A chloride channel from human placenta reconstituted into giant liposomes

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OBJECTIVE: Ion channels play important roles in epithelial transport, but they are difficult to access for conventional electrophysiologic studies in intact placenta. The purpose of this work was to explore the suitability of purified trophoblast plasma membrane as a source of ion channels for reconstitution in artificial lipid membranes.

STUDY DESIGN: Human placental brush border membranes were purified by differential and gradient centrifugation and fused with small liposomes. Giant liposomes were then generated by a cycle of dehydration and rehydration. These giant liposomes are suitable for electrophysiologic studies and were probed for the presence of active ion channels by the patch-clamp method.

RESULTS: The results reported here indicate the presence of a high conductance chloride channel showing some similarities with "maxi" chloride channels described in secreting and absorbing epithelia. The channel had a slight outward rectification with conductances of 232 and 300 pS at negative and positive potentials, respectively.

CONCLUSIONS: For the first time successful reconstitution of a human placental ion channel is achieved in a system suited for electrophysiologic studies. The chloride channel described might play a role in transplacental transport. (Am J Obstet Gynecol 1995;173:733-8.)

Key words: Chloride channel, human placenta, ion channel reconstitution, patch-clamp method

Transport functions in the placenta have great importance for fetal growth and development. The trophoblast separates the maternal and fetal blood and may be viewed as an epithelium effectively segregating two fluid compartments. Because it is a syncytium, however, the trophoblast differs from other mammalian epithelia. Thus there is no paracellular route and all the solutes (water, nutrients such as amino acids, electrolytes, etc.) must pass directly through both trophoblast membranes, which constitute the primary barriers to maternal-fetal transport. Placental transport mechanisms have been extensively studied by means of plasma membrane preparations isolated from both the brush border (maternal-facing) and basal (fetal-facing) surface of the trophoblast.[1] However, there is little information regarding the ion channels present on these membranes.[1] Ion channels are known to play important roles in the regulation of cellular volume, maintenance of membrane potential, secretion and absorption processes, and many other functions.[2-7] It is reasonable to assume that ion channels in placental membranes have functions similar to those of other epithelia. Considerable interest has been focused on the chloride conductance in apical membranes of epithelial cells, which mediates secretion under the control of hormonal stimuli.[2-4]

Chloride transport has been studied with vesicles prepared from brush border membranes of the placenta. In this preparation about 50% of chloride transport occurs by Cl^-/HCO_3^- exchange[8, 9]; the remaining flux is dependent on membrane potential and probably takes place through a conductive pathway.[1, 8-10] Subsequent detailed studies of chloride fluxes and ionic dependence of membrane potential have confirmed the presence of chloride conductive pathways in the microvillous membrane.[11,12,14,15] Recently Brown et al.,[16] applying the patch-clamp technique, have presented the first direct demonstration of a chloride channel of high conductance in apical membranes of the intact trophoblast epithelium.

The aim of the current study was to explore the suitability of purified trophoblast plasma membrane preparations as a source for reconstituted ion channels that might play a role in placental transport. A method that uses highly purified trophoblast brush border

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Table I. Enrichment of alkaline phosphatase in purified apical membrane preparations

	Tissue homogenate	*Crude apical cell membrane*	*Purified apical cell membrane*
Protein (mg/100 gm of tissue)	7530 ± 1166 (n = 3)	236.8 ± 19.9 (n = 3)	20.13 ± 1.41 (n = 3)
Tissue homogenate (%)	100	3.1	0.003
Alkaline phosphatase (μmol/mg/min)	0.023 ± 0.011 (n = 6)	0.408 ± 0.054 (n = 6)	0.726 ± 0.217 (n = 3)
Relative activity	1	17.7	31.6

Values are means ± SD. Number of preparations in parentheses.

membrane incorporated into giant liposomes was used. Patch-clamp single-channel analysis demonstrated the presence of cation and anion channels. The most frequently encountered channel activity corresponded to a chloride channel of large unitary conductance. The approach described here could be useful to circumvent some of the difficulties in studying ion channels in a complex epithelium such as the placenta.

Material and methods

Preparation of placental brush border membrane. Human placental brush border membrane vesicles were prepared by a method similar to that described by Glazier et al.[17] Placentas (700 to 800 gm) were obtained immediately after delivery from the university hospital and transported to the laboratory on ice. The maternal decidua was removed, and the central portion between the maternal and fetal surfaces was used in the preparation. Maternal villous tissue (110 to 150 gm), was chopped into small pieces and washed in buffer (150 mmol/L sodium chloride, 20 mmol/L Tris-HEPES [N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid], pH 7.4). The tissue was homogenized in three volumes of ice-cold buffer (300 mmol/L mannitol, 1 mmol/L magnesium sulfate, and 20 mmol/L Tris-HEPES, pH 7.4). After homogenization 10 mmol/L magnesium chloride was added, and the homogenate was stirred on ice for 10 minutes before centrifugation at 2200g for 15 minutes. The supernatant was spun at 23,500g for 40 minutes. The resulting pellet was resuspended in the mannitol buffer and the Mg^{++} treatment repeated, followed by centrifugation as before to yield the microvillous membrane pellet.

Sucrose gradient centrifugation. Microvilli were further purified on a sucrose step gradient made up with 10 ml of 45%, 7 ml of 37%, and 8 ml of 25% sucrose. All the solutions were buffered with 20 mmol/L Tris-HEPES, pH 7.4. A portion (2 to 3 ml) of the microvillous-enriched preparation containing about 10 to 15 mg of protein was overlayed on the gradient and centrifuged overnight at 80,000g at a temperature of 4° C. The band at the 37%/45% sucrose interface was collected and diluted tenfold with 20 mmol/L Tris-HEPES, pH 7.4, before centrifugation at 100,000g for 60 minutes. The pellet was resuspended in 300 mmol/L sucrose, 20 mmol/L Tris-HEPES, pH 7.4, and stored at −80° C.

Marker enzyme assays: alkaline phosphatase. Alkaline phosphatase activity, a brush border membrane marker, was measured by the method of Lansing et al.[13] with p-nitrophenylphosphate used as a substrate. The p-nitrophenol produced was measured spectrophotometrically at 420 nm. Enzyme activity was expressed as micromoles of p-nitrophenol formed per milligram of protein per minute. To test for contamination of basal membranes, propranolol-sensitive tritiated dihydroalprenolol binding was measured by the method of Kelley et al.[18]

Reconstitution of the brush border membrane into giant liposomes. Giant liposomes were prepared by submitting a mixture of the reconstituted brush border membrane vesicles and asolectin lipid vesicles to a partial dehydration-rehydration cycle, as reported by Riquelme et al.[19] A membrane aliquot containing 100 to 150 μg of protein was routinely mixed with 2 ml of a 13 mmol/L (in terms of lipid phosphorus) suspension of the asolectin vesicles. After the partial dehydration-rehydration cycle,[19] the diameter of the resulting giant multilamellar liposomes ranged from 5 to 100 μm.

Patch-clamp measurements. Aliquots (3 to 15 μl) of giant liposomes were deposited into 3.5 cm Petri dishes, mixed with 1 ml of buffer of choice for electrical recording (bath solution) and incubated for 10 to 15 minutes at room temperature. Single-channel recordings were obtained by patch-clamp techniques as described by Hamill et al.[20] Giga seals (10 to 20 GΩ) were formed on giant liposomes with microelectrodes of 10 to 20 MΩ resistance. After sealing, withdrawal of the pipette from the liposome surface resulted in an excised patch ("inside-out" configuration). Current was recorded with an EPC-7 patch-clamp amplifier (List Medical Electronics, Darmstadt, Germany), at a gain of 50 to 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. Currents flowing from the bath to the pipette interior are represented as positive. The signal was monitored on a storage oscilloscope and stored on a videotape with a modified pulse-code modulator. The signal was analyzed off-line by means of programs kindly provided by Dr.

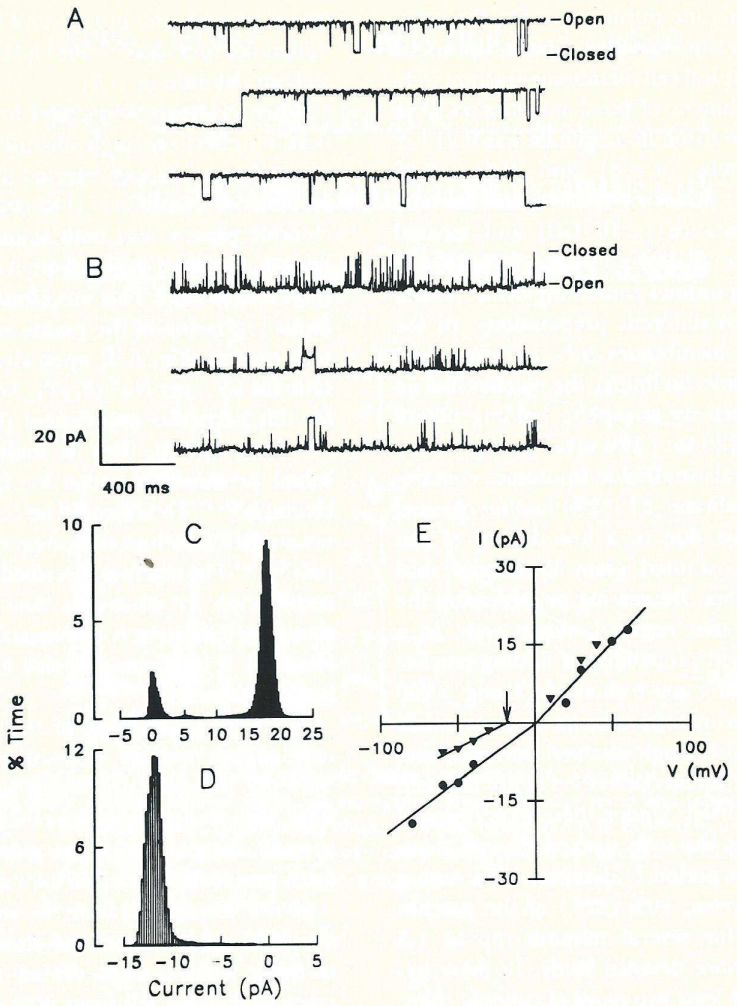

Fig. 1. A and **B,** Single-channel currents recorded in excised, inside-out patch from brush border membrane reconstituted into giant liposomes. Currents were recorded at +60 and −60 mV with bath and pipette solutions containing (in millimoles per liter) 140 NMDGCl, 2.6 calcium chloride, 1.3 magnesium chloride, 10 sodium HEPES, pH 7.4. **C** and **D,** Current amplitude histograms corresponding to records shown in **A** and **B,** respectively. **E,** Current-voltage relationship *(filled circles)* for channel illustrated in **A** and **B.** Data points in this plot are means of several measurements made in same patch. Current-voltage relationship *(filled triangles)* for channel recorded under asymmetric sodium chloride concentrations (equilibrium potential for chloride −32 mV). Pipette solution contained 140 mmol/L and the bath 40 mmol/L sodium chloride. Points at negative potentials were used to extrapolate zero-current voltage by linear regression (*arrow,* −20 mV, extrapolated reversal potential).

J. Dempster (University of Strathclyde, Scotland). The signal was filtered on an eight-pole Bessel filter at 2 kHz (−3 dB) and sampled at 10 kHz. The open probability was calculated with a 50% threshold method from records lasting at least 1 minute. All measurements were made at room temperature.

The solutions used for the patch clamp experiments were the following for the pipette and the bath (in millimoles per liter): 140 N-methyl-D-glucamine chloride (NMDGCl), 140 sodium chloride or 140 potassium chloride, and, in all cases, 2.6 calcium chloride, 1.3 magnesium chloride, and 10 sodium HEPES, pH 7.4.

Unless stated otherwise all reagents and chemicals were from Sigma, St. Louis.

Results

The specific activity of alkaline phosphatase in brush border membranes and in whole tissue homogenates is given in Table I. Compared with placental homogenates, the enrichment in alkaline phosphatase activity was 18-fold for a crude apical membrane fraction and 32-fold in a purified apical membrane fraction obtained from the 37%/45% sucrose gradient interface. A 32-fold increase in alkaline phosphatase activity indicates a high degree

of brush border membrane purification. On the other hand, propranolol-sensitive tritiated dihydroalprenolol binding of the crude apical cell membrane fraction indicated a lack of enrichment of basal membranes. The specific binding to the tissue homogenate was 0.213 ± 0.05 pmol/mg protein ($n = 4$) and 0.31 ± 0.09 pmol/mg protein ($n = 4$) for the crude apical fraction.

Seals of high resistance (≥10 GΩ) and excised patches in an "inside-out" configuration were obtained from cell-size, giant liposomes containing brush border membrane from seven different preparations. In the absence of placental membranes (i.e., in giant liposomes made only of asolectin lipids), the patches had no detectable electrical activity at pipette holding potentials ranging from −120 to +120 mV.

Of a total of 109 seals studied in liposomes containing brush border membrane, 57 (52%) had no channel activity. This could be due to a low density of ion channels in the reconstituted giant liposomes, thus explaining silent patches. Decreasing or increasing the initial amount of protein resulted, respectively, in a decreased and increased probability of finding patches of membranes containing active channels. Using >200 μg of protein in the reconstitution assay, however, made it difficult to distinguish single-channel currents, and therefore for the experiments described below between 100 and 150 μg was used. Under these experimental conditions 28% (30) of the seals studied showed channel activity for a short period (<1 minute), becoming silent thereafter. However, 20% (22%) of the patches had active channels for several minutes (up to 1.5 hours), allowing for more detailed study. In these experiments we found at least two types of cationic channels. These channels are similarly permeable to Na^+ and K^+ ions and have a conductance around 290 and 60 pS (data not shown). We also found a large conductance chloride channel, which was the most frequently observed activity in stable patches (11 over 22 patches).

Single-channel currents for the chloride channel reconstituted from placental membranes are shown in Fig. 1, *A* and *B*, at holding potentials of +60 and −60 mV, respectively. The corresponding amplitude distribution histograms are shown in Fig. 1, *C* and *D*. Both traces showed a primary conductance state, but substates were also evident in the traces and the histogram in Fig. 1, *C*. Fig. 1, *E* (filled circles), depicts the current versus voltage plot obtained from amplitude histograms at different holding potentials. Only the dominant current level was used, ignoring any subconductance levels. Slope conductances for outward currents (232 pS) and inward currents (300 pS) were determined separately by linear regression. In three separate experiments the respective conductances were 225 ± 10 pS and 314 ± 16 pS (mean ± SD). This large conductance was observed in experiments done with 158 mmol/L symmetric concentrations of chloride independently of the cation present (the pipette and the bath containing (in millimoles per liter): 140 NMDGCl ($n = 4$) or 140 sodium chloride ($n = 7$).

When Na^+ was substituted for K^+ in the bath solution, no effect on single-channel current amplitude or reversal potential was detected (data not shown). Under asymmetric conditions (140 and 40 mmol/L sodium chloride pipette and bath solutions, respectively), the reversal potential shifted from 0 mV to around −20 mV (Fig. 1, *E*, arrow). This was estimated extrapolating the linear regression of the points at negative potentials to zero current (Fig. 1, *E*, open circles). The permeability ratio for Cl^- over Na^+ (P_{Cl}/P_{Na}) of 5.3 can be calculated for this particular experiment (Mean $P_{Cl}/P_{Na} = 7.2 \pm 1.4$ SEM, $n = 3$). This is similar to previously published permeability ratios for large conductance Cl^- channels.[21, 22] Thus it could be concluded that the channel studied falls into the category of "maxi" Cl^- channels.[2, 21-24] The channel described here presents outward rectification in symmetric solutions (see above). Although rectification is an unusual property of maxi Cl^- channels, it has been reported in human colonic carcinoma cell lines.[25]

The open probability of this chloride channel was only slightly voltage dependent. Between −50 and +50 mV the open probability was approximately constant, with respective values of 0.92 and 0.97. There was a small decrease at more polarized potentials, with open probability values of 0.69 at −80 mV and 0.79 at +60 mV. This differs from the voltage dependence of a maxi Cl^- channel described elsewhere in intact placental tissue, which is markedly bell-shaped.[15] In that study open probability fell from 90% within the range ±30 mV to 25% at ±50 mV. The discrepancy between these two studies could be due to an effect of the lipid environment or to the reconstitution procedure in the current work. Although voltage had no marked effect on open probability, it was apparent that the kinetics changed with voltage. Fig. 1 shows that closures at +60 mV were long lasting when compared with those at −60 mV. Analysis of the distribution of dwell times (not shown) indicated two types of closure at +60 mV with time constants of 1.7 and 16 ms, respectively. Only one time constant, 0.34 ms, was required to describe the distribution of closed intervals at −60 mV. The presence of short-lived closures was more evident at −80 mV, as shown in Fig. 2, *A*. This impression is further illustrated in the transitions evoked by a voltage ramp (Fig. 2, *C*). A different pattern of activity was generated at the positive end of the ramp with longer closures, flickering being observed at the negative end of the pulse.

The channel also displayed a number of intermediate subconductance states. The current trace illustrated in Fig. 2, *A*, and its corresponding amplitude histogram (Fig. 2, *B*) suggests that there are at least four states of

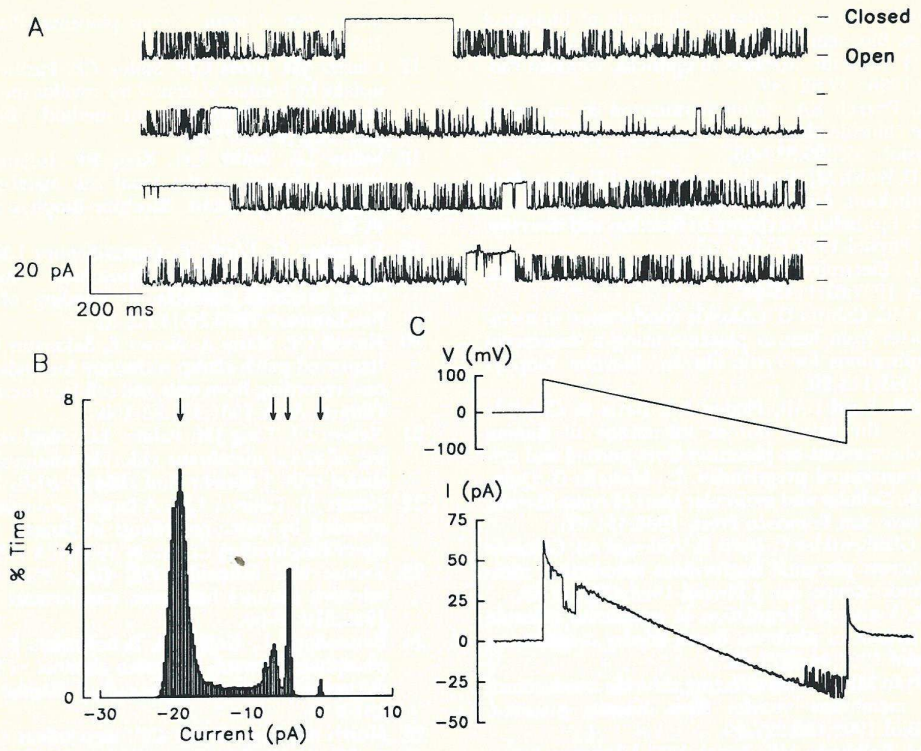

Fig. 2. Voltage dependence of placental chloride channel transitions. **A,** Record at −80 mV holding potential. **B,** Corresponding current amplitude histogram. *Arrows,* Primary states and substates (0, 4, 6, and 19 pA). **C,** Current record *(lower panel)* obtained from voltage ramp illustrated in *upper panel* (duration 830 ms). All experiments were done under symmetric 140 mmol/L NMDGCl. The recordings illustrated were obtained in single patch, but similar behavior was observed in all recordings of this type of anion channel.

conductance of the channel, including closed and fully open. The largest peak in the histogram corresponds to 19 pA, indicating that the channel was most frequently open to the full conductance state. The two conducting substates were at 4 and 6 pA. The presence of subconductance states is also a reported characteristic feature of maxi Cl^- channels.[15, 23]

Comment

The data presented here demonstrate the presence of a chloride channel in apical membranes of the placenta. The channel is selective for anions over cations, has large conductance (>200 pS), and multiple subconductance states. Some of these properties are similar to those described for a family of chloride channels identified in secreting and absorbing epithelia,[2-4, 21, 25] other cell types,[22-24, 26, 27] and more recently in small fragments of villous tissue dissected from human placenta.[15] These channels are generally referred to as maxi Cl^- channels. Interestingly, Hardy and Valverde[28] demonstrated that this type of chloride channel is under regulation of estrogens and antiestrogens acting through a plasma membrane receptor. In view of the importance of these agents in regulating placental function, it is tempting to speculate that the maxi Cl^- channel described here might play a physiologic role under the control of estrogens.

The results reported in this work are the first description of an ion channel from human placental trophoblast reconstituted into giant liposomes, a preparation suitable for patch-clamp recording of single-channel ion currents. The precise role of these channels in placenta has yet to be determined. The electrophysiologic approach described here could contribute to advance our understanding of the biophysical properties, function, and regulation of the ion channels in different membranes of this tissue.

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