Annexins in Cell Membrane Dynamics: Ca^{2+}-regulated Association of Lipid Microdomains

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Abstract. The sarcolemma of smooth muscle cells is composed of alternating stiff actin-binding, and flexible caveolar domains. In addition to these stable macrodomains, the plasma membrane contains dynamic glycosphingolipid- and cholesterol-enriched microdomains, which act as sorting posts for specific proteins and are involved in membrane trafficking and signal transduction. We demonstrate that these lipid rafts are neither periodically organized nor exclusively confined to the actin attachment sites or caveolar regions. Changes in the Ca^{2+} concentration that are affected during smooth muscle contraction lead to important structural rearrangements within the sarcolemma, which can be attributed to members of the annexin protein family. We show that the associations of annexins II, V, and VI with smooth muscle microsomal membranes exhibit a high degree of Ca^{2+} sensitivity, and that the extraction of annexins II and VI by detergent is prevented by elevated Ca^{2+} concentrations. Annexin VI participates in the formation of a reversible, membrane–cytoskeleton complex (Babiychuk, E.B., R.J. Palstra, J. Schaller, U. Kämpfer, and A. Draeger. 1999. *J. Biol. Chem*. 274:35191–35195). Annexin II promotes the Ca^{2+}-dependent association of lipid raft microdomains, whereas annexin V interacts with glycerophospholipid microcompartments. These interactions bring about a new configuration of membrane-bound constituents, with potentially important consequences for signaling events and Ca^{2+} flux.

Key words: DIGs • smooth muscle • rafts • caveolae • contraction

Introduction

The eukaryotic cell plasma membrane consists of a well ordered array of proteins and lipids. Discrete domains therein either furnish sites for extra- and intracellular attachments or generate a favorable environment for the lodgment of receptor and signaling molecules. These specialized regions have a highly characteristic protein structure that is maintained by mutual cross-linking mechanisms. In contractile cells, such as smooth muscle, these regions become periodic and display a highly sophisticated organization. Owing to functional demands, the sarcolemma is segregated into force-transmitting regions, which are directly linked to the cytoskeleton, and flexible vesicular domains, which contain numerous caveolae. Since these cells are required to adapt rapidly and continuously to changes in length, sarcolemmal and cytoskeletal protein reorganization must needs be highly precise and flexible. Members of the annexin protein family have been recently assigned a role in smooth muscle membrane organization: they are believed to represent a reversible, Ca^{2+}-dependent link between the sarcolemma and the cytoskeleton (Babiychuk et al., 1999). The present study delineates the distinct characteristics of individual annexins (II, V, and VI) in their Ca^{2+}-dependent association with, and regulation of, specialized lipid microdomains (rafts) within smooth muscle plasma membranes and their impact on contraction.

Rafts represent lateral plasma membrane assemblies enriched in cholesterol and sphingolipids (Parton and Simons, 1995; Harder and Simons, 1997; Brown and London, 1998a,b). Acting as platforms for receptors and components of signal transduction cascades, they are associated with glycosyl phosphatidylinositol–anchored proteins on the cell surface and with members of the src-family kinases on their cytoplasmic face. This localization serves to increase the concentration or stability of complexes, with a direct enhancing effect on signaling levels (Kholodenko et al., 2000). Lipid rafts are resistant to solubilization by nonionic detergents and, therefore, can be isolated as deter-

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gent-insoluble glycosphingolipid–enriched membrane domains (DIGs;[1] Parton and Simons, 1995; Brown, 1998; Brown and London, 1998a,b). Annexins II and XIII previously have been associated with lipid rafts (Oliferenko et al., 1999; Lecat et al., 2000).

In the present study, we demonstrate that annexins II and VI translocate to the detergent-resistant sites of the smooth muscle cell plasmalemma in a Ca^{2+}-dependent manner. We also show that in the presence of Ca^{2+}, annexin II is responsible for the association of rafts. The assemblage of individual rafts is believed to be critical for raft-dependent signaling (Harder and Simons, 1997; Brown and London, 1998a,b; Schroeder et al., 1998; Pralle et al., 2000). We postulate that the membrane organizer caveolin is destined to hold lipid microdomains in place within caveolae for extended periods, and that the annexins might constitute a more subtle and flexible means of regulating Ca^{2+}-dependent raft-assemblage. In smooth muscle, the assemblage of signaling components into macromolecular complexes could permit an increase in the velocity and efficiency of force transduction as well as render possible the precise modulation of specific downstream effectors.

Materials and Methods

Tissue Preparation

Since several of the antibodies employed in this study have a restricted cross-reactivity, it was necessary to use human material for immunolabeling. Consent for working with this tissue was obtained from the Medical Ethical Commission of the University of Bern.

Thin longitudinal strips of taenia coli were obtained during surgery or within 12 h of death. Processing for contraction/relaxation experiments, thin cryosectioning and immunolabeling were performed as described previously (Babiychuk et al., 1999). In analogy to our biochemical experiments, some muscle strips were incubated for 2–8 h either in ice-cold, Ca^{2+}-free Na^+-Tyrode's solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Hepes, pH 7.4) containing 2 mM EGTA or in K^+-Tyrode's buffer (140 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$, 10 mM glucose and 10 mM Hepes, pH 7.4) containing 2 mM $CaCl_2$. Both solutions were additionally supplemented with 0.8% NP-40. The tissue was fixed in Na^+- or K^+-Tyrode's solution containing 4% paraformaldehyde and processed as described previously (Babiychuk et al., 1999). For electron microscopy, the muscle strips were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer, postfixed in osmium tetroxide, embedded in epoxy resin, sectioned, and viewed as described by Zaugg et al. (1999).

Immunohistochemistry

Immunolabeling was performed as described by Jostarndt-Fögen et al. (1998). mAbs against annexins II and VI and a polyclonal antibody against caveolin were purchased from Transduction Laboratories. An mAb against 5′-nucleotidase was provided by Professor H.G. Mannherz (University of Bochum, Bochum, Germany; Mehul et al., 1992). For dual labeling purposes, a polyclonal antibody against annexin II was generated in a rabbit; annexin II was purified according to the method described by Babiychuk et al. (1999); and subcutaneous immunization was performed in compliance with the protocol established by Harlow and Lane (1988). The antibody thereby generated was purified by ammonium sulfate precipitation and ionic exchange chromatography. It was specific for annexin II, cross-reactivity with other proteins being undetected by Western blotting.

Fluorescent labeling was performed using Cy3- (Jackson ImmunoResearch Laboratories) or Alexa-conjugated (Molecular Probes) secondary antibodies. Negative controls were generated by absorbing the antibody with purified antigen (for annexins II and VI) or by applying a nonbinding primary antibody. Tissue sections were examined in a Zeiss Axiophot fluorescent microscope, and images were collected using a digital CCD camera (Ultrapix; Astrocam).

Isolation of Smooth Muscle Microsomal and DIG Membranes

Smooth muscle microsomal membranes were isolated from porcine stomach smooth muscle according to a modified version of the protocol described by Parkin et al. (1996). Unless otherwise stated, all procedures were performed at 4°C or on ice. Minced muscle (100 g) was routinely extracted in 300 ml of buffer A (60 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 20 mM imidazole, pH 7.0). When indicated, the extraction was performed in the presence of 2 mM EGTA. After low speed centrifugation at 10,000 *g* for 30 min, the supernatant was filtered through glass wool and subjected to high speed centrifugation at 50,000 *g* for 90 min. The pellets obtained were washed three times (with intervening centrifugations at 10,000 *g* for 30 min) in 10 vol of buffer B (120 mM KCl, 0.2 mM $CaCl_2$, and 20 mM imidazole, pH 7.0) and finally resuspended in 10 ml of the same buffer. The DIG membrane fraction was obtained by following a similar procedure to that described above for microsomal membranes, the extraction step, in this case, being performed in the presence of nonionic detergent (0.5% NP-40).

Extraction of Microsomal Membranes with NP-40

100-μl aliquots of the microsomal membrane preparations (protein concentration = 4 mg/ml) were incubated for 10 min at ambient temperature in buffer B containing different concentrations of NP-40. When indicated, 2 mM EGTA was added to the preparations. The suspensions were subjected to low speed centrifugation (10 000 *g*, 30 min), and the resulting supernatants and pellets (made up to 100 μl) were characterized by TLC, for lipid components, and electrophoresis (SDS-PAGE), for protein components; their 5′-nucleotidase activity was also monitored.

EGTA Extraction of Microsomal and DIG Membranes, Turbidity Measurements, and Annexin binding

10 μl of a 100-mM solution of EGTA was added to 990-μl aliquots of smooth muscle microsomal or DIG membrane preparations (suspended in buffer B; protein concentration = 4 mg/ml). After a 10-min incubation period at ambient temperature, the suspensions were subjected to low speed centrifugation (10 000 *g* for 30 min). Ca^{2+} and EGTA concentrations in the supernatants (soluble membrane) were adjusted to 1.1 and 2 mM, respectively. 0.5-μl aliquots of a 50-mM solution of $CaCl_2$ were added stepwise (with intervening 2-min incubations at ambient temperature) to 500 μl of the resulting preparations. The Ca^{2+}-dependent increase in absorbance (turbidity) was measured at 400 nm (A_{400}) at ambient temperature. In separate experiments, the soluble membrane preparations (100 μl; protein concentration = 0.2–0.4 mg/ml) were incubated in buffer B containing 2 mM EGTA and $CaCl_2$ ranging from 1.1 to 2.1 mM. After a 10-min incubation at ambient temperature, the suspensions were centrifuged (10, 000 *g* for 30 min), and the resulting supernatants and pellets (made up to 100 μl) were characterized by TLC and SDS-PAGE as well as for 5′-nucleotidase activity.

Sucrose Gradient Centrifugation

DIG membrane preparations (protein concentrations = 2 mg/ml) or soluble DIG membrane preparations (protein concentrations = 0.5 mg/ml) were resuspended in buffer B containing either 0.2 mM $CaCl_2$ or 5 mM EGTA. These concentrations of $CaCl_2$ or EGTA were retained in the Ca^{2+}- or EGTA gradients, respectively. 1.5-ml aliquots were layered at the base of ultracentrifuge tubes and adjusted to 40% (wt/vol) sucrose by mixing with an equal volume of 80% sucrose in buffer B. Linear sucrose gradients (5–30%, buffer B) were formed over the membrane samples. Centrifugation was performed at 120, 000 *g* for 16 h in an SW27 rotor (Beckman Instruments).

Miscellaneous

5′-nucleotidase activity was monitored as P_i release, which was measured spectrophotometrically at a wavelength of 820 nm (A_{820}) according to a modification of the method described by Fiske and Subbarow (1925), using a 5′-AMP as a substrate (Babiychuk et al., 1999). TLC was performed using a modified version (Babiychuk et al., 1999) of the protocol pub-

[1]*Abbreviation used in this paper:* DIG, detergent-insoluble glycosphingolipid.

lished by Macala et al. (1983). SDS-PAGE was carried out using the procedure described by Laemmli (1970). Polypeptides were visualized by staining with Coomassie blue. Blotting of gels on Immobilon-P membranes (Millipore Corp.) was performed as detailed by Towbin et al. (1979). Immunoreactivity was detected using a secondary antibody conjugated to HRP (Amersham Pharmacia Biotech) and visualized with metal-diaminobenzidine. Protein concentrations were determined according to the method described by Bradford (1976) using BSA as a standard. Free Ca^{2+} concentrations ($[Ca^{2+}]_{free}$) were calculated using MAX CHELATOR software (Chris Patton, Stanford University, Hopkins Marine Station). TLC plates and SDS-PAGE gels were scanned and analyzed using IMAGEQUANT 3.3 software (Molecular Dynamics; Amersham Pharmacia Biotech).

Results

Macro- and Microdomains within the Smooth Muscle Sarcolemma

The smooth muscle cell sarcolemma consists of alternating vesicular and actin-binding domains, which are characterized by their respective marker proteins, caveolin and vinculin (North et al., 1993). When in a relaxed state, the cell displays a smooth surface contour (Fig. 1 a); but, when stimulated by an increase in intracellular Ca^{2+} concentration, it assumes an undulated appearance with bulging caveolar domains and caved actin attachment sites (Gabella, 1976; Fig. 1 c). It is obvious that such changes in cell shape have an impact on the caveolar form (Fig. 1, b and d). Nevertheless, the overall domain structure of the sarcolemma remains unchanged during contraction (Draeger et al., 1989), as evidenced by the failure of caveolin and vinculin immunoreactivity profiles to colocalize (Fig. 2 a).

In addition to this unvarying macrodomain organization, smooth muscle cells also possess less well defined microdomains, such as lipid rafts, which are not confined to one particular region of the plasma membrane. Dual labeling with an antibody against the 5′-nucleotidase, a marker protein for lipid rafts, reveals no preferential codistribution with caveolin (Fig. 2 b). This finding accords with data evidencing the existence of caveolin-containing and noncaveolar detergent-insoluble microdomains (Schnitzer et al., 1995b).

Annexin VI translocates to the sarcolemma at elevated intracellular Ca^{2+} concentrations (Babiychuk et al., 1999). Superimposition of images dual-labeled with antibodies against annexin VI and caveolin reveals a strong, albeit incomplete, colocalization of the two proteins, annexin VI not being exclusively restricted to one distinct macrocompartment (Fig. 2 c). In smooth muscle strips treated with NP-40 in the presence of Ca^{2+}, annexins II and VI colocalize within the sarcolemma (Fig. 3, a and b). Annexin II is also abundantly expressed in capillary endothelial cells (Figs. 2 d and 3 c). Both annexins are extracted from smooth muscle in the absence of Ca^{2+} (Fig. 3, c and d), although residual endothelial staining for annexin II is occasionally observed (Fig. 3 c). This latter finding can be explained in terms of a Ca^{2+}-independent binding of annexin II, as described by Harder et al. (1997) and König et al. (1998).

Highly Ca^{2+}-sensitive Interaction of Annexins II, V, and VI with Smooth Muscle–derived Microsomes

The Ca^{2+}-dependent interactions between annexins and the sarcolemma are the result of smooth muscle stimulation (Babiychuk et al., 1999). These membrane associations lead to the preferential accumulation of annexins II, V, and VI in microsomal preparations obtained in the

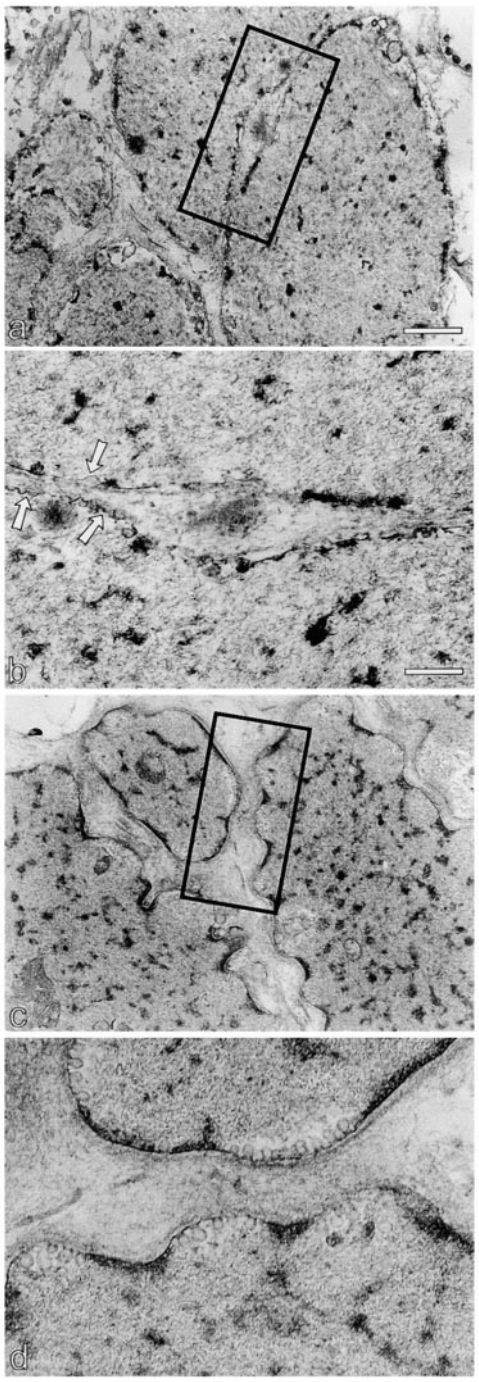

Figure 1. Sarcolemmal organization in relaxed and contracted smooth muscle cells. Electron micrographs of transverse sections through taenia coli relaxed by incubation in Ca^{2+}-free solutions (a and b) or in rigor mortis (c and d). Dense actin-binding regions (focal contacts) alternate with vesicular domains. In relaxed cells, the cell surface has a smooth contour (a), with the caveolae being wide-necked and open in some places (b, arrows). During contraction, the actin attachment sites are drawn in, whereas vesicular regions bulge out (c and d). Bars: (a and c) 1 μm; (b and d) 0.2 μm.

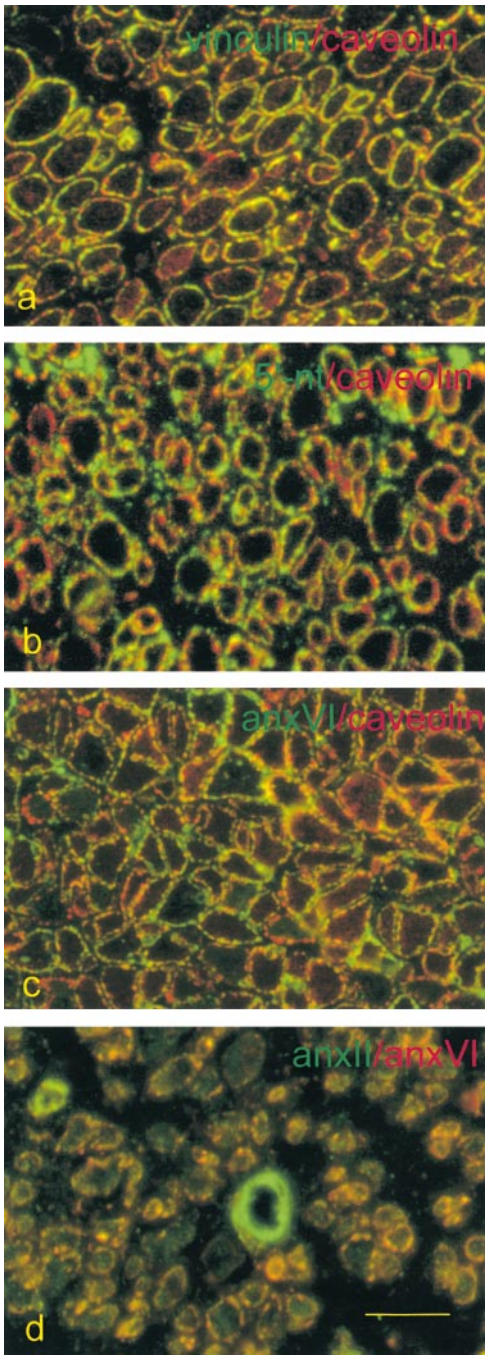

Figure 2. Smooth muscle membrane domains. Fluorescent micrographs of transverse ultra cryosections through taenia coli in rigor (a–c), or treated with NP-40/Ca^{2+}–containing Na-Tyrode's solution (d). (a) Stable macrodomains, with no overlap of regularly alternating actin attachment sites, labeled with an mAb against vinculin (green) and vesicular regions that are depicted with a polyclonal antibody against caveolin (red). (b and c) Partial colocalization of macro- and microdomains. Dual labeling with mAbs against 5′-nucleotidase (b, green) or annexin VI (c, green) and a polyclonal one against caveolin (b and c, red). (d) Dual labeling with a polyclonal antibody against annexin II (green) and a monoclonal one against annexin VI (red). Both proteins colocalize at the sarcolemma. Note the annexin II delineation of capillary endothelium. Bar, 10 μm.

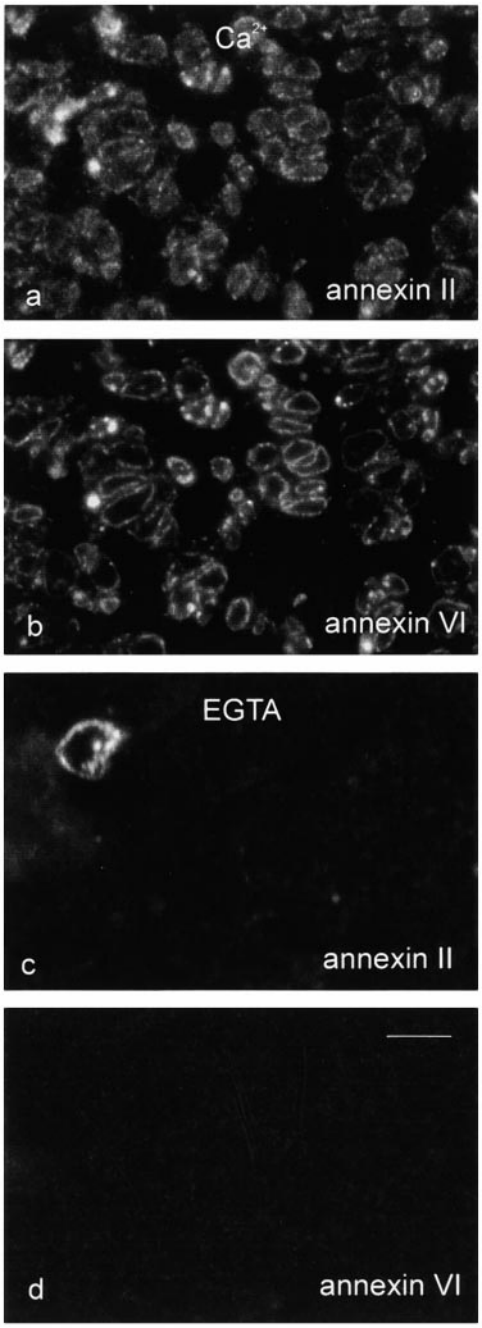

Figure 3. Extraction of annexins II and VI by detergent is prevented by elevated Ca^{2+} concentrations. Fluorescent micrographs of ultra cryosectioned taenia coli, dual-labeled with a polyclonal antibody against annexin II (a and c) and a monoclonal one against annexin VI (b and d). Annexins II and VI localize to the sarcolemma at elevated Ca^{2+} levels. Their extraction is prevented by the presence of this divalent cation (a and b). In the absence of Ca^{2+}, both proteins are extracted from smooth muscle (c and d). Annexin II is retained in some capillary endothelial cells, which might indicate that, in these cells, its lipid binding is at least partially Ca^{2+}-independent. Bar, 15 μm.

presence of Ca^{2+} (Fig. 4 a). By decreasing the Ca^{2+} concentration, it is possible to reextract these annexins (Fig. 4 d) together with membrane lipids (Fig. 4 b, inset), membrane protein markers (5′-nucleotidase [Fig. 4 c] and cave-

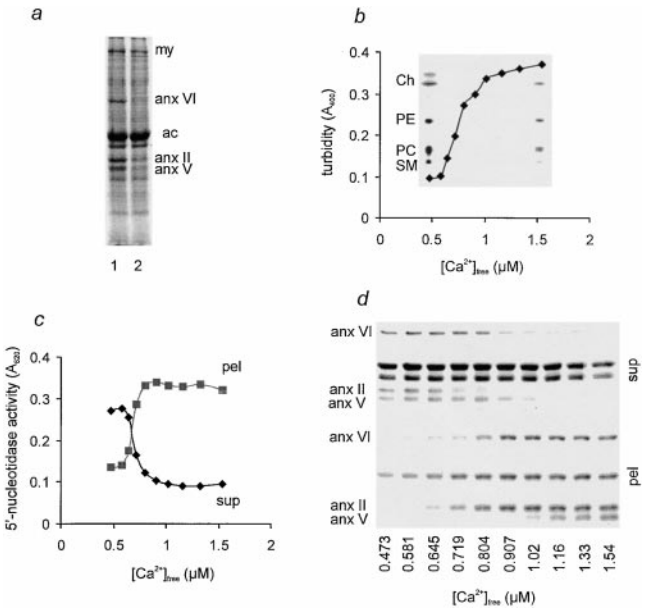

Figure 4. The interaction of annexins II, V, and VI with smooth muscle microsomal membranes exhibits a high Ca^{2+} sensitivity. (a) SDS-PAGE (30 μg of protein) of smooth muscle microsomal membrane preparations obtained in the presence of 0.2 mM $CaCl_2$ (lane 1) or 2 mM EGTA (lane 2). The positions of myosin heavy chains (my), annexin VI (anx VI), actin (ac), annexin II (anx II), and annexin V (anx V) are indicated. (b) The turbidity (A_{400}) of EGTA extracts (soluble membranes) of smooth muscle microsomal membrane preparations was measured as a function of $[Ca^{2+}]_{free}$. (b–d) EGTA extracts of smooth muscle microsomal membrane preparations, supplemented with $CaCl_2$ to obtain the indicated final $[Ca^{2+}]_{free}$, were subjected to low speed centrifugation. Equal volumes of the resulting pellets (pel) and supernatants (sup) were analyzed. (b, insets) TLCs of lipids remaining in the supernatant at a $[Ca^{2+}]_{free}$ of 0.47 and 1.54 μM; (c) 5′-nucleotidase activity and (d) an SDS-PAGE of the resulting pellets and supernatants. The positions of cholesterol (Ch), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM) are indicated.

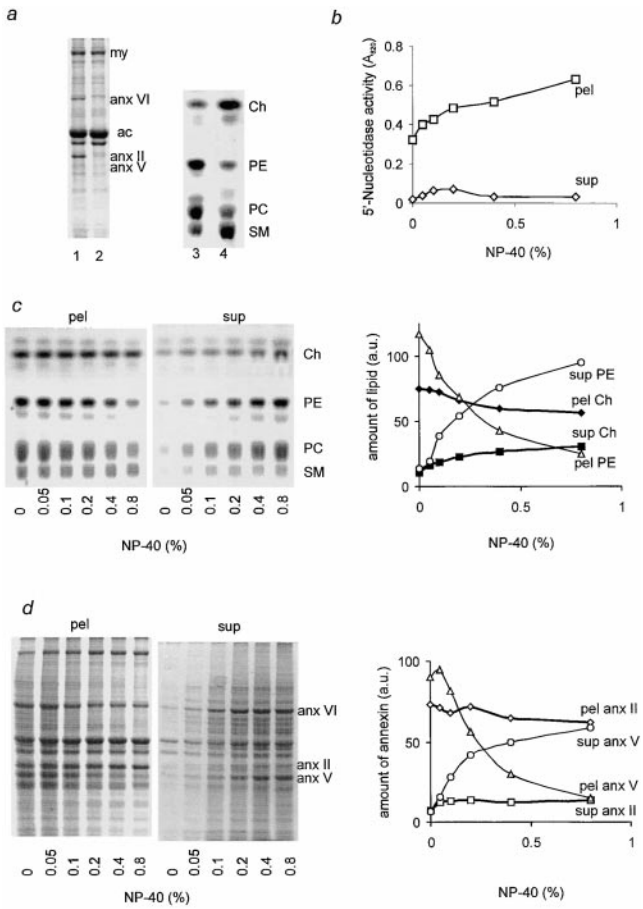

Figure 5. Annexin II and annexin V interact with different membrane lipid microcompartments. (a) SDS-PAGE (lanes 1 and 2; 20 μg of protein) of smooth muscle DIG membrane preparations obtained in the presence of 0.2 mM $CaCl_2$ (lane 1) or 2 mM EGTA (lane 2). TLC of smooth muscle microsomal (lane 3, 30 μg of protein) or DIG- (lane 4, 20 μg of protein) membrane preparations. (b–d) Microsomal membrane preparations (0.2 mM $CaCl_2$) were incubated in the presence of NP-40 at the indicated concentrations. After low speed centrifugation, equal volumes of the resulting pellets (pel) and supernatants (sup) were analyzed for 5′-nucleotidase activity (b) by TLC (c) and by SDS-PAGE (d). TLC plates and SDS-PAGE gels were scanned, and the amounts (arbitrary units) of PE, Ch, anx II, and anx V in the pellets and supernatants were plotted as a function of NP-40 concentrations (c and d, right panels).

olin [not shown]). The resulting soluble microsomal preparations (additionally containing actin and tropomyosin; Fig. 4 d) underwent aggregation upon increasing the Ca^{2+} concentration to within the physiological range (Fig. 4 b). Note that the term "soluble" as used here, indicates that the protein or lipid of interest is present within the supernatant under the centrifugation conditions employed in the present study. It is our understanding that the soluble part of the preparations is represented by small, nonaggregated microsomes or DIGs, whereas the insoluble part consists of large aggregated entities. This aggregation resulted in an accumulation of membrane lipids (Fig. 4 b, insets), protein markers (Fig. 4 c), and annexins (Fig. 4d) within the low speed centrifugation pellets. That actin and tropomyosin remained largely in the supernatant, irrespective of the Ca^{2+} concentration (Fig. 4 d), suggests that their presence in the soluble microsomal preparations is an artefact. The aggregation was highly Ca^{2+}-sensitive, with half-maximum occurring at $[Ca^{2+}]_{free}$ of 700 nM (Fig. 4, b and c), which corresponds to a half maximal accumulation of annexin II in the low speed pellet (Fig. 4 d). Annexins VI and V also accumulated in the pellets, but only at higher Ca^{2+} concentrations, with half-maximal effects occurring at ~800 nM and 1 μM, respectively (Fig. 4 d).

Different Annexins Interact with Distinct Microdomain Compartments within the Sarcolemma

In smooth muscle tissue, the extraction performed in the presence of the nonionic detergent, NP-40, resulted in a preferential solubilization of glycerophospholipids (Fig. 5 a, lanes 3 and 4) and DIG formation (Babiychuk et al., 1999). Under these conditions, annexin V, in contrast to annexins II and VI, was effectively excluded from the high speed pellet, even in the presence of Ca^{2+} (compare Figs. 4

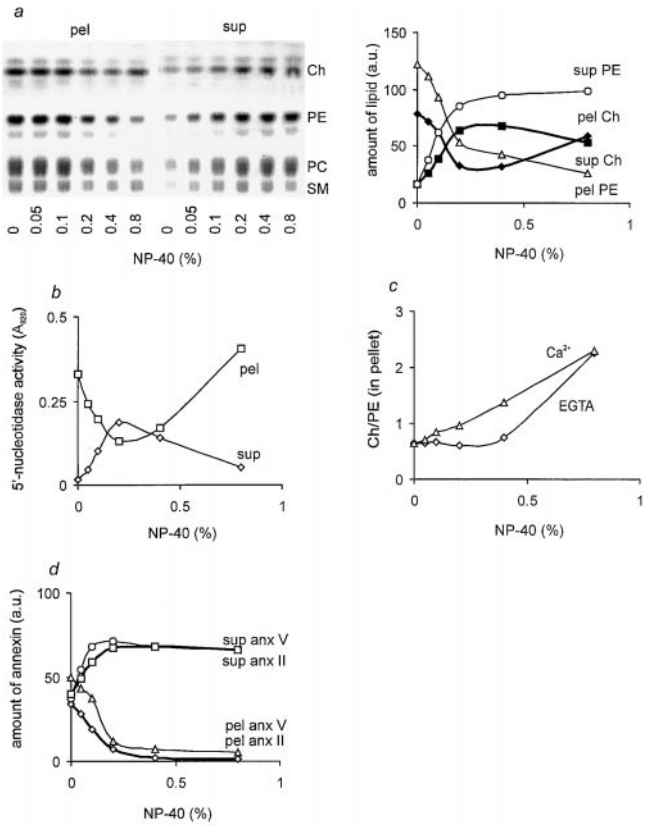

Figure 6. The formation of DIGs is a Ca^{2+}-dependent process. Microsomal membrane preparations (1 mM EGTA/0.2 mM $CaCl_2$) were incubated in the presence of the indicated NP-40 concentrations. After low speed centrifugation, equal volumes of the resulting pellets (pel) and supernatants (sup) were analyzed (a) by TLC, (b) for 5′-nucleotidase activity; and (d) by SDS-PAGE TLC plates and SDS-PAGE gels were scanned, and the amounts (arbitrary units) of PE, Ch, anx II, and anx V in the pellets and supernatants were plotted as a function of NP-40 concentration (a, right panel, and d). The relative ratios of Ch/PE in the resulting pellets that were obtained in the presence of EGTA or Ca^{2+} (Fig. 5) are given in c.

a and 5 a, lane 1). This implies that in stimulated smooth muscle, annexins II and VI interact with raft microdomains and, thus, remain in the detergent-insoluble DIG pellet after biochemical fractionation. At the same time, annexin V interacted preferentially with the glycerophospholipid membrane compartment and, therefore, was extracted together with the glycerophospholipids after NP-40 treatment.

For a more detailed investigation of these interactions, we monitored the redistribution of annexins and raft-specific markers between soluble (supernatant) and insoluble (pellet) fractions of smooth muscle microsomal preparations extracted with NP-40. In the presence of Ca^{2+}, annexin II (Fig. 5 d) pelleted with the raft markers, cholesterol, sphingomyelin (Fig. 5 c), and 5′-nucleotidase (Fig. 5 b), whereas annexin V was gradually extracted by the detergent (Fig. 5 d) after the removal of glycerophospholipids (Fig. 5 c). Annexin VI was only partially extracted with the glycerophospholipids, a portion of it remaining within the pellet even at high detergent concentrations (Fig. 5 d).

The Formation of DIGs Is a Ca^{2+}- and Annexin II–dependent Process

As was to be expected from the detergent insolubility of lipid rafts, cholesterol and sphingomyelin were only partially extracted by NP-40 from smooth muscle microsomal preparations in the presence of Ca^{2+} (Fig. 5 c). A preferential extraction of glycerophospholipids (Fig. 5 c) and, as a result, the formation of DIGs (Fig. 6 c), was observed. In the absence of Ca^{2+}, the amount of cholesterol/sphingomyelin and 5′-nucleotidase extracted by NP-40 was, surprisingly, relatively high at intermediate concentrations of detergent (0.2–0.4% of NP-40; Fig. 6 a). Consequently, in the presence of EGTA, DIG's formation was observed only at the highest detergent concentrations used in the present study (Fig. 6, a–c).

As demonstrated in Figs. 5–7, the Ca^{2+}-dependent formation of DIGs corresponded to the accumulation of an-

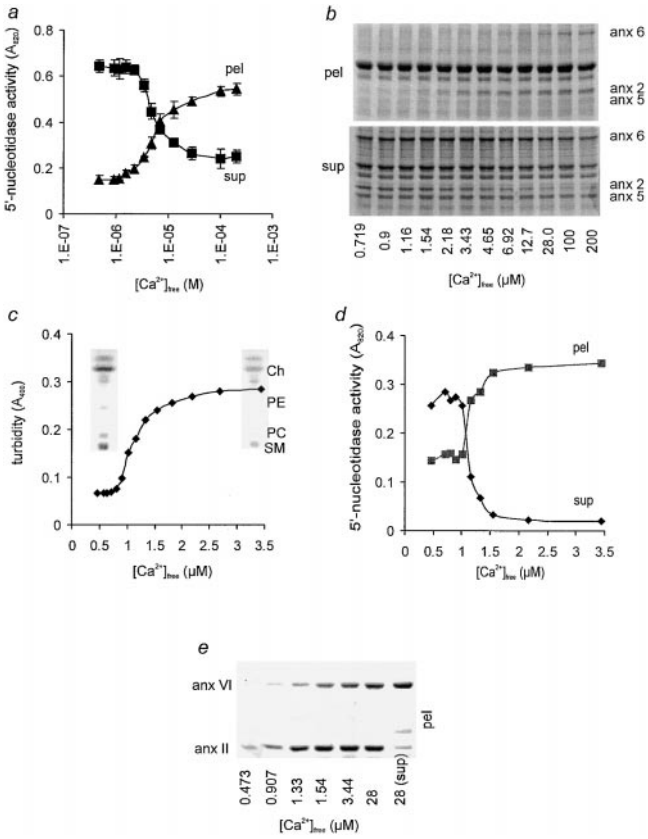

Figure 7. The Ca^{2+} sensitivity of DIG association corresponds to that of their interaction with annexin II. Microsomal membrane preparations (0.4% NP-40) were incubated in the presence of the indicated $[Ca^{2+}]_{free}$. After low speed centrifugation, equal volumes of the resulting pellets (pel) and supernatants (sup) were analyzed for 5′-nucleotidase activity (a) and by SDS-PAGE (b). The turbidity (A_{400}) of EGTA extracts of smooth muscle DIG membrane preparations (soluble DIGs) was measured as a function of $[Ca^{2+}]_{free}$ (c). Soluble DIGs, supplemented with $CaCl_2$ to obtain the indicated final $[Ca^{2+}]_{free}$, were subjected to low speed centrifugation. Equal volumes of the resulting pellets (pel) and supernatants (sup) were analyzed. TLC of lipids (c, insets) remaining in the supernatant at a $[Ca^{2+}]_{free}$ of 0.47 and 3.44 μM; (d) 5′-nucleotidase activity, and (e) SDS-PAGE of resulting pellets and supernatants.

nexin II in the DIG pellet. At low Ca^{2+} concentrations, when no interactions between DIGs and annexin II (Figs. 6 d and 7 b) were observed, raft markers (Fig. 6, a and b, and Fig. 7 a) were more readily extracted by NP-40 than at Ca^{2+} concentrations sufficiently high to bring about annexin II–DIG binding (Fig. 5, b–d, and Fig. 7).

The extraction of rafts in the absence of Ca^{2+} is surprising, but nonetheless readily explicable. It is our understanding that the Ca^{2+}-dependent formation of DIGs, observed at low detergent concentrations, occurs because of annexin II–driven cohesion of small-nonaggregated detergent-insoluble rafts initially present in microsomal preparations. Such big, associated raft structures are pelleted at low speed centrifugation, whereas small nonassociated rafts that are present at low $[Ca^{2+}]$, insufficient to ensure annexin II–raft interaction, remain in the supernatant.

While at high concentrations of detergent (Figs. 5 and 6), raft aggregation occurs independently of $[Ca^{2+}]_{free}$ and annexin II, the detergent itself is not responsible for the Ca^{2+}- and annexin II–dependent association of the rafts. As shown in Fig. 7 (c–e), in preparations of purified DIGs, aggregated and unaggregated rafts can be separated at low $[Ca^{2+}]_{free}$ by low speed centrifugation even in the absence of detergent. Moreover, the nonaggregated (soluble) rafts were able to associate in a Ca^{2+}-dependent manner (Fig. 7, c and d). The Ca^{2+} sensitivity of this association corresponded to that of annexin II–raft interaction (Fig. 7 e). Fig. 7 also demonstrates that the raft association occurs independently of cytoskeletal constituents since the pellets of soluble rafts are free of actin or other cytoskeletal proteins (Fig. 7 e).

The Ca^{2+}- and annexin II–dependent association of rafts was confirmed in density gradient centrifugation experiments. In agreement with the data of Parkin et al. (1996), the position of DIGs in the sucrose gradients depended on $[Ca^{2+}]_{free}$. At low $[Ca^{2+}]_{free}$, the centrifugation gave rise to a broad peak of DIGs (Fig. 8, a–c), which were separated from the annexins (Fig. 8 d). At high $[Ca^{2+}]_{free}$, the DIGs (Fig. 8, a–c) cofractionated with the annexins (Fig. 8 d) in a more clear-cut peak, its maximum shifted towards the base of the gradient. Such a Ca^{2+}-dependent shift in the peak position is indicative of processes associated with the change of the size of its constituents, such as occur during oligomerization of proteins. It is our understanding that in the absence of Ca^{2+}, DIGs are represented by different populations varying in their size: the aggregated (oligomeric) DIGs are located in a more basal position of the sucrose gradient, whereas smaller nonassociated (monomeric) ones float closer to the top. At a $[Ca^{2+}]_{free}$ sufficient to ensure annexin–DIG interaction, the annexin II–driven association leads to the disappearance of monomers, thus, shifting the peak of the DIGs towards the base of the gradient. The Ca^{2+}-dependent aggregation of DIGs was more pronounced in the fractions recovered from the descending (monomeric) shoulder of the peak, and occurred only when annexins were present (Fig. 8 e).

The Ca^{2+}-dependent changes in the position of DIGs within the sucrose gradient were even more pronounced in the preparations of soluble DIGs (see above); monomeric DIGs initially separated from the aggregated ones. An increase in $[Ca^{2+}]_{free}$ leads to aggregation of such preparations (Figs. 7 and 9 a), and results in their pelleting together with annexin II, even in the sucrose gradients (Fig. 9, b and c). We understand that the pelleting of soluble DIGs, observed in the presence of Ca^{2+} within sucrose gradients, is a result of high protein/lipid ratio introduced into the rafts by interacting annexin II molecules. At low $[Ca^{2+}]_{free}$, soluble DIGs do not interact with annexins, and therefore, remain floating with a peak at fraction 11 of the gradient (Fig. 9, b and c). Recombination of soluble DIGs and the annexins, which were separated by sucrose gradient centrifugation, resulted in the Ca^{2+}-dependent DIG association (Fig. 9 d).

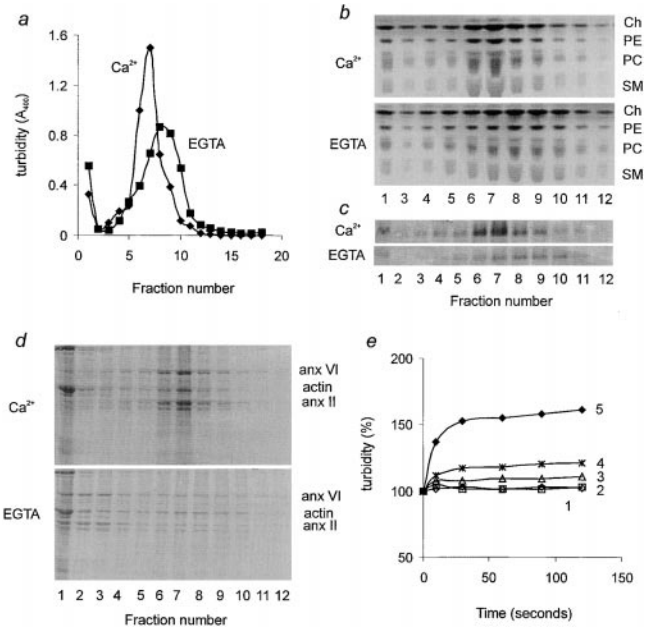

Figure 8. Ca^{2+}- and annexin II–dependent association of rafts as revealed by sucrose density gradient centrifugation: DIGs. DIG membrane preparations were subjected to sucrose gradient centrifugation either in the presence of 200 μM of $[Ca^{2+}]_{free}$ or 5 mM EGTA. The gradients were harvested in 1-ml fractions (fraction 1, base of gradient), the turbidities of which were measured at 400 nm (a). The pellet, which was obtained as a result of centrifugation, was resuspended in fraction 1. Subsequently, the fractions were analyzed by TLC for and by Western blotting for lipid (b) and caveolin (c) content, respectively, and by SDS-PAGE for protein composition (d). In e, 200 μl of fraction 6 (ascending shoulder of the DIG peak) and fraction 11 (descending shoulder of the DIG peak) of the EGTA gradient was recombined with 200 μl of the annexin-containing fraction 2 of the EGTA gradient. The turbidity of the samples (lanes 4 and 5) was measured at indicated times after raising the $[Ca^{2+}]$ to 200 μM (zero time). In control experiments, the Ca^{2+}-dependent changes in turbidity of fraction 11 of the EGTA gradient recombined either with 40% sucrose (lane 1) or the annexin-free fraction 2 of the Ca^{2+} gradient (lane 2) as well as fraction 2 of EGTA gradient alone (lane 3) were measured as described above. Note that the Ca^{2+}-dependent aggregation of DIGs occurs only in samples containing both DIGs and annexins. Moreover, the aggregation is more prominent in fractions taken from the descending shoulder of the DIG peak (DIG monomers) than in ascending ones (DIG oligomers).

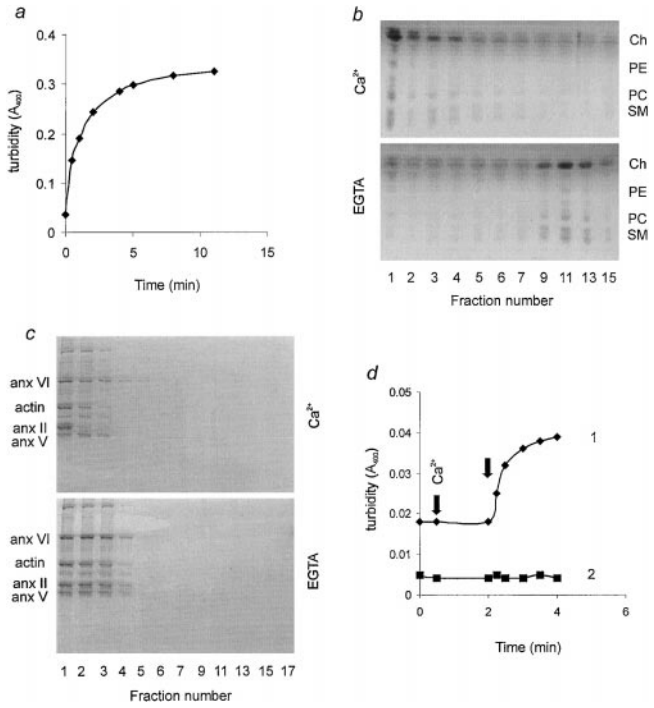

Figure 9. Ca^{2+}- and annexin II–dependent association of rafts as revealed by sucrose density gradient centrifugation: soluble DIGs. Soluble DIG preparations were subjected to sucrose gradient centrifugation either in the presence of 200 μM of $[Ca^{2+}]_{free}$ or 5 mM EGTA. The addition of Ca^{2+} before centrifugation resulted in the rapid association of soluble DIGs (a). The gradients were harvested in 1-ml fractions (fraction 1, base of gradient). The pellet, which was obtained as a result of centrifugation, was resuspended in fraction 1. Subsequently, the fractions were analyzed by TLC for lipid (b) and by SDS-PAGE for protein (c) composition. Note that in the presence of Ca^{2+}, most of the DIGs coprecipitate together with annexin II, but not annexin V in the pellet, whereas in the absence of Ca^{2+}, the DIGs separate from the annexins and float on the gradient with a peak in fraction 11. In d, the $[Ca^{2+}]_{free}$ in the DIG-containing fraction 11 of the EGTA gradient (400 μl) was adjusted to 200 μM (first arrow). Subsequently, 200 μl of the annexin-containing fraction 2, derived from the EGTA gradient, was added to the sample (second arrow, lane 1). The turbidity of the samples was measured at the indicated times. In control experiments, the Ca^{2+}-dependent changes in the turbidity of fraction 2 of the EGTA gradient recombined with the DIG-free fraction 11 of Ca^{2+} gradient (lane 2) was measured as described above. Note that a Ca^{2+}-dependent aggregation of soluble DIGs occurs only in the presence of annexins.

Discussion

Annexins as Membrane Organizers

In spite, or possibly because, of their universal distribution, with several family members residing within any one cell, the physiological role of annexins has remained an enigmatic one. Although their distinct biological function may differ according to the cell type investigated, this is always intimately connected with their lipid-binding properties. The Ca^{2+}-dependent binding of annexins II, V, and VI to microsomal and DIG smooth muscle membrane preparations, which was observed in the present study, is in keeping with their role as principal cell membrane organizers (Gerke and Moss, 1997). However, Ca^{2+} concentrations far exceeding the physiological limit have been required for their binding to cell membranes (with the exception of the annexin II tetramer), a circumstance which has represented a major challenge to scientists in evaluating their biological roles (Raynal and Pollard, 1994). In the present study, each of the three annexins investigated, interacted with smooth muscle microsomes with a very high, yet clearly distinct, Ca^{2+} sensitivity. This observation goes some way towards accounting for the presence, in any given cell, of several proteins with barely differing basic biochemical properties. Our findings suggest that different members of the annexin family permit a cell to sense and respond to the fluctuations in intracellular Ca^{2+} levels, that occur during stimulation.

Another challenging problem is presented by the well documented preference of annexins for Ca^{2+}-dependent binding to negatively charged glycerophospholipids (for review see Raynal and Pollard, 1994; Gerke and Moss, 1997). According to specific biochemical traits, such as Ca^{2+} affinities and lipid specificities, all annexins readily bind to membranes containing negatively charged glycerophospholipids (e.g., phosphatidic acid, phosphatidylserine, and phosphatidylinositol). Since these negatively charged glycerophospholipids represent ubiquitous structural components of all biological membranes, an explanation for the preferential interaction of different annexins with specific target membranes is not immediately forthcoming (for review see Gerke and Moss, 1997). The present study demonstrates that preferential binding of the annexins to negatively charged glycerophospholipids as observed in artificial membrane preparations does not necessarily reflect conditions pertaining in vivo. Our data suggest that whereas the interactions with negatively charged glycerophospholipids are presumably critical for annexin V binding to biological membranes, annexin II exhibits a preference for different lipid moieties. Since DIGs contain only trace amounts of negatively charged glycerophospholipids (Parkin et al., 1996), the annexin II–driven aggregation of these structures that were observed in the present study must be governed by an unrelated mechanism. We have not been able to ascertain whether this is based on a direct interaction between annexin II and specific lipids (cholesterol and sphingomyelin), or whether this protein recognizes structural differences in the lipid organization between raft- and nonraft regions (Brown and London, 1998b). Since no highly Ca^{2+}-specific interactions between annexins and lipids (other than negatively charged ones) have been observed to date in model experiments with artificial membranes, we favor the latter explanation.

Annexins and Lipid Raft Dynamics

Lipid rafts, which represent platforms for the recruitment of glycosyl phosphatidylinositol–anchored and acylated proteins, are involved in a multitude of membrane trafficking and signaling events. They can associate to form larger stable complexes or move to preexisting membrane invaginations, namely, the caveolae (Parton and Simons, 1995; Schnitzer et al., 1995a; Harder and Simons, 1997; Anderson, 1998).

The plasma membrane can be envisaged as a mosaic composite of raft- and nonraft regions, the dynamics of which is determined by the cohesion of glycosphingolipid–cholesterol rafts and their entropy-driven dispersal in the glycerophospholipid surroundings (Parton and Simons, 1995; Harder and Simons, 1997; Brown and London, 1998a,b). The size of lipid microdomains is variable since individual rafts can dissociate or fuse to form larger complexes. As raft size is dependent on dissociation kinetics, larger entities are more stable than smaller ones. Pralle et al. (2000) have recently demonstrated that individual rafts are small structures containing a limited number of associated proteins. For this reason, they proposed that only by clustering is the concentration of associated molecules elevated sufficiently to elicit a signal above the activating threshold. Hence, the regulatory mechanisms underlying lipid microdomain oligomerization are critical for raft-dependent signaling.

One obvious means of effecting raft oligomerization is already known to be operative in caveolae: cholesterol-binding caveolin generates oligomers that create a lattice for raft binding and association (Parton and Simons, 1995). This kind of raft association might lead to the formation of stable structures, which are subject to long-term regulation by modifications in the levels of cholesterol or caveolin production within the cell. However, it is also conceivable that raft association is regulated by short-term mechanisms, such as the fluctuations in cytosolic Ca^{2+} concentration occurring during cell stimulation. Here, we have demonstrated that annexin II aggregates DIG preparations in a Ca^{2+}-dependent manner.

Together with a cascade of other proteins, including receptors, membrane transporters, and signal transducers (Parton and Simons, 1995; Anderson, 1998), annexins II and VI have been localized to purified DIGs and caveolae (Sargiacomo et al., 1993; Lisanti et al., 1994; Harder and Gerke, 1994; Schnitzer et al., 1995a; Parkin et al., 1996; Stan et al., 1997; Babiychuk et al., 1999). In vitro, one annexin II tetramer might aggregate lipids, providing a physical link between two neighboring vesicles. But in vivo, such an aggregation is likely to be spatially impossible, the size of one annexin II molecule being insufficient to bridge the distance between two adjacent rafts. However, annexins can self-associate on membrane surfaces, forming a two-dimensional lattice (Lambert et al., 1997). As in the case of caveolin oligomers, these Ca^{2+}-dependent annexin oligomeric structures might serve as platforms for raft recruitment and association. That cell-surface receptors localized within lipid rafts have been observed to cocluster upon submembranous aggregation of mutant annexin II (Oliferenko et al., 1999) is strongly supportive of this mechanism.

Given its wide distribution, we propose that the annexin II–driven, Ca^{2+}-dependent association of rafts is not restricted to smooth muscle tissue, but represents a general regulatory mechanism. Parkin et al. (1996) have demonstrated that DIGs isolated from porcine lung membranes float on sucrose gradients in the presence of detergent as two distinct bands, both enriched in raft markers and annexin II, but lacking annexin I, IV, or V. In accordance with our findings, annexin VI was found to be evenly distributed between the detergent-soluble and insoluble fractions. Introduction of EGTA into the sucrose gradients prevented the formation of the lower detergent-insoluble fraction and led to the dissociation of annexins II and VI from DIGs. Our results suggest that the two distinct bands observed by Parkin et al. (1996) represent aggregated and nonaggregated DIGs, the former dissociating in the presence of EGTA. Their observation confirms the validity of our data and demonstrates that DIG association is a reversible process, which is governed by Ca^{2+}/annexin II.

Our data suggest that raft-dependent signaling in vivo might be linked to Ca^{2+}-dependent signaling pathways via annexin II. Various molecular mechanisms can be envisaged for such a coordinated action of two vital signaling cascades. Here, we deliberate on two, which might be involved in signal transduction from the inside of the cell across the plasma membrane (Fig. 10). Oligomerization of raft microdomains could either alter the activity of peripheral enzymes by promoting changes in their lipid environments, or induce clustering of cell-surface receptors by forcing their coercion. In response to an initial stimulation, this would lead to membrane compartmentalization and a corresponding effect on a multitude of signaling events connecting the cell to the outside world.

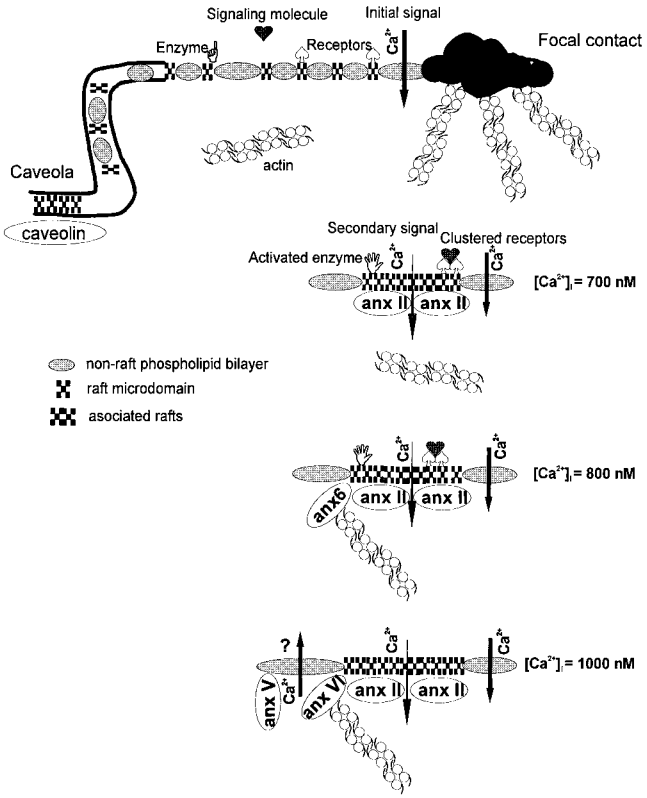

Figure 10. Ca^{2+} dependency of annexin–membrane interactions in smooth muscle cells. An initial signal raises the intracellular $[Ca^{2+}]_{free}$ to 700 nM. This triggers the translocation and binding of annexin II to DIGs. The clustering of these lipid microdomains alters the spatial organization of membrane receptors, activates enzymes, and triggers the firing of secondary signals, which further elevate intracellular Ca^{2+} concentration. At $[Ca^{2+}]_{free}$ of 800 nM, annexin VI translocates to the sarcolemma and forms a bond between cytoskeleton and the plasma membrane. Elevation of $[Ca^{2+}]_{free}$ to 1,000 nM induces annexin V–binding to glycerophospholipids. This mechanism may possibly instigate cell relaxation.

Annexins and Smooth Muscle Contraction

The cytoplasmic $[Ca^{2+}]_{free}$ is the principal determinant of contractility in smooth and striated muscle. Triggered by the activation of surface receptors, a transient rise in Ca^{2+} initiates a series of protein interactions that lead to cell shortening. Several members of the annexin family have been implicated in the regulation of Ca^{2+} homeostasis, either because of their intrinsic Ca^{2+} channel activity or their ability to regulate the activity of unrelated channels and pumps (for review see Gerke and Moss, 1997). Cardiomyocyte-directed overexpression of annexin VI results in a decrease in the resting levels of Ca^{2+} and the generation of smaller Ca^{2+} spikes on electrical stimulation of murine mutants (Gunteski-Hamblin et al., 1996)

In the present study, we describe annexin–membrane interactions that might lead to major changes in Ca^{2+} homeostasis within another contractile cell type. We have ascertained the existence of distinct levels of Ca^{2+} sensitivity for the interaction of different annexins with microsomal membranes. Translated into functional consequences during smooth muscle contraction, we propose that the initial signal will elevate intracellular $[Ca^{2+}]_{free}$ to ~700 nM, which might initiate annexin II–linked oligomerization of lipid microdomains (Fig. 10). This event might, in turn, give rise to a secondary increase in intracellular $[Ca^{2+}]_{free}$, to ~800 nM, which would establish an annexin VI–dependent link between the contractile apparatus and the sarcolemma to meet the cell's increasing demand for efficient force transduction (Babiychuk et al., 1999). Should the rise in $[Ca^{2+}]_{free}$ persist and attain micromolar levels, the sarcolemma could react by using annexin V–dependent signaling events. It is not inconceivable that the latter, being localized to sarcolemmal microcompartments distinct from those associated with annexin II, could elicit a decrease in intracellular $[Ca^{2+}]_{free}$ to prevent overcontraction and induce the commencement of relaxation.

In smooth muscle cells, caveolae are stable components within a well defined sarcolemmal domain, to which the main elements of contraction (e.g., Ca^{2+}-ATPase, IP_3 receptors, and calmodulin) have been localized (for review see Anderson, 1998). Caveolae are also believed to be sites for Ca^{2+} storage and entry, and two potent smooth muscle relaxants, namely, nitric oxide and adenosine, are produced by caveolae-associated enzymes (Rizzo et al., 1998; Ludwig et al., 1999; Lasley et al., 2000).

Constant changes in the shape of smooth muscle cells during contraction-relaxation cycles require an effective coordination of cytoskeletal and sarcolemmal rearrangements to protect the cells from mechanical damage. Although smooth muscle cells do not possess the highly developed, stretch-sensing organs of skeletal muscle, the contractile state of individual cells is, nonetheless, subject to constant monitoring and fine tuning. Endothelial cell caveolae derived from the pulmonary vasculature have been accredited recently with a stretch sensor function (Rizzo et al., 1998). And it is conceivable that in the smooth muscle cell sarcolemma, caveolar regions act in a similar capacity. Members of the annexin protein family, with their closely related lipid-binding properties, but distinct features as regards Ca^{2+} sensitivity and actin-binding properties, would be ideal candidates to act as intermediaries between the cytoskeleton and plasma membrane in the subtle regulation of sarcolemmal tension.

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