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Identification and Biochemical Localization of a Na-K-Cl Cotransporter in the Human Placental Cell Line BeWo

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Several transport systems mediating the placental transport of Na, K and Cl have been described, but whether the trophoblast membrane also expresses a Na-K-Cl cotransporter that mediates the coupled movement of all three ions remains unclear. Here we show that BeWo cells, a human trophoblastic cell line, exhibit bumetanide-sensitive ^{86}Rb (a K surrogate) uptake. Entry via this route accounts for ~17% of the ^{86}Rb influx with the remainder being mediated largely via the Na,K-ATPase. The activity of the bumetanide-sensitive transporter was rapidly elevated (>40%) upon subjecting cells to an acute hyperosmotic challenge signifying a potential role in cell volume regulation. Antibodies to the Na-K-Cl cotransporter identified a single band of ~200 kDa on Western blots of fractionated BeWo membranes. This immunoreactivity colocalised with that of the Na,K-ATPase (a basal membrane marker), but was absent from membranes enriched with placental alkaline phosphatase (an apical membrane marker). These findings show for the first time, that a Na-K-Cl cotransporter is expressed in a human placental cell line which may be involved in regulating trophoblast cell volume.

Key Words: **triple cotransporter; bumetanide; placenta; cell volume; Na pump; Na,K-ATPase; membrane; transport; placenta; trophoblast.**

The syncytiotrophoblast layer of the human placenta plays an essential role in foetal growth and development. It is in direct contact with maternal blood and forms a continuous multinucleated cell layer that mediates materno-foetal exchange of nutrients (1). Several ion transporters and conductances have been identified in both the maternal-facing microvillous plasma membrane (e.g., Na/H and Cl/HCO_3 exchangers, sodium-coupled amino acid transporters as well as chloride and potassium conductances) and in the foetal-facing basal plasma membrane (e.g., Na/K-ATPase and Ca-ATPase) (1, 2). Of these the Na/K-ATPase is considered to play a key role in helping to establish electrochemical gradients across the mature syncytiotrophoblast and its activity has important functional implications for numerous other membrane transporters that utilise the Na gradient for the co- and counter-transport of both organic and inorganic ions (e.g., amino acids and H^+) (3).

Unlike sodium and potassium our current understanding of the transcellular mechanisms involved in chloride transport across the placenta remains weak. Much of the available data concerning placental transfer of chloride has been obtained from studies with microvillous (apical) membrane vesicles which have identified three routes of chloride transport. The first involves an electroneutral diisothiocyano-2-2′-disulfonic stilbene (DIDS)-sensitive anion-exchanger, the second is a chloride conductance which is sensitive to diphenylamine 2-carboxylate (DPC) (4, 5) and the third is a DIDS-insensitive chloride conductance (6). In contrast, considerably less is known about how chloride is transported across the basal membrane of the trophoblast although the presence of both DIDS- and DPC-sensitive chloride conductance pathways have recently been proposed (7). In many epithelial cells, chloride enters the cell via a basolateral Na-K-Cl cotransporter and exits down its electrochemical gradient by passing through apical chloride channels. However, no direct evidence has thus far been presented supporting the presence of a Na-K-Cl cotransporter in the basal membrane of the human trophoblast. In an attempt to address this issue we have used a monolayer-forming human trophoblast cell line known as BeWo. These cells display many of the biochemical and morphological characteristics reported for the *in utero* cytotrophoblast during the last trimester of pregnancy (8) and have been used extensively in the study of amino acid uptake (9, 10) and transport of inorganic ions, such as iron and copper (11–13). Here, we show that BeWo cells express a functional Na-K-Cl cotransporter that localises in membranes ostensibly of basal origin and that its activity can be rapidly modulated in response to changes in the trans-membrane osmotic gradient.

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MATERIALS AND METHODS

BeWo cells were obtained from the European Tissue Culture Centre. F-12 (HAM) culture medium, foetal bovine serum and antimycotic/antibiotic solution for cell culture were purchased from GIBCO (Paisley, Scotland). Polycarbonate tissue culture inserts were purchased from Millipore (Watford, Herts). Sterile trypsin-EDTA, ouabain and bumetanide were all obtained from Sigma (Poole, Dorset). D-Glucose and all other reagent grade chemicals for buffers were obtained from BDH (Poole, Dorset). ^{86}Rb was obtained from New England Nuclear (Stevenage, UK). Reagents for enhanced chemiluminescence (ECL) were obtained from Amersham Life Science (Buckinghamshire, England). An antibody directed to the Na-K-Cl cotransporter (T4) was obtained from the Developmental Studies Hybridoma bank (University of Iowa, IA) and Dr. T Pressley (University of Texas, TX) kindly provided an antibody against the α1-subunit of the Na-K-ATPase (anti-LEAVE). Polyclonal antibodies against the Na-K-ATPase β1-subunit were purchased from UBI (Buckingham, England) and that against human placental alkaline phosphatase from Dako (Glostrup, Denmark).

BeWo cell culture. For analyses of $^{86}Rb^+$ uptake, BeWo cells were cultured on polycarbonate filter inserts (0.4 μm pore size, 30 mm diameter) placed within wells of a six-well multi-dish (Nunc). Cells were grown to confluence and maintained as cytotrophoblasts as described previously (14). In some experiments, BeWo cells were cultured on 15 cm diameter dishes and grown to confluence prior to being harvested for subcellular fractionation.

Subcellular fractionation. Total membranes from confluent BeWo cells were prepared using a procedure adapted from that reported previously for L6 muscle cells (15). Briefly, cells from five (15 cm) dishes were harvested using a rubber policeman, pooled and pelleted gently by centrifugation (700 g, 5 min). The cell pellet was resuspended in ice-cold buffer (250 mM sucrose, 20 mM HEPES, 5 mM NaN_3, 2 mM EGTA, 100 μM phenylmethylsulphonyl fluoride, 10 μM *trans*-epoxysuccinyl-L-leucyl amido [4-guanidino]butane (E64), 1 μM pepstatin-A and 1 μM leupeptin, pH 7.4) and homogenised using a Dounce homogeniser. Crude membranes were isolated by centrifuging the cell homogenate at 31,000 g for 1 h at 4°C (16). In some experiments crude membranes which were fractionated subsequently on a continuous 10–40% sucrose (w/w) gradient by centrifugation at 210,000 g for 2.5 h. After centrifugation, 200 μl aliquots were collected sequentially from the top of the gradient. A small volume of each fraction was retained for protein analyses using the method of Bradford (17) and a 25 μl aliquot reconstituted subsequently in Laemmli buffer (18) prior to immunoblot analyses. In some experiments human brain microsomes were run in a parallel gel lane as a positive immunoreactive control for the expression of the Na-K-Cl cotransporter and Na,K-ATPase subunits. Human brain tissue was obtained at post-mortem and microsomes prepared as described previously (19).

Western blot analysis. Crude membranes or membranes isolated following fractionation were subjected to SDS–PAGE on 7.5% or 9% resolving gels and separated proteins transferred (electrophoretically) onto nitrocellulose membranes (Bio-Rad). Following transfer, membranes were blocked with 3% (w/v) bovine serum albumin in Tris-saline buffer (50 mM Tris/HCl, 150 mM NaCl, pH 7.5) for 1 h at room temperature prior to incubation with antibodies directed against placental alkaline phosphatase (1:100), Na-K-Cl cotransporter (anti-T4, 1:500), Na-K-ATPase α1 subunit (anti-LEAVE, 1:100) and the β1 subunit (1:500). Following primary antibody incubation, nitrocellulose membranes were washed in Tris-saline buffer containing Tween 20 (0.05% v/v) and incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (1:1000) or HRP anti-mouse IgG (1:500) as appropriate. Membranes were washed subsequently three times in Tris-saline-Tween 20 for 15 min and immunoreactive proteins visualised using ECL. Signal intensities on immunoblots were quantified using a Biorad 670 densitometer.

$^{86}Rb^+$ uptake assays. Monolayers of BeWo cells grown on polycarbonate filter inserts were subjected to four successive washes in HEPES-buffered saline solution (HBS, 140 mM NaCl, 2.4 mM $MgSO_4$, 5 mM KCl, 1 mM $CaCl_2$ and 20 mM Na-HEPES, pH 7.4) containing 10 mM D-glucose. Cells were then pre-incubated for 15 min with HBS in the absence or presence of ouabain or bumetanide, which were also present throughout the uptake assay at concentrations indicated in the figure legends. In some experiments Na and Cl in the HBS were substituted with *N*-methyl-D-glucamine (NMDG) or gluconate, respectively. Uptake of $^{86}Rb^+$ (1 μCi/ml, used to trace K^+ transport) was measured over 5 min. In some experiments, BeWo cells were preincubated in hypertonic HBS (420 mOsm kg^{-1}) prior to assaying $^{86}Rb^+$ uptake. Buffer osmolarity (normally 300 mOsm kg^{-1}) was manipulated by the addition of sucrose and was verified using an osmometer (Gonotec, Germany). Uptake was terminated by rapid aspiration of the radioactive incubation solution, followed by four successive washes of cells with ice-cold isotonic saline solution (0.9% NaCl, w/v). Cells were lysed by removing the filters from their plastic cases and immersing them in 0.05 N NaOH. 1 ml of the cell lysate was removed and cell associated radioactivity determined using a Beckman LS 60001C scintillation counter. The protein content in cell lysates was determined using the method of Bradford (17).

Statistical analysis. Statistical analysis was carried out using a two-tailed Student's *t*-test. Data were considered statistically significant at P values ≤ 0.05. Curve fitting was performed using GraphPad Prism software.

RESULTS

Ouabain and bumetanide inhibit $^{86}Rb^+$ uptake in BeWo cells. In order to determine whether BeWo cells take up potassium by a resident Na-K-Cl cotransporter we assessed the effects of ouabain and bumetanide, which respectively inhibit the Na,K-ATPase and Na-K-Cl cotransporter, upon potassium ($^{86}Rb^+$) uptake. Figure 1 shows that both ouabain and bumetanide induced a dose-dependent inhibition in $^{86}Rb^+$ uptake. The inhibition exerted by ouabain was maximal at a concentration of 1 mM and occurred with an IC_{50} of ~1 μM. Bumetanide suppressed $^{86}Rb^+$ uptake with an IC_{50} of ~0.1 μM, with maximal inhibition observed at a concentration of 10 μM (Fig. 1b). The effect of both inhibitors on cellular $^{86}Rb^+$ uptake was monitored over a period of 5 min for convenience as in separate experiments we were able to establish that $^{86}Rb^+$ uptake was linear over a period of at least 20 min (data not shown).

Having established the maximal concentrations at which ouabain and bumetanide were effective we next investigated the extent to which $^{86}Rb^+$ uptake was suppressed when cells were exposed simultaneously to both inhibitors. Figure 2a shows that $^{86}Rb^+$ uptake was inhibited by ~73% in the presence of 1 mM ouabain, ~17% by 10 μM bumetanide and ~90% in the presence of both inhibitors. The residual $^{86}Rb^+$ influx (~10%) may represent a small leak component or an undefined K^+ channel/transporter. Since Na-K-Cl cotransporter activity is dependent on the presence of all three ions we investigated the effects of removing extracellular sodium or chloride on bumetanide-sensitive $^{86}Rb^+$ uptake. Figure 2b shows that removal of either extracellular sodium or chloride led to a substantial loss in

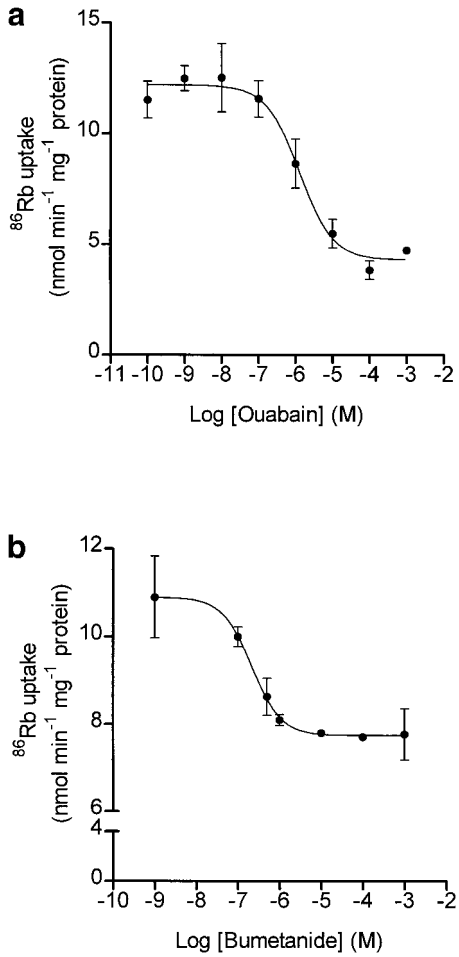

FIG. 1. Dose-response relationship for the effects of (a) ouabain and (b) bumetanide on $^{86}Rb^+$ uptake in BeWo cells. BeWo cells were incubated with HBS/10 mM D-glucose for 15 min in the presence of different ouabain or bumetanide concentrations prior to $^{86}Rb^+$ uptake. Results are the means ± SEM of six experiments.

uptake via the cotransporter. Another characteristic feature of the Na-K-Cl cotransporter is that its activity can be modulated by subjecting cells to an acute osmotic challenge. When BeWo cells were subjected to hyperosmotic shock we observed a rapid, and significant increase in the bumetanide-sensitive uptake of $^{86}Rb^+$. The data shown in Fig. 2c indicate that the Na-K-Cl cotransporter was activated upon cell shrinkage (by 42%) during the first 20 min of cell incubation in hypertonic media and starts to subside thereafter.

BeWo cells express the α1- and β1-subunit isoforms of the Na-K-ATPase and a Na-K-Cl cotransporter. Crude membranes from BeWo cells were subjected to SDS–PAGE and immunoblotting using isoform-specific antibodies recognising the Na,K-ATPase and the Na-K-Cl cotransporter. Western blot analysis (Fig. 3) revealed that BeWo cells expressed only the $\alpha 1$ and $\beta 1$ subunit isoforms of the Na-K-ATPase as antibodies directed against other Na,K-ATPase isoforms ($\alpha 2$, $\alpha 3$

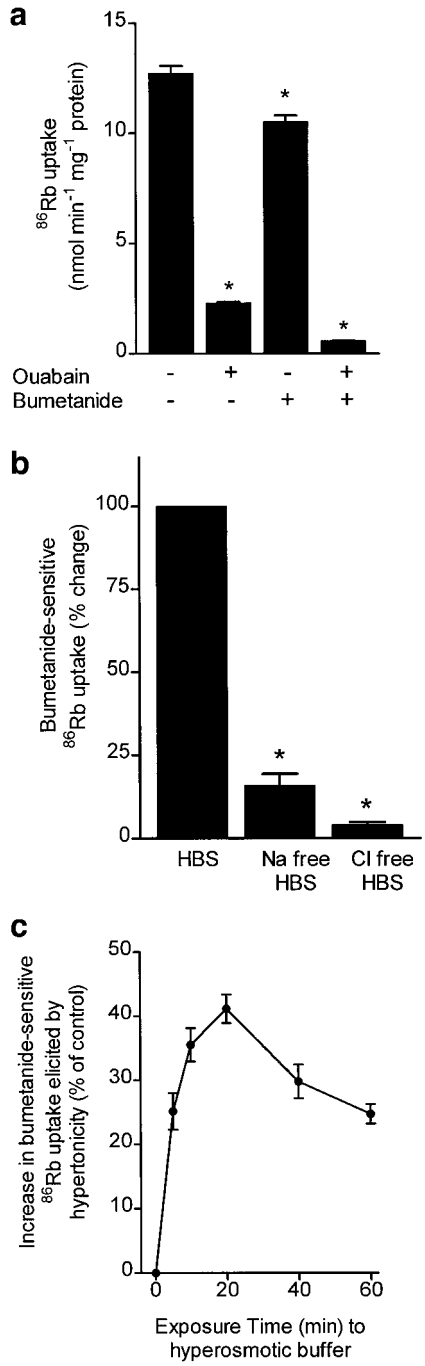

FIG. 2. (a) Effect of ouabain and bumetanide on $^{86}Rb^+$ uptake in BeWo cells. BeWo cells were incubated for 15 min ± 1 mM ouabain or 10 μM bumetanide or both inhibitors together prior to uptake. (b) Effect of replacing extracellular Na^+ (with NMDG) or Cl^- (with gluconate) on bumetanide-sensitive $^{86}Rb^+$ uptake in BeWo cells. Uptake was assayed over 5 min in Na^+ or Cl^--free media. (c) Bumetanide-sensitive $^{86}Rb^+$ uptake in BeWo cells following exposure of cells to hyperosmotic (430 mOsm kg^{-1}) HBS/10 mM D-glucose. Uptake data are expressed as a percentage of that measured in cells assigned as controls that were maintained in isotonic (300 mOsm kg^{-1}) HBS/10 mM D-glucose. Results are mean ± SEM for between five to eight experiments. Asterisks represent significant differences ($P < 0.01$) from the control value.

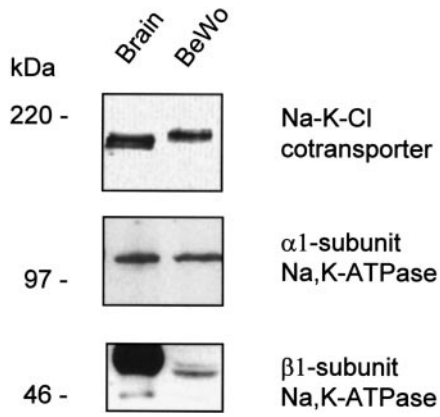

FIG. 3. Representative immunoblots showing the expression of the Na-K-Cl cotransporter and the α1- and β1-subunit isoforms of Na-K-ATPase in BeWo cells. Crude membranes prepared from BeWo cells (50 μg) and human brain microsomes (30 μg, used as a positive immunoreactive control) were subjected to SDS-PAGE and immunoblotting using isoform-specific antibodies to the Na-K-ATPase subunits and Na-K-Cl cotransporter.

and β2) did not react with BeWo membranes (data not shown). When BeWo membranes were probed with an antibody against the Na-K-Cl cotransporter, a single immunoreactive band (~200 kDa) was observed. This protein band migrated with a slightly higher molecular mass than the cotransporter expressed in human brain, possibly reflecting differences in *N*-linked glycosylation.

Subcellular localisation of the Na-K-Cl cotransporter. In order to determine the subcellular localisation of the Na,K-ATPase and Na-K-Cl cotransporter we fractionated crude plasma membranes isolated from BeWo cells on a 10–40% (w/w) continuous sucrose gradient. Fractions (200 μl aliquots) were collected sequentially from the top of the gradient and membranes from every seventh fraction were subjected to SDS–PAGE and immunoblotting using antibodies against the Na-K-Cl cotransporter, the α1 subunit of the Na,K-ATPase (a marker of the basal membrane) and placental alkaline phosphatase (PALP; a protein marker of the apical membrane). Immunoreactivity against the Na-K-Cl cotransporter and the α1 subunit of the Na,K-ATPase was colocalised and largely confined to fractions 22 to 50. In contrast, the less dense fractions (1 to 15) were devoid in both of these proteins, but enriched in PALP. Interestingly, the latter could not be detected in membrane fractions that reacted positively with antibodies against the α1 subunit of the Na,K-ATPase and Na-K-Cl cotransporter (Fig. 4a). When the relative signal intensities of PALP and Na,K-ATPase were quantified and expressed per μg of membrane protein there was little evidence for any major overlap in the distribution of the two proteins in the fractions immunoblotted (Fig. 4b). This finding indicates that the fractionation procedure used was able to resolve BeWo membranes of basal and apical origin. Similar quantitative analyses of the distribution of the Na-K-Cl cotransporter substantiated its strong colocalisation with the Na,K-ATPase (Fig. 4b).

DISCUSSION

The major aim of the work described here was to evaluate whether BeWo cells, a human trophoblast cell line, express a Na-K-Cl cotransporter and to gain some insight into its cellular localisation and regulation by changes in extracellular osmolarity. In addressing this issue we have also assessed the relative contributions made by the Na-K-Cl cotransporter and Na,K-ATPase towards $^{86}Rb^{+}$ (potassium) uptake and obtained infor-

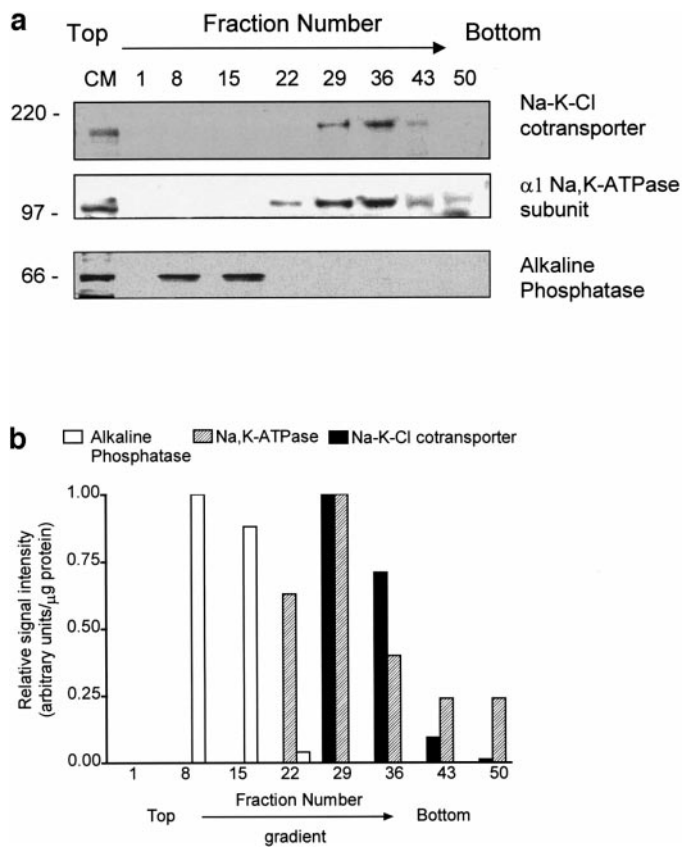

FIG. 4. (a) Distribution of the Na-K-Cl cotransporter and Na-K-ATPase in crude membranes isolated from BeWo cells fractionated on a 10–40% (w/v) continuous sucrose gradient. Membranes (25 μl aliquots) isolated from the gradient in every seventh fraction were subsequently resolved on a 7.5% polyacrylamide gel. Separated proteins were transferred to nitrocellulose membrane and blotted using antibodies against α1-subunit of Na-K-ATPase, Na-K-Cl cotransporter and placental alkaline phosphatase. (b) Relative signal intensity (expressed in arbitrary units/μg membrane protein) of alkaline phosphatase, Na,K-ATPase and the Na-K-Cl cotransporter in membranes immunoblotted in (a). A value of 1.0 for each protein was given to that fraction in which it was most abundant.

mation on the molecular identity of Na,K-ATPase isoforms expressed in this trophoblastic cell line.

Our results indicate that BeWo cells express only the $\alpha 1$ and $\beta 1$ subunits of the Na,K-ATPase as antibodies to other subunit isoforms failed to react with BeWo membranes. It is also noteworthy that the dose-response inhibition for the effects of ouabain on $^{86}Rb^+$ uptake (Fig. 1a) was mono-phasic, consistent with the suggestion that a single ouabain-binding (α subunit) component is expressed in BeWo cells. These observations are in agreement with the recent work of Clarson *et al.* (20) who also reported that the choricarcinoma cell line, JAr, and cytotrophoblasts isolated from term human placenta express mRNA encoding only the $\alpha 1$ and $\beta 1$ Na,K-ATPase subunits (20). Thus the active holoenzyme or 'Na pump' in BeWo cells is an $\alpha 1\beta 1$ heterodimer, which accounts for most ($\sim$73%) of the cellular $^{86}Rb^+$ uptake. However, our findings signify that rubidium (used here as a surrogate ion for K^+) is also taken up by a carrier that mediates the coupled transport of this ion with that of Na and Cl. This proposition is based on (i) the finding that the loop diuretic bumetanide, which is known to inhibit the Na-K-Cl cotransporter, causes a significant inhibition in $^{86}Rb^+$ uptake in BeWo cells, (ii) removal of extracellular Na or Cl promotes a substantial reduction in bumetanide-sensitive $^{86}Rb^+$ uptake and (iii) antibodies against the Na-K-Cl cotransporter recognise a protein of the appropriate molecular size in BeWo cell membranes. Previous studies using isolated microvillous membranes prepared from human placenta have been unable to detect any significant coupling of rubidium uptake to that of sodium and chloride (21) or any sensitivity of rubidium or chloride uptake to bumetanide or furosemide (4, 6). These reports would argue against the presence of a Na-K-Cl cotransporter in the apical (maternal facing) membrane, but do not exclude the possibility that the carrier may be resident in the basal or foetal facing membrane.

It has been shown previously that BeWo cells form a polarised monolayer with regular microvillous projections on the apical membrane which morphologically resemble the late gestational (third trimester) human trophoblast (22). We postulated that since BeWo cells exhibit bumetanide-sensitive $^{86}Rb^+$ uptake that the Na-K-Cl cotransporter may be localised to the basal membrane. Our finding that the cotransporter is present in membrane fractions enriched with Na,K-ATPase, a protein widely accepted as being localised in the basal membrane of most epithelial cells, but not in fractions containing alkaline phosphatase, a marker of the apical membrane, provides strong support for this view. It is also noteworthy that in this study BeWo cells were cultured on polycarbonate inserts thus allowing measurement of $^{86}Rb^+$ uptake across both the apical and basal membrane. However, when BeWo cells are cultured on plastic as confluent monolayers the basal membrane is largely inaccessible and under these culture conditions we observe a significantly diminished ouabain- and bumetanide-sensitive ^{86}Rb uptake (data not shown); this observation is consistent with the localisation of both the Na,K-ATPase and Na-K-Cl cotransporter in the basal membrane of BeWo cells.

Powell and coworkers (7) have reported the presence of DIDS- and DPC-sensitive chloride conductances in the basal membrane of the human syncytiotrophoblast, which they suggest may contribute towards transcellular chloride movement. Whilst these authors did not assess the presence of a bumetanide-sensitive component, it has been proposed that chloride transporting mechanisms may participate in volume regulation of the trophoblast (1, 5). In this regard it is well established that in both epithelial and non-epithelial cells, the Na-K-Cl cotransporter plays a key role in cell volume regulation (23, 24); a function it is also likely to perform in BeWo cells based on its activation when cells were subjected to hyperosmotic stress (Fig. 2c). The observed activation is rapid but transient, consistent with the suggestion that the cotransporter helps bring about a net increase in salt uptake that would facilitate a regulatory volume increase. As cells begin to recover their volume cotransporter activity would be expected to decline towards normal levels as observed in our studies.

In summary, our data demonstrate that BeWo cells express the $\alpha 1$ and $\beta 1$ subunits of the Na,K-ATPase and a Na-K-Cl cotransporter, which participate in potassium (Rb) uptake across the basal membrane. The activity of the Na-K-Cl cotransporter is enhanced significantly when cells are subjected to a hyperosmotic shock indicating that it may play a role in the regulation of trophoblast cell volume. The precise nature of the signalling mechanisms that instigate an increase in cotransporter activity under these circumstances remain unknown currently, but represents an interesting topic for further study.

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