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Cystic fibrosis transmembrane regulator (CFTR) in human trophoblast BeWo cells and its relation to cell migration

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ABSTRACT

Introduction: ENaC and CFTR are coexpressed in epithelia and have positive or negative functional interactions. In addition, ENaC and CFTR promote migration in placental trophoblastic cells and human airway cells, respectively. Here we tested the idea if CFTR is functionally expressed in BeWo cells, a trophoblastic cell line, and if it is involved in their migratory behavior.
Methods: CFTR expression was studied in BeWo cells with RT-PCR, biotinylation and Western blot. Ion currents were analyzed with patch clamp, and cell migration with the wound healing method.
Results: The mature CFTR 160-kDa band was present, and its localization at the surface membrane was confirmed. Forskolin (20 μM), an adenylate cyclase activator, was used for channel activation, and subsequently $CFTR_{inh}$-172 (2 μM) for its inhibition. The conductances in the presence of $CFTR_{inh}$-172 plus forskolin (16.0 ± 0.7 pS/pF and 32.6 ± 1.5 pS/pF) were significantly lower than in presence of only forskolin (29.7 ± 0.9 and 47.0 ± 2.0 pS/pF). The conductance of $CFTR_{inh}$-172 inhibited currents was 14.9 ± 0.7 pS/pF with a linear I-V relationship illustrating the nonrectifying properties of the CFTR. Cell migration was measured and covered 11.2 ± 0.4, 24.0 ± 1.7 and 13.9 ± 1.0% of the wound when cells were cultivated under control, forskolin, and forskolin plus $CFTR_{inh}$-172, respectively. Proliferation was not changed by any of the treatments.
Conclusions: Our results shows that BeWo cells functionally express the CFTR which plays a role in the wound healing increasing the cell migration process.

1. Introduction

The cystic fibrosis transmembrane regulator (CFTR) is a cAMP-activated, ATP-dependent Cl^- channel that mainly participates in the electrolyte and fluid transport in several epithelia [1,2]. CFTR is also involved in HCO_3^- transport from the intracellular to the extracellular side of the membrane [3] and controls the activity of other membrane transport proteins, such as ATP secretion, the outwardly rectifying anion channel (ORCC) and the epithelial sodium channel (ENaC) [4,5]. CFTR also responds to Ca^{2+} mobilizing secretagogues, and contributes to cholinergic and purinergic responses [6].

ENaC and CFTR are coexpressed at the apical surface of epithelia and have positive or negative functional interactions that are tissue specific [2]. In addition to function as an anion channel, CFTR is also involved in cell migration and proliferation [7,8]. Schiller et al. [1] demonstrated that the inhibition of CFTR transport produced delays in wound closure in human airway epithelial that correlated with a reduction in lamellipodia surface area.

CFTR is also found in the trophoblast from human normal placenta [9] and its expression as well as that of ENaC is diminished in preeclamptic placentas [10,11, see below]. Since we showed that ENaC and ORCC are present in trophoblast BeWo cells [12–15], we tested the idea if CFTR is also functionally expressed in BeWo cells and is involved in their migratory behavior.

The BeWo cell line is a human hormone-synthesizing trophoblastic cell line which displays many biochemical and morphological properties similar to those reported for the *in utero* proliferative cytotrophoblast during the last trimester of pregnancy and they have been used as a model to investigate the placental transport mechanisms [16]. Molecular biology and patch clamp techniques were used to study the expression and function of CFTR channels and the scratch wound assay for the migration process under normal conditions, and also the use of blockers of CFTR channels. Our results show that CFTR participates in the migration of BeWo

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cells for the following reasons: 1) CFTR is expressed functionally in BeWo cells; 2) there is an increase in wound healing in forskolin activated CFTR channels compared to cells in the absence of the drug, being this effect blocked by the use of $CFTR_{inh}$-172, a specific CFTR inhibitor. Cell proliferation, assessed with the reagent MTT, was not affected.

2. Methods

2.1. Cell culture

The BeWo and T84 (positive control, [17]) cell lines (American Type Culture Collection, USA) were maintained in Ham's F12 medium (Gibco BRL, Life Technologies, Buenos Aires, Argentina) with 10% fetal bovine serum (FBS, Natocor Biotechnology, Buenos Aires, Argentina) [14,15].

2.2. Reagents

Forskolin (Alomone Labs, Jerusalem, Israel) and $CFTR_{inh}$-172 (Calbiochem, San Diego, CA, USA) were dissolved in DMSO. DMSO final concentration per se did not affect the experiments [18].

2.3. RT-PCR

RT-PCR was performed as described before [18] with 5 μg of total RNA. PCR (35 cycles) was performed at 94 °C for 60 s, 59 °C for 60 s, and 72 °C for 60 s, followed by a final extension of 10 min at 72 °C. The reaction was carried out using two specific oligonucleotide primers coding for nucleotides 4354–4374 and 4650–4630, respectively, of CFTR exon 24 (COOH terminus) sequence (sense 5′-ACTATTGCCAGGAAGCCATT-3′, antisense 5′CACCGGAACTCAAGCAAGTG-3′).

2.4. Immunoblotting

Total proteins (75 μg) from the cells were resolved on 6% polyacrylamide gel and electrotransferred onto nitrocellulose membrane. We employed polyclonal antibodies against CFTR (Alomone Labs. or Santa Cruz Biotechnology (CA, USA), dilution 1:2500 overnight) and a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:5000) (Vector Lab., Burlingame, USA) [15].

2.5. Cell surface biotinylation

Cell-surface expression levels of the CFTR protein in BeWo and T84 cells were examined using the membrane-impermeant biotinylation reagent (Pierce Chemical, Rockford, IL, USA). Cells were seeded onto T75 cm^2 flasks up to 90–95% confluence. After this, the medium was removed and the cells were washed twice with ice-PBS, pH 8.0. Each flask of cells was incubated with 10 ml of sulfo-NHS-SS-biotin (0.5 mg/ml in PBS; Pierce) and gently agitated on ice for 30 min at 4°C. The reagent was freshly prepared for incubation. After biotinylation, each flask was incubated with 500 μl of quenching solution (192 mM glycine, 25 mM Tris-Cl [pH 7.4]) for 20 min on ice to ensure complete quenching of the unreacted sulfo-NHS-SS-biotin. Cells were scraped into solution and pelleted at 500 rpm for 5 min. Pellets were solubilized for 30 min in 150 μM of lysis buffer (500 mM NaCl, 50 mM Tris-Cl, 1% Triton X-100 and 5 mM EDTA) containing protease inhibitors. Unlysed cells were removed by centrifugation at 13,000 rpm at 4°C. Biotinylated proteins were precipitated with NeutrAvidin-agarose beads (50 μl, Pierce Chemical), added to the supernatant to isolate cell membrane protein and incubated overnight in an end-over-end mixing rotator. Membrane proteins were dissolved in loading buffer with 50 mM DTT. The CFTR was detected in the pool of membrane proteins by polyacrylamide gel electrophoresis and immunoblotting as described above with CFTR H-182-Santa Cruz Biotechnology antibody. The immunoblots were quantified by densitometric analysis. As input controls 75 μg of nonbiotinylated cell lysates proteins were used (input). The CFTR expression at the cell surface (same protein loading) was quantified by densitometric analysis. The intensities of the surface signals (each band) were normalized to the corresponding intensities of nonbiotinylated lysate signals (input). The calculated values for immature CFTR were set as 100%. After normalization the values were plotted as intensity of the biotinylated mature CFTR with respect to immature CFTR (mean percent change ± SE). Each experiment was repeated five times in BeWo cells and 3 times in T84 cells.

2.6. Whole-cell recordings

Electrical activity was recorded in the whole cell configuration as described previously [15,18] with standard patch clamp technology. The protocol consisted in pulses from −80 to +100 mV in steps of 20 mV, from a holding potential of 0 mV and with a 10 ms interval between each pulse. Solutions: Pipette solution (intracellular) contained (mM): 125 CsCl, 1 $MgCl_2$, 10 HEPES, 0.2 BAPTA (pH 7.4). The bath solution contained (mM): 150 NaCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH 7.4). CsCl was the main salt in the intracellular solution in order to diminish K^+ conductances. The osmolarity was 260 ± 5 mOsm/l to the solution of CsCl, and 292 ± 6 mOsm/l for NaCl solution.

Current–voltage (I–V) plots were obtained from currents measured in a stationary state and were expressed as current densities (current per unit cell capacitance) [14,15]. The capacitance of the cells (C_m) was measured applying 100 ms, 10 mV, depolarizing pulses from a holding potential of 0 mV. The series resistances (Rs) were determined (14.7 ± 1.1 MΩ, $n = 4$), the currents were fitted to an exponential function and the time constant (τ) was measured; thus $C_m = \tau/Rs$. The Rs and capacitances were compensated during experiment using the analog circuit of the amplifier. Although, not been taken note of the exact values of the used seal resistances, only patches with seal resistances greater than 1 GΩ were recorded. All experiments were done at room temperature (20–24 °C). Cells were exposed to 20 μM forskolin for 10 min and then to 2 μM $CFTR_{inh}$-172 (in the presence of forskolin) until maximal blockage was achieved.

2.7. Wound-healing assay

Wound healing assay was performed as previously [1,15]. Briefly, monolayers were manually scraped and Ham's F12 + 1% FBS was added to attenuate cellular proliferation without impairing cell survival, with different treatments at pH 7.4: (1) a control group, (2) 20 μM forskolin, (3) 20 μM forskolin plus 2 μM $CFTR_{inh}$-172, and (4) $CFTR_{inh}$-172. At this time was considered time 0. All treatments were incubated for 5–6 h straight and images were taken.

Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay as described previously [15] at the same conditions of wound healing experiments.

2.8. Statistical analysis

Data were expressed as mean values ± standard error (SE) (n = number of cells and repetitions). We used either a Student's test or one-way and repeated measures ANOVA for multiple data comparison, followed by a *post hoc* test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. CFTR expression in BeWo cells

To determine whether CFTR is expressed in BeWo cell line, total RNA was extracted and RT-PCR analysis was performed using primers to amplify human CFTR (see Methods). Cells from the T84 line from a colonic human cancer were used as a positive control [17,19]. An expected band of 295 bp corresponding to CFTR product was obtained in both, BeWo and T84 cells. No product was detected in the absence of reverse transcriptase (negative control). A band of 289 bp was detected, corresponding to β-actin, showing positive cDNA integrity (Fig. 1A).

Further determination of CFTR protein in BeWo cells was conducted by Western blot analysis of whole cell lysates. Fig. 1B shows blots of BeWo cells. The membranes were incubated with two types of antibodies generated against different parts of the protein. One of them was a polyclonal antibody generated against aminoacids 1-182 mapping at the N-terminus of human CFTR (H-182, Santa Cruz Biotechnology). A band of about 160 kDa was observed, that corresponds to mature CFTR. In addition, another band of a lower molecular weight was evident representing an immature, deglycosylated form of CFTR. This is in agreement with previous results indicating that the immunoreactive bands in the 140- to 180-kDa regions represent various forms of the CFTR protein [20,21]. A unique band of 160 kDa was observed in T84 cells used as positive control, where CFTR is constitutively expressed [17] (left panels). It is consistent with the idea that the maturation of CFTR protein is complete in T84 cells, so with an efficient processing of endogenous CFTR [22]. The other used antibody was against aminoacids residues 1468–1480 corresponding to C-terminal part of human CFTR (Alomone Labs LTD). The 160-kDa band was present in BeWo and T84 cells (right panels). No bands were detected with nonimmune mouse IgG (data not shown).

Biotinylation of the BeWo membrane was used to estimate whether the CFTR is expressed on cell-surface. The biotinylated CFTR protein was detected as the two forms, mature glycosylated and immature unglycosylated CFTR. A unique band corresponding to mature glycosylated CFTR was found in T84 cells used as positive