Barium, Tea and Sodium Sensitive Potassium Channels are Present in the Human Placental Syncytiotrophoblast Apical Membrane

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Keywords:
Placenta
Syncytiotrophoblast
Apical membrane
Potassium channels

A B S T R A C T

The human placental syncytiotrophoblast (hSTB) is a polarized epithelial structure, without paracellular routes, forming the main barrier for materno–fetal exchange. There is ample evidence suggesting the presence of potassium (K^+) channels in the placental apical membrane; which could contribute to membrane potential and volume regulation. We have therefore examined the K^+ currents of isolated apical membranes from human term placenta using electrophysiological methods: reconstitution of ion channels from apical membranes into giant liposomes (single channel recordings, patch clamp method) or their functional transplantation into *Xenopus laevis* oocytes (total currents recording, voltage clamp method).

Single channel recording experiments show the presence of K^+ channels in the hSTB microvillous membrane sensitive to Tetraethylammonium (TEA) and Barium (Ba^{+2}). Patch current activity was diminished 50% and 70% by 20 mmol/L TEA and 5 mmol/L Ba^{+2} respectively. The more frequent conductance was approximately 73 pS, however several levels of current were detected suggesting the presence of more than one type of K^+ channel. In addition, sodium (Na^+) sensitivity was detected in the patch current thus, over 10 mmol/L Na^+ reduced the seal current to 38%. These results were corroborated by the total current experiments where the K^+ current elicited in injected oocytes with apical purified membrane was blocked by Ba^{+2} and TEA. The total current was also affected by Na^+, becoming larger when a Na^+-free solution was used. Our results show the existence of at least two types of Ba^{+2}-sensitive K^+ channels including a TEA sensitive sub-population, and some of them Na^+ sensitive K^+ channels. These channels could be the conductive pathways proposed previously for this cation in placental hSTB. Our novel contribution has been to successfully obtain K^+ channel recordings in systems suitable for electrophysiological studies of isolated apical membranes.

1. Introduction

Potassium (K^+) channels conduct K^+ ions across the cell membrane, down their electrochemical gradient. K^+ conduction underlies different cellular processes including membrane potential, cell volume regulation and hormone secretion, amongst many other functions in epithelial and non-epithelial cells. The human placental syncytiotrophoblast (hSTB) is a continuous epithelial layer without paracellular routes which forms the main barrier for materno–fetal exchange. In common with all epithelia, the activity of K^+ channels present in the hSTB will have a major influence on membrane potential, electrogenic solute transport and volume regulation. However, their biophysical characterization and their specific function in trophoblast is poorly understood in contrast with the non-placental epithelia, where K^+ channels have been identified and their contribution to physiological processes is well characterized. K^+ transport has been studied in vesicles prepared from apical membranes of the placenta suggesting that at least part of the flux is dependent on membrane potential, and probably takes place through a conductive pathway [1]. Subsequent detailed studies of K^+ or rubidium (Rb^+) fluxes and ionic dependence of membrane potential have confirmed the presence of K^+ conductive pathways in the microvillous membrane in isolated placental villi (explants) or in cytotrophoblast cells isolated from term placentae maintained in primary culture [2,3]. Birdsey et al. (1999) have demonstrated that Ba^{+2}-sensitive K^+ conductances contribute to the resting potential of the hSTB microvillous membrane in isolated villi from term human placentae, as well they have shown that the hSTB microvillous membrane responds to a hyposmotic stimulus by activating both Ba^{+2}-sensitive K^+ and DIDS-sensitive anion

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conductances; however, the specific ion channels involved are currently unknown. In cytotrophoblast cells, three approaches have been used to identify K^+ conductances: membrane potential measurements, $^{86}Rb^+$ (K^+) efflux, and patch clamp studies [2]. Clarson et al. in 2001 demonstrated that cytotrophoblast cells express an inwardly rectifying K^+ current typical of Kir 2.1, whose incidence increases with cytotrophoblast cell differentiation. In addition, stimulation of cytotrophoblast cells by hyposmotic swelling or extracellular nucleotides, promotes $^{86}Rb^+$ efflux. This K^+ permeability is due, partly to the stimulation of Ca^{+2}-activated K^+ current, sensitive to charibdotoxin but insensitive to apamin and iberiotoxin, implicating an intermediate conductance Ca^{+2}-activated K^+ channel [4]. Recently Bai et al. (2005 and 2006) [5,6] have shown the expression and activity of the two pore domain K^+ channels (K_{2p}) TASK (1 and 2) and TREK in villous tissue from human placenta, and in cultured human cytotrophoblast cells at multinucleate and mononucleated stages of culture. It is likely that K_{2p} channels may therefore have a role in epithelial K^+ homeostasis, transport or in setting membrane potential.

Although there is evidence which indicates that different K^+ channel types are expressed in cytotrophoblast cells, there are presently no equivalent studies in the hSTB apical membrane from term placenta. We thought that an electrophysiological study of these K^+ channels would help solve the issue of whether they participate in the swelling process or in the maintenance of the membrane potential.

Previously, using differential sucrose density migration, we described the apical (maternal-facing) membrane with two isolated fractions: the classical apical fraction (MVM) used by us and others to study transport mechanisms, and another apical fraction termed light microvillous membrane (LMVM) [7]. Ion channels by electrophysiological methods have been studied in MVM fraction [8–15]. We described a Maxi chloride channel and a non-specific cation channel, but we did not find a K^+ channel. On the other hand, LMVM until now has been unexplored from the point of view of channel activity. The aim of our present work has been to detect K^+ channels in the light microvillous hSTB plasma membrane (LMVM) and to study their electrophysiological and pharmacological behavior. These results will hopefully aid in the understanding of the role of K^+ as an important physiological factor in placental epithelium.

2. Materials and methods

2.1. 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.

2.2. Preparation of placental apical membrane (LMVM)

The human placental apical membrane vesicles were prepared from fresh placentae by a method that we have described previously allowing simultaneous isolation of apical and basal membranes from the same placenta [7]. Purification method involved different steps such as precipitation of non-microvillous membrane with magnesium ions, differential centrifugation and a sucrose step gradient; this assured that isolated fractions were enriched and free of contamination. All solutions were buffered with 20 mmol/L Tris-Maleate, pH 7.4. A portion (2–3 mL) of the microvillous-enriched preparation containing about 10–15 mg of protein was overlayed on the sucrose gradient. As described in our previous results reported in Jimenez et al. 2004 [7], two isolated apical fractions were obtained from a sucrose gradient. The bands obtained at the concentration 10/37% and 37/45% sucrose interfaces, corresponding to an apical fraction named light microvillous membrane (LMVM) and to the classical apical fraction named apical microvillous membrane (MVM) respectively, resulted in adequate enrichment of apical membrane markers. Both purified apical fractions have low basal membrane and intracellular membrane contamination markers. The purity and cross-contamination of the purified membranes were comparable to those reported for single or paired apical and basal membrane preparations [24–26], as we demonstrated in Jimenez et al. [7]. In the present work we used only the LMVM. This fraction was collected and diluted 10 fold with 20 mmol/L Tris-Maleate, pH 7.4 before centrifugation at $110{,}000 \times g$ for 30 min. The final pellet was resuspended in 300 mmol/L sucrose, 20 mmol/L Tris-Maleate, pH 7.4 solutions, and stored in liquid nitrogen. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Inc.) for the colorimetric detection and quantification of total protein [16,17]. The purity and enrichment of the LMVM membrane fraction was determined routinely by assaying for alkaline phosphatase activity, an apical membrane marker; adenylate cyclase as a basal membrane marker and cytochrome-*c* oxidase/succinate dehydrogenase as mitochondrial membrane markers [7].

2.3. Oocyte microinjection of LMVM vesicles

Xenopus laevis oocytes at stages V and VI [18] were isolated from ovary segments of 25–100 mg/L tricaine anesthetized frogs, under aseptic conditions. The protocol was approved by the Committee for Animal Research Bioethics at the Faculty of Medicine, University of Chile. Collected oocytes were enzymatically defolliculated by treatment with 0.5 mg/mL collagenase (Sigma-Aldrich, Inc.), as previously described by Miledi [19,20]. Oocytes were injected with LMVM vesicles (~34 nL; 200–300 μg of proteins) using a Nanoliter Injector (World Precision Instruments, Inc.), and maintained at 15–16 °C in a modified Barth's solution plus antibiotics (in mmol/L: 88 NaCl, 1 KCl, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 2.4 $NaHCO_3$, 10 Na-HEPES, pH 7.4, 100 IU/mL of penicillin and 0.1 mg/mL of streptomycin). Uninjected oocytes and oocytes injected with ~34 nL of buffer (in mmol/L: 300 sucrose, 20 Tris-Maleate, pH 7.4) were used as controls. A morphologic criteria of inclusion was evaluated (i.e. spherical form, turgidity, unharmed cellular surface, poles clearly delineated) previous to the functional recording of macroscopic currents of uninjected and injected oocytes.

2.4. Oocyte recordings

The functional incorporation of foreign channels into the oocyte membrane was assessed 16–24 h after injection by recording membrane currents from voltage clamped oocytes. The voltage clamp recording methodology (two-electrode voltage clamp technique) is described in detail elsewhere [21]. Briefly, oocytes were placed in a small chamber and superfused with Normal Ringer (NR) solution at room temperature (21–25 °C). Functional incorporation of foreign voltage dependent channels into oocyte plasma membrane was evaluated using an Oocyte Clamp 75-C amplifier (World Precision Instruments, Inc.). Intracellular electrodes (1–4 MΩ resistance) were filled with 3 mmol/L KCl. The membrane currents were recorded in response to voltage pulses from −120 to +40 mV at 20 mV steps (−60 mV holding potential; V_h). In case of the blocking solutions used, we applied a +40 mV voltage pulse ($V_h = -60$ mV). Uninjected oocytes were considered in these experiments as control cells. Control oocyte currents at +40 mV were taken as reference to normalize LMVM injected oocyte currents. The WCP for Windows program V3.5.8 provided by John Dempster (University of Strathclyde, UK), was used for data acquisition and analysis.

2.5. Voltage clamp solutions

Normal Ringer solution (RN), in mmol/L: 115 NaCl, 2 KCl, 1.8 $CaCl_2$, 5 Na-HEPES, pH 7.0) and a Na^+-free solution was prepared by substituting NaCl by equimolar amounts of *N*-methyl-D-glucamine chloride (R-NMDGCl). The K^+ channel-blocking agents Tetraethylammonium chloride (TEA; Sigma-Aldrich, Inc.) and Barium chloride ($BaCl_2$; Sigma-Aldrich, Inc.) were added to R-NMDGCl from stock solutions up to final concentrations of 20 and 5 mmol/L respectively. Chloride (Cl^-) channel blocking agent 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS; Sigma-Aldrich, Inc.) was added from stock solution up to a final concentration of 1 mmol/L.

2.6. Reconstitution of the LMVM into giant liposomes

Giant liposomes were prepared by submitting a mixture of the isolated LMVM vesicles and asolectin lipid vesicles to a partial dehydration/rehydration cycle, as reported by Riquelme et al. [22]. An aliquot containing 100–150 μg of membrane protein was 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, the diameter of the resulting giant multilamellar liposomes ranged from 5 to 100 μm accessible to patch clamp recording.

2.7. Patch clamp measurements

The electrical activity of K^+ channels was studied in excised patches in an "inside out" configuration obtained from cell-size giant liposomes containing LMVM from normal term placentae. Aliquots of 1–3 μL of giant liposomes were deposited into an excised Patch chamber (RC-28, Warner Instruments Corporation, USA) mixed with 0.4 mL of the buffer of choice for electrical recording (bath solution). Single channel recordings were obtained by patch clamp technique as described by Hamill et al. [23]. Giga seals were formed on giant liposomes with glass microelectrodes of 5–10 MΩ resistance. 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 when necessary. The signal was analyzed off-line by means of the TAC (Bruxton Corporation) and Pulse Fit (Heka, Lambrecht/Pfalz, Germany) software. All measurements were made at room temperature.

2.8. Patch clamp solutions

The pipette and bath solutions had the following composition (in mmol/L): 140 KCl (or K-gluconate), 10 Na-HEPES, pH 7.4, unless stated otherwise. The K^+ channel-blocking agents Tetraethylammonium chloride (TEA; Sigma-Aldrich, Inc.) and Barium chloride ($BaCl_2$; Sigma-Aldrich, Inc.) were added to bath solution up to final concentrations of 20 mmol/L (TEA) and 5 mmol/L ($BaCl_2$). Cl^- blocking agent 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS; Sigma-Aldrich, Inc.) was added from stock solution up to a final concentration of 1 mmol/L.

2.9. Statistical analysis

Results are expressed as means ± SEM. Measures of statistical significance were obtained using Student's *t*-test. A *p*-value of less than 0.05 was considered significant. Measures of statistical significance were also obtained using one-way ANOVA plus Bonferroni multiple comparisons test. A *p*-value of less than 0.01 was considered significant. The n for statistical analysis corresponds to the total number of experiments.

3. Results

The following results were obtained from single channel recordings by the path clamp method in giant liposomes containing reconstituted placental apical membrane and total current recordings by the voltage clamp method in *Xenopus* oocytes containing transplanted healthy term placenta purified apical membranes (LMVM). Purification of apical membranes was achieved through the protocol described in Section 2. In agreement with our previous results reported in Jimenez et al. 2004 [7], the specific activity of alkaline phosphatase (PLAP) in LMVM compared with placental homogenates (H), showed an elevated enrichment in alkaline phosphatase activity indicating a high degree of apical membrane purification. The enrichment factors for $n = 9$ independent placentae, assessed using the enzyme marker PLAP, was 29.7 ± 5.6 fold, calculated as the ratio of activity in membrane fractions compared to that in the H. The enrichment in PLAP activity in the purified apical membrane fraction obtained from the 37%/45% sucrose gradient interface, MVM, was 21.7 ± 3.2 fold for the same nine membrane preparations from equal number of independent placentae. Both apical membrane fractions have high enrichment factors, although these data suggests a higher value for LMVM than MVM in agreement with previous data.

3.1. K^+ current recordings from LMVM reconstituted into cell-size giant liposomes

LMVM from $n = 12$ normal term placentae and a total of $n = 40$ high-resistance excised patches were used for the results described below.

3.2. Single K^+ channels recording and conductance

Several levels of current were detected when K^+-rich solutions were used; this suggests the presence of one channel with subconductance states or of more than one type of channel from LMVM, either cationic or anionic. However, the most frequent electrical activity pattern observed in stable patches is shown in Fig. 1A. In symmetrical K^+ solutions the current record obtained from a voltage ramp pulse between −120 and +120 mV (40 mV/s) has a reversal potential of 0 mV. Under asymmetrical K^+ concentrations (140 and 70 mmol/L K^+ bath and pipette, respectively) the reversal potential shifted from 0 to −20.5 ± 3.7 mV either in KCl or

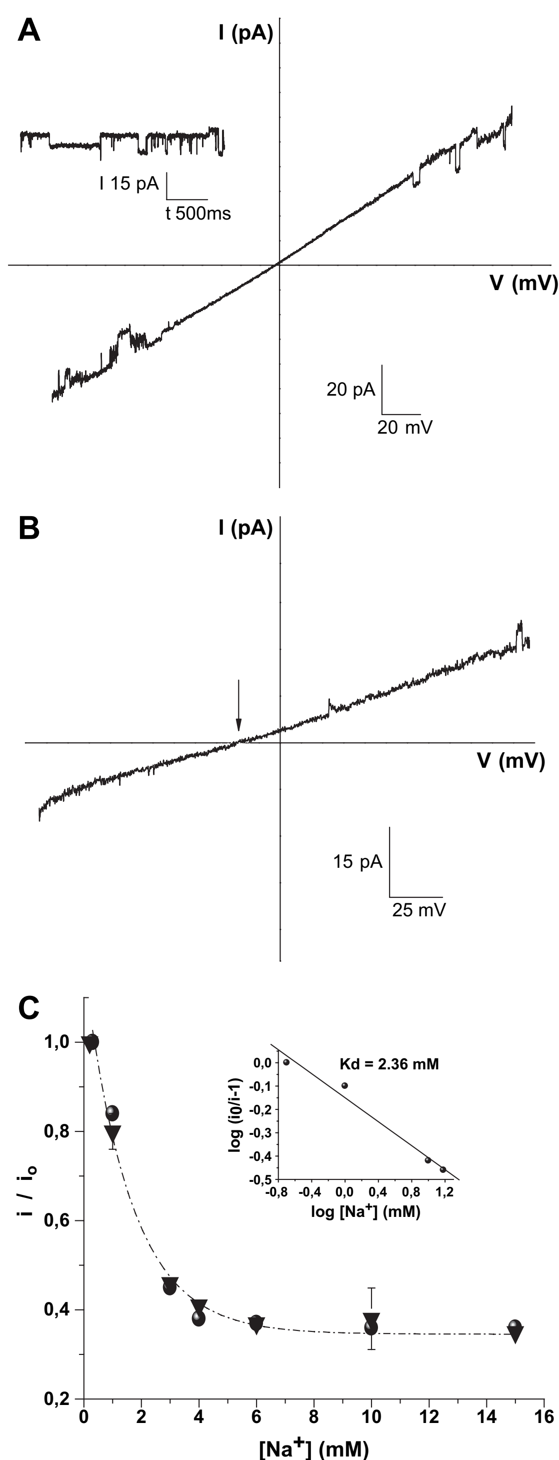

Fig. 1. K^+ single channel recording from LMVM in excised patches from giant liposomes. (A) *I*/*V* curve obtained from voltage ramp between −120 and +120 mV in symmetrical solutions (bath and pipette solutions containing 140 mmol/L KCl). **Inset** represents a recording of potential pulse at +80 mV. (B) *I*/*V* curve obtained from voltage ramp between −120 and +120 mV recording in gradient conditions of KCl solutions (70 mmol/L KCl pipette, 140 mmol/L KCl bath), the arrow shows reversal potential displacement at −22.8 mV. (C) Effect of Na^+ on K^+ channels from LMVM. K^+ channels were exposed in a range from 0 to 15 mmol/L Na^+ concentration in excised patch from LMVM reconstituted into giant liposomes at −80 mV (circles) and at +80 mV potentials (triangles). Each point indicates mean ± S.E.M. ($n = 10$ seals, $n = 3$ placentae). The current (i) is normalized relative to current amplitude in absence of Na^+ (i_0) and expressed as i/i_0 ($n = 10$ seals, $n = 3$ placentae). Superimposed fit is a single exponential decaying function. **Inset**, the same data are plotted on a double logarithmic scale to provide a better estimate of the Na^+ required to produce half-maximal inhibition of the current (K_d); the indicated K_d value of 2.4 mmol/L was derived from the slope of linear regression fit superimposed on the data points.

K-gluconate solutions (Fig. 1B, arrow), close to the theoretical equilibrium potential for K^+ (−16.1 mV), indicating that all electrical activity observed corresponded to K^+ channels in this group of seals.

Single channel current traces, at the indicated holding potential, are shown in Fig. 1A inset. Both traces showed a primary conductance state, but sub-states were also evident. Only the higher current level was used, ignoring any sub-conductance levels. The slope conductance was of 72.9 ± 7.6 pS ($n = 8$ seals, $n = 5$ placentae) in symmetrical KCl or K-gluconate solutions (140 mmol/L). Additionally, we obtained two seals in symmetrical KCl where the current presented an inward rectification. The linear part had a similar conductance to that mentioned above.

3.3. *Block of K^+ currents by Ba^{+2} and TEA*

In general, the record of electrical activity could be due to the presence of either one channel with several sub-conductance states, or several populations of K^+ channels. For this purpose, we planned pharmacological strategies to determine the presence of one or more populations of these channels. Among the numerous K^+ channel blockers, we chose Ba^{+2} because it can block independent populations of K^+ channels, whose effects have been previously detected in human trophoblast tissue by other authors [2,3]; and a classical K^+ channel blocker, TEA. Membrane excised patches in 140 mmol/L KCl pipette-to-bath were studied using voltage ramps between +120 and −120 mV to obtain total patch currents in control conditions and subsequent to the presence TEA and Ba^{+2} (Fig. 2A,B). An inhibition in a range 35–45% of the total seal current was achieved when TEA was added to the control bath solution in concentrations of 20 mmol/L ($n = 5$ seals, $n = 2$ placentae). The difference observed between negative and positive potentials suggests a weakly voltage dependent blockade. With TEA present, the addition of Ba^{+2} (5 mmol/L), diminished the K^+ current at a supplementary range between 48 and 50% ($n = 2$ seals, $n = 2$ placentae; Fig. 2A). In contrast, when the sequence of addition of blockers was changed, TEA being added to the bath after Ba^{+2}, the level of inhibition was not modified (Fig. 2B). As shown in Fig. 2C ($n = 6$ seals, $n = 4$ placentae), 5 mmol/L Ba^{+2} by itself diminished the total current in the patch close to 80%. At −80 mV the total block of K^+ current was 77.2 ± 5% and 72.2 ± 8.1% at +80 mV, although the effect is slightly voltage-dependent, this dependence is not significant. Additionally, there was a group of experiments that showed a similar behavior in presence of the K^+ channels blockers, but the percentage of blockage was only 38.2 ± 5.6% at −80 mV and 51.8 ± 2.3% at +80 mV from the total seal current ($n = 4$ seals, $n = 3$ placentae). These seals probably contain another type of channel, where a natural candidate would be a Cl^- channel, since in two of them DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid), a Cl^- channel blocker, diminished the seal current (data not shown).

3.4. *Effect of Na^+ on K^+ channels*

Application of Na^+ ions produces an effect in some types of K^+ channels [27–33], including TASK-2, a member of the two-pore domain K^+ channel family that is expressed at high levels in several epithelia, including the placenta [5,6,34].

The effect of Na^+ ions on the placental K^+ current was investigated in excised patches using a voltage ramp to obtain total patch currents in K^+ symmetrical solutions. When Na^+ was added to the bath solution, a voltage independent reduction of the patch electrical current was observed; however, this reduction was Na^+ concentration dependent. To quantify this observation, we examined the effect of a range of concentrations between 0 and 30 mmol/L Na^+ on seal current amplitude at +80 and −80 mV.

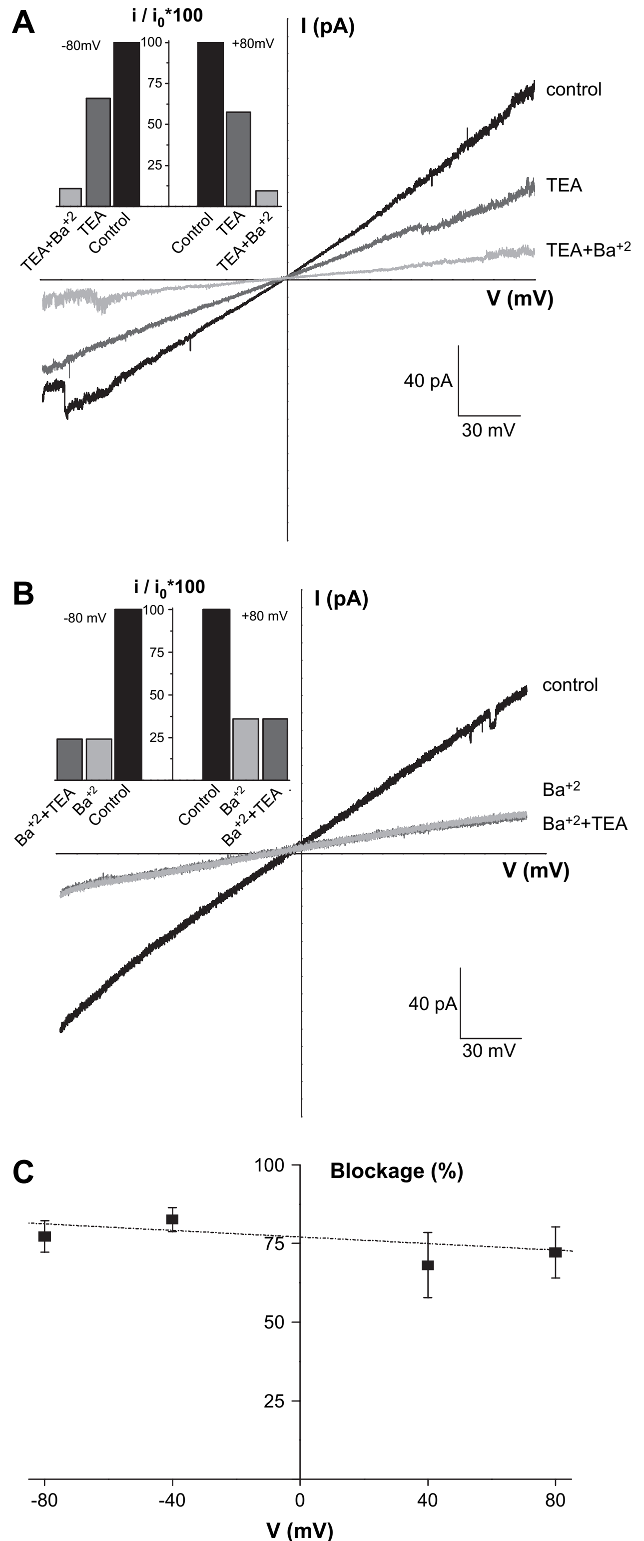

Fig. 2. Block of single K^+ currents from LMVM by Ba^{+2} and TEA in excised patches from giant liposomes. Representative *I*/*V* curves obtained from voltage ramps between −120 and +120 mV. (A) *I*/*V* ramp represents a control situation (0 blocker), with 20 mmol/L TEA first and second with 5 mmol/L Ba^{+2}. **Inset** represents bar graph of current blockage at −80 and +80 mV, control conditions (black bar), 20 mmol/L TEA (dark gray bar) and 20 mmol/L TEA plus 5 mmol/L Ba^{+2} (gray bar). (B) *I*/*V* ramp represents a control situation (0 blocker) and the addition of 5 mmol/L Ba^{+2} first and in second place, 20 mmol/L TEA. **Inset** represents bar graph of current blockage at −80 and +80 mV, control conditions (black bar), 5 mmol/L Ba^{+2} (gray bar) and 5 mmol/L Ba^{+2} plus 20 mmol/L TEA (dark gray bar). (C) Blockage-voltage curve in the presence of 5 mmol/L Ba^{+2}, each point indicates mean ± S.E.M. ($n = 6$ seals, $n = 4$ placentae).

Mean values for patch current normalized to the value in the absence of Na^+ are shown in Fig. 1C for both voltages tested. Addition of Na^+ up to concentrations of 10 mmol/L (15, 20, 30 mmol/L) reduced total current to $36.7 \pm 4.7\%$ at −80 mV and $38.9 \pm 6.6\%$ at +80 mV ($n = 10$ seals, $n = 3$ placentae) reaching a plateau. The K_d for Na^+ block at ±80 mV was 2.36 mmol/L (Fig. 1C, inset). The preceding data indicates that Na^+ modulates K^+ channels from LMVM and this effect is voltage-independent and behaves in a dose-dependent manner.

In summary, the data from the patch clamp experiments demonstrated the presence of K^+ channels in LMVM, which are blocked by Ba^{+2} and a sub-population that is also blocked by TEA. Interestingly these currents are sensitive to Na^+.

3.5. *Exogenous K^+ currents from LMVM transplanted into* Xenopus laevis *oocytes*

In order to corroborate the results described above, total current experiments were performed in LMVM appertaining to the same set of placentae from normal term pregnancies and transplanted into *Xenopus* oocytes suitable for voltage clamp method. This method was developed by Miledi [19,20] to incorporate in the oocyte membrane foreign proteins that had already been assembled in their native cells and has been used by many authors and membranes from different cell sources [19,35–42], including placenta [14]. Previously we have reported that the oocyte plasma membrane incorporates the placental apical membrane (MVM) and efficiently acquires functional channels [14].

The total current was studied in 173 out of 1039 oocytes that fulfilled the morphologic and functional criteria of inclusion (described in Section 2), being registered by the voltage clamp method. These oocytes were from $n = 24$ independent frog donors injected with LMVM vesicles, obtained from $n = 12$ normal term human placentae. In 47% of them, the injection of LMVM vesicles elicited significant changes in the membrane properties of the cells, i.e. changes in membrane potential, conductance and permeability as showed in Table 1, suggesting that these oocyte group exhibited exogenous macroscopic currents in NR solution. Fig. 3 shows a current intensity/potential relationship (I/V) for the functional behavior of a population of control (without injection) and LMVM injected oocytes, when applying voltage pulses from −120 to +40 mV, in 20 mV steps (with holding potential $V_h = -60$ mV). The intensity current magnitude is approximately four times higher (~450%; *t-test $p < 0.05$) at +40 mV in LMVM injected oocytes ($n = 72$) compared to controls ($n = 56$) from 19 donors. This difference could also be established at −120 mV potential with $\sim -230\%$ (*t-test $p < 0.05$). Additionally, there was a significant reversal potential (V_{rev}) displacement towards depolarizing potentials from -34.5 ± 1.6 mV in control oocytes to -25.3 ± 0.7 mV (*t-test $p < 0.05$). The inset shows membrane currents recorded from individual uninjected (control) and LMVM injected oocyte: both cells were obtained from the same donor. In oocytes injected with buffer there were no differences compared to uninjected oocytes (data not shown).

3.6. *Effect of Na^+ removal in exogenous currents from LMVM transplanted to* Xenopus laevis *oocytes*

Following the experiments shown by the patch clamp technique, where Na^+ affects the K^+ currents from LMVM, the effect of Na^+ on the total current from LMVM elicited in oocytes was investigated. A voltage pulse protocol was applied initially when the oocyte was bathed in NR solution and afterwards, in Na^+-free/R-NMDGCl solution, Fig. 4 shows the I/V relationship in both conditions. An increase of positive and negative currents was observed in LMVM injected oocytes when removing Na^+ from bath solution ($n = 38$; 11 donors). First, a higher chord conductance at −120 and +40 mV, and a V_{rev} displacement towards hyperpolarizing potentials compared to the value presented in NR solution. This displacement in V_{rev} indicated a change in the plasma membrane relative permeability when removing Na^+ from bath solution; specifically, this value was near to the Cl^- equilibrium potential at first, and moved slightly towards K^+ equilibrium potential in Na^+-free solution where the equilibrium potentials previously described for the oocytes are $V_{eqCl-} = -26.2$ mV and $V_{eqK+} = -102.6$ mV [43]. Table 2 shows the membrane properties of LMVM injected and control oocytes in Na^+-free solution. Due to the high K^+ concentration inside the oocytes, the increase of outward currents (at positive potentials) could be due essentially to an efflux of K^+ ions with the contribution of a Cl^- influx to the oocyte. Considering our interest in K^+ currents we focused the study on the outward exogenous currents expressed in Na^+-free solution. In control oocytes ($n = 27$; 11 donors) Na^+ removal did not produce changes in membrane conductance as shown in Fig. 4, inset A, behaving as described previously by Ivorra et al. [14]. On the other hand, the increase of inward currents (at negative potentials) could be due essentially to an efflux of Cl^- anion from the oocyte; and secondarily, at more hyperpolarized potentials, it would be possibly due to K^+ influx; however, since the external concentration of K^+ is only 2 mmol/L, this would be a small contribution. As we commented above, the outward current could include an important K^+ component and in Na^+-free solution this current increase indicates an inhibitory effect of Na^+ in agreement with the results obtained in single channels experiments. Then Na^+-free solution was the better experimental condition used in order to obtain the outward K^+ current component.

3.7. *K^+ conductances are mainly present in LMVM exogenous oocyte currents*

To analyze the components of outward LMVM exogenous currents, we studied the blocking effect of DIDS and TEA, a known Cl^- channel blocker and a non-specific K^+ channel blocker respectively, in Na^+-free solutions. The explicit exogenous current blocking effect was calculated by subtracting the blocking percentage of endogenous control current in all cases at a +40 mV voltage pulse ($V_h = -60$ mV). Fig. 4, inset B shows DIDS (1 mmol/L) effect on a group of control ($n = 5$) and LMVM injected oocytes ($n = 4$) from three donors where it can be observed that DIDS did not produce significant inhibition on exogenous LMVM outward currents. On the contrary, when applying 20 mmol/L TEA on control

Table 1
Membrane properties of uninjected (control) and LMVM injected oocytes

	Vm (mV)	V_{rev} (mV)	g −120 mV(μS)	g +40 mV(μS)
Control	-48.3 ± 2.3 (71;24)	-34.5 ± 1.6 (56;19)	3.0 ± 0.3 (56;19)	4.2 ± 0.3 (56;19)
LMVM injected	-32.6 ± 1.8* (87;24)	-25.3 ± 0.7* (72;19)	$8{,}2 \pm 0{,}7$* (72;19)	13.2 ± 0.7* (72;19)

Values of resting membrane potential (Vm), reversal potential (V_{rev}) and slope conductance (g) at −120 and +40 mV in cells bathed in NR solution are given. *t-test $p < 0.05$ compared to controls. Adjacent to each value, the numbers of oocytes and their respective donors are shown between parentheses.

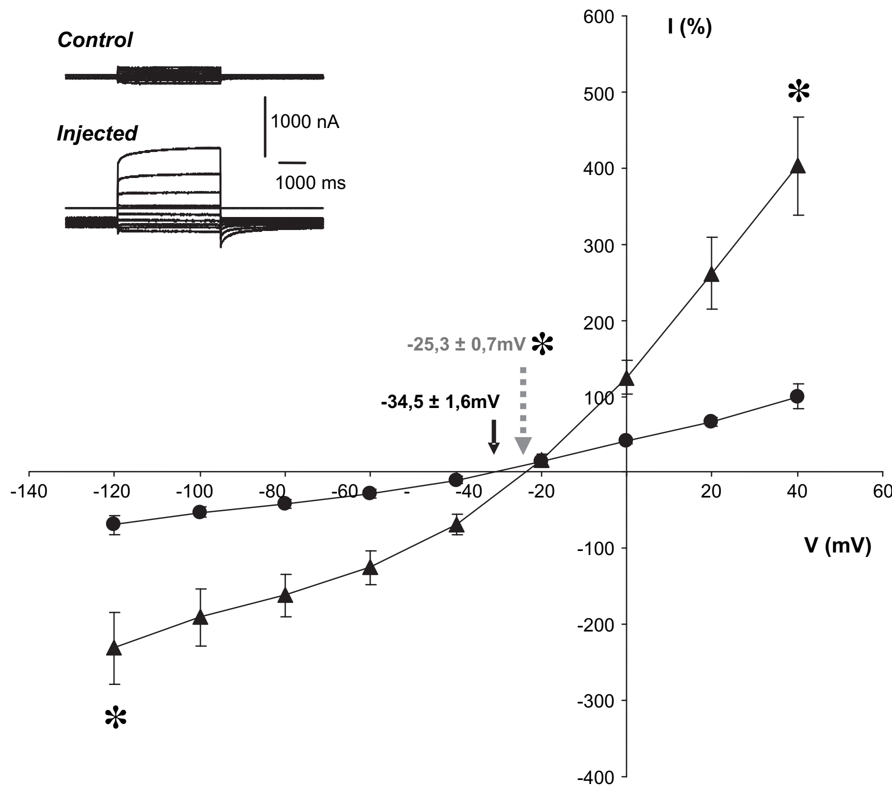

Fig. 3. Functional incorporation of human syncytiotrophoblast LMVM channels into the *Xenopus* oocyte plasma membrane. *I*/*V* relationship for the membrane currents elicited in a population of control uninjected oocytes ($n = 56$, circles) and LMVM injected oocytes ($n = 72$, triangles) pertaining to 19 donors when submitted under voltage pulses from −120 to +40 mV in 20 mV steps ($V_h = -60$ mV) in NR solution. $I = I * 100/I_{control + 40 mV}$ *t-test $p < 0.05$ for statistical significance of total current. The black arrow indicates the average value of the reversal potential (V_{rev}) for the curve of control oocytes. The gray dotted arrow indicates the respective average V_{rev} for the LMVM injected oocytes curve. *t-test $p < 0.05$ for statistical significance of V_{rev}. **Inset** shows membrane currents generated in a control (top) and a LMVM injected (bottom) oocyte in response to the voltage pulses described above. Dotted line indicates zero level. Both oocytes were obtained from the same donor.

($n = 6$) and LMVM injected oocytes ($n = 7$) from three donors, an inhibition effect of 39% was observed in LMVM exogenous current (*t-test, $p < 0.05$; Fig. 5A). These results suggest that the main component of exogenous outward LMVM currents is a K^+ current.

3.8. Ba^{+2} and TEA blockade of LMVM exogenous oocyte currents

Susceptibility to inhibition by 20 mmol/L TEA and 5 mmol/L Ba^{+2}, was studied on LMVM exogenous currents on injected and uninjected (control) oocytes in Na^+-free solution. Fig. 5C shows, in control oocytes, the inhibition effect in the order: TEA-Ba^{+2} ($n = 3$), and vise versa: Ba^{+2}-TEA ($n = 3$). With respect to TEA and Ba^{+2}, control oocytes behaved as previously described in literature [43,44]. Fig. 5B shows the effect of these blockers on LMVM positive exogenous outward currents in Na^+-free solution, where the K^+ channel blockers were applied sequentially: TEA-Ba^{+2} ($n = 3$) or Ba^{+2}-TEA ($n = 3$). Specifically when adding TEA first, the blocking percentage was 39% over exogenous currents on injected oocytes and afterwards, with Ba^{+2} an additional 10%; on the other side, when adding primarily Ba^{+2}, the inhibition was 27% and afterwards, with TEA, a 17%; confirming the existence of TEA and Ba^{+2}-sensitive K^+ currents on LMVM, in agreement with those obtained for single channel currents from the patch clamp experiments. However, there were some differences in the total percentage of block with the blockage results obtained from single current recordings; firstly, there could exist more than one type of current in the oocyte, second, we do not know the orientation of the exogenous protein in the oocytes or in the liposomes, thus, the access to the blocker binding site could be different in both experimental systems.

4. Discussion

Our data, from single channel experiments and total current measurements, demonstrate functional K^+ channels in the apical hSTB membrane from normal term human placenta. The blocking effect of K^+ currents by TEA and Ba^{+2} suggests the presence of K^+ channels with a high sensitivity to Ba^{+2}, a sub-population that is also sensitive to TEA, with at least some of these currents being sensitive to Na^+.

It is important to note the difficult access to intact placenta in order to characterize channel activity by conventional electrophysiological procedures, since the hSTB from normal term human placenta is a large multinucleated cell. A good alternative methodology is the reconstitution of ion channels from purified trophoblast plasma membranes, extracted from either normal or pathological placental tissue, into artificial lipid membranes [8–13,15,45]; or their functional transplantation into *Xenopus laevis* oocytes [14]. Both methods require highly purified hSTB plasma membranes, and we have developed improved methods for isolation and purification as we described in Jimenez et al. 2004. In that work, we have described the apical (maternal-facing) membrane composed by two isolated fractions, the classical MVM and the LMVM [7]. We have found recently that both fractions from apical membrane have different lipid and protein composition [46,47].

There are several studies by electrophysiological methods of different types of ion channels in the MVM fraction [8–13,15,45,48–52], but these do not include K^+ channels. The LMVM fraction has been unexplored from the point of view of channel activity, thus LMVM was a candidate for containing the K^+ channels which are

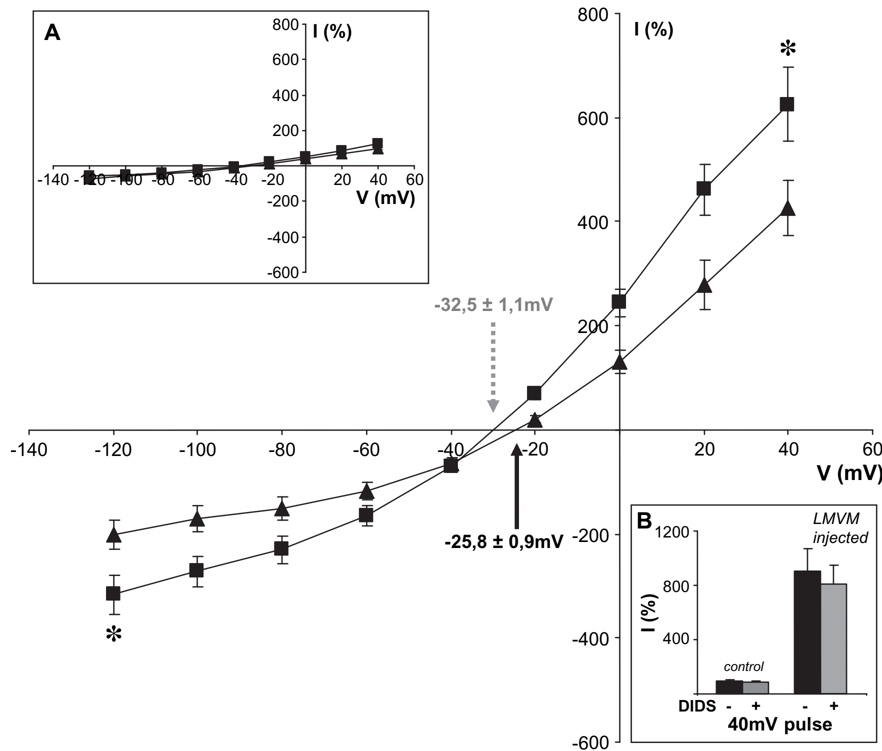

Fig. 4. Effect of Na^+ removal in the exogenous currents from LMVM transplanted to *Xenopus laevis* oocytes. *I/V* relationship for the membrane currents elicited in a population of 38 LMVM injected oocytes (11 donors) when submitted under voltage pulses from −120 to +40 mV in 20 mV steps ($V_h = -60$ mV) in NR solution (triangles) and in Na^+-free solution (squares), $I = I * 100/I_{control+40\ mV}$. The black arrow indicates the average value of V_{rev} for the curve of injected oocytes in NR. The gray dotted arrow indicates the respective average V_{rev} for the injected oocytes in Na^+-free solution. *t-test $p < 0.05$. **Inset A** shows the behavior of a population of 27 control uninjected oocytes (11 donors) when the same voltage pulse protocol is applied in NR solution (triangles) and Na^+-free solution (squares). **Inset B** shows the blocking effect of 1 mmol/L DIDS in a group of $n = 5$ control oocytes and $n = 4$ LMVM injected oocytes pertaining to three donors when a +40 mV voltage pulse ($V_h = -60$ mV) was applied. $I = I * 100/I_{control+40\ mV}$.

necessarily present in the apical plasma membrane. There is electrophysiological and molecular evidence regarding the presence of K^+ channels in cytotrophoblast at different stages of differentiation [3,5,6], nevertheless there are no electrophysiological studies in placentae from term pregnancies. Ample indirect evidence has shown K^+ flux via a conductive pathway in apical hSTB plasma membranes from human term placenta [2–4,6], however the biophysical characteristics of the ion channels underlying these K^+ conductances, using electrophysiological methods, were unknown until now. In the present work the activity of single K^+ channels was detected in excised patches obtained from cell-size, giant liposomes containing placental LMVM from human term placenta. The single channel conductance was around 70 pS for one type of channel observed. Similar to several other K^+ channels [27–34], it was sensitive to Na^+ with a maximum blockade over 10 mmol/L where the patch current decreased to 40% of the total initial current. Our patch clamp data showed that TEA and Ba^{+2} block K^+ channels in LMVM. Susceptibility to inhibition by blockers such as TEA and Ba^{+2} are a characteristic of K^+ conductances described in a wide variety of cell types [31,33,34,53].

Table 2
Effect of Na^+-free solution on membrane properties of uninjected (control) and LMVM injected oocytes

	V_{rev} (mV)	g −120 mV (μS)	g +40 mV (μS)
Control	−38.7 ± 2.8 (27;11)	2.3 ± 0.4 (27;11)	5.3 ± 0.6 (27;11)
LMVM injected	−32.5 ± 1.1* (38;11)	12.0 ± 1.3* (38;11)	30.2 ± 2.9* (38;11)

Values of reversal potential (V_{rev}) and slope conductance (g) at −120 and +40 mV in cells bathed in R-NMDGCl (Na^+-free) solution are given. *t-test $p < 0.05$ compared to NR solution. Adjacent to each value, the numbers of oocytes and their respective donors are shown between parentheses.

The results obtained from total currents from injected oocytes by voltage clamp were in agreement with the patch clamp results. Our results suggest that these single channels are underlying the outward exogenous currents from LMVM detected in *Xenopus* oocytes.

The analysis of the total currents elicited by the injection of LMVM vesicles into *Xenopus laevis* oocytes induced significant changes in the membrane properties of these cells. The fact that the current increased significantly together with a V_{rev} displacement in LMVM injected oocytes with respect to the value observed in uninjected oocytes, demonstrates the integration of exogenous ionic channels that change the relative permeability of the oocyte plasma membrane. This is the first demonstration in which *Xenopus laevis* oocytes functionally incorporate foreign ionic channels originated from the hSTB LMVM fraction, as an exogenous current. A significant increase in macroscopic exogenous currents was also observed when replacing Na^+ in the bath solution, and a change in V_{rev} towards hyperpolarizing potentials. Specifically, when analyzing the increase of outward currents (at positive potentials), in Na^+-free solution, it would be composed mainly by an efflux of K^+ ions from the oocyte. These facts indicate that Na^+ could be regulating a sub-population of exogenous channels originated from LMVM, according to the results obtained in single current experiments where part of the patch current was blocked by Na^+.

The action of TEA and Ba^{+2} was studied on LMVM exogenous currents of injected and control uninjected oocytes in Na^+-free solution. Addition of TEA and Ba^{+2} blocks *Xenopus* oocyte endogenous K^+ channels according to literature reports [43,44]. This TEA-sensitive endogenous currents and Ba^{+2}-sensitive endogenous currents were subtracted when analyzing the blocking effect of TEA or Ba^{+2} on the LMVM exogenous oocyte currents. TEA and Ba^{+2}

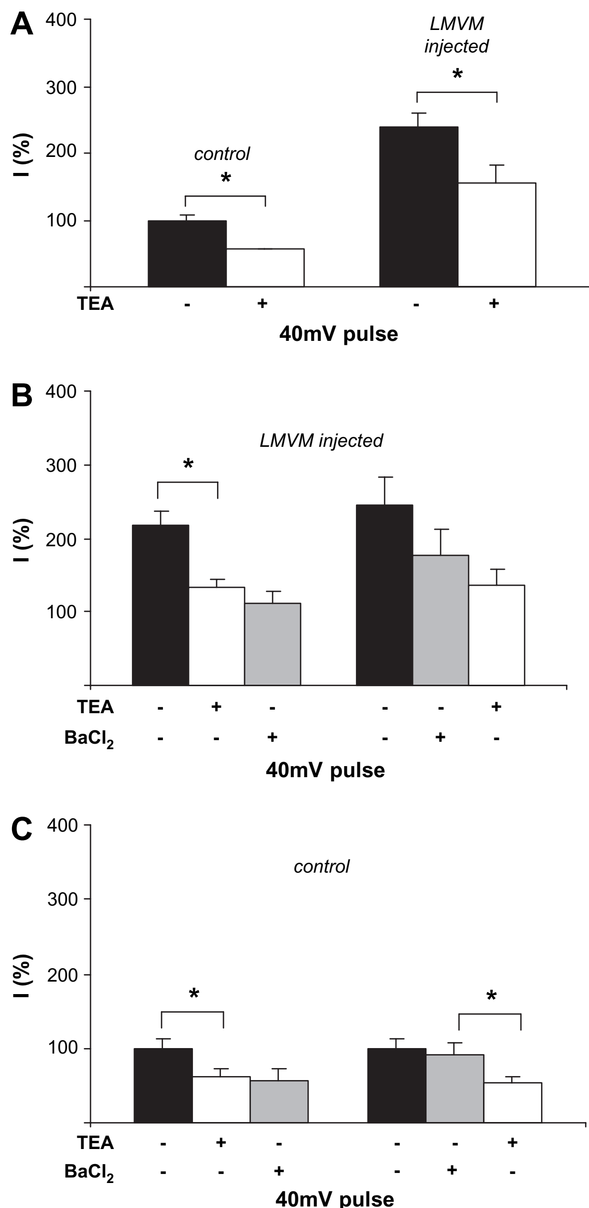

Fig. 5. Blocking effect of Ba^{+2} and TEA on LMVM outward currents (A) Shows the blocking effect of 20 mmol/L TEA on a group of $n = 6$ control uninjected and $n = 7$ LMVM injected oocytes (three donors) when applying a +40 mV voltage pulse ($V_h = -60$ mV) in Na^+-free solution; *t-test $p < 0.05$. (B) Shows the additive effect of K^+ channel blockers TEA (20 mmol/L) and Ba^{+2} (5 mmol/L) on LMVM exogenous currents (injected oocytes) when applying the same voltage pulse in Na^+-free solution. Left graph in order: TEA and Ba^{+2} ($n = 3$), and right graph: Ba^{+2} and TEA ($n = 3$). (C) Shows the blocking effect of TEA and Ba^{+2} in control uninjected oocytes when applying same voltage pulse in Na^+-free solution. Left graph in order: TEA and Ba^{+2} ($n = 3$), and right graph: Ba^{+2} and TEA ($n = 3$). *t-test $p < 0.05$.

induced blockade of outward LMVM exogenous currents in injected oocytes, demonstrating the presence of functionally expressed Ba^{+2}-sensitive K^+ conductances and a sub-population TEA-sensitive. This significant blocking effect of TEA and Ba^{+2} on exogenous LMVM currents in oocytes was observed in Na^+-free solution. However, we do not discard a non-Na^+ sensitive component in LMVM K^+ conductances, since there was a blocking response with 20 mmol/L TEA in NR solution, i.e. with Na^+ 115 mmol/L (data not shown). This behavior was observed in single channel current recordings where 40% of the patch current was insensitive to Na^+, however Ba^{+2} and TEA together blocked close to the 80% of the current activity in the patch.

In conclusion, our data from both experimental approaches demonstrates the presence of various types of K^+ channels in LMVM from the point of view of their conductance and pharmacological response (Na^+, Ba^{+2} and TEA).

Our novel results and biophysical characterization of K^+ channels from syncytium apical membrane could contribute to the understanding of their specific function in trophoblast. K^+ channels present in the hSTB have been involved in membrane potential generation, cell volume regulation and electrogenic solute transport. Up to now, several studies of K^+ transport from purified vesicles of apical membranes, cytotrophoblast or from isolated placental villi (explants) report the presence of K^+ conductive pathways in the microvillous membrane [1–3]. We believe that our data, at least partly, characterizes the populations of K^+ channels that underlie those conductive pathways for K^+ ions.

Birdsey et al. (1999) [2] have demonstrated that Ba^{+2}-sensitive K^+ conductances contribute to the resting potential of the hSTB microvillous membrane, in isolated villi from term human placenta. They have also shown that the hSTB microvillous membrane responds to a hyposmotic stimulus by activating Ba^{+2}-sensitive K^+ conductances. Considering the indirect evidence for the presence of K^+ channels in the hSTB apical membrane, we suggest that this Ba^{+2}-sensitive K^+ channels described by electrophysiological methods, could be the channels that participate in the physiological processes such as membrane potential and volume regulation in placental hSTB.

Clarson et al. (2001) [3] demonstrated that cytotrophoblast cells express an inwardly rectifying K^+ current typical of Kir 2.1. However, in the isolated apical membrane from term placenta the most frequent K^+ channel was not a rectifier channel; we obtained an inward rectifier K^+ conductance only twice. We are not ruling out the existence of this type of channel in our preparation. This point needs to be studied in depth in the future.

Recently, Bai et al. (2005 and 2006) [5,6] demonstrated the expression and activity of the two pore domain K^+ channels (K_{2p}) TASK (1 and 2) and TREK in villous tissue from human placenta, and in cultured human cytotrophoblast cells at multinucleate and mononucleated stages of differentiation. Remarkable is the work of Morton et al. (2005) [34], where these authors demonstrated that Na^+ reduces the amplitude of TASK-2 single channel currents in excised inside-out patches, indicative of rapid block and unblock of the pore. The authors proposed that this sensitivity to Na^+ may be an additional potential regulatory mechanism of TASK-2 channels.

The correlation of our results with different types of channels described in cytotrophoblast cells and suggested by molecular techniques needs to be studied in detail. The aim of this work was to detect K^+ channels in the hSTB apical membrane functionally, however in the future it is necessary to investigate the biophysical properties and pharmacological effects of the different types of K^+ channels present in this tissue.

We are convinced that our results and the electrophysiological approach described here could contribute to the advancement in the understanding of the biophysical properties, function and regulation of K^+ channels as an important physiological factor in the placental epithelium.

Acknowledgements

We are grateful to Dr. M. Pérez and the staff at The San José Hospital Maternity Unit for assistance in obtaining the biological material. We also thank V. Illanes M.D. and Edna Mancilla M.D. for critically reading the manuscript and Mr. Aldo Valdebenito for technical assistance. This research was supported by grant Fondecyt – Chile 1070695.

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