Annexin 6 Modulates the Maxi-chloride Channel of the Apical Membrane of Syncytiotrophoblast Isolated from Human Placenta*

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The syncytiotrophoblast separates the maternal and fetal blood and constitutes the primary barrier for maternal-fetal transport. The Maxi-chloride channel from the apical membrane of the syncytiotrophoblast plays a role in the chloride conductance. Annexins can play an important role in the regulation of membrane events. In this study we evaluate the role of annexin 6 in the Maxi-chloride channel properties. The results showed that annexin 6 is bound in the apical placenta membranes in a calcium-dependent phospholipid-binding manner but also in a calcium-independent fashion. The neutralization of annexin 6 decreased the total current by 39 ± 1.9% in the range of ±80 mV, and the currents decrease with the time. The single-channel slope conductance was decreased from 253 ± 7.4 pS (control) to 105 ± 13 pS, and the amplitude decreased by 50%. The open probability was also affected when higher voltage steps were used, changes in either the positive or negative direction induced the channel to close, and the open probability (P_o) did not decrease. In channels with neutralized annexin 6, it was maintained at 1 at ±40 mV and at ±80 mV. These results suggest that endogenous annexin 6 could regulate the Maxi-chloride channel. The results obtained with normal placentae, in which annexin 6 was neutralized, are similar to those described for the Maxi-chloride channel isolated from pre-eclamptic placenta. Together these data suggest that annexin 6 could play an important role in ion transport of the placenta.

The human placental syncytiotrophoblast is a polarized epithelial structure that results from the fusion of precursor cytotrophoblast cells producing a syncytium. It is the main barrier for materno-fetal exchange. Ionic transport in the syncytiotrophoblast involves conductive pathways that are associated with numerous epithelial functions, such as maintenance of membrane voltage, cell volume regulation, solute transport, and others. In placenta the 4,4′diisothiocyanostilbene-2,2′-disulphonic acid (DIDS)-sensitive Cl^- conductances and K^+ conductances contribute to the resting potential of the syncytiotrophoblast microvillous membrane and are involved in volume regulation (1, 2). Chloride is the main anion of extracellular fluid in the fetus, as it is in the adult, but at all gestational ages fetal Cl^- is 5–6 mM higher than in maternal blood. There are no maternal-fetal differences in either Na^+ or K^+ concentration (3). There has been considerable interest focused on the chloride conductance of the human placenta apical membrane and how it is regulated. However, the specific ion channels involved in these and other placental processes are as yet unknown. A possible molecular candidate for the 4,4′diisothiocyanostilbene-2,2′-disulphonic acid-sensitive anion conductance in apical syncytiotrophoblast plasma membrane is a Maxi-chloride channel. Maxi-chloride channels have been identified in secreting and absorbing epithelia (4), in non-epithelial cell types (5–8), and in the apical membrane from human placenta using electrophysiological methods by our group and by others (9–11). This channel may play a role in complex cellular regulation involving inhibition through phosphorylation by protein kinase C, activation by a Ca^{2+}-dependent process, and in volume regulation. More recently, Sabirov *et al.* (12) concluded that this channel serves as a pathway for swelling-induced ATP release. In normal human term placentae, the Maxi-chloride channel from the apical membrane of the syncytiotrophoblast has similar biophysical characteristics to the other Maxi-chloride channels in epithelial and non-epithelial cells.

There is evidence that suggests that the cytoskeleton participates in the regulation of the Maxi-chloride channels involved in volume regulation decrease. In the non-swelling rabbit cortical collecting cell line RCCT-28A, agents that depolymerize F-actin enhance the chloride current by activation of the Maxi-chloride channel. On the other hand, F-actin-stabilizing agents prevent the activation of this channel. This evidence suggests that desegregation of the cytoskeleton activates Maxi-chloride channels and could be the reason why the activity of the Maxi-chloride channel is detected mainly in isolated patches. In 1999 Babiychuk and collaborators (13) found that annexin 6 participated in the formation of reversible membrane cytoskeleton complexes in smooth muscle cells. Annexins are membrane organizers that interact with actin and could be determining factors in complex formation between the cytoskeleton and the plasma membrane.

Annexins are a family of calcium-dependent phospholipid-binding proteins. Calcium reversibly shifts the annexins from a soluble to a membrane-associated state (14, 15). Several reports show that specific members of the annexin family participate in the regulation of ionic channels in different cells (16–19). Naciff *et al.* (20) showed that annexin 6 regulates the potassium and calcium currents of sensorial neurons. By using nonspecific affinity-purified anti-annexin 6 antibody they found an increase in the magnitude of the K^+ current and an increase in the Ca^{2+} current in sensory neurons. These reports suggest that endogenous annexin 6 regulates the Ca^{2+} conductance, which indirectly modifies Ca^{2+}-dependent ionic conductances in neurons. Diaz-Muñoz *et al.* (17) demonstrated

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that annexin 6 increased the mean open time and opening frequency of the sarcoplasmic reticulum ryanodine-sensitive calcium channels. In placenta, annexin 6 is expressed in cytotrophoblasts, syncytiotrophoblasts, fetal vascular endothelium, and chorionic trophoblasts throughout pregnancy (21).

The aim of the present study was to evaluate the role of annexin 6 in the regulation of the Maxi-chloride channel of apical membranes isolated from placentae from normal subjects and reconstituted in giant liposomes. The results showed that the electrophysiological characteristics of chloride channels with neutralized annexin 6 are different from those with intact annexin 6. These results suggest that annexin 6 has the ability to regulate the Cl^- conductance in human placenta.

EXPERIMENTAL PROCEDURES

Placenta Collection—Placentae obtained from normal pregnancies were collected immediately after delivery from the San José Hospital Maternity Unit and transported to the laboratory on ice.

Immunohistochemistry of Human Placentae—Archival formalin-fixed and paraffin-embedded placental blocks were used. Paraffin-embedded placentae tissue sections were cut at 7 μm. To detect annexin 6, we used the Vectastain ABC Peroxidase Elite IgG goat kit for the polyclonal antibody against annexin 6 (dilution 1:750, Santa Cruz Biotechnology) with aminoethyl carbazole, which produces a red stain, as the peroxidase substrate (Vector Laboratories, Burlingame, CA). All sections were counterstained with hematoxylin for 1 min and then mounted in 1:9 phosphate-buffered saline/glycerol. Preimmune goat serum was used as a negative control. All sections were examined by one observer who was blinded to tissue identity, and localization and intensity of the immunostaining were recorded.

Preparation of Placental Apical Membrane Vesicles—The human placental microvillous membrane (MVM)[1] vesicles were prepared by a method we have described (22) that allows simultaneous isolation of apical and basal membranes from the same placenta. This method is a modification of the method described by Illsley *et al.* (23). We added one step to isolate plasma membrane free of mitochondrial membranes (24) and have preserved the conditions used in our apical membrane isolation protocol when working with this simultaneous isolation protocol.

The purification method involved precipitation of a non-microvillous membrane with magnesium ions, differential centrifugation, and a sucrose step gradient. All solutions were buffered with 20 mM Tris-HEPES, pH 7.4. A portion (2–3 ml) of the microvillous-enriched preparation containing about 10–15 mg of protein was overlaid on the sucrose gradient. The band at the 37/45% sucrose interface was collected and diluted 10-fold with 20 mM Tris-HEPES, pH 7.4, before centrifugation at 110,000 $\times$ *g* for 30 min. The final pellet was resuspended in 300 mM sucrose, 20 mM Tris-maleate, pH 7.4, and stored at −150 °C (liquid nitrogen).

The purity and enrichment of the MVM membrane fraction was evaluated routinely using enzymatic assays, binding studies, Western blotting, and immunohistochemistry for mitochondrial membrane markers. Enrichment of alkaline phosphatase activity (an apical membrane marker) for MVM was 17- to 21-fold, with 13–16% protein recovery relative to the homogenate, and was essentially free of basal membranes and mitochondrial membranes. Lack of contamination of purified apical membranes from basal and mitochondrial membranes was confirmed by quantification of adenylate cyclase activity and binding of [^{3}H]dihydroalprenolol to β-adrenergic receptors both basal membrane markers, and cytochrome-*c* oxidase/succinate dehydrogenase as mitocondria markers.

Western Blot Analysis—Samples from the placenta preparation fractions were diluted in sample buffer, containing 0.25 M Tris (pH 6.8), 20% glycerol, 5% β-mercaptoethanol, and 0.02% bromphenol blue and heated at 100 °C for 5 min. Protein samples were loaded (30 μg per lane) and separated using 12% Tris/glycine gels (Invitrogen) run at 100 mV. Low molecular weight markers (Bio-Rad) were loaded as standards. The gels were then electroblotted onto nitrocellulose membranes (Osmonics, Inc). The blots were blocked for 1 h in Tris-buffered saline consisting of 100 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 0.1% Tween 20 (v/v), and 5% (w/v) nonfat dried milk at room temperature with agitation. The blots were then incubated with 1:2000 dilution of anti-annexin 6 antibody (200 μg/ml, Santa Cruz Biotechnology) overnight at 4 °C. The blots were washed three times in Tris-buffered saline containing 0.1% Tween 20, incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-goat at 1:2000 dilution, and washed three times with 0.1% Tween 20. Western blot was developed with enhanced chemiluminescence (ECL) using an ECL kit (Pierce).

Reconstitution of the Apical Membrane into Giant Liposomes—Giant liposomes were prepared by submitting a mixture of the isolated apical membrane vesicles and asolectin lipid vesicles to a partial dehydration/rehydration cycle, as reported by Riquelme *et al.* (25). An aliquot containing 100–150 μg of membrane protein was mixed with 2 ml of a 13 mM (in terms of lipid phosphorus) suspension of the asolectin vesicles. After the partial dehydration/rehydration cycle, the diameter of the resulting giant multilamellar liposomes ranged from 5 to 100 μm.

Patch Clamp Measurements—Aliquots of 3–6 μl of giant liposomes were deposited into the excised Patch chamber (RC-28, Warner Instruments Corp.) and mixed with 0.4 ml of the buffer of choice for electrical recording (bath solution). Single-channel recordings were obtained by patch clamp techniques as described by Hamill *et al.* (26). Giga seals were formed on giant liposomes with glass microelectrodes of 5 to 10 MΩ resistances. After sealing, withdrawal of the pipette from the liposome surface resulted in an excised patch. Current was recorded with an EPC-9 patch clamp amplifier (Heka Elektronic, Lambrecht/Pfalzt, Germany) at a gain of 50–100 mV/pA and a filter setting of 10 kHz. The holding potential was applied to the interior of the patch pipette, and the bath was maintained at virtual ground ($V = V_{bath} - V_{pipette}$). The bath was grounded via an agar bridge, and the junction potential was compensated for when necessary. The signal was analyzed off-line by means of the analysis of single-channel recordings (Bruxton Corp.) and Pulse Fit (Heka Elektronic) software. All measurements were made at room temperature.

Solutions—The pipette and bath solutions had the following composition (in mM): 140 *N*-methyl-D-glucamine chloride, 2.6 calcium chloride, 1.3 magnesium chloride, and 10 sodium HEPES, pH 7.4, unless otherwise stated.

Statistical Analysis—Results are expressed as mean ± S.E. Measures of statistical significance were obtained using Student's *t* test. A *p* value of less than 0.05 was considered significant.

RESULTS

Cellular Localization of Annexin 6 in Normal Human Placenta—Our results show that every section of placental villous tissue from normal pregnant subjects at term exhibited immunostaining for annexin 6 in the apical and basal syncytiotrophoblast membranes (Fig. 1). The level of staining varied from mild to strong. We also observed expression in the fetal vascular endothelium (Fig. 1).

Annexin 6 in MVM Isolated from Human Placenta—To demonstrate that annexin 6 is present in the purified MVM vesicles used in the electrophysiological studies, in the presence of the contaminating calcium and in the absence of EGTA, we performed Western blot analysis using a specific annexin 6 polyclonal antibody. As can be observed in Fig. 2, all the annexin 6 is located in the MVM fraction (pellet). These data suggest that during the normal isolation of the MVM, annexin 6 is bound to the membrane and it is not released into the media.

To evaluate the calcium-specific properties of annexin 6 in the purified MVM, membranes were incubated 30 min on ice in the presence of a calcium chelator, EGTA. Pellets were washed and centrifuged, and annexin 6 levels were evaluated by SDS-PAGE followed by Western blot analysis, in the supernatant and in the new pellets (Fig. 2, *EGTA*). The results showed that the treatment with EGTA released some of the annexin 6 into the supernatant, which also suggests that there is a percentage of annexin 6 that binds the membranes in a calcium-independent fashion. To release all the annexin 6 it was necessary to use a detergent, saponin (Fig. 2, *EGTA + saponin*). It is known that saponin acts by removing cholesterol from the membrane. When apical membranes were isolated in the presence of 10 mM EGTA throughout the isolation, they contained almost 80% less annexin 6 than those isolated in the absence of the chelator

[1] The abbreviations used are: MVM, microvillous membrane; ECL, enhance chemiluminescence; VDAC, voltage-dependent anion channel; pS, picosiemens.

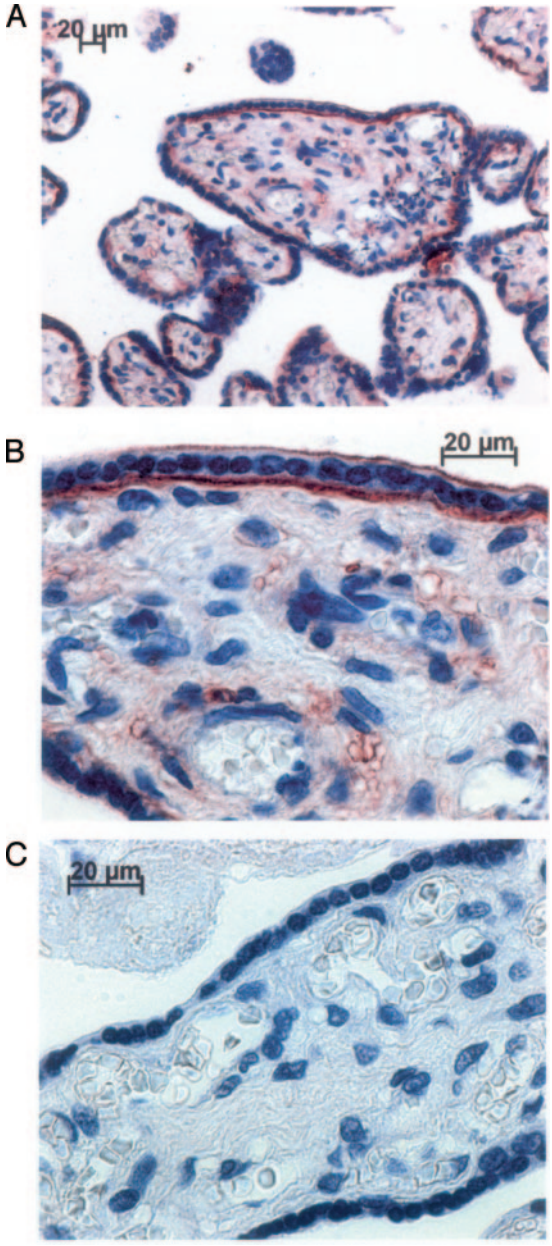

FIG. 1. **Immunolocalization of annexin 6 in placenta from normal subjects.** Paraffin sections of placenta tissue were immunostained with annexin 6 polyclonal antibody as primary antibody (*A* and *B*) or with preimmune sera (*C*), using the Vectastain elite ABC kit with aminoethyl carbazole as the peroxidase substrate (shown in *red*). Samples were counterstained with hematoxylin. Magnification is as labeled in the figure.

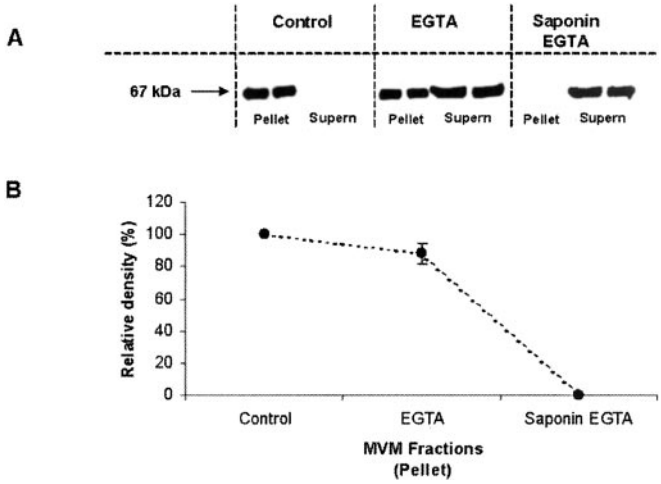

FIG. 2. **Western blot analysis of annexin 6 in isolated MVM incubated in different conditions.** In *A*: *Control*, MVM purified in buffer containing only contamination free calcium; *EGTA*, MVM control were incubated in the presence of 10 mM EGTA for 30 min on ice, mixed, then centrifuged at 100,000 × *g* for 60 min. The annexin 6 concentration was then evaluated in the supernatant, and the pellet was analyzed by SDS-PAGE followed by Western blotting using a polyclonal anti-annexin 6 antibody (see "Experimental Procedures"); *EGTA+Saponin*, MVM control was incubated in the presence of 10 mM EGTA plus saponin for 30 min on ice, mixed, then centrifuged at 100,000 × *g* for 60 min, and samples were processed as described for samples treated with EGTA. *B*, densitometry measures from annexin 6 present in the pellet.

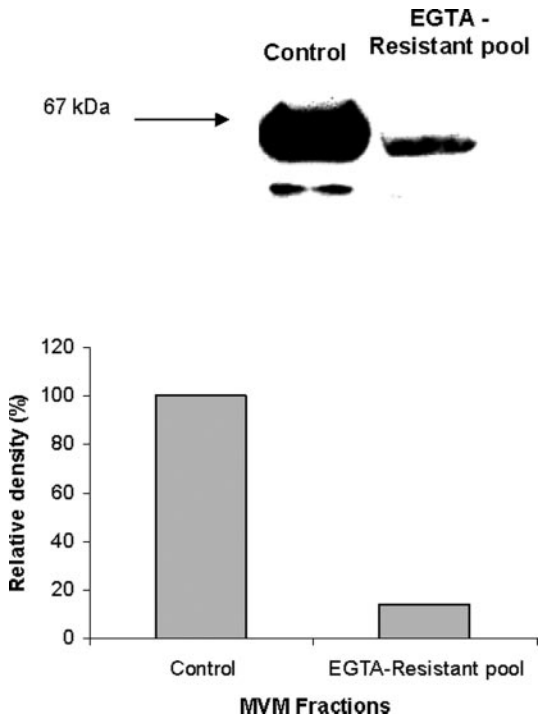

FIG. 3. **Comparative Western blot analysis of annexin 6 in isolated MVM from membrane preparations control and in the presence of 10 mM EGTA.** *Top panel*: *Control* is MVM from routine protocol (see "Experimental Procedures"). *EGTA-Resistant pool* is MVM purified with a modified protocol, in this case 10 mM EGTA was added during the purification procedure. *Bottom panel*: densitometry measures from fractions in the *top panel*.

(Fig. 3, *A* and *B*). These results confirm that annexin 6 is bound in the MVM placenta membranes in a calcium-dependent phospholipid binding manner but also in a calcium-independent fashion.

Anti-annexin 6 Antibody Modulates the Maxi-chloride Channels from Human Placental MVM—The fact that annexin 6 is present in MVM suggested a possible role for annexin 6 in modulation of the Maxi-chloride channel present in these membranes. To explore this possibility, we evaluated the effect of anti-annexin 6 antibody on the activity of the Maxi-chloride channel by adding the antibody to the bath in excised patch recording of the Maxi-chloride channels. The Maxi-chloride channel was examined prior to and following the exposure of the seal to the antibody. The specificity of the inhibitory effect exerted by annexin 6 antibody was evaluated by comparing it to the inhibition produced by the preimmune serum. The experiments were carried out in symmetrical solutions where Cl^- was the major permeated species. Each seal served as its own control with the current measured prior to and following expo-

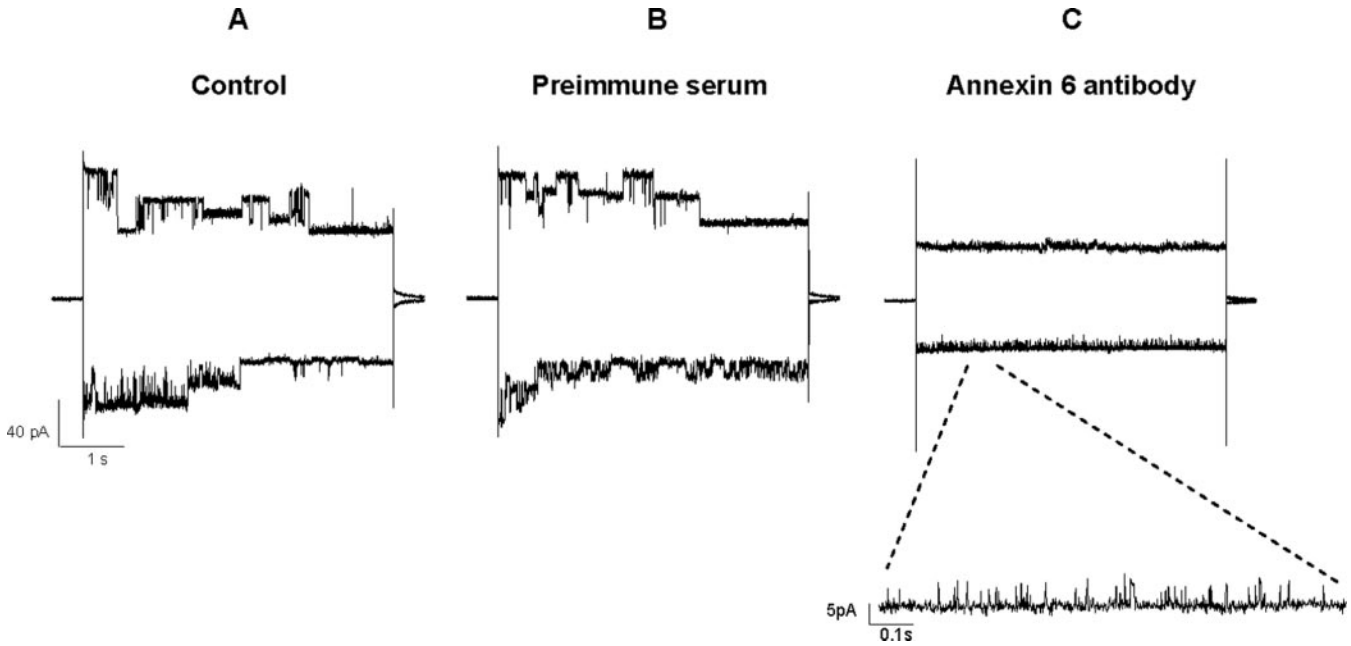

FIG. 4. **Recording of the Maxi-chloride channel from human placenta.** Single-channel currents recorded in an excised patch from MVM reconstituted into giant liposomes. Currents were recorded at ±80 mV with bath and pipette solutions containing (in mM): 140 NMDGCI, 2.6 $CaCl_2$, 1.3 $MgCl_2$, 10 Na-HEPES, pH 7.4. *A*, control. *B*, preimmune serum. *C*, annexin 6 antibody.

sure to either preimmune serum (normal goat serum) or annexin 6 antibody.

The results presented here support the results previously reported in which we showed a typical activity of the large conductance of the chloride channels with different subconductance levels for the Maxi-chloride channel from MVM. Single channel current traces, at the indicated holding potential, for chloride channels are shown in Fig. 4. The *trace* in Fig. 4*A* shows control recordings of normal Maxi-chloride channels, and *traces* in Figs. 4*B* and 4*C* show the two more frequent behaviors for the recorded chloride currents with 5 μg/ml preimmune serum (normal goat serum) and with 5 μg/ml annexin 6 antibody in the bath solution, respectively, at 15 min following application. Both records show two types of electrical activity patterns for the Maxi-chloride channel in these conditions. The first one is similar to the control, suggesting that preimmune serum did not affect the electrical activity in the patch. However, when annexin 6 is presumably neutralized by antibody, there was a substantial change in the biophysical properties of the channel. To identify the biophysical characteristic of the affected conductance, the channel open probability (P_o) was studied with neutralized and non-neutralized annexin 6 conditions.

Effect of Annexin 6 Antibody on Total Current (I_{total}) in the Patch—Addition of 5 μg/ml annexin 6 antibody to the bath solution decreased the total current in the patch by 39 ± 1.9% in the range of ± 80 mV (n = 4). However, the presence of 5 μg/ml preimmune serum (normal goat serum) did not affect the total current in the patch (n = 3). Fig. 5 show the results at the different experimental voltages applied in independent seals. The time course of total current (I_{total}) in the patch shows that the current did not change with time either under control conditions (Fig. 6*A*) or in the presence of preimmune serum (5 μg/ml) (Fig. 6*B*). However, when the annexin 6 antibody (5 μg/ml) was added to the bath solution, the currents decreased with time (Fig. 6*C*).

Unitary Conductances for Maxi-chloride Channels from Control versus Neutralized Annexin Condition—A linear current-potential relationship was obtained with reversal at 0 mV (Fig. 7*A*) for the control, the preimmune serum, or for annexin 6 antibody. Only the dominant current level was used, ignoring any subconductance levels for current-voltage relationships in each case. These conductances were observed in experiments performed with the bath and pipette solutions containing (in mM) 140 *N*-methyl-D-glucamine chloride, 2.6 $CaCl_2$, 1.3 $MgCl_2$, 10 Na-HEPES, and pH 7.4. As expected (27, 10, 11) the single-channel slope conductance was 253 ± 7.4 pS (n = 5) for the Maxi-chloride channel in control conditions. The activity recorded in the presence of preimmune serum in the bath did not show changes in conductance (251 ± 14 pS; n = 3). However the single-channel slope conductance in the presence of annexin 6 antibody decreased to 105 ± 13 pS (n = 4). The single-channel amplitude was affected by annexin 6 antibody, with a decrease of 50% (Fig. 7*B*).

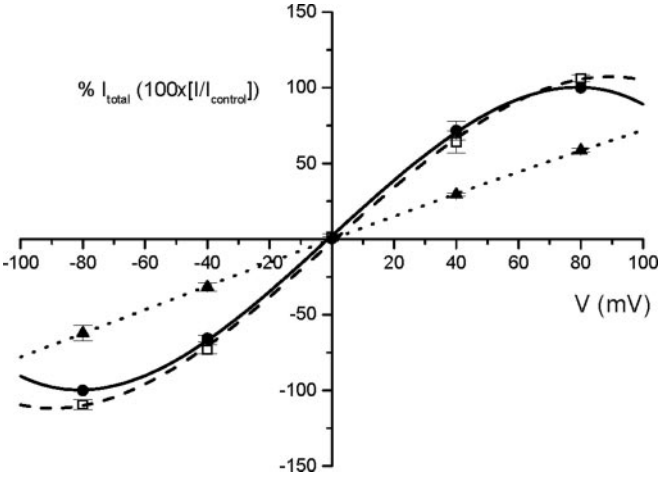

FIG. 5. **Effect of annexin 6 antibody on total current (I_{total}) in the patch.** Addition of 5 μg/ml annexin 6 antibody to the bath solution decreased the total current in the patch in the range of ±80 mV (▲, n = 4), and 5 μg/ml preimmune serum did not affect the total current in the patch (□, n = 3). Control (■, n = 5).

Open Probability (P_o) for Maxi-chloride Channels from Control versus Neutralized Annexin Condition—The voltage dependence of open channel probability of apical chloride chan-

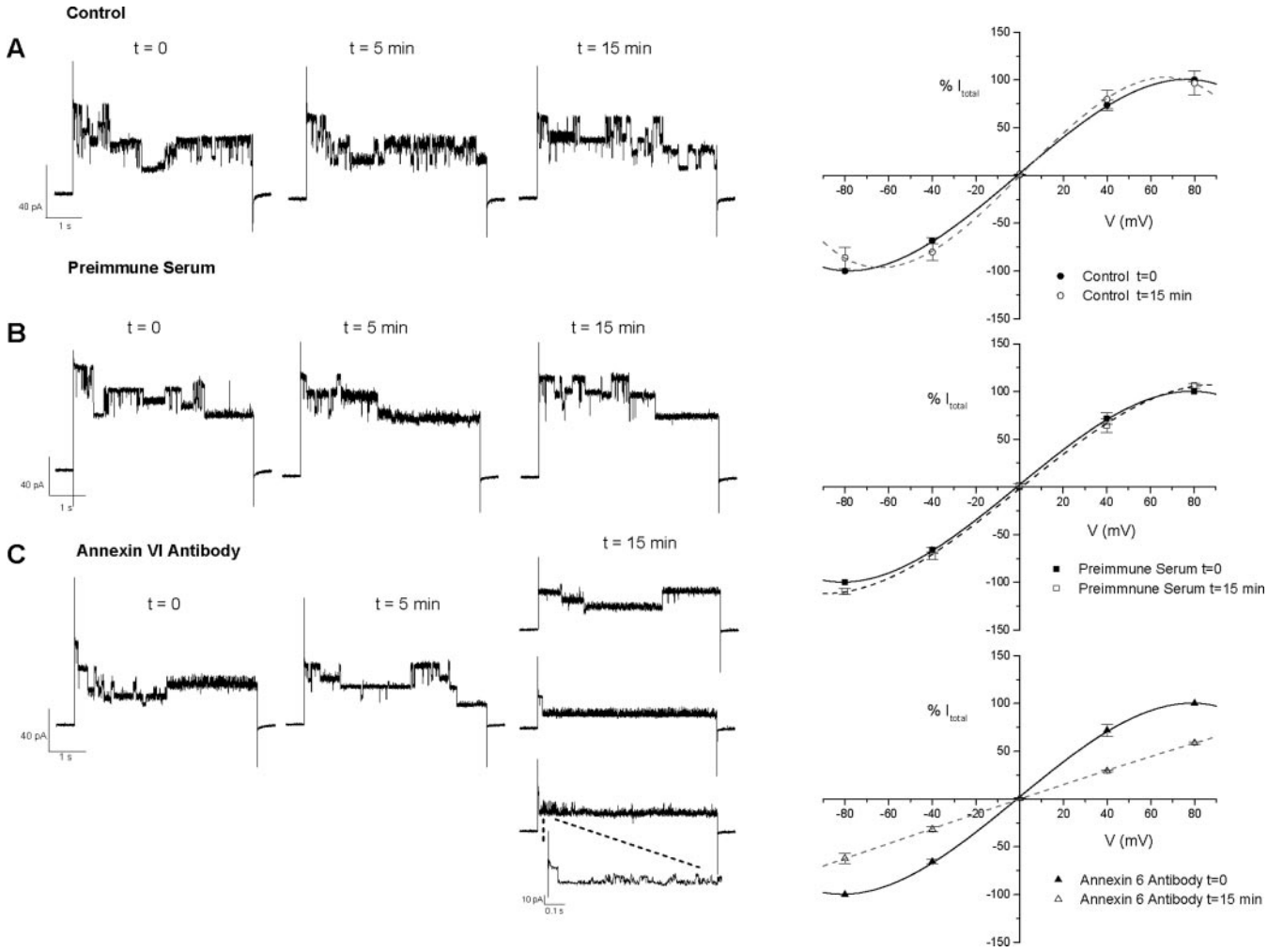

FIG. 6. **Time course on total current (I_{total}) of the Maxi-chloride channel.** *A*, control conditions; *B*, in the presence of 5 μg/ml preimmune serum; and *C*, in the presence 5 μg/ml annexin 6 antibody, to the bath solution at the indicated time.

nels has been obtained as the ensemble-averaged current of 4–30 consecutive current responses to a voltage ramp pulse applied in independent seals. These P_o values were calculated as $P_o = (I/V)/G_{max}$, where I is the patch current, V is the voltage, and G_{max} is the maximal patch conductance near 0 mV. A voltage ramp pulse was usually applied from −120 mV to +120 mV, at a rate of 40 mV/s. Fig. 8 shows the curve for P_o *versus* voltage for these results. In agreement with the characteristics of P_o reported before for the placental Maxi-chloride channel from normal placenta (9, 27, 10) the open probability (P_o) *versus* voltage relationship for our normal contemporaneous controls ($n = 5$) could be described by a bell-shaped curve. The channel was normally open at potentials between −50 and +50 mV, and voltage increases in either a positive or negative direction induced channel closure. A similar situation was observed in the presence of preimmune serum in the bath. In the presence of annexin 6 antibody the channel was also normally open at potentials between −50 mV and +50 mV; however, at higher voltage steps, in either a positive or negative direction, no channel closures were induced. P_o did not decrease; it is maintained at 1 at ±40 mV and 1 at ±80 mV in channels with neutralized annexin 6, compared with P_o that diminished from ~1 at ±40 mV to 0.6 at ±80 mV in the control condition. As shown in Fig. 8, the bell-shaped curve of voltage dependence of P_o for neutralized annexin 6 is flattened with respect to that of control, *i.e.* chloride-conducting channels with neutralized annexin 6 lost a voltage dependence of P_o.

DISCUSSION

The immunohistochemistry results presented in this study support previous published data by other investigators that showed that annexin 6 is expressed in the syncytiotrophoblast of the placenta. However, in distinction to other studies, we find that annexin 6 is specifically localized in the apical and basal membrane of the placenta syncytiotrophoblast. The expression of annexin 6 at the plasma membrane level suggests a potential role in the plasma membrane activity. Because we have previously identified Maxi-chloride channels in the apical membrane of the human placentae, we decided to evaluate whether annexin 6, which is highly expressed in the apical membrane, regulates the electrophysiological properties of the Maxi-chloride channel. Our approach was to use a polyclonal anti-annexin 6 antibody to neutralize the annexin 6 protein bound to the apical membrane phospholipids. The use of specific antibodies to block intracellular function of annexin family members has previously been reported (16, 17, 20). We used apical membrane reconstitution into giant liposomes to evaluate the role of this protein on the regulation of the Maxi-chloride channel.

We first evaluated whether expression of the protein was affected by the isolation protocol for apical and basal placental membranes. Our results showed that the apical membrane fraction contained high levels of annexin 6. However, when we tried to isolate an apical membrane in the absence of annexins

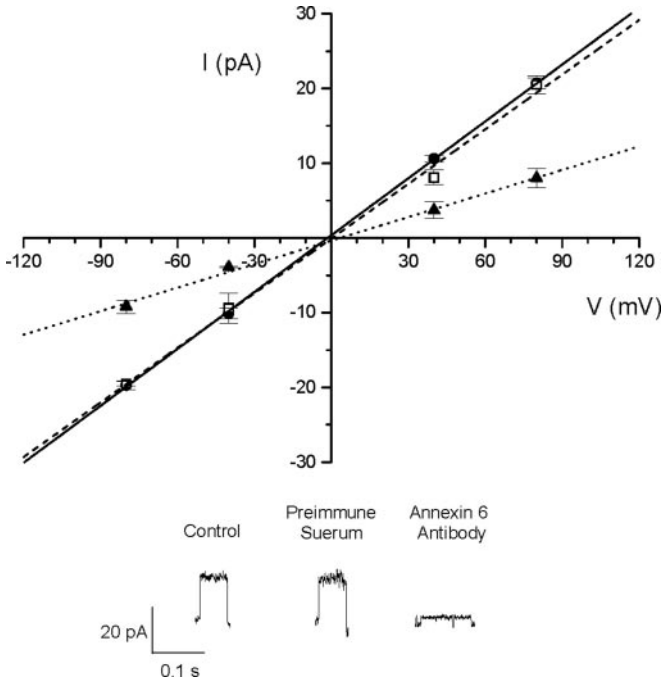

FIG. 7. **The conductance of the Maxi-chloride channel is decreased with annexin 6 antibody.** *A*, the conductance of the major (most frequent) substrate Maxi-chloride channel in control conditions was 253 ± 7.4 pS (*black squares*; $n = 5$), in the presence of preimmune serum in the bath it was 251 ± 14 pS (*empty squares*, $n = 3$), and conductance in the presence of annexin 6 antibody was 105 ± 13 pS (*black triangles*; $n = 4$). All experiments were performed in symmetric concentration conditions. Data are shown as mean ± S.E. *B*, example of single channel transitions at +80 mV and the indicated conditions. The amplitude with annexin 6 antibody, in this case, decrease 60–70% in respect to the control.

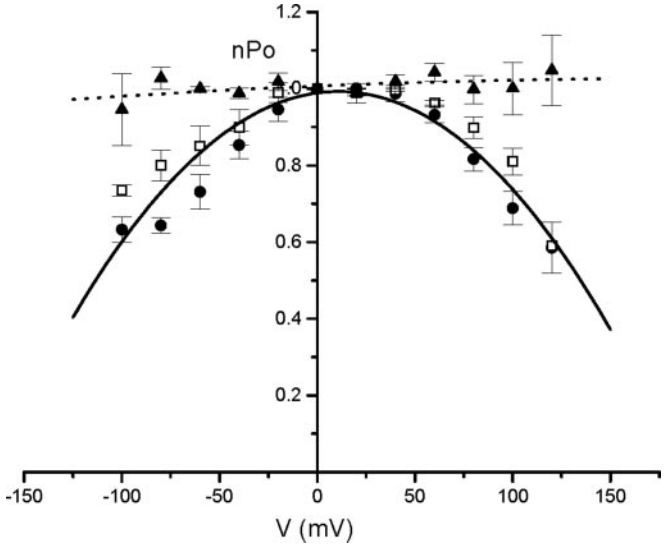

FIG. 8. **Effect of annexin 6 antibody on open probability (nP_o) of the Maxi-chloride channel.** The classical bell-shaped curve of nP_o in control conditions (*black circles*; $n = 5$) was flattened by addition of 5 μg/ml annexin 6 antibody to the bath (*black triangles*; $n = 4$). In contrast, the preimmune serum did not induce significant changes in nP_o (*empty squares*; $n = 3$).

by adding EGTA to the preparation (28, 15, 29), we observed both calcium-dependent and calcium-independent binding of annexin 6. When the levels of annexin 6 were compared between samples prepared in the absence and in the presence of EGTA, we observed an ~85% release of annexin 6 suggesting that 15% of the annexin 6 is bound to the apical membrane in a calcium-independent fashion. This observation agrees with results previously reported by de Diego and collaborators who showed that changes in the distribution and concentration of cholesterol within the endosomal compartment regulate, together with phospholipids, the annexin 6 localization and potentially its function (30). This calcium-independent binding could be also attributed to a decrease on the intracellular pH as was shown for Golzak and collaborators (31). They showed that lowering the pH increased the hydrophobicity of annexin 6, allowing its interaction with membrane lipids in a calcium-independent manner. Another alternative is that annexin 6 binds to the cytoskeleton, as reported by Babiychuk and collaborators (13). Their results demonstrate that an increase in intracellular calcium, induced by smooth muscle stimulation, induces the formation of an annexin 6-dependent membrane cytoskeleton to appear. This interaction induces the translocation of annexin from the cytoplasm to the membrane in a calcium-dependent manner. Smart and collaborators (32) postulated that this interaction may take place in the caveolae. It has been suggested that the interaction between annexin 6 and membrane domains may regulate the activity or the functioning of various integral membrane proteins, for example ion channels and enzymes (33) such as some protein kinase C isoforms. In fact Dubois and collaborators (34–36) showed association between annexin 6 and a 56-kDa protein kinase in T cells. Other proteins that affect the annexin binding to the membrane are the phospholipases (37, 38). In smooth muscle cell membranes, the organization of raft or non-raft microdomains is regulated by annexin 6, and this association is mediated by interaction with the cytoskeleton (13, 39–41).

To evaluate whether annexin 6 was associated with or bound to cholesterol, saponin, a detergent that specifically solubilizes cholesterol, was added to our membrane preparation. Saponin is a type of glycoside widely distributed in plants (sapogenin glycosides) that form a soapy lather when agitated in water, a characteristic that gives this group of biological compounds its name. A variety of biological properties have been attributed to saponins, including cytotoxicity and various actions on the cardiac muscle (42). In our experiments, when the saponin was added to the membrane preparation, it induced complete release of the annexin 6 from the membrane, which suggested binding of annexin 6 to cholesterol.

Once we evaluated the presence of annexin 6 in the apical membranes, we determined the effect of neutralization of this protein on the Maxi-chloride channel activity. Initially we evaluated the characteristics of the channel in the absence of neutralizing antibody and in the presence of the specific immune serum, to assure the absence of a nonspecific effect due to one of the sera components. The results showed no difference between the control and the samples treated with the pre-immune serum. However, when the anti-annexin 6 antibody was added to the bath solution, a dramatic effect was observed in the voltage-dependent properties of the Maxi-chloride channel activity. Furthermore, the annexin 6 antibody also drastically decreased the conductance of the channel and affected the open probability of the channel. These results suggest that the direct interaction between annexin 6 and the annexin 6 antibody may disturb the binding of annexin 6 to the phospholipids or may inhibit the interaction between the protein and the Maxi-chloride channel. Because the interaction of the antibody is specific to annexin 6 and the pre-immune sera did not have any effect, we propose that this interaction is directly related to annexin 6 and not to another protein. Our data also suggest that annexin 6 is a potent regulator of the Maxi-chloride channel. Because placental transport is essential for the optimal development of the fetus, these results are of high importance and may open new avenues for the study of the role of annexin 6 expressions,

and its regulation, in pregnancy-associated complications such as pre-eclampsia and anti-phospholipid syndrome. Previous results from Riquelme's laboratory (43) have shown a decrease in the conductance state, a decrease in the open probability, and a flattened curve when the Maxi-chloride channel was evaluated in membrane fractions isolated from patients with moderate pre-eclampsia, similar to the experiments presented herein where the annexin 6 was neutralized. These results suggest that annexin 6 may play a significant role in the anionic transport of normal and pathological pregnancies.

The fact that annexin 6 was found to redistribute at acidic pH also suggests that the function and distribution of annexin 6 may have an impact in pathological pregnancies. For example, it is known that in pre-eclampsia the oxidative stress is increased, inducing a decrease in pH (44, 45, 31), and this change in intracellular pH may induce the binding of annexin 6 to cholesterol and thus reduce its binding to phospholipids. This change in affinity of annexin 6 may produce a change in the properties of the Maxi-chloride channel. Further experiments will be needed to fully evaluate the role of annexin 6 in this pathological state.

Our results have shown that the Maxi-chloride channel from normal placenta has a large conductance (>240 pS), multiple subconductance states, and voltage dependence of open probability, being open between −50 and +50 mV and closed at more extreme potentials. However, the addition of anti-annexin 6 antibody, which neutralizes the annexin 6-phospholipid interaction, in the bath modified the biophysical behavior of the Maxi-Cl^- channel in excised patches. Voltage-dependent open probability showed a flattened curve compared with control values when 5 μg/ml antibody was added to the bath solution. These results indicate that endogenous annexin 6 regulate the Maxi-Cl^- channel.

The complementary experiments could be the biochemical demonstration of an association of Maxi-Cl channels with annexin 6, but this question is difficult to answer now because of the elusive molecular identity of Maxi-Cl channels. Large conductance chloride (Maxi-Cl) currents have been recorded in several types of cells, but there is still little information on the molecular nature of the channels underlying this conductance. These channels were first identified in excised patches from the plasma membrane of skeletal muscle 20 years ago. The search for their molecular identity has continued with irregular success over all these years. The fact that Maxi-Cl channel electrophysiological properties resemble those of the mitochondrial voltage-dependent anion channel (VDAC) (46) encouraged some investigators to assume that the two channels were one and the same protein. This hypothesis was based on early observations suggesting the presence of VDAC protein on the plasma membrane (47, 48) but was questioned by others (49). The proposal suggesting the presence of VDAC in extramitochondrial locations has received recent strong support: Buettner *et al.* (50) reported the identification of a VDAC isoform (pl-VDAC) that contains a leader sequence for its trafficking to the plasma membrane, Bathori *et al.* (51) reported the presence of VDAC in caveolae, and Bahamondes *et al.* (52) have evaluated the presence of VDAC in the plasma membrane of C1300 neuroblastoma cells and its contribution to the Maxi-Cl currents activated by anti-estrogens in these cells.

Recently, Makoto Suzuki and Atsuko Mizuno (53) have reported that a gene located in *Drosophila flightless*, has a structure similar to those of known channels and that human homologues of *tweety* (hTTYH1–3) are novel Maxi-Cl channels. hTTYH3 mRNA was found to be distributed in excitable tissues. Like a Maxi-Cl channel from human syncytiotrophoblast, the hTTYH3 single channel showed 260-pS linear current voltage, 4,4-diisothiocyanatostilbene-2,2-disulfonic acid sensitivity, subconductance levels. However, the current-voltage relation of the hTTYH3 channel did not show a bell-shape. Similarly, hTTYH2 encoded an ionomycin-induced Maxi-Cl channel. In this study, the authors showed that the hTTYH family possesses five or six transmembrane segments encoding a large conductance Cl^- channel. In addition, the author with the Northern blot analysis of hTTYH3 in human tissues shows that it was found in the brain, heart, skeletal muscle, colon, spleen, kidney, and peripheral blood leukocytes, among other tissues, but not in placenta.

In the future it will be necessary to take a biochemical approach to identify the proteins that underlie the Maxi-Cl channel from human placenta to determine whether annexin 6 copurifies with the Maxi-Cl channel. To confirm that annexin 6 associates with the Maxi-chloride channel in human syncytiotrophoblast, coimmunoprecipitations will be performed using the anti-annexin 6 antibody and anti-Maxi-Cl channel antibody in a similar way as has been done in other studies to show the interactions between the two proteins (54).

The results presented here suggest that annexin 6 may be a regulator of placental transport and that this is dependent on the metabolic state of the syncytiotrophoblast cells. An alteration in the lipid domain can modulate the regulatory effect of annexin 6 on the Maxi-chloride channel. This alteration can be one of the multiple pathological changes found in pregnancy complicated with pre-eclampsia and/or intrauterine growth restriction. Studies are under way to evaluate the role of the annexin 6 in pregnancy-associated complication.

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