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An outwardly rectifying chloride channel in BeWo choriocarcinoma cell line

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ABSTRACT

In this study, an outwardly rectifying chloride channel was characterized in the trophoblastic cell line BeWo, a human hormone-synthesizing cell which displays many biochemical and morphological properties similar to those reported for the human cytotrophoblast. Ion channel activity was recorded in the cell attached and inside-out configurations with standard patch-clamp technology. In most of the BeWo cells studied, the channel under symmetrical *N*-methyl-D-glucamine (NMDG-Cl) concentration (Na^+ free solution) in both sides of the membrane exhibited spontaneous activity, an outwardly rectifying current/voltage relationship and single-channel conductances of 15 pS and 48 pS for inwards and outwards currents, respectively. The channel has a low permeability for gluconate with a relative permeability $P_{gluconate}/P_{Cl}$ of 0.23, and a higher permeability to I^-.

The open probability (Po) of the channel exhibited dependence with the applied membrane potential with greater activity at positive pulses. The channel activity was inhibited by the sulphonylurea hypoglycemic agent glibenclamide (50 μM) or by diphenylamine-2-carboxylate (DPC, 500 μM) added to the cytoplasmic side of the patch whereas conductances remained unchanged. The blockade with glibenclamide and DPC was independent of the applied membrane potential. All these results are characteristic of the outwardly rectifying Cl channel (ORCC) found in other types of cells. Neither Po, conductances nor reversal potential (Er) values were affected by the absence of intracellular Ca^{2+}, suggesting that the channel is not sensitive to Ca^{2+}.

1. Introduction

The human placental syncytiotrophoblast is a polarized epithelial structure which represents the main barrier for maternal-fetal exchange and this process requires the operation of several specific transport mechanisms. The electrophysiological studies by patch clamp techniques on these cells are extremely difficult due to their syncytial nature. Ion channels were described in human placental cytotrophoblast and syncytiotrophoblast cells in culture [1–3], in membranes reconstituted in liposomes [4–6], in lipid-bilayer reconstituted membranes [7,8] or transplantation of channels to *Xenopus laevis* oocytes [9].

The BeWo cell line is a human hormone-synthesizing trophoblastic cell line established in continuous culture by Pattillo and Gey [10]. These cells comprise a human trophoblast cell line which displays many biochemical and morphological properties similar to those reported for the in uterus proliferative cytotrophoblast during the last trimester of pregnancy [11]. Even though these cells represent an immortalized and modified cell line, the membrane transport systems expressed in BeWo cells are similar to those reported in normal human trophoblasts; therefore, they have been widely used as a model to investigate the placental transport mechanisms of amino acids, immunoglobulins and fatty acids [12,13]. Some channels and transporters have been studied in BeWo cells, e.g., the L-type $Ca2^+$ channel, the calcium transporter protein type 1, Ca^{2+}-ATPases, Na^+/Ca^{2+} exchangers, Na^+/H^+ exchangers and the Na–K–Cl cotransporter [12,14,15]. Recently we described the functional presence of the three subunits of the amiloride sensitive Na channel (ENaC) [16,17].

In this study we performed patch clamp measurements to analyze the chloride currents in BeWo cells. Our results indicate that this cell line expresses an outward rectifier channel compatible with the outwardly rectifier chloride channels (ORCC) recorded in different cell lines [18,19].

2. Material and methods

2.1. Cell culture

The BeWo cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Ham's F12 medium (GIBCO BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Natocor Biotechnology, Córdoba, Argentina), 2 mM L-glutamine (Sigma, St. Louis, MO), 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were harvested once

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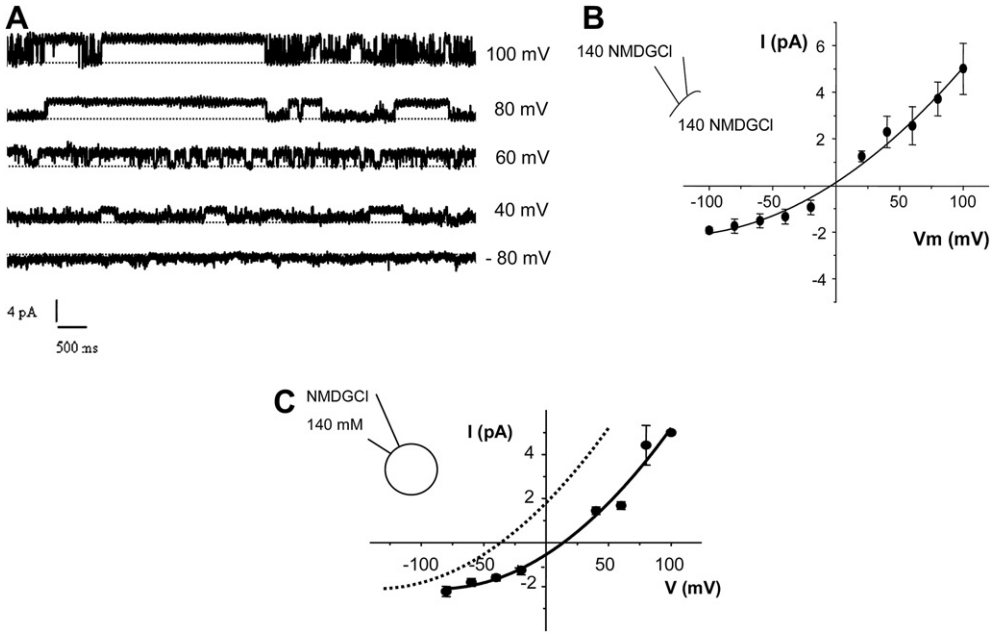

Fig. 1. A. Representative recordings showing the channel activity observed from an inside-out membrane patch under symmetrical 140 NMDG-Cl conditions at several membrane holding potentials. Dotted lines indicate the closed state of the channels. Each trace contains 5 s of recording. B. Current–voltage (I–V) relationship averaged from twelve patches. Bars are SD. C. I–V plot from averaged currents recorded under cell-attached configuration before excising the patch ($n = 6$). The dotted line represent the hypothetical I–V curve correcting the applied voltage ($V = V_{bath} - V_{pipette}$) for the likely membrane potential of BeWo cells (Em = ~−50 mV).

a week with 0.25% trypsin–EDTA (GIBCO BRL, Life Technologies) and incubated at 37 °C in humid air with constant 5% CO_2.

2.2. Single channel recordings

The activity of ion channels in BeWo cells was recorded under cell-attached and inside-out configurations of the patch clamp technique, using a Warner PC-501A amplifier (Warner Instruments, Hamden, Conn., USA) with a 10 GΩ feedback resistor. Standard glass micropipettes were used with a tip resistance of 5–10 MΩ when filled with pipette solution. Electrical signals were filtered at 2 kHz and digitized at 5 Hz using pCLAMP v. 8.2 software (Molecular Devices, Union City, CA, USA). Single-channel records were further filtered for display purposes only. Potentials and currents are referred in the conventional manner.

The membrane potential was recorded between 100 and −100 mV returning to 0 in each application. Since the noise level of our setup is close to 0.5 pA, we have only included recordings with current amplitudes higher than this level. Data were corrected for junction potentials at the bath reference electrode with the various test solutions using JPCalc software written by Dr Peter Barry (University of New South Wales, Australia). Text and figures from inside-out experiments show membrane potentials ($V = V_{bath} - V_{pipette}$) corrected accordingly. Current–Voltage plots (I–V) represent the relationship between the applied voltages and the recorded amplitude currents obtained from the amplitude histograms. The Goldman-Hodgkin-Katz (G-H-K) equation adjustment of I–V plots were used to calculate the individual Er values. The inward and outward conductances derived from the slopes of linear regression lines fitted separately to the currents at −40 to −100 mV and 40–100 mV, respectively, and were expressed in pS (S = current/voltage). The open Po of the channel was estimated using the complete opening levels [20]. Relative ion permeabilities were estimated from the corrected Er for current flux using the modified G-H-K equation. All experiments were carried out at room temperature (20–25 °C).

2.3. Solutions

All concentrations are given in mM. The pipette solution contained: NMDG-Cl 140, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, Hepes 10 (pH 7.4), and the bath solution was the same as in the pipette. For the substitution experiments, NMDG-Cl in the bath was replaced by Na-Cl, Na-gluconate, Na-iodide or Na-fluoride 140 mM. Glibenclamide or DPC (Sigma, St. Louis, Mo., USA) were added to the bath solution (cytoplasmic side of the channel) containing the membrane patch in the inside-out configuration. Glibenclamide and DPC were diluted in DMSO and absolute ethanol, respectively. The final concentration of these solvents had no affect per se on the ionic activity (trials in our laboratory with up to 0.2% on various cell types; data not shown).

2.4. Statistical methods

Data are given as means ± SD. A paired or unpaired Student's *t*-test or ANOVA were performed. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Outwardly rectifier chloride channel

We examined ionic activity by measuring single currents in response to depolarizing and hyperpolarizing pulses under inside-out conditions. As it is shown in Fig. 1A, the channel that we observed is characterized by a disparity between the amplitudes of inwards and outwards current values in Na-free symmetrical solution. Panel B shows the I–V plot where it is evident the nonlinear current–voltage relationship with mean conductance from 15.1 ± 7.4 pS ($n = 10$) in the negative voltage range to 48.0 ± 11.9 pS ($n = 12$) in the positive voltage range, indicating an outward rectification. Er obtained from twelve patches was −2.3 ± 5.5 mV. This value is consistent with the theoretical Nernst equilibrium potential for a Cl^- selective current in symmetrical Cl^- concentrations used on both sides of the membrane. In some patches the channel activity could be recorded under cell attached configuration before excising the patch. In this configuration the membrane potential could be estimated from $Vm = Em - (V_{bath} - V_{pipette})$. Fig. 1C shows an outwardly rectifying current with 12.7 ± 0.6 mV of Er ($n = 6$). The hypothetical I–V plot correcting the applied voltage for the likely resting membrane potential of BeWo cells (Em = −50 mV) were superimposed. Replacing bath NMDG by equimolar concentrations of Na^+ did not change the conductance, Er or Po (Fig. 2). The outward and inward conductances and the Er in this condition were: 45.1 ± 9.0 pS ($n = 7$), 21.7 ± 2.5 pS ($n = 5$) and −5.7 ± 1.6 mV ($n = 7$), respectively. As an example, the Po at 60 mV was 0.53 ± 0.06 ($n = 7$). All these values were not different with respect to patches in the absence of Na ions ($p > 0.05$). The Cl^- sensitive nature of this current was confirmed by evaluating the dependence of the reversal potential with the modifications in the intracellular chloride

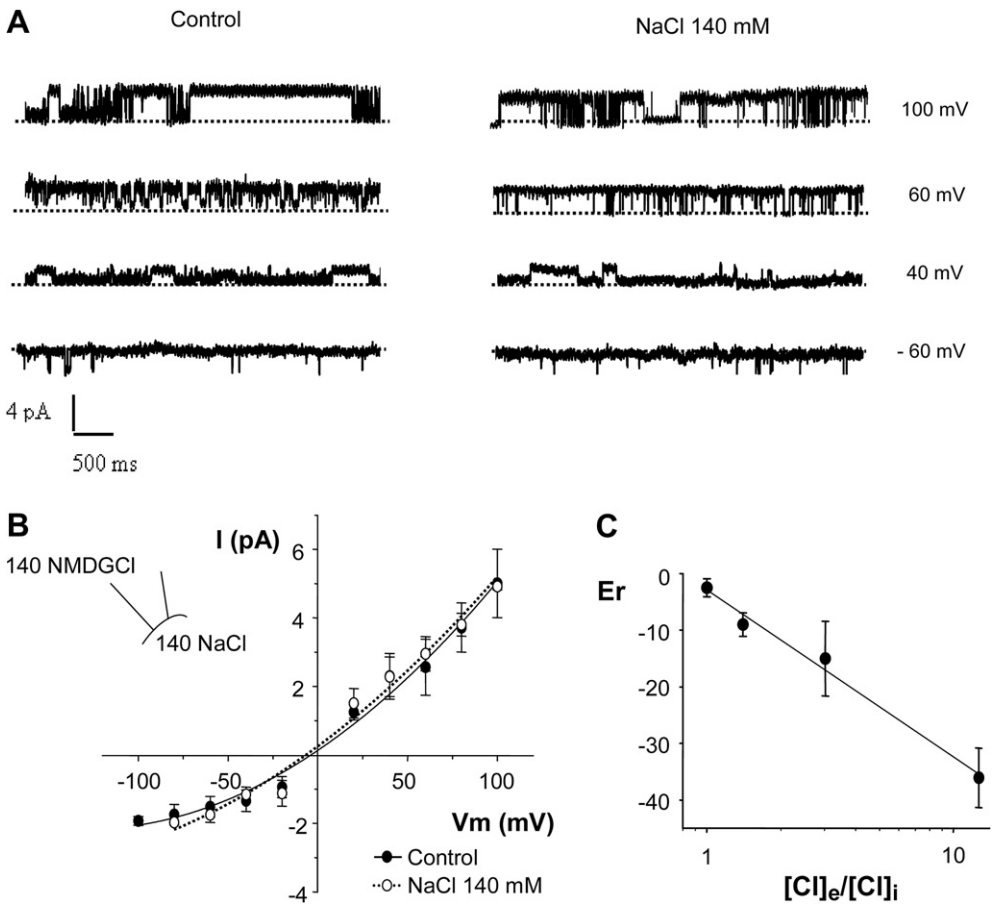

Fig. 2. A. Representative current tracings obtained from an inside-out patch. The currents on the left hand side are with 140 mM NMDG-Cl in bath and pipette and on the right hand side with 140 mM NaCl in the bath (pipette contained the 140 mM NMDG-Cl standard solution). Each trace contains 3 s of recording. Dotted lines represent the closed state. B: Corresponding I–V relationship averaged before (*solid line*, $n = 12$) and after (*dotted line*, $n = 7$, $p > 0.05$) NMDG replacement for Na^+ at the internal solution (bath). Reversal potential was -5.71 ± 1.73 mV. Means $\pm$ SD. C. Relationship between mean reversal potential and log ($[Cl]_e/[Cl]_i$) adjusted to a linear function ($n = 4$).

concentration (bath solution). The Er appeared to be a linear function of log ($[Cl]_e/[Cl]_i$), where $[Cl]_e$ is the concentration of extracellular Cl^-, and $[Cl]_i$ is the concentration of intracellular Cl^- (Fig. 2C). These results suggest that the observed currents are due to Cl^- movement through an outwardly rectifier channel.

3.2. Anion selectivity

To evaluate the anionic selectivity of the channel, Cl^- in the bath solution (in contact with the intracellular membrane surface) was replaced by different anions. The Er values (means $\pm$ SD) for each anion were obtained from the individual I–V plots adjusted to the G-H-K equation and corrected by junction potential. The permeability ratios (P_X/P_{Cl}) were calculated from the shifts of the corrected Er using the modified G-H-K equation:

$$\Delta Er = Er_{(X)} - Er_{(Cl)} = 58.\log\frac{P_X[X]_i + P_{Cl}[Cl]_{Cl}}{P_{Cl}[Cl]_0}$$

where, ΔEr is the shift in Er induced by replacing 140 mM of the internal Cl^- (bath solution) by the anion X (gluconate, F^- or I^-); $Er_{(Cl)}$ is the reversal potential in the presence of Cl^-. Fig. 3 shows the current recordings at two holding potentials, -60 and 60 mV and the I–V relationship when bath solution was 140 mM Na-gluconate and Table 1 shows the average values obtained with different anions. The shift in the Er to more negative potentials is indicative of a lower permeability for gluconate or F^- compared with Cl^-, and the shift to less negative potentials corresponds to a higher permeability for I^- compared with Cl^-, after correction for the junction potential at the bath reference electrode. These values indicate that the anion permeability sequence of the current through the channel was $I^- > Cl^- > F^- >$ gluconate. None of these ions applied to the inside of the membrane modified significantly the channel conductance (Table 1).

3.3. Effect of glibenclamide and DPC

We assessed the effect of glibenclamide, a sulphonylurea hypoglycaemic agent that blocks several outwardly rectifying chloride channels (ORCC) [21–23]. Fig. 4 shows a representative record in which channel activity in the patch held at 100 mV is reduced by 50 μM glibenclamide (left panel, see also the expanded trace shown below). The drug had no effect on single-channel current amplitude (see Fig. 5A) but increased the open-channel noise. Amplitude histograms in the absence or presence of glibenclamide were constructed for patches held at 100 mV (right panel of Fig. 4) from 5 s records. It is clear that in the presence of glibenclamide the open level distribution is shift towards the closed levels. For the calculation of Po of the channel, records with only one level active channel were analyzed. The values of Po determined at various membrane potentials (Fig. 5B) indicate that channel activity under control conditions increased at positive potentials ($n = 6$). Cytoplasmic application of 50 μM glibenclamide reduced Po regardless of the

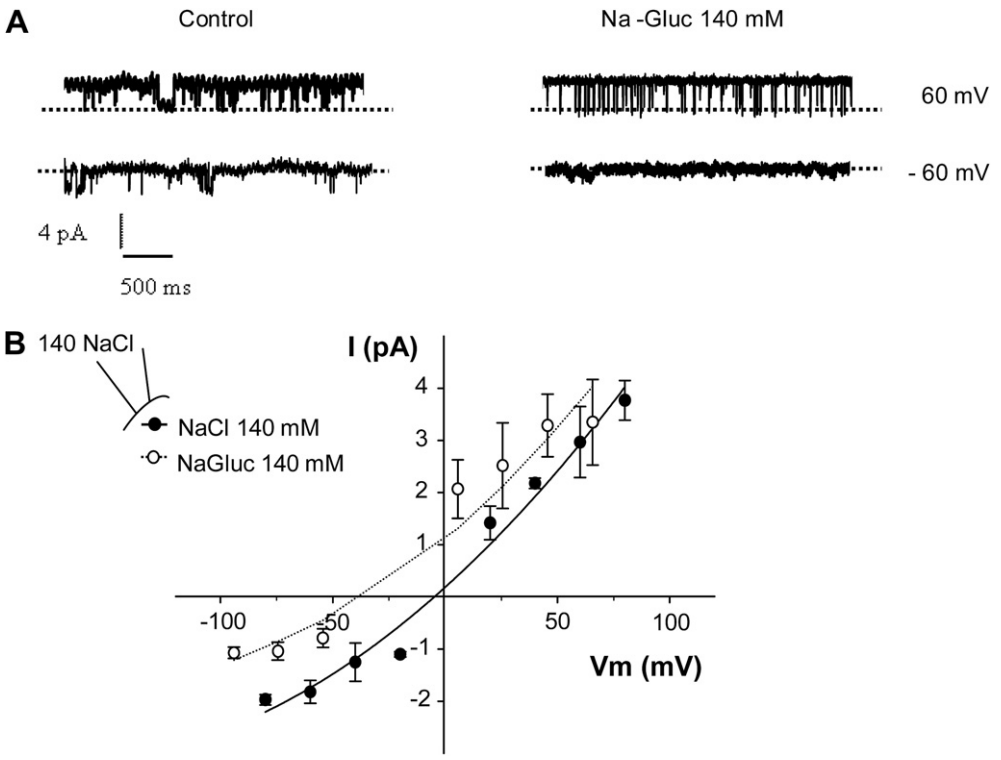

Fig. 3. Anion selectivity of ORCC. Representative recordings and current–voltage relationship obtained from four patches where 140 mM NaCl was replaced by 140 mM Na-gluconate. The I–V plot shows the currents and voltages after correction for the junction potential at the bath reference electrode. The dotted line represents the best fit according Goldman-Hodgkin-Katz equation. The corrected reversal potential was −36.1 ± 9.1 mV (Mean ± SD, $n = 4$).

applied membrane potential, which was about 40% at all tested potentials except for 20 and −20 mV (Fig. 5B, $n = 6$). In addition, the channel Po was affected by glibenclamide in a dose dependent way (Fig. 5C, $n = 5$).

The effect of DPC, another chloride channel blocker, upon the single channel activity was also tested. Fig. 6 shows that the presence of 500 μM DPC reduced the channel activity by decreasing the Po while the single conductances or Er were not affected (Fig. 6A) ($n = 6$). Cytoplasmic addition of DPC reduced the Po by 30–50% at all tested potentials following the dependence of membrane potential observed in control cells (Fig. 6B) ($p < 0.05$; $n = 6$).

3.4. Effect of intracellular calcium

Due to the existence of Ca^{2+}-sensitive Cl^- channels and ORCC channels regulated by Ca^{2+} [23,24], bath Ca^{2+} (2.5 mM) was removing (0 Ca^{2+} plus 0.2 mM BAPTA) at inside patches to evaluate the activity of the channel under this condition. The outward and inward conductance and the Er in this condition were: 39.4 ± 13.5 pS, 14.6 ± 5.5 pS, and −6.1 ± 1.14 mV, respectively ($n = 5$; $n = 5$; $p > 0.05$). Thus, in five paired experiments in which patches were held at −80 and 80 mV, Po values in Ca^{2+} free solution were 0.23 ± 0.05 and 0.68 ± 0.10 ($n = 5$, $p > 0.05$), respectively. The removing of intracellular Ca^{2+} neither affected the Po, conductances nor Er suggesting that the channel is not sensitive to internal Ca^{2+}.

Table 1
Current–voltage relationships were determined with bath solutions containing the test anion as a sodium salt (140 mM). The relative permeability (P_{anion}/P_{Cl}) was derived from the mean reversal potential (Er) after correction for the junction potential, from the G-H-K equation ($n = 4$).

Anion	Er (mV)	Panion/PCl	g outward (pS)	g inward (pS)
Iodide	+17.4 ± 3.2	2.76	41.4 ± 2.7	17.0 ± 7.8
Chloride	−5.7 ± 1.6	1.00	39.8 ± 7.7	21.1 ± 4.3
Fluoride	−28.5 ± 4.9	0.34	28.7 ± 2.7	12.1 ± 1.0
Gluconate	−36.1 ± 9.1	0.23	31.2 ± 2.5	14.9 ± 2.0

4. Discussion

We employed the patch clamp technique to characterize the ion channel in BeWo cells. Our experiments demonstrated the presence of an outwardly rectifying chloride channel (ORCC) with a lower permeability to gluconate and F^- than to Cl^-, a higher permeability to I^-, and inhibited by glibenclamide and DPC, all characteristics that resemble the ORCC described in epithelial and nonepithelial cells. These channels have been identified at the single-channel level in symmetrical Cl^- solution in different epithelial cells: airway epithelia [25], T84 and HT29 human colon cell line [26,27] as well as in other type of cells such as cardiomyocytes [28].

Healthy cells express both the cystic fibrosis transmembrane regulator (CFTR) and the ORCC channels whereas cystic fibrosis affects the expression or the properties of ORCC [29,30]. CFTR and ORCC are distinct proteins with a regulatory relationship [31,32] and it has been proposed that CFTR controls ORCC by an autocrine mechanism involving CFTR-dependent ATP release [26,34]. In this sense it is interesting to note that CFTR is found in human placenta [33] and its expression decreased in preeclamptic placentas [35].

We showed that the channel is selective for Cl^- over Na^+ with a conductance consistent with the reported ORCC characteristics [18,19,48]. When Cl^- was replaced by gluconate or F^- the Er shifted to more negative potentials due to the fact that the driving force decrease and so the current too. Thus, to stop the inwards flow of Cl^- a more negative internal potential is required. The permeability ratios (P_X/P_{Cl}) for I^-, Cl^-, F^- and gluconate obtained here for BeWo

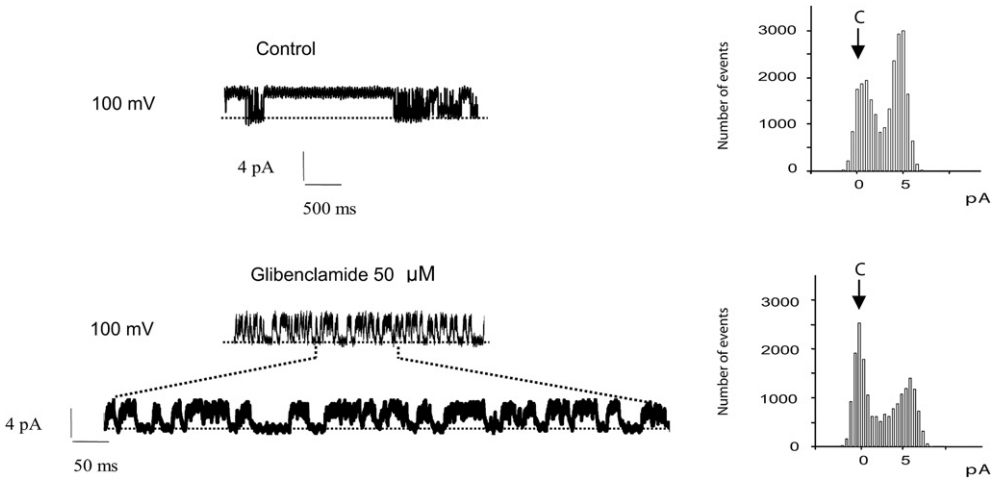

Fig. 4. Effect of chloride channel inhibitor glibenclamide on ORCC. *Left*: Representative single channels recording in excised, inside-out patch held at 100 mV in standard solution (*upper*) and in the presence of 50 µM of glibenclamide added to the cytoplasmic side of the patch (*lower*). Addition of the drug decreased Cl^- channel activity. Dotted lines indicate closed state of the channel. *Right*: Analysis of distribution of current amplitudes for corresponding records shown at left. The patch was held at 100 mV in the absence (*upper histogram*) or 4 min after addition of 50 µM glibenclamide (*lower histogram*).

cells are consistent with the values reported previously in other tissues [18,19]. We also found that after Ca^{2+} removing this channel displayed identical biophysical properties to the basal currents suggesting that is not sensitive to internal Ca^{2+} as reported previously in other tissues [18,19].

The sulphonylurea drug glibenclamide is widely used to block several types of ionic channels, including ORCC [36]. In the present study we observed a reduction in channel activity as a decrease in Po that was independent of the membrane potential when drug was added to the cytoplasmic side of the patch. Although, we found

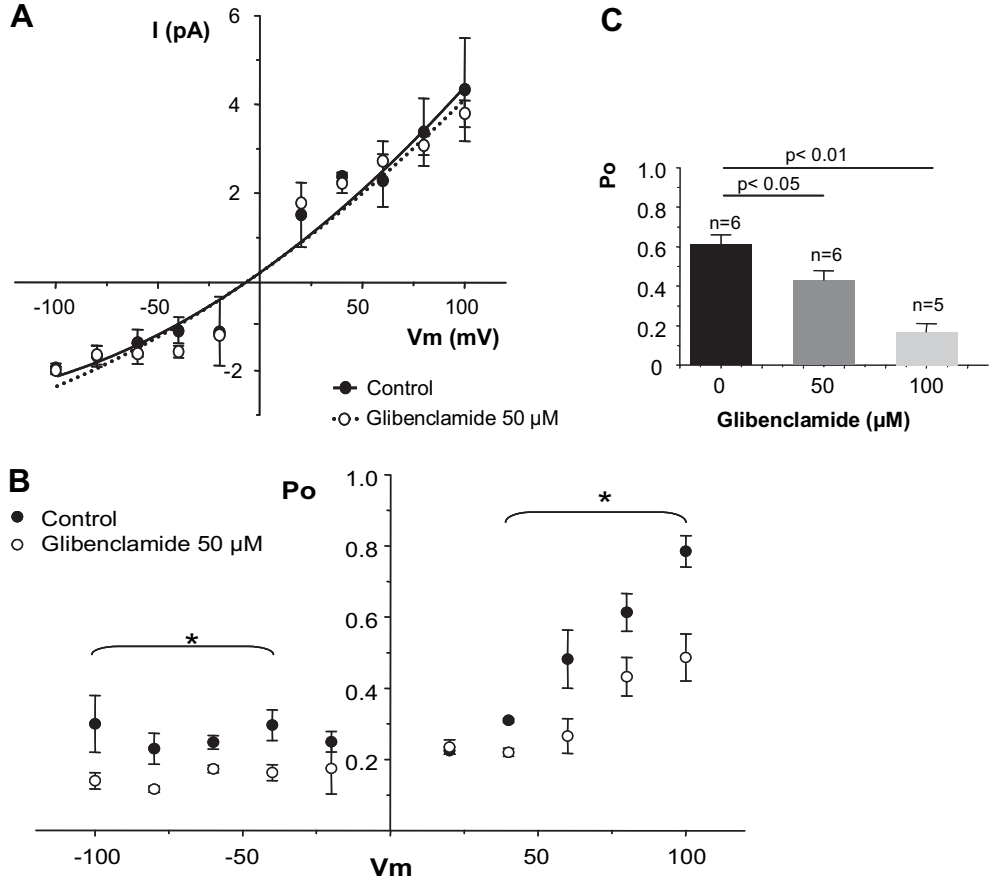

Fig. 5. Effect of glibenclamide on the open probability (Po) of the channel. A. I–V relationship in the absence (*solid line*) or presence (*dotted line*) of 50 µM of glibenclamide obtained in symmetrical 140 mM NMDG-Cl solution ($n = 6$). B. Relationship between Po and voltage in the absence or presence of 50 µM glibenclamide when the patch was bathed in symmetrical 140 mM NMDG-Cl solutions. Means ± SD, $n = 6$. C. Effect of different concentrations of glibenclamide on Po measured at +80 mV. The asterisk indicates statistical significance respect to control ($n = 6$).

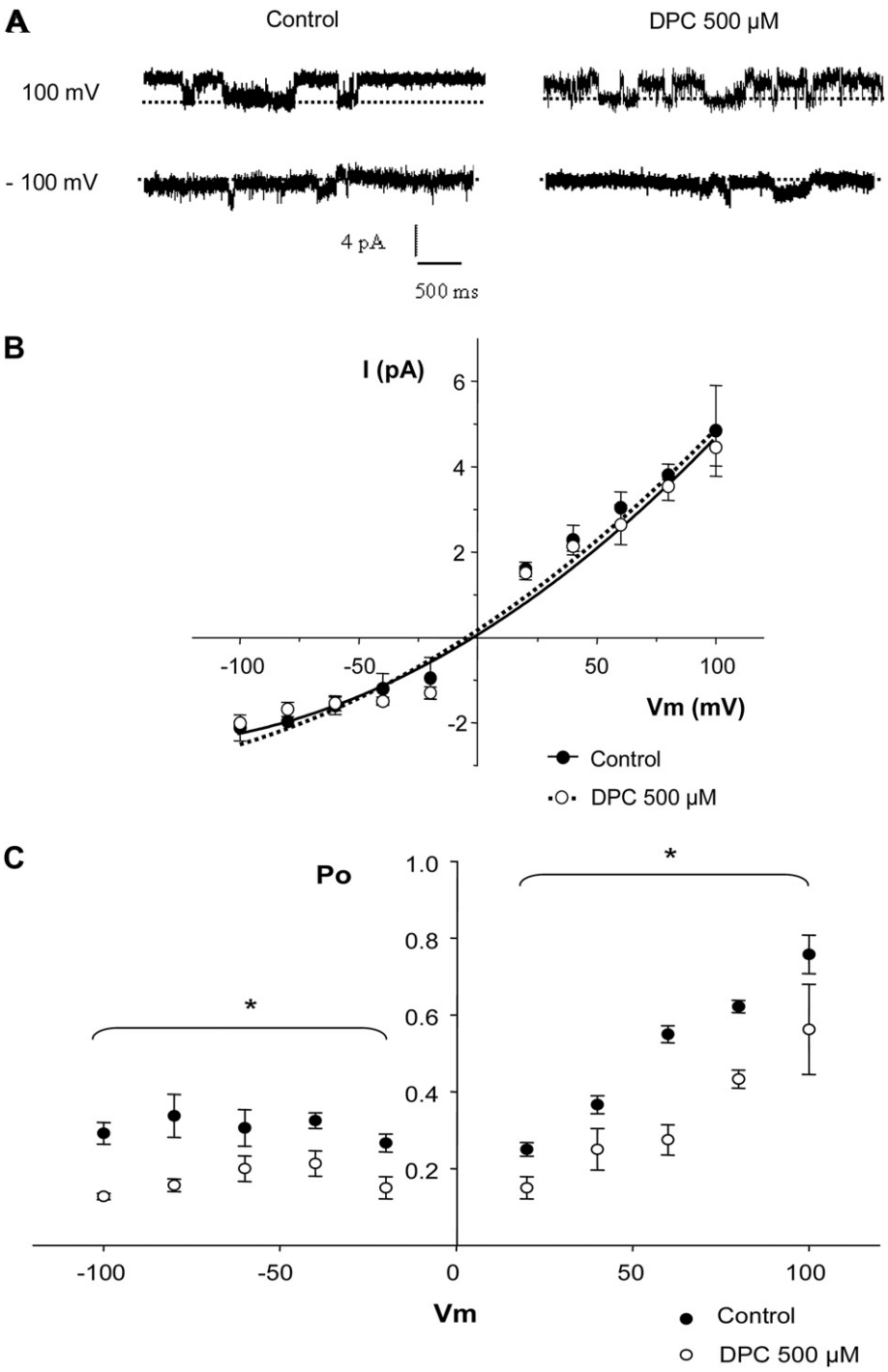

Fig. 6. A. *Left*: Representative single-channel currents in excised, inside-out patches held at 100 and −100 mV in the absence or presence of 500 μM DPC in symmetrical 140 mM NMDG-Cl solutions. Dotted lines indicate the closed state of the channels. *Right*: I–V relationship in the absence or presence of 500 μM DPC. B. Relationship between Po and voltage in the absence or presence of DPC. Means ± SD, $n = 6$.

no significant reduction in Po at 20 and −20 mV, this result could be due to low current amplitude at these pulses that could hinder the calculation of Po. It has been reported that glibenclamide inhibits CFTR channel by occluding the pore in a voltage-dependent way with the drug gaining access to its binding site located within a large intracellular vestibule that is part of the CFTR pore [37]. On the other hand, Rabe et al. [36] demonstrated that the type and magnitude of glibenclamide blockade on ORCC was independent of membrane potential but they suggested that drug diffuses across the patch membrane acting from the extracellular surface by virtue of its lipophilicity. Moreover, it was suggested that the expression of CFTR confers glibenclamide sensitivity to the ORCC in Hi-5 cells [38]. The activity of ORCC was also inhibited with DPC by reducing the open probability as previously reported [18]. Singh et al. [39] established a decrease of channel amplitude after addition of DPC, although the Po was not determined.

Ion transport in placental syncytiotrophoblast has been associated with several functions such as cell volume regulation, nutrient

transport and maintenance of membrane potential, among others. The maxi Cl^- is the most frequently anionic channel described in human placental syncytiotrophoblast [40] and also reported in human term placental trophoblast reconstituted into giant liposomes but its role is still unclear [41] The maxi Cl^- channel is characterized by a high single conductance (200–500 pS), a bell-shape voltage-dependent activity and its sensitivity to DIDS but not to DPC [42]. Despite not using a protocol designed to study this type of channel, we have detected in several patches a channel with a high conductance consistent with the maxi Cl^- (data not shown). Although its characterization is beyond the scope of this work, this evidence suggests that the Cl^- channels expressed in BeWo cells might not differ from that of native trophoblast.

The molecular structure of ORCC is not known as well as its physiological function. ORCC may play a role in the volume-regulated anion current (VRAC) in myocytes because it shares many similarities in biophysical properties [19,44]. ORCC and other Cl^- channels participate in the cell volume homeostasis [6,45] and may be relevant for cystic fibrosis therapy because it could function as an alternative Cl^- channel [46,47]. From our experiments under cell attached condition before excising the patch we speculate that the channel is active at the resting membrane potential (Em). Under this condition we observed rectifying currents with an Er of 12.7 ± 0.6 mV in some patches consistent to previous findings [19]. As far as we know the Em of BeWo cells has not been reported, although Greenwood et al. [43] found a progressive fall in Em associated with trophoblast cell differentiation. The Em values for JAr, mono- and multinucleate cytotrophoblast cells were: −57, −48 and −40 mV, respectively. Thus, considering a value of −50 mV for BeWo cells, the estimated reversal potential in the cell attached configuration is −37 mV. The small inward current observed at this voltage would suggest that intracellular chloride activity is above electrochemical equilibrium in BeWo cells. In addition we found that incubation with increasing concentrations of glibenclamide for 72 h reduce BeWo viability and cell proliferation. A similar result was obtained using DPC, suggesting that the ORCC channel, as other Cl^- channels could contribute to the trophoblastic cells proliferation (Marino GI, Assef YA and Kotsias BA, unpublished results). However, we can not rule out the contribution of other types of channels on this effect such as the ATP-sensitive K^+ channels that are also inhibited by glibenclamide [36].

In conclusion, our experiments demonstrated the presence of an ORCC in BeWo cells with conductances of 15 pS and 48 pS for inwards and outwards currents, respectively and inhibited by glibenclamide and DPC.

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