and expressed in yeast, using a vector provided by Dr D. Pompon (CNRS, Gif sur Yvette, France). The recombinant protein was purified by procedures to be published elsewhere. The light chain, p11, was purified by a procedure to be published, after expression in *Escherichia coli* of a vector kindly provided by Dr V. Gerke (University of Münster, Germany). The heterotetramer, p90, was obtained by mixing the heavy and the light chains at a molar ratio of 1:1. The properties of the reconstituted recombinant protein were identical to those of the bovine lung tetramer in the chromaffin granule aggregation assay. p36 Edman degradation assay indicated that the N-terminal extremity is blocked, suggesting acetylation of the protein in yeast, as previously observed for annexin 1 [26]. p33 was a spontaneously formed degradation product and sequence analysis indicated that residues 1–29 were deleted.

2.3. Preparation of EGTA-treated chromaffin granule membranes

Bovine chromaffin granules were prepared as described, using a Percoll density gradient [27]. Purified granules (25 mg of protein) were homogenized in 25 ml of 20 mM HEPES pH 7.4. Membranes were collected by centrifugation at $100\,000 \times g$ for 20 min at 4°C. The pellet was resuspended in 25 ml of 20 mM HEPES buffer pH 7.3 containing 150 mM NaCl and 2 mM EGTA at 4°C with gentle stirring for 20 min, then collected by centrifugation at $100\,000 \times g$ for 10 min at 4°C. The particulate fraction was resuspended in 3 ml of buffer A (50 mM HEPES, 10 mM $MnCl_2$ pH 7.3).

2.4. Treatment of chromaffin granule membranes with MBCD

EGTA-treated granule membranes (400 μg of protein) were incubated with increasing concentrations of MBCD in buffer A in a total volume of 1 ml. The fractions were incubated for 30 min at room temperature with rotatory shaking. Following centrifugation for 20 min at $100\,000 \times g$ at 4°C, the pellets were washed, resuspended in 500 μl of buffer A and kept at −20°C. The protein, phospholipid and cholesterol contents of membranes were estimated using the Coomassie protein assay reagent (Pierce), the colorimetric assay of hydrolyzed phosphate and the cholesterol oxidase method (Sigma). The annexin 2 content was estimated by SDS/PAGE and immunoblotting with immunopurified polyclonal anti-bovine annexin 2 antibody at 1 μg/ml. Gels and Western blot quantifications were performed by numerization of images and analysis with the NIH Image 1.62 software.

2.5. Treatment of cholesterol-depleted membranes with cholesterol/methyl-β-cyclodextrin inclusion complexes

Granule membranes (6 mg of protein) were depleted of cholesterol by incubation with MBCD (75 mM), resuspended in 1.4 ml of buffer A and kept at −20°C. These membranes were incubated with a tritium-labeled cholesterol inclusion complex [28], obtained by incubation of 3.5 μCi [^{3}H]cholesterol and 3 mg of non-labeled cholesterol with 100 mg of MBCD in 0.3 ml of H_2O with stirring for 60 min at room temperature, until clarification of the solution. Membranes (50 μl) were incubated with increasing concentrations of cholesterol–MBCD inclusion complex (0–3.5 mM cholesterol) in 250 μl buffer A, with shaking for 30 min at room temperature. The fractions were centrifuged twice to remove excess inclusion complex, resuspended in 500 μl buffer A and stored at −20°C.

2.6. Annexin 2 binding to membranes reconstituted with cholesterol

Aliquots (5 μl) of the suspension of membranes reconstituted with increasing concentrations of cholesterol were incubated with 195 μl of buffer B (30 mM KCl, 1 mM EGTA, 40 mM HEPES pH 7) containing 0.06 μg of tetrameric annexin 2 (3.3 nM). After incubation for 30 min at room temperature, the samples were centrifuged for 20 min at $170\,000 \times g$ in a Beckman Airfuge. The pellets were dissolved in the SDS/PAGE sample buffer and assayed for annexin 2 by electrophoresis and immunoblotting with a monoclonal anti-human annexin 2 antibody, at 1/10 000 dilution.

2.7. Preparation of liposomes

PC-, PS- and cholesterol-containing liposomes were obtained by extrusion. Lipids in chloroform were mixed in different proportions, subjected to solvent evaporation and resuspended by vortexing vigorously in buffer B to a total lipid concentration of 2.5 mg/ml. The multilamellar liposomes were then extruded by filtration through 0.4, 0.2 and 0.1 μm pore size polycarbonate filters (Sciema Technical Services Ltd, Richmond, BC, Canada), the latter filtration being repeated three times.

2.8. Annexin 2 binding to liposomes and EGTA-treated chromaffin granule membranes

Liposomes of different composition or EGTA-treated chromaffin granule membranes were added to different amounts of monomeric or tetrameric annexin 2 in buffer B containing the indicated free Ca^{2+} concentrations, as calculated with the calcv.22

program [29], in 50 μl final volume. The mixtures were incubated for 30 min at room temperature, then centrifuged in a Beckman Airfuge for 30 min at 170 000 × *g*, or in a Beckman 70.1 Ti rotor operated at 40 000 rpm for 30 min at 4°C. The pellets were resuspended in 25 μl of electrophoresis loading solution. Pellets and supernatants were analyzed for annexin 2 content by SDS–PAGE and scanning of Coomassie-stained gels or Western blot using a monoclonal anti-human annexin 2 antibody. Results were corrected by subtracting the insoluble annexin 2 background observed in membrane-free controls.

2.9. Aggregation of liposomes by annexin 2

Liposome aggregation was monitored in buffer B at different free Ca^{2+} concentrations as calculated with the calcv.22 program [29]. Liposomes were added at a phospholipid concentration of 20 μg/ml. To initiate the aggregation reaction, monomeric or tetrameric annexin 2 was added at a concentration of 20 μg/ml. Aggregation was taken as the absorbance increase at 340 nm, at 28°C, 7 min after annexin 2 addition.

3. Results

3.1. Calcium-independent binding of annexin 2 to chromaffin granules is cholesterol-dependent

Consistent with a previous observation [30], 15% of the annexin 2 (p36) associated with purified chromaffin granule membranes is not solubilized by incubation with 1 mM EGTA. In order to investigate the role of cholesterol in this type of binding, the EGTA-treated membranes were incubated with different cholesterol sequestering agents. Digitonin and filipin extracted a small fraction of the membrane cholesterol (30 and 40% respectively), moreover, these agents solubilized 40 and 20% of the granule proteins. These results indicate both low specificity and low efficiency of cholesterol extraction. We then incubated the EGTA-treated membranes with increasing concentrations of methyl-β-cyclodextrin (MBCD), another reagent which extracts cholesterol from membranes [28,31]. In the range of concentration tested, MBCD solubilized a large fraction of the

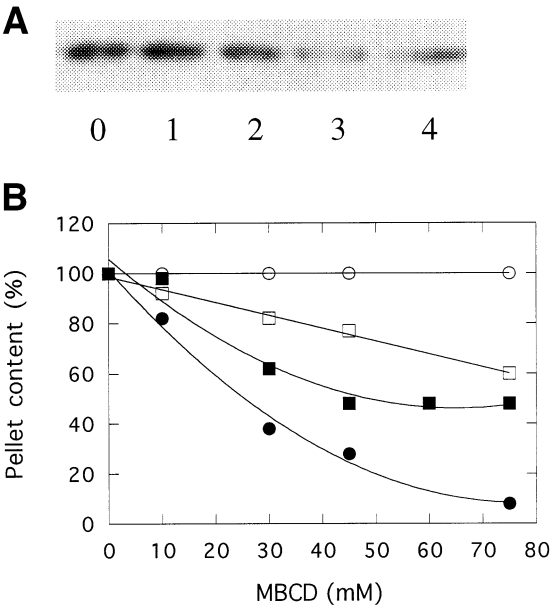

Fig. 1. Characterization of chromaffin granule membranes treated with methyl-β-cyclodextrin. Granule membranes were treated with increasing amounts of MBCD for 30 min at room temperature. After centrifugation, removal of the supernatant and washing, the pellets were resuspended and assayed for total protein, phospholipid, annexin 2 and cholesterol contents. (A) Western blot of annexin 2 in MBCD-treated membranes (50 μl of extract). Lane 0, no MBCD; lanes 1–4, 10, 30, 45 and 75 mM MBCD. (B) Contents of MBCD-treated membranes expressed as percentage of untreated membranes: protein (○), phospholipid (□), annexin 2 (■) and cholesterol (●). Values are means calculated on two independent experiments.

bound annexin 2 (Fig. 1A) and extracted quantitatively the cholesterol of the membrane (Fig. 1B). It should be noted that extraction of 90% of the cholesterol required a concentration of 75 mM MBCD and that this treatment resulted in the solubilization of 35–40% of membrane phospholipid. The use of such concentrations of MBCD [28,32] and the partial solubilization of phospholipid by MBCD has been reported previously [31]. That annexin 2 solubilization is selective is shown by the electrophoretic profile of the treated membranes (Fig. 2). MBCD treatment did not induce visible changes.

Since MBCD solubilized not only cholesterol but also phospholipid, the role of cholesterol was ascertained by testing the reversibility of solubilization (Fig. 3). Membranes depleted by treatment with 75 mM MBCD were incubated with increasing concentrations of [^{3}H]-labeled cholesterol–MBCD inclusion

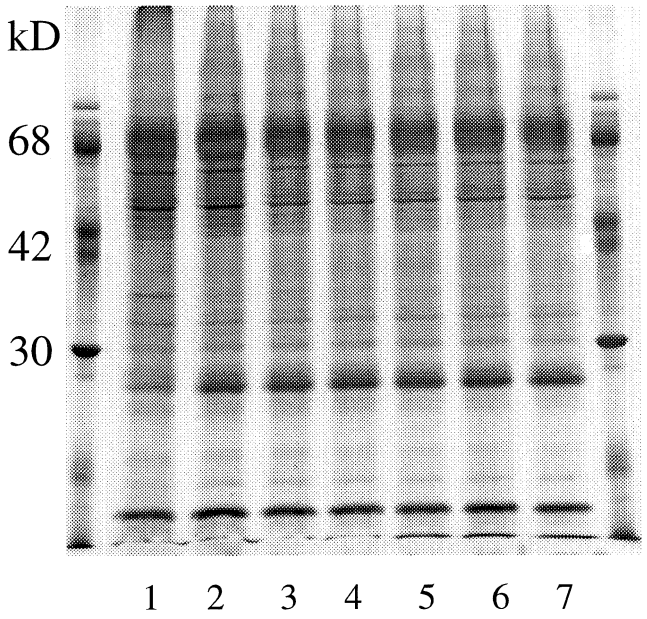

Fig. 2. SDS–PAGE analysis of chromaffin granule membranes. Purified chromaffin granule membranes (lane 1) were treated with EGTA (lane 2) and then by increasing MBCD concentrations in buffer A: lane 3, buffer A alone; lane 4, 10 mM MBCD; lane 5, 30 mM MBCD; lane 6, 45 mM MBCD; lane 7, 75 mM MBCD. Aliquots (35 μg of protein) were analyzed on 12% acrylamide gels.

complexes. The treatment restored the cholesterol content of the membranes to 65–70% of the initial value. Membranes with increasing cholesterol content were then incubated with a constant concentration of tetrameric annexin 2. Annexin 2 binding paralleled cholesterol content and reached a plateau value corresponding to 170% of that observed in EGTA-treated membranes, thus clearly indicating a role for cholesterol in the calcium-independent binding of annexin 2 to chromaffin granule membranes.

3.2. *Effect of cholesterol on annexin 2 binding to liposomes*

In order to test the hypothesis that annexin 2 Ca^{2+}-independent binding was supported by cholesterol and/or phospholipids in the absence of a receptor protein, the binding of monomeric p36 (Fig. 4A) and tetrameric p90 (Fig. 4B) annexin 2 was analyzed on liposomes of different composition, in the absence (open bars) or in the presence of 200 μM free Ca^{2+} (closed bars). Liposomes containing either only PC or PC/cholesterol (60/40, w/w) did not bind significantly annexin 2 (monomer or tetramer) either in the absence or in the presence of Ca^{2+} ions (experiments 1 and 3). On the other hand, liposomes containing PS (PC/PS, 60/40) did bind annexin 2, even in the absence of Ca^{2+} ions (experiment 2). Under the experimental conditions used in this experiment, 11% of added p36 and 23% of added p90 were bound, and these fractions increased in the presence of Ca^{2+} ions to 30 and 45%, respectively. Interestingly, the addition of cholesterol to PC/PS-containing liposomes (PC/PS/cholesterol, 50/25/25 w/w, 40/20/40 mol/mol) resulted in a marked increase of both p36 and p90 binding, which is clearly seen in the absence of Ca^{2+} ions (compare experiments 2 and 4 in Fig. 4).

From these experiments it can be concluded that cholesterol-mediated Ca^{2+}-independent annexin 2 binding can be observed in liposomes, but that this binding requires the presence of PS in these liposomes. This observation would explain the failure previously noted with PC/cholesterol liposomes [18]. Similar results were observed with the monomer and the tetramer, although Ca^{2+}-independent binding to PC/PS liposomes is more intense with the tetramer.

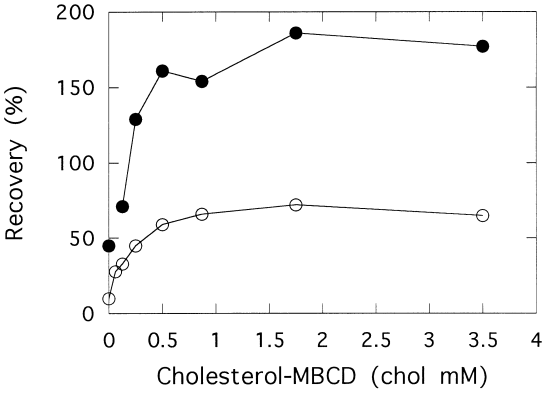

Fig. 3. Binding of annexin 2 to cholesterol-depleted chromaffin granule membranes incubated with increasing amounts of cholesterol–MBCD inclusion complex. Granule membranes pretreated by 75 mM MBCD for 30 min were incubated with increasing concentrations of [^{3}H]cholesterol–MBCD. [^{3}H]cholesterol incorporated into membranes (○) was determined by liquid scintillation counting on an aliquot. Another aliquot of resuspended membranes was incubated with tetrameric annexin 2. After centrifugation, annexin 2 was analyzed by SDS–PAGE and Western blotting (●). Results are expressed as percentage of recovery compared to MBCD-untreated membranes. Similar results were obtained in two different experiments.

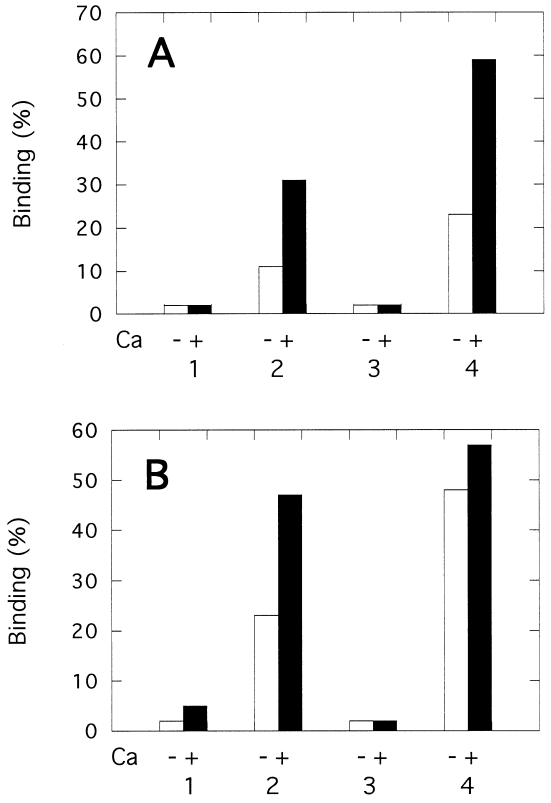

Fig. 4. Binding of annexin 2 to liposomes of various compositions. Annexin 2 (3 μg of protein) in the monomeric (A) or in the tetrameric (B) form was incubated with liposomes (25 μg of lipid) of various compositions (w/w): 1, PC alone; 2, PC/PS 60/40; 3, PC/cholesterol, 60/40; 4, PC/PS/cholesterol, 50/25/25, in the absence (open bars) or in the presence of 200 μM free Ca^{2+} ions (closed bars). After incubation, the mixtures were centrifuged and the supernatants and the pellets were assayed for their p36 content. Results were expressed as percentage of annexin 2 found in the pellet and are representatives of two independent experiments.

3.3. Cholesterol dose-dependence of annexin 2 binding to liposomes

Experiments were performed with PC/PS/cholesterol liposomes in the absence of Ca^{2+} ions. In a preliminary experiment, different concentrations of PS were tested at constant cholesterol concentration. A minimal PS fraction of 15% (w/w) was required to induce cholesterol-mediated annexin 2 binding and at PS concentrations larger than 40% (w/w), the cholesterol effect was masked by the high PS-induced annexin 2 binding. Optimal binding was obtained with liposomes containing 25% PS and 25% cholesterol (w/w).

Cholesterol dose-dependence was then investigated in PC/PS/cholesterol liposomes containing 25% PS (w/w), using p36, p90 and p33, a proteolysed form of p36 lacking residues 1–29 (Table 1). With p36 and p90, a minimal cholesterol concentration of 15% (w/w) was required to observe an effect and the effect was more marked at 25%. As noted previously, there is a significant binding of p90 to PC/PS liposomes, which is not observed with p36 under the experimental conditions used. In the absence of Ca^{2+}, no binding was observed with p33 either to PC/PS or PC/PS/cholesterol liposomes. However, p33 bound efficiently (90%) to PC/PS liposomes at 500 μM Ca^{2+} (data not shown).

3.4. Comparison of the calcium-independent binding of annexin 2 to liposomes and to chromaffin granule membranes

The preceding experiments indicated that annexin 2 can bind to liposomes in the absence of a protein receptor. However, comparison of binding to liposomes and to chromaffin granule membranes re-

Table 1
Effect of cholesterol concentration on the binding of p90, p36 and p33

Cholesterol content (%)	0	5	15	25
	Binding (%)			
p90	38.2 ± 4.4	35.0 ± 1.0	43.3 ± 4.4	56.0 ± 4.6
p36	2.7 ± 1.7	3.0 ± 3.0	14.3 ± 4.2	19.0 ± 4.5
p33	0	0	0	0

Liposomes containing 25% PS (w/w) and with different contents of PC and cholesterol were incubated with p33, p36 or p90 and assayed for bound annexin 2. Results (means ± S.E.M. of three–four experiments) are expressed as the percentages of annexin 2 bound to liposomes.

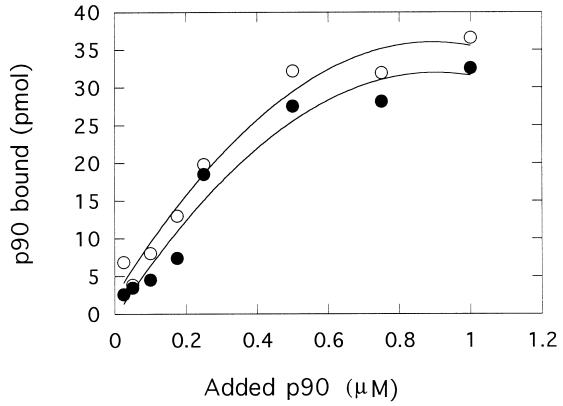

Fig. 5. Binding of annexin 2 tetramer to EGTA-treated chromaffin granule membranes and liposomes. Tetrameric annexin 2 was incubated with liposomes (1.25 μg of PC, 0.625 μg of cholesterol, 0.625 μg of PS) or EGTA-treated chromaffin granule membranes (containing 3.4 μg of protein, 3.6 μg of cholesterol, and 0.78 μg of acidic phospholipids) in 500 μl. Liposomes (○); chromaffin granule membranes (●). Results are representatives of three experiments.

quired analysis of saturation isotherms. For these experiments (Fig. 5), the lipid composition of our chromaffin granule membranes was calculated to be 1.06 mg (2.65 μmol) of cholesterol and 0.23 mg (0.33 μmol) of acidic phospholipids per mg of protein using our cholesterol and phospholipid assay and assuming that 10% of the phospholipids are acidic ones [33]. Liposomes (PC/PS/cholesterol 50/25/25, w/w/w) and chromaffin granule membranes at a similar concentration of acidic phospholipids were compared. Very similar binding curves were obtained in three experiments performed at different membrane concentrations. Though experimental difficulties limited the quality of the data and the derivation of binding parameters, the equilibrium dissociation constant appeared to be in the micromolar concentration range for both granule membranes and liposomes.

3.5. *The aggregation of liposomes by annexin 2 is affected by their cholesterol content*

Aggregation of liposomes with or without cholesterol was measured as a function of Ca^{2+} concentration, for p36 and p90. In preliminary experiments, annexin 2 was unable to aggregate PC and PC/cholesterol liposomes in the absence or in the presence of 100 μM Ca^{2+}, a result consistent with binding experiments. On the other hand, PC/PS and PC/PS/cholesterol liposomes were easily aggregated in the presence of Ca^{2+} ions. In the absence of Ca^{2+} ions, p90 induced some aggregation which was larger in the presence of cholesterol (Fig. 6). As for binding experiments, this effect was weaker at high PS concentration: when the PS content of liposomes was 25%, addition of 25% cholesterol increased aggregation by a factor of 2.3 whereas addition of 33% cholesterol to liposomes containing 33% PS induced only a 1.5-fold increase of aggregation.

Analysis of the Ca^{2+}-dependence of aggregation was thus performed on liposomes containing either PC/PS, 75/25 or PC/PS/cholesterol, 50/25/25 (Fig. 7). Without calcium, the monomeric protein p36 did not aggregate liposomes. In cholesterol-containing liposomes, aggregation by p36 started at a free Ca^{2+} concentration of about 2.5 μM (pCa 5.6), and half maximal aggregation was obtained at 25 μM (pCa 4.5). These values were different from those obtained with cholesterol-free liposomes in which half maximal aggregation was obtained at 100 μM (pCa 4). For the tetramer p90, the difference was more marked, since an efficient aggregation was observed at low Ca^{2+} concentration, which was superimposed on the curve obtained with liposomes containing PC/PS (Ca^{2+}-dependent aggregation starting at pCa of 7.5, 32 nM, and half maximal aggregation at 0.25 μM of Ca^{2+}, pCa 6.6). This effect is likely to reflect

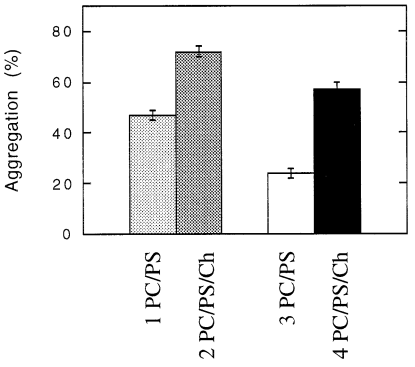

Fig. 6. Effect of liposome composition on p90-induced liposome aggregation. Tetrameric annexin 2 was added at 20 μg/ml final concentration to a medium containing 20 μg/ml of liposomes of different compositions (w/w): 1, PC/PS, 67/33; 2, PC/PS/cholesterol, 34/33/33; 3, PC/PS, 75/25; 4, PC/PS/cholesterol, 50/25/25, in the absence of added Ca^{2+} ions. Aggregation was monitored at 340 nm for 7 min at 28°C. Results (means ± S.E.M. of six experiments) are expressed as percentages of the aggregation observed in the presence of 10 μM free Ca^{2+}.

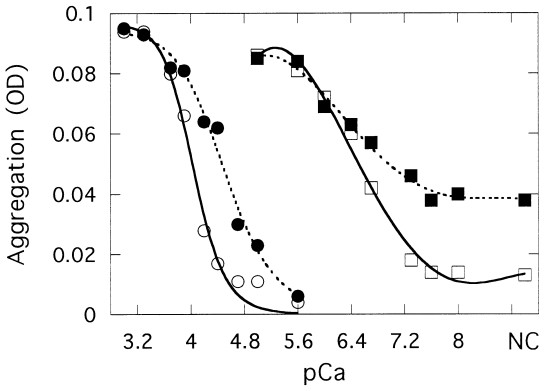

Fig. 7. Calcium-dependence of aggregation of cholesterol-containing and cholesterol-free liposomes by monomeric and tetrameric annexin 2. Liposome composition was PC/PS, 75/25 (open symbols) and PC/PS/cholesterol, 50/25/25 (closed symbols). p36, circles; p90, squares. NC, no calcium added. Results are representatives of three independent experiments.

the modulation of p90 binding by cholesterol and the fact that the bound tetramer can bind two opposing membranes in the absence of Ca^{2+}.

Finally, the effect of cholesterol concentration was analyzed with PC/PS/cholesterol liposomes containing 25% PS (Fig. 8). For the p36 monomer, the experiment was performed at pCa 4.4 and the results were expressed as percentages of maximal aggregation, observed at pCa 3.0. Similarly, for the p90 tetramer, the experiment was performed at pCa 8.0 and the results expressed as percentages of maximal aggregation at pCa 5.0. The two experiments indicate a clear correlation between aggregation and the cholesterol content of the liposomes.

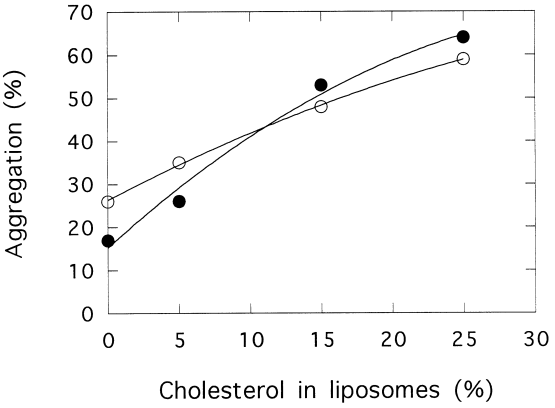

Fig. 8. Annexin 2 induced aggregation of liposomes containing increasing amounts of cholesterol. Liposomes, PC/PS/cholesterol, containing 25% PS and the indicated percentage (w/w) of cholesterol were incubated with p36 at pCa 4.4 (●) or p90 at pCa 8.0 (○). Results are expressed as percentages of the aggregation observed with p36 at pCa 3.0 or with p90 at pCa 5.0. Results are representatives of two experiments.

4. Discussion

In early endosomes, calcium-independent binding of annexin 2 to membranes has been demonstrated [17,18] and attributed to the presence of cholesterol in the membrane [18]. Calcium-independent binding of annexin 2 has also been observed in alveolar type II cells [34] and chromaffin granule membranes [30] and, in this work, we show that cholesterol is involved in this binding. Our demonstration takes advantage of the properties of MBCD, a compound which complexes specifically cholesterol and which can be used, not only to deplete membranes, but also to introduce variable amounts of cholesterol in depleted membranes. The fact that there is a correlation between cholesterol content and annexin 2 binding is strong evidence for the involvement of cholesterol in Ca^{2+}-independent annexin 2 binding. In the reconstitution experiment (Fig. 3), the plateau value of annexin 2 binding is higher than the initial value in EGTA-treated membranes, an observation which might be accounted for by the low affinity of this binding (Fig. 5). It should be noted that, in the solubilization experiment (Fig. 1), a fraction of annexin 2 remained in the membrane after removal of 90% of the cholesterol, suggesting that cholesterol is not directly involved in annexin 2 binding.

In the case of endosomes, it has been proposed that Ca^{2+}-independent annexin 2 binding might occur through a proteinaceous receptor [17]. In this context, it is interesting to note that a 200 kDa protein from clathrin-coated vesicles was found to associate with annexin 2 [35]. However, for chromaffin granules, the existence of a proteinaceous receptor requiring cholesterol to interact with annexin 2 is unlikely for two reasons. First, annexin 2 binds to liposomes in the absence of Ca^{2+} (Figs. 4 and 5). Moreover, saturation curves indicate that the equilibrium dissociation constant is very similar for liposomes and chromaffin granule membranes, when concentrations were normalized on the basis of the acidic phospholipid content. Second, from Fig. 5, the p90 binding capacity (B_{max}) of chromaffin granule

membranes can be approximated to 10 nmol/mg of membrane protein. The protein composition of this membrane is known [33] and its content in cytochrome b561, the most abundant component is 6 nmol/mg of protein, DBH, the second most abundant component is 0.7 nmol/mg, too low to account for annexin 2 binding. It should be noted that the B_{max} for the Ca^{2+}-dependent binding of annexin 2 of chromaffin granules has been estimated to 2.2 nmol/mg of total chromaffin granule protein [36] corresponding to a value of 11 nmol/mg of membrane protein. That the two values are in the same range suggests the binding of annexin 2 to acidic phospholipids, in the absence of Ca^{2+}. From experiments with liposomes and assuming a similar B_{max} (Fig. 5), it can be calculated that one mol of annexin 2 binds to 17 PS molecules a value consistent with previous experiments on the Ca^{2+}-dependent binding of annexin 2 to chromaffin granules [36]. In the experiments on Ca^{2+}-dependent binding [36], K_d values were in the nanomolar concentration range (10–30 nM), whereas values in the micromolar range are suggested by the data of Fig. 5. We thus propose that both Ca^{2+}-dependent and Ca^{2+}-independent binding occur on PS, but that the affinity is decreased by two orders of magnitude in the absence of calcium.

What might be the basis for the Ca^{2+}-independent interaction between annexin 2 and PS-containing liposomes? Until now, PS, a negatively charged phospholipid, has been implicated in the binding of annexin 2 via Ca^{2+} coordination, at the level of the three class II Ca^{2+} binding sites on repeats 2, 3 and 4 [37]. However, structural studies of annexin 5 have indicated some direct interactions between the protein and phospholipids [38,39]. Mutagenesis experiments in which residues 72, 144, 228 and 303 (highly conserved serines and threonines) on the D/E loop at the convex face of annexin 5 change its membrane binding capacity without changing the Ca^{2+} binding sites, thus suggesting that the side chains contribute to membrane binding by contact with phospholipids [39]. In addition, the structure of annexin 5 crystallized in the presence of Ca^{2+} and of glycerophosphoserine (as a model for PS) has indicated some direct interaction between the protein and the ligand, at the level of the highly conserved amino acids Thr-187 and Glu-226 [38]. Structural complementarity between the protein and the phospholipid head group might be more important than net phospholipid charge in determining selectivity.

Taking these observations into account, we can speculate on the role of cholesterol in annexin 2 binding. In biological membranes, cholesterol association with phospholipids is not only controlled by the nature of the fatty acids but also by that of the polar head groups [40]. By a mechanism to be determined, cholesterol might induce the formation of PS-rich microdomains, which in turn would favor the interaction of this phospholipid with annexin 2, at the level of the core. Such a hypothesis is consistent with the facilitation effect of cholesterol, observed at low PS content. It accounts also for the fact that MBCD solubilized fully the cholesterol of chromaffin granule membrane, and only partially annexin 2. According to this hypothesis, the N-terminal domain of annexin 2 would have an indirect role, probably interacting with the core of the protein and modulating its affinity for acidic phospholipids. The p33 protein lacking residues 1–29 did not bind to PS/cholesterol liposomes in the absence of calcium (Table 1), thus suggesting a positive regulation by the terminal domain. The results of Jost et al. [17] showing that a deletion mutant (1–24 residues) did not associate with endosomes when expressed in BHK cells might be interpreted in a similar way. On the other hand, the fact that the tetramer, in which the N-terminal extremity of the heavy chain is engaged in a complex with the p11 light chain, interacts more strongly with PS than the p36 monomer (Figs. 4 and 7 and references [16,41]) suggests also the presence of domains or conformations with a negative regulatory effect. The existence of different domains (or conformations) of the N-terminal tail with opposing effects on both membrane binding and aggregation has been noted in the case of annexin 1 [42,43].

The cholesterol/PS ratio is important for the Ca^{2+}-independent binding of annexin (Table 1). It might be noted that in chromaffin granules, cholesterol is 38% (mol/mol) of the membrane lipid [33], corresponding to 24% (w/w), a value very close to the concentration used in our experiments with liposomes, thus suggesting that these experiments are physiologically relevant. The present work shows that cholesterol decreases the calcium concentration required for the binding of annexin 2 to phospholi-

pidic membranes and their aggregation, particularly by the tetrameric form of the protein. In chromaffin cells, the percent by weight of cholesterol in total membrane lipids is 13, 18 and 24%, in the endoplasmic reticulum, Golgi complex and chromaffin granule membranes respectively [44], thus suggesting that in vivo, in resting cells (where Ca^{2+} concentration has been estimated to be 100 nM), the cholesterol content of the different intracellular membranes might regulate the localization of annexin 2, and possibly of other annexins [30,45]. In different cell types, the annexin 2 tetramer might be localized at the level of cholesterol-enriched membranes forming clusters which might be important for organelle organization and secretion.

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