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Annexin VI Participates in the Formation of a Reversible, Membrane-Cytoskeleton Complex in Smooth Muscle Cells*

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The plasmalemma of smooth muscle cells is periodically banded. This arrangement ensures efficient transmission of contractile activity, via the firm, actin-anchoring regions, while the more elastic caveolae-containing "hinge" regions facilitate rapid cellular adaptation to changes in cell length. Since cellular mechanics are undoubtedly regulated by components of the membrane and cytoskeleton, we have investigated the potential role played by annexins (a family of phospholipid- and actin-binding, Ca^{2+}-regulated proteins) in regulating sarcolemmal organization. Stimulation of smooth muscle cells elicited a relocation of annexin VI from the cytoplasm to the plasmalemma. In smooth, but not in striated muscle extracts, annexins II and VI coprecipitated with actomyosin and the caveolar fraction of the sarcolemma at elevated Ca^{2+} concentrations. Recombination of actomyosin, annexins, and caveolar lipids in the presence of Ca^{2+} led to formation of a structured precipitate. Participation of all 3 components was required, indicating that a Ca^{2+}-dependent, cytoskeleton-membrane complex had been generated. This association, which occurred at physiological Ca^{2+} concentrations, corroborates our biochemical fractionation and immunohistochemical findings and suggests that annexins play a role in regulating sarcolemmal organization during smooth muscle contraction.

The unique role of smooth muscle cells residing within any organ wall is their propulsive activity. Irrespective whether they belong to the gastrointestinal, the urogenital tract, or the vascular system, their contraction exerts a force, which is transmitted along their entire length to the surrounding extracellular matrix via the sarcolemma. The smooth muscle cell sarcolemma is segregated into domains of rib-like *adherens* junctions alternating with regions containing vesicular invaginations (1, 2).

Actin filaments are assembled within the submembranous *adherens* junctions or dense plaques, and are coupled to the sarcolemma via a complex set of molecules; these, in turn, are linked to the extracellular matrix by transmembrane integrin receptors (3). The "non-junctional" regions are comprised of caveolae which occur in close proximity to Ca^{2+}-storage sites in the sarcoplasmic reticulum (4, 5). During contraction, the sarcolemma displays a sequential arrangement of firm, inward-caving anchoring regions and flexible, outward bulging, hinge-like domains, an organization which is reminiscent of a barrel when viewed in three-dimensions (6, 7).

Recently lipid-binding, Ca^{2+}-regulated proteins purified by us from porcine stomach smooth muscle were identified as belonging to the annexin protein family. Implicated in membrane organization, these proteins have also been assigned roles in the regulation of Ca^{2+} homeostasis and signal transduction (for review, see Ref. 8). The purpose of the present study was to pinpoint the intracellular location of these annexins and to elucidate the mode of their action in smooth muscle cells. Antibody labeling of thin tissue sections of human *taenia coli* revealed the intracellular distribution of annexin VI in smooth muscle to depend upon the contraction state of the cells. Consequently, our biochemical experiments revealed the formation of a Ca^{2+}- and annexin-dependent membrane-cytoskeleton complex. We propose that such a complex is involved in sarcolemmal organization during smooth muscle contraction.

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 post-mortem, within 12 h of death. These muscle strips were secured at both ends, in a slightly extended state, and then either fixed immediately ("native" tissue) in 4% paraformaldehyde (see below) or plunged into 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, for periods between 15 min and 12 h to ensure complete relaxation. Isometric contraction of a number of the "relaxed" muscle strips (still secured) was elicited by incubation either 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) or in Na^+-Tyrode's solution containing 2 mM $CaCl_2$ and 10 nM angiotensin II, for 1–30 min in each instance. In some cases, smooth muscle strips were subjected to contraction-relaxation-contraction cycles. Native, relaxed or contracted, tissue was fixed in Na^+- or K^+-Tyrode's solution containing 4% paraformaldehyde for 2–3 h at ambient temperature. After several washes in phosphate-buffered saline, muscle strips were cryprotected by immersion overnight in a polyvinylpyrrolidone/sucrose mixture (9). They were then mounted on aluminum cryopins and plunge-frozen in liquid nitrogen. Semi-thin (~0.25 μM thick) cryosections were prepared according to the method described by Tokuyasu (10), retrieved on droplets of 2 M sucrose containing 0.75% gelatin, transfered to siliconized glass slides, and then maintained in phosphate-buffered saline for no longer than 1 h prior to immunolabeling.

Immunohistochemistry

Immunolabeling was performed as described by Jostarndt-Fögen *et al.* (11). Monoclonal antibodies against annexins II and VI and a polyclonal antibody against caveolin were purchased from Transduction Laboratories (Lexington, KY). Fluorescent labeling was performed using Cy3- or Cy2-conjugated secondary antibodies (Jackson Laboratories, Baltimore, MD). No difference in pattern or intensity of staining was observed between surgically derived material and that obtained up

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to 12 h post-mortem. Tissue sections were examined in a Zeiss Axiophot fluorescent microscope and images collected using a digital CCD camera (Ultrapix, Astrocam).

Purification of a Smooth Muscle Membrane-Cytoskeleton Complex and Its Annexin and Lipid Fractions—Unless otherwise stated, all procedures were performed at 4 °C or on ice. Minced porcine stomach muscle (100 g) was extracted in 300 ml of buffer A (60 mM KCl, 2 mM $MgCl_2$, and 20 mM imidazol, pH 7.0) containing 0.5% Triton X-100 and 0.2 mM $CaCl_2$. After centrifugation at 6,000 × *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 thereby obtained were washed 3 times (with intervening centrifugations at 6,000 × *g* for 30 min) in 10 volumes of buffer B (120 mM KCl and 20 mM imidazol, pH 7.0) containing 0.2 mM $CaCl_2$ and finally resuspended in 10 ml of the same buffer. This represented the purified membrane-cytoskeleton complex.

For purification of annexin and lipid fractions, the membrane-cytoskeleton complex was extracted with 5 volumes of buffer B containing 1 mM EGTA. The extracts were centrifuged at 6,000 × *g* for 30 min. To the annexin/lipid supernatant 1.2 mM $CaCl_2$ was added. The pellet obtained after centrifugation at 6,000 × *g* for 30 min was washed in buffer B, 0.2 mM $CaCl_2$ (this step led to removal of most of contaminating actin/tropomyosin and low-Ca^{2+}-sensitive annexin V) and re-extracted with 3 volumes of buffer B containing 1 mM EGTA. The resulting pellet was washed 3 times (intervening centrifugation 6,000 × *g* for 30 min) and finally resuspended in buffer B. This represented the purified lipid fraction of membrane-cytoskeleton complex. The supernatant was centrifuged at 20,000 × *g* for 60 min; the resulting supernatant consisted of the purified annexin fraction (annexin II and VI). For N-terminal amino acid sequencing, annexin VI was further purified by anion-exchange chromatography and gel filtration (Q-Sepharose FF and Sephacryl S-200 HR; Amersham Pharmacia Biotech, Uppsala, Sweden).

Purification of Smooth Muscle Actomyosin—As the actomyosin fraction of the membrane-cytoskeleton complex was contaminated by lipids, smooth muscle actomyosin was purified according to the method described by Sobieszek and Bremel (12) for use in recombination experiments. In short, minced porcine stomach muscle (100 g) was extracted in 300 ml of buffer A containing 0.5% Triton X-100. The homogenate was centrifuged at 6,000 × *g* for 30 min and the pellet washed 3 times in the same buffer (with intervening centrifugations at 6,000 × *g* for 30 min). This was then homogenized in 3 volumes of buffer A containing 2 mM EDTA, 2 mM EGTA, and 10 mM ATP and the whole centrifuged at 25,000 × *g* for 30 min. The supernatant was filtered through glass wool, and its $MgCl_2$ concentration raised to 25 mM. This fraction was incubated overnight at 4 °C and then centrifuged at 6,000 × *g* for 30 min. The resulting pellet was washed extensively in buffer A containing 1 mM EGTA; it consisted of purified actomyosin.

5′-Nucleotidase Activity—5′-Nucleotidase was assayed using 5′-AMP as substrate. In brief, samples (2–5 μl) were incubated in 100 mM Tris-HCl (pH 8.5) containing 10 mM AMP and 10 mM $MgCl_2$ (final volume, 300 μl), for 10–30 min at 37 °C. For control runs, the protein was inactivated prior to incubation by treatment with 5% trichloroacetic acid. The reaction products were quantified spectrophotometrically (A_{820}) as described by Parkin *et al.* (13).

N-terminal Amino Acid Sequence Analysis and Mass Spectrometry—N-terminal amino acid sequence analysis was performed in a pulsed liquid-phase sequenator 477A (Applied Biosystems) using the Edman degradation technique and a program adapted from Hunkapillar *et al.* (14). The released amino acids were analyzed on-line by reverse-phase high performance liquid chromatography. The masses of the samples were determined by electrospray ionization mass spectrometry using a VG Platform single-stage quadruple mass spectrometer (Micromass, Manchester, United Kingdom).

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—SDS-PAGE[1] was performed according to the procedure described by Laemmli (15). Polypeptides were visualized by Coomassie staining. Blotting of gels on Immobilon-P membranes (Millipore Corp., Bedford, MA) was carried out according to the method described by Towbin *et al.* (16). Monoclonal antibodies against α-smooth muscle and β-cytoplasmic actin, smooth muscle myosin, and vinculin were obtained from Sigma. Immunoreactivity was detected using a secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) and visualized with diaminobenzidine.

Thin-layer Chromatography (TLC)—Samples (10–50 μl) were extracted in a 1:1 mixture (1 ml) of chloroform and methanol for 10 min on ice. 0.25 ml of water was then added and the emulsion centrifuged for 5 min. The lower organic layer was carefully removed and dried. Samples were resuspended in 50 μl of chloroform and spotted onto a silica gel TLC plate (Merck, Darmstadt, Germany), which was developed according to Macala *et al.* (17). Lipid concentration was estimated by the simultaneous application of lipid standards of known concentration. Lipids were detected by spraying TLC plates with a 5% (w/v) ethanolic solution of phosphomolybdic acid, followed by heating at 120 °C for 10 min.

Miscellaneous—Protein concentrations were determined according to the method described by Bradford (18) using bovine serum albumin as a standard. Free Ca^{2+} concentrations ($[Ca^{2+}]_{free}$) were calculated according to Fabiato and Fabiato (19) and Harrison and Bers (20) using the MAX CHELATOR computer program designed by Chris Patton (Stanford University, Hopkins Marine Station).

RESULTS

Constant changes in the shape of smooth muscle cells during contraction-relaxation cycles require effective regulatory mechanisms for co-ordinated cytoskeleton and sarcolemma rearrangement to protect the cells from mechanical damage. It is not inconceivable that these changes are regulated by calcium-dependent pathways. In our quest for Ca^{2+}-dependent interactions between plasma membrane and actin-based cytoskeleton, we were investigating a redistribution of membrane lipids and actin in smooth muscle extracts in the presence or absence of Ca^{2+}.

Formation of a Smooth Muscle-specific, Ca^{2+}-dependent Membrane-Cytoskeleton Complex in Smooth Muscle Extracts—Porcine stomach smooth muscle (100 g) was homogenized in 3 volumes of buffer A in the presence of 2 mM EGTA and the supernatant collected after low-speed centrifugation (6,000 × *g*, 30 min) was then subjected to a high-speed centrifugation (50,000 × *g*, 90 min) to remove insoluble material (first high-speed pellet). One-half of the supernatant was left on ice while to the other 2.2 mM $CaCl_2$ was added and after incubation of both fractions at ambient temperature for 30 min they were further centrifuged at 50,000 × *g* for 90 min. Resulting high-speed Ca^{2+} and EGTA pellets as well as the first high-speed pellet were extensively washed (10,000 × *g*, 30 min) in buffer B containing additionally 0.2 mM $CaCl_2$ (Ca^{2+} pellet) or 1 mM EGTA (EGTA pellet) and finally resuspended in 10 ml (20 ml for the first high speed pellet) of buffer B per 0.2 mM $CaCl_2$.

We observed an accumulation of cytoskeletal and contractile elements (α-smooth muscle and β-cytoplasmic actin isoforms, smooth muscle myosin), annexins (II and VI), and membrane lipids in the high-speed Ca^{2+} pellet (Fig. 1*a* and *b, lane 4*) in comparison to that obtained in the presence of EGTA (Fig. 1, *a, lanes 5–5′*, and *b, lane 5*). Polypeptides of molecular mass of 32 and 38 kDa present in the Ca^{2+} pellet were identified by mass-spectrometry as annexin V and tropomyosin (not shown).

Incubation of the Ca^{2+} pellet at 0.47 or 12.2 μM $[Ca]_{free}$ led to separation of both suspensions into two phases (Fig. 2*a*). At 0.47 $[Ca]_{free}$ lipids were evenly distributed over the total volume, while actomyosin formed the lower phase. At 12.2 μM $[Ca]_{free,}$ the smaller upper phase did not contain lipids nor actomyosin, both of which constituted the lower phase (Fig. 2*a*). At elevated $[Ca^{2+}]_{free}$, the recombination of the lipid and annexin fractions of the Ca^{2+} pellet with purified actomyosin (Fig. 2*b*), led to the formation of a structured precipitate, identical to that shown in Fig. 2*a*. At low $[Ca^{2+}]_{free}$ or in the absence of either annexin or lipid fractions, actomyosin rapidly accumulated at the bottom of the tube (see Fig. 2*b*). Thus, both lipid and annexin fractions are responsible for the Ca^{2+}-dependent changes in actomyosin precipitation pattern. This observation, in concert with the Ca^{2+}-dependent co-fractionation of actomyosin, annexins, and lipids in smooth muscle extracts (Fig. 1)

[1] The abbreviation used is: PAGE, polyacrylamide gel electorphoresis.

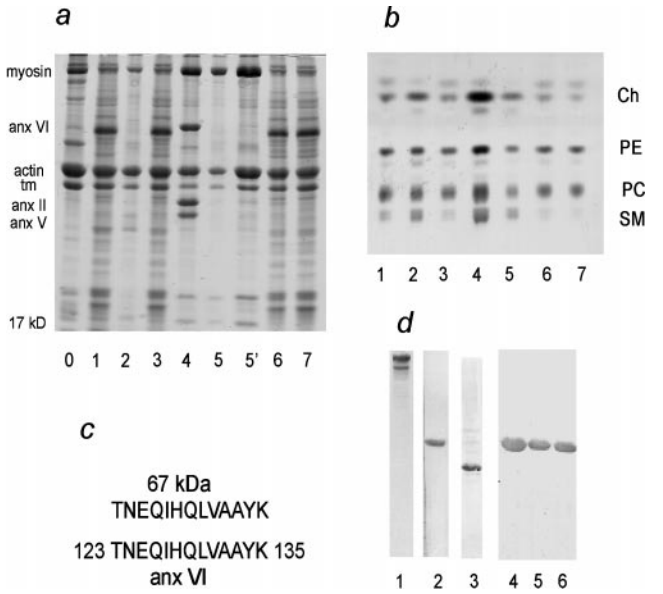

FIG. 1. **Ca^{2+}-dependent co-precipitation of actomyosin, membrane lipids, and annexins in smooth muscle extracts.** *a* and *b,* equal amounts of protein (*0, 1, 3, 4, 5′, 6*, and *7*) or equal aliquots (*2, 4*, and *5*) of each sample: *0*, 6000 × *g* pellet; *1,* 6000 × *g* supernatant; *2,* first high-speed pellet; *3,* first high-speed supernatant; *4,* high-speed Ca^{2+}-pellet; *5*, and *5′*, high-speed EGTA pellet; *6*, high-speed Ca^{2+}-supernatant; *7,* high-speed EGTA supernatant, were analyzed for protein composition by SDS-PAGE (*a*) or lipid composition by TLC (*b*). The positions of major polypeptides identified in high-speed Ca^{2+} pellet and cholesterol (*Ch*), phosphatidylcholine (*PC*), phosphatidylethanolamine (*PE*), and sphingomyelin (*SM*) are indicated. Note an absence of Ca^{2+}-dependent accumulation of calmodulin (17 kDa) in the Ca^{2+}-pellet (the Ca^{2+}-independent polypeptide of the same M_r is myosin light chain). *c,* the partial amino acid sequence of rat annexin VI (123–135) is identical with that of the 67-kDa polypeptide enriched in the Ca^{2+} pellet. *d,* Western blotting of Ca^{2+} pellet (*1–3, 5*), 6000 × *g* pellet (*4*), and 50,000 × *g* Ca^{2+} supernatant (*6*). Equal amounts of protein from each sample were subjected to SDS-PAGE and blotted with monoclonal antibodies raised against smooth muscle myosin, (*1*), α-smooth muscle actin (*2*), annexin II (*3*), and β-smooth muscle actin (*4–6*).

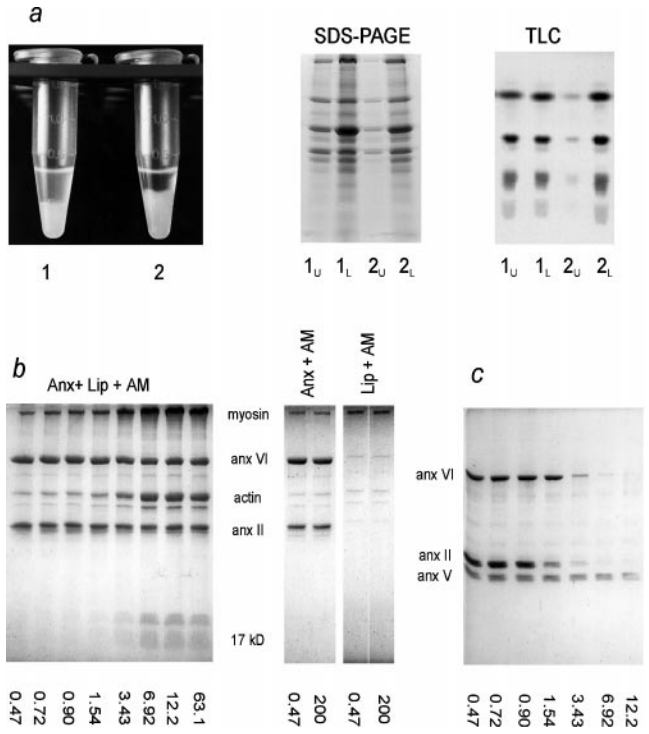

FIG. 2. **Ca^{2+}- and annexin-dependent formation of a membrane-cytoskeleton complex.** *a,* suspensions of the Ca^{2+} pellet (1.5 mg/ml protein) were incubated for 60 min on ice in buffer B at 0.47 (*1*) or 12.2 (*2*) μM $[Ca]_{free}$. 50 μM of each upper (*U*) and lower (*L*) phases were withdrawn and equal aliquots analyzed by SDS-PAGE and TLC. *b,* purified actomyosin (100 μg) was recombined (final volume: 50 μl) either with the purified annexin fraction (25 μg) (*Anx + AM*), the purified lipid fraction (50 μg) (*Lip + AM*), or both annexin, and lipid fractions (*Anx + Lip + AM*), at the indicated concentrations of free Ca^{2+}. After incubation for 20 min on ice the fractions separated into 2 phases, as shown in *a*. Subsequently, 30-μl aliquots were carefully withdrawn from the *top* of each sample and analyzed by SDS-PAGE. The volume of the lower phase increases in parallel with the increase in Ca^{2+} concentration due to formation of a structured precipitate (as shown in *a*). Therefore, at rising Ca^{2+} concentrations, the analyzed samples (*b*) contain a higher proportion of proteins present in the lower phase. Note the absence of calmodulin (molecular mass = 17 kDa) in the annexin or lipid fractions. *c,* the Ca^{2+} pellet was resuspended in buffer B containing 1 mM EGTA, and supplemented with $EGTA/CaCl_2$ to obtain the indicated final concentrations of $[Ca^{2+}]_{free}$ (μM), final protein concentration being 1.5 mg/ml. After incubation for 15 min at ambient temperature, the suspension was separated by centrifugation at 6,000 × *g* for 30 min, and equal aliquots of supernatant analyzed by SDS-PAGE. The positions of annexins II (mass = 34 kDa) and VI (mass = 67 kDa) are indicated. Note that annexin V was the least Ca^{2+}-sensitive among the annexins and required unphysiologically high concentrations of Ca^{2+} for its binding within the complex.

suggested the formation of a lipid-annexin-actomyosin (membrane-cytoskeleton) complex at elevated Ca^{2+} concentrations. The Ca^{2+} sensitivity of this complex formation (Fig. 2*b*) corresponded to that of annexin II and VI binding within the complex (Fig. 2*c*), suggesting that these proteins act as Ca^{2+}-sensitive linkers between actomyosin (actin-based cytoskeleton) and lipids (sarcolemma).

Detailed analysis of the lipid composition revealed that the lipid fraction of the Ca^{2+} pellet was enriched in cholesterol and sphingomyelin (Fig. 1*b*) properties attributed to the so-called "detergent insoluble glycosphingolipid-enriched membrane domains," characteristic for caveolae (21). In addition, these structures contain glycosylphosphatidylinositol-anchored proteins (*i.e.* 5′-nucleotidase) (21). Western blotting and 5′-nucleotidase activity measurements demonstrated that in contrast to vinculin, a specific marker for *adherens* junctions, both caveolar-specific marker proteins, caveolin and 5′-nucleotidase, were enriched in the Ca^{2+} pellet (Fig. 3, *a* and *b*).

A comparative study, performed on smooth, skeletal, and cardiac muscle extracts points to the specificity of the membrane-cytoskeleton complex for smooth muscle (Fig. 4). Neither the annexins, nor actomyosin were present in skeletal or heart muscle high speed pellets (Fig. 4*a*). Besides, 5′-nucleotidase activity and an elevated cholesterol, sphingomyelin contents were observed exclusively in smooth muscle samples (Fig. 4, *b* and *c*).

Annexin VI Translocates to the Sarcolemma in Response to Ca^{2+} Elevation—In agreement with Ca^{2+} -dependent translocation of annexin VI observed in biochemical experiments its distribution in smooth muscle cells depended upon their state of contraction. When fixed in rigor (native tissue), this protein was located exclusively within the plasmalemma (Fig. 5*a*). After relaxation of cells in a Ca^{2+} free solution for periods between 15 min and 12 h, annexin VI became diffusely distributed within the cytosol (Fig. 5*b*). Subsequent incubation of this relaxed muscle in a contraction solution, prompted relocation of annexin VI to the plasmalemma (Fig. 5*c*). Stimulation of muscle strips which had been maintained in a relaxed state for longer than 12 h led to an increasingly patchy plasmalemmal staining for annexin VI, which was accompanied by enhanced labeling of the nuclear lamina and spotted cytosol (Fig. 5*d*). The low intensity of staining of annexin II in smooth muscle cells rendered difficult an assessment of its potential redistribution during contraction.

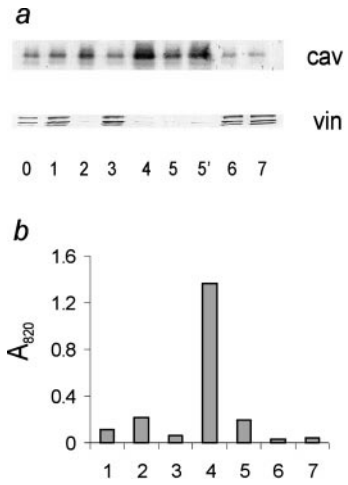

FIG. 3. **The caveolar domain of smooth muscle sarcolemma is a component of the membrane-cytoskeleton complex.** Samples prepared and numbered as described in the legend to Fig. 1 were analyzed by Western blotting for caveolin and vinculin (*a*) or by measurement of specific enzymatic activity for 5′-nucleotidase (*b*) contents.

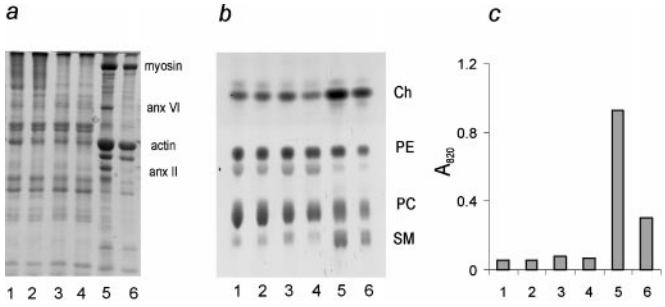

FIG. 4. **The membrane-cytoskeleton complex is specific for smooth muscle.** High-speed Ca^{2+} pellets (*1, 3*, and *5*) were obtained from skeletal (*1*), cardiac (*3*), and smooth (*5*) muscle as described under "Materials and Methods." The corresponding high-speed EGTA pellets (*2, 4*, and *6*) were obtained by the same procedure except that $CaCl_2$ in the initial extracts was substituted by 1 mM EGTA. Equal aliquots of each resulting pellet were analyzed for protein (SDS-PAGE) (*a*) and lipid (TLC) composition (*b*) and for 5′-nucleotidase activity (*c*).

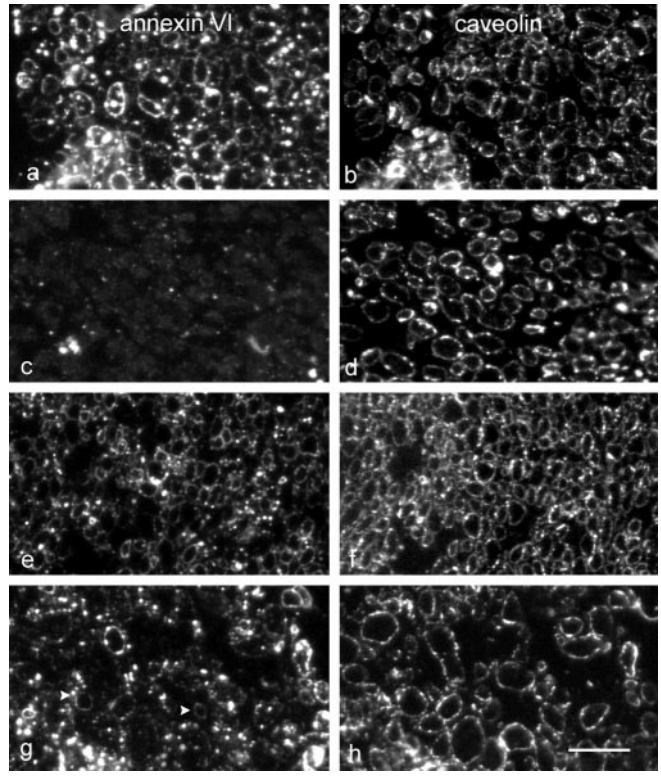

FIG. 5. **Redistribution of annexin VI in smooth muscle cells during contraction-relaxation cycles.** Transverse sections of human *taenia coli* double-labeled with antibodies against annexin VI (*a, c, e*, and *g*) and caveolin ((*b, d, f*, and *h*) for the demarcation of cell borders). Within smooth muscle cells fixed in *rigor* (*a* and *b*), annexin VI is localized to the plasmalemma (*a*): after relaxation of cells for 2 h, this protein becomes diffusely distributed (*c*). Depolarization of the sarcolemma (with ensuing cell contraction), elicits a reversion to the plasmalemmal localization of annexin VI (*e*). Incubation of cells in the relaxing solution for longer than 12 h renders the plasma membranes leaky. Subsequent stimulation of such cells leads to more sporadic plasmalemmal labeling for annexin VI and an increased frequency of stained nuclear laminae (*g*) (*arrowheads*). *Bar* = 20 μm.

DISCUSSION

Annexins II and VI Act as Ca^{2+}-sensitive Linkers between the Caveolar Domain of the Sarcolemma and the Actin-based Cytoskeleton—The mechanical link coupling cytoskeletal and contractile proteins to the sarcolemma of smooth muscle cells is essential for transmitting tension from the cell's interior to the exterior. The sarcolemma is segregated into regularly spaced domains of rib-like *adherens* junctions alternating with caveolae containing regions (1, 2). In *adherens* junctions or plasmalemma-associated dense plaques, actin filaments are coupled to transmembrane integrins via a cascade of submembranous proteins. While the mechanisms regulating the assembly of dense plaques are still the subject of intense investigation, it is generally taken for granted that these structures exist in a fixed, assembled state throughout the entire contraction-relaxation cycle.

Our work postulates on the existence of an additional, and reversible, cytoskeleton-membrane complex, which is forged in the caveolar domain of the sarcolemma in response to a rise in Ca^{2+} concentration following smooth muscle cell stimulation. The Ca^{2+} sensitivity of this complex indicates that it can be formed *in vivo* under the conditions prevailing during smooth muscle contraction (22).

The plasmalemmal sites are obvious loci for the formation of such reversible, cytoskeletal links, since the plasmalemma is closely apposed to cytoskeletal elements. Apart from the *adherens* junction, known to serve as attachment site for the actin filaments, actin and myosin subfragment 1 have been identified in fractions of purified caveolae (23–26) and the caveolar-specific plasmalemmal inositol 1,4,5-triphosphate receptor-like protein is aligned along actin filaments in bovine aortic endothelial cells (27). In addition, annexins II and VI were found to be components of different membranous preparations, usually also containing significant amounts of actin (24, 25, 28, 29).

The particular distribution of membrane domain markers such as caveolin and 5′-nucleotidase, together with high amounts of cholesterol and sphingomyelin in the Ca^{2+} pellet, suggests that the caveolar domain of the sarcolemma takes part in the formation of the Ca^{2+}-regulated membrane-cytoskeleton complex. In agreement with these data, the Ca^{2+} pellet did not include vinculin, the main component of the *adherens* junctions.

A property shared by all members of the annexin protein family is their capacity to bind acidic phospholipids and actin-based cytoskeletal elements in a Ca^{2+}-dependent manner (30–36). These characteristics render them ideal candidates to play a role as Ca^{2+}-sensitive linkers between the cell's membranous structures and cytoskeleton. However, while the well documented interactions between annexins and acidic phospholipids or actin-based cytoskeletal elements could play a role in formation of the membrane-cytoskeleton complex described here, the precise mechanisms responsible have not been elucidated to date.

The Redistribution of Annexins in Response to Stimulation of Smooth Muscle Cells—Even in unstimulated cells, the localization of annexins and their precise allocation to a distinct intracellular compartment remain a subject of controversy (for reviews, see Refs. 8 and 37). Observations concerning the translocation of annexins in stimulated cells are thus often contradictory. These apparent contradictions reflect the great complexity and diversification of the responses to stimulation manifested by different cell and tissue types and the multitude of different annexins present within a given cell (36, 38). Discrepancies can also be accounted for by the developmental state of a cell or by experimental variables, such as fixation, type of antibody used, or the duration of stimulation (39). Therefore, it is not surprising that, while Kaufman *et al.* (40) ascribed the translocation of annexin I from the cytosol of resting neutrophils to the plasma membrane to a rise in intracellular Ca^{2+} concentration, Raynal *et al.* (41), on the other hand, demonstrated that in human fibroblasts annexins I, II, VI, and VII do not redistribute during treatment with the Ca^{2+} ionophore A23187. And in myogenic cell lines, as well as in human fibroblasts, annexins II, IV, V, VI, or VII have been shown to relocate to the plasma membrane, endoplasmic reticulum, cytoplasmic vesicles, or other intracellular membranes, in response to rises in Ca^{2+} concentration (39, 41, 42).

In our study, human smooth muscle of surgical or autoptic origin was always in a state of *rigor* upon our receipt of it, this being recognized by the plasmalemmal localization of annexin VI's. After inducing relaxation by incubation in an EGTA-containing solution, a diffuse redistribution of annexin VI to the cytosol was observed. In the only previous investigation, annexin VI has been localized to the sarcolemma of smooth muscle cells indicating that the tissue the authors used for their study was fixed in the state of *rigor* (43). In the present paper we, for the first time, demonstrate that stimulation of smooth muscle led to a rapid and reversible relocation of annexin VI from the cytosol to the plasma. However, if smooth muscle was relaxed for periods longer than ~12 h prior to contraction or subjected to repeated contraction-relaxation cycles, annexin VI labeling of the plasmalemma became increasingly patchy and the number of cells revealed a nuclear laminal signal. We ascribe this increasingly irregular pattern of redistribution in patches of irregular size and distribution to a gradual decay in plasmalemmal integrity, with an accompanying increase in Ca^{2+} influx and spurious binding of annexin VI to detached lipid-membrane structures.

Annexin II, a vigorously investigated member of this protein family, was indeed but scantily stained in smooth muscle cells with available antibody making difficult an assessment of its potential redistribution during contraction. However, our biochemical data suggest that a relocation of annexin II could, indeed, take place upon smooth muscle stimulation. Other authors have reported such a relocation to occur (from cytoplasmic to membrane sites) after stimulation of a number of cell types (42, 44–47).

In conclusion, we suggest that annexins II and VI play a determinant role in smooth muscle contraction and sarcolemmal organization providing a Ca^{2+}-sensitive link between sarcolemma and underlying actin-based cytoskeleton. A physiological function for such a link would be a co-operative regulation of cytoskeleton and plasmalemma rearrangements during contraction-relaxation cycles to prevent the cell membrane from mechanical damage and to ensure a better force transduction from contractile apparatus to extracellular matrix.

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