Functional demonstration of Na$^+$-K$^+$-2Cl$^-$ cotransporter activity in isolated, polarized choroid plexus cells

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Wu, Qiang, Eric Delpire, Steven C. Hebert, and Kevin Strange. Functional demonstration of Na$^+$-K$^+$-2Cl$^-$ cotransporter activity in isolated, polarized choroid plexus cells. *Am. J. Physiol.* 275 (*Cell Physiol.* 44): C1565–C1572, 1998.—The function of the apical Na$^+$-K$^+$-2Cl$^-$ cotransporter in mammalian choroid plexus (CP) is uncertain and controversial. To investigate cotransporter function, we developed a novel dissociated rat CP cell preparation in which single, isolated cells maintain normal polarized morphology. Immunofluorescence demonstrated that in isolated cells the Na$^+$-K$^+$-ATPase, Na$^+$-K$^+$-2Cl$^-$ cotransporter, and aquaporin 1 water channel remained localized to the brush border, whereas the Cl$^-$/HCO$_3^-$ (anion) exchanger type 2 was confined to the basolateral membrane. We utilized video-enhanced microscopy and cell volume measurement techniques to investigate cotransporter function. Application of 100 µM bumetanide caused CP cells to shrink rapidly. Elevation of extracellular K$^+$ from 3 to 6 or 25 mM caused CP cells to swell 18 and 33%, respectively. Swelling was blocked completely by Na$^+$ removal or by addition of 100 µM bumetanide. Exposure of CP cells to 5 mM BaCl$_2$ induced rapid swelling that was inhibited by 100 µM bumetanide. We conclude that the CP cotransporter is constitutively active and propose that it functions in series with Ba^{2+}-sensitive K$^+$ channels to reabsorb K$^+$ from cerebrospinal fluid to blood.

potassium transport; cerebrospinal fluid secretion; cerebral edema; intracranial hypertension; bumetanide

MAINTENANCE OF CEREBROSPINAL fluid (CSF) volume and composition is crucial for normal function of the nervous system. The bulk of the CSF is produced by the choroid plexus (CP), which lines the ventricles in the brain. CP epithelial cells play diverse roles in regulating brain function and metabolism. They supply neurons and glia with micronutrients, remove waste products and toxins from the nervous system, and provide a pathway for neuroendocrine communication within the brain (13, 31).

One of the most important functions of CP cells is secretion of the CSF and control of its ionic composition. The mechanisms and regulation of CSF secretion are incompletely understood. In CP cells, unlike most epithelia, the Na$^+$-K$^+$-ATPase is located on the apical membrane, facing the CSF (22). The Na$^+$-K$^+$-ATPase maintains a low intracellular Na$^+$ concentration. Secondary active transport of Na$^+$ occurs at the basolateral cell membrane and appears to be mediated in part by a Na$^+$/H$^+$ exchanger (24–26). A Cl$^-$/HCO$_3^-$ exchanger is also located at the basolateral membrane (1, 17). It has been proposed that these two exchangers transport NaCl and osmotically obliged water into the CP cell (13, 14). At the apical membrane, Na$^+$ is extruded into the CSF via the Na$^+$-K$^+$-ATPase. Cl$^-$ transport into the CSF is thought to be mediated in part by an apical anion channel (6, 7, 10).

A number of studies have implicated the Na$^+$-K$^+$-2Cl$^-$ cotransporter as having an important role in the secretion and regulation of CSF ionic composition, but the precise function of the cotransporter is uncertain and controversial. In most models of CP function, the cotransporter has been postulated to be localized to the basolateral membrane and is thought to play a central role in CSF and NaCl secretion (12, 13, 30). Such a model is consistent with the well-established function and localization of the cotransporter in numerous other secretory epithelia (8). There are, however, no unequivocal data to support this model in the CP. Administration of the loop diuretics bumetanide and furosemide has been shown to either inhibit or have no effect on CSF secretion in several animal models (12). Interpretation of these discrepant findings is obscured by a variety of significant methodological concerns (12, 17). Studies of isolated tissues have shown clear effects of loop diuretics on CP ion transport (2), but the membrane localization of the cotransporter cannot be deduced from these experiments.

Based on their studies of isolated rat CP, Keep and co-workers (19, 20) proposed that the cotransporter is localized to the apical cell membrane and that it is operating in a reverse mode to secrete salt and water into the CSF. Delpire and co-workers have recently examined the distribution of the cotransporter in the brain. These investigators cloned the ubiquitous isoform of the cotransporter, NKCC1 (3), and demonstrated that it was expressed abundantly in the nervous system (29). The bulk of the signal in the brain is derived from the CP. Immunofluorescence studies of the CP demonstrated that the cotransporter is expressed predominantly, if not exclusively, on the apical cell membrane (29). The abundant apical expression of the cotransporter raises important questions regarding its role in CP function.

Intracranial hypertension and cerebral edema are common and very serious clinical problems. Administration of drugs that reduce CSF secretion is a standard approach for reducing brain swelling (12). An understanding of the precise mechanisms and regulation of CSF secretion therefore has significant clinical importance. However, detailed cellular and molecular investi-

gations of solute and fluid transport in the mammalian CP are difficult, due to the complicated morphology of the tissue and the small quantities that can be isolated and studied. To circumvent these difficulties, we developed a novel isolated CP cell preparation that maintains a normal polarized morphology and distribution of transport proteins. These cells are amenable to detailed study using optical and electrophysiological techniques. In the present investigation, we demonstrate that the Na^+-K^+-$2Cl^-$ cotransporter is constitutively active, that it functions to reabsorb NaCl and KCl from the CSF, and that it is the major pathway for concentration gradient-driven K^+ uptake in the CP.

MATERIALS AND METHODS

Isolation of single, polarized rat CP epithelial cells. The method for isolating single, polarized CP cells was similar to that described by Torres et al. (35) for the *Necturus* gallbladder. Briefly, 3- to 7-wk-old Sprague-Dawley rats were anesthetized by ether inhalation. After decapitation of the rats, CP were dissected from the lateral and fourth ventricles. Isolated CP were washed with Hanks' balanced salt solution (HBSS) and then treated with 1 mg/ml collagenase IV (Sigma Chemical, St. Louis, MO) and 1 mg/ml protease XIV (Sigma) in HBSS for 60 min at room temperature. The epithelium was disrupted, and single cells suspensions were produced by gentle pipetting. Suspensions were centrifuged at 300 *g* for 5 min, and pellets were resuspended in artificial CSF (aCSF; see Table 1). The pH and gas content of the aCSF in which the cells were suspended was kept constant by continuously blowing a stream of 5% CO_2-95% air over the surface of the medium. Cells were utilized for experiments within 6 h after isolation.

Several criteria were utilized in selecting isolated cells for experimentation. Cells that were obviously swollen and cells that had blebbing membranes, intracellular vacuoles, or disrupted microvilli were discarded.

Antibodies. Rabbit polyclonal antibodies against a carboxy-terminal region of the mouse bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter (mNKCC1) were produced as described previously by Kaplan et al. (18). Polyclonal antibodies to the rat Na^+-K^+-ATPase α_1-subunit were purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies to the mouse aquaporin 1 water channel and the mouse anion exchanger type 2 (AE2) were generous gifts of Dr. Dennis Brown (Massachusetts General Hospital, Boston, MA) and Dr. Seth Alper (Beth Israel Hospital, Boston, MA), respectively.

Immunofluorescence. Isolated CP cells were attached to glass coverslips coated with Cel-Tak (Collaborative Biomedi-cal Products, Bedford, MA) and fixed with 2% paraformaldehyde in PBS for 30 min at room temperature. After fixation, cells were permeabilized with 0.075% saponin in PBS for 10 min. Permeabilized cells were then exposed for 30 min at room temperature to a blocking solution containing 0.075% saponin and 0.2% BSA in PBS and incubated overnight at 4°C with primary antibodies diluted in PBS containing 0.075% saponin and 0.2% BSA. After a wash in PBS, cells were incubated for 90 min at room temperature with indocarbocyanine-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:600 in PBS containing 0.2% BSA. The cells were then washed with PBS and mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Cells were visualized using a Nikon Eclipse E800 microscope equipped with a Nikon Plan Apo ×100 objective lens [1.4 numerical aperture (NA)] and an Optronics DEI-750 color charge-coupled device (CCD) camera (Optronics Engineering, Goleta, CA). Montages were generated from digitized images using Adobe PhotoShop 4.0 and printed with a Tektronix Phaser 450 color printer (Tektronix, Wilsonwill, OR).

Measurement of relative cell volume changes. Video-enhanced differential interference contrast (DIC) microscopy (33) was used to measure relative volume changes in isolated, polarized CP cells. Briefly, cells were attached to the poly-L-lysine-coated coverslip bottom of a bath chamber (model R-26G, Warner Instrument, Hamden, CT) that was mounted onto the stage of a Nikon TE 300 inverted microscope. The bath chamber was perfused continuously at room temperature with experimental solutions gassed with 5% CO_2-95% air. The compositions of various solutions used in these studies are shown in Table 1.

Cells were visualized using a Nikon ×60 objective lens (0.7 NA) and a long working distance condenser lens (0.52 NA). Images were continuously recorded using a super VHS video cassette recorder (model SVO-2000, Sony Electronics, San Jose, CA) and a Hamamatsu CCD camera (model C2400, Hamamatsu Photonics, Hamamatsu City, Japan). The cross-sectional area (CSA) of single cells was quantified by digitizing recorded video images with an image-processing computer board (MV-1000, MuTech, Woburn, MA) with 512 × 480 × 8-bit resolution and a 200-MHz Pentium computer (Dimension XPS M200s, Dell Computer, Austin, TX). Digitized images were displayed on the computer monitor, and cell borders were traced using a mouse and a computer-generated cursor. CSA of the traced regions were determined by image analysis software (Optimas, Bioscan, Edmonds, WA). Isolated CP cells have a spherical morphology, and relative volume changes were calculated as (Experimental CSA/control CSA)$^{3/2}$.

Table 1. *Composition of bath solutions*

	aCSF	25 mM K^+	6 mM K^+	0 Na^+, 25 mM K^+	5 mM $BaCl_2$	5 mM $BaCl_2$, 0 Na^+	0 Na^+	0 Cl^-
Na^+	127.75	127.75	127.75	0	127.75	0	0	127.75
NMDG	22.0	0	19.0	127.75	14.5	139.75	149.75	22.0
K^+	3.0	25.0	6.0	25.0	3.0	3.0	3.0	3.0
Ba^{2+}	0	0	0	0	5.0	5.0	0	0
Cl^-	135.4	135.4	135.4	135.4	135.4	135.4	135.4	0
Gluconate	0	0	0	0	2.5	2.5	0	130.5
NO_3^-	0	0	0	0	0	0	0	4.9

Values are in mM. NMDG, *N*-methyl-D-glucamine. Solutions were gassed with 95% air-5% CO_2 and had a pH of 7.35–7.4 and osmolality of 285–295 mosM. In addition to listed constituents, all solutions also contained (in mM) 1.15 Ca^{2+}, 0.8 Mg^{2+}, 21 HCO_3^-, 0.25 $H_2PO_4^-$, 3.4 glucose, and 2.0 L-glutamine. aCSF, artificial cerebrospinal fluid.

Statistics. Data are reported as means $\pm$ SE (n = number of cells, number of CP). Statistical significance was assessed using Student's *t*-test.

RESULTS

Isolated CP cells maintain functional polarity. Reuss and co-workers (34, 35) have shown that certain epithelial cells maintain morphological and functional polarity when isolated from their native tissues. Figure 1 demonstrates clearly that CP cells behave in a similar fashion. A distinct apical pole, marked by a prominent brush border, and a basolateral pole are readily visible by DIC microscopy in fixed (Fig. 1, *left*) and living cells (not shown).

Previous studies in intact CP have demonstrated that the Na⁺-K⁺-ATPase (22), the aquaporin 1 water channel (27), and the ubiquitous isoform of the Na⁺-K⁺-2Cl⁻ cotransporter, NKCC1 (29), are localized to the apical brush border. In contrast, the nonerythrocyte Cl⁻/HCO$_3^-$ exchanger (AE2) is localized exclusively to the basolateral membrane (1). As shown in Fig. 1,

middle and *right*, these transport proteins maintain their polarized distribution in isolated CP cells.

The CP Na⁺-K⁺-2Cl⁻ cotransporter is constitutively active and reabsorbs solute from the CSF. Cell volume is held constant under steady-state conditions by a precise matching of rates of solute influx and efflux across the plasma membrane. Alterations in the activity of either solute influx or efflux pathways can result in net gain or loss of solute and osmotically obliged water, with resultant cell swelling or shrinkage. Experimental manipulation of transport pathway activity and quantification of associated cell volume changes can therefore provide insight into the nature and regulation of transporters and channels (5, 33).

We utilized cell volume measurements in an effort to begin defining the role of the Na⁺-K⁺-2Cl⁻ cotransporter in CP function. As shown in Fig. 2, application of 100 µM bumetanide, a relatively selective cotransporter inhibitor, caused CP cells to shrink $-9 \pm 0.4\%$ (measured 5 min after bumetanide addition) at an initial rate of -2.2 ± 0.6 %/min (n = 20,6). Cell volume

Na-K-2Cl cotransporter (NKCC1)

Aquaporin 1 water channel

Na,K-ATPase α-1 subunit
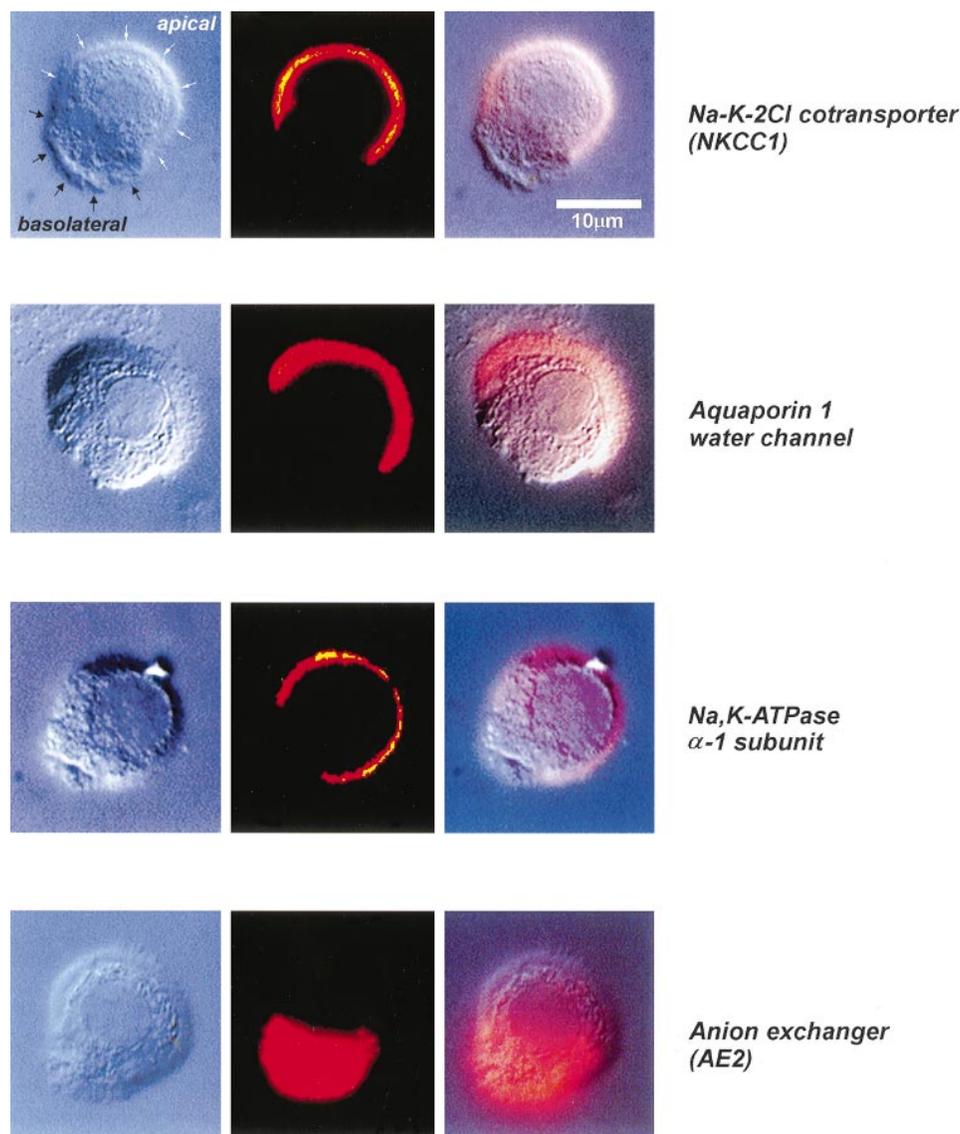
Anion exchanger (AE2)

Fig. 1. Isolated choroid plexus (CP) cells maintain morphological and functional polarity. Cells were fixed, treated with antibodies to various transport proteins, and then imaged by differential interference contrast (DIC; *left*), fluorescence (*middle*), or combined DIC-fluorescence microscopy (*right*). Apical microvilli and basolateral pole are readily discernible in cells imaged by DIC microscopy (*left*). Previous studies of intact CP have demonstrated that Na⁺-K⁺-2Cl⁻ cotransporter, aquaporin 1 water channel, and α$_1$-subunit of Na⁺-K⁺-ATPase are localized exclusively to apical microvilli (22, 27, 29). In contrast, anion exchanger type 2 is found only in basolateral cell pole (1). Apical and basolateral localization of these transport proteins is maintained in isolated cells (*middle* and *right*).
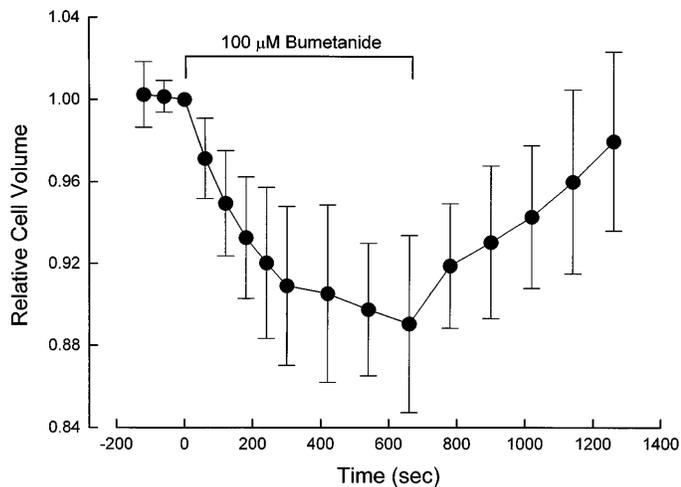
Fig. 2. Na^+-K^+-$2Cl^-$ cotransporter in CP is constitutively active and functions to reabsorb Na^+, K^+, and Cl^- from cerebrospinal fluid (CSF). Exposure of cells to 100 μM bumetanide (*time 0*) inhibits cotransporter and induces a rapid cell shrinkage. Cell shrinkage indicates that cotransporter is transporting solute into cell from CSF (see RESULTS and DISCUSSION). Values are means ± SE ($n = 20,6$).

remained stable for at least 3 min after completion of the shrinkage phase. Application of 10 μM bumetanide induced a similar cell shrinkage (data not shown). Washout of bumetanide caused the cells to reswell to their original volume at an initial rate of 1.5 ± 0.5 %/min ($n = 11,3$).

The bumetanide-induced shrinkage indicates that the cotransporter in CP cells is constitutively active and functioning to move solute into the cell. To further test for cotransporter function, we exposed CP cells to a bath solution with elevated extracellular K^+ (Table 1). Increasing extracellular K^+ concentration from 3 to 25 mM caused CP cells to swell $33 \pm 0.5\%$ (measured 5 min after elevation of K^+ concentration) at an initial rate of 12.7 ± 3.7 %/min ($n = 19,4$). Reduction of extracellular K^+ concentration to 3 mM caused the cells to shrink back toward their original volume (Fig. 3*A*). K^+-induced swelling was prevented completely by 100 μM bumetanide or by removal of extracellular Na^+ (Fig. 3*B*). These results demonstrate clearly that the cotransporter is a major pathway for concentration gradient-driven K^+ uptake into CP cells.

The increased influx of K^+ via the cotransporter in response to an elevation of CSF K^+ concentration has important physiological implications (see DISCUSSION). However, even under extreme pathophysiological conditions, CSF K^+ levels are unlikely to rise to 25 mM. We therefore examined the effect on CP cell volume of raising extracellular K^+ from 3 to 6 mM. Exposure to 6 mM K^+ caused CP cells to swell $18 \pm 6\%$ at an initial rate of 7.2 ± 2.4 %/min ($n = 4,1$). This volume change was blocked completely by 100 μM bumetanide (the cell volume change measured 3 min after exposure to 6 mM K^+ and 100 μM bumetanide was $0.9 \pm 2.3\%$, $n = 6,1$). Thus even small, physiologically relevant increases in extracellular K^+ result in an immediate and rapid uptake of K^+ into the CP cell via the Na^+-K^+-$2Cl^-$ cotransporter.

Keep and co-workers (19, 20) have proposed that the rat CP cotransporter is localized to the apical cell membrane and that it functions to transport Na^+, K^+, and Cl^- from the cytoplasm to the CSF. Data shown in Fig. 2 are inconsistent with this hypothesis. To further examine the directionality of net solute uptake by the cotransporter, we used cell volume measurements to estimate intracellular Na^+ and Cl^- concentrations for calculation of the net cotransporter driving force. In other epithelial cell types, more direct measurements with methods such as electron microprobe analysis and ion-sensitive fluorescent dyes have demonstrated that ion concentrations estimated by cell volume changes provide reasonable estimates of actual intracellular concentrations (see for example, Refs. 5 and 32).

When extracellular Na^+ was replaced with N-methyl-D-glucamine or Cl^- was replaced by gluconate, CP cells
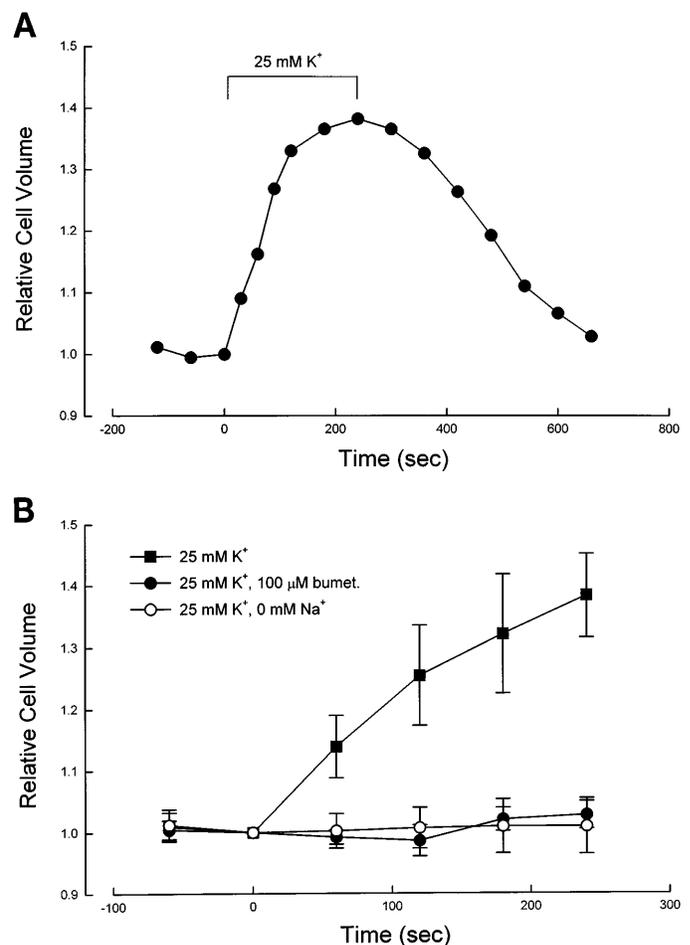

A

B

Fig. 3. Na^+-K^+-$2Cl^-$ cotransporter is major pathway in CP for concentration gradient-driven K^+ uptake. *A*: elevation of extracellular K^+ from 3 to 25 mM (*time 0*) induces a rapid and reversible cell swelling. Data shown were obtained from a single cell. *B*: K^+-induced swelling is inhibited completely by 100 μM bumetanide (bumet) or by replacement of extracellular Na^+ with N-methyl-D-glucamine. Cells were exposed to Na^+-free medium or 100 μM bumetanide for 4–6 min before elevation of extracellular K^+ to 25 mM (*time 0*). Both these experimental manipulations induced cell shrinkage (see Fig. 1 and text). For clarity, initial relative volume of these 2 groups of cells was normalized and plotted as 1.0 at *time 0*. Values are means ± SE ($n = 5$–19,2–4).

shrank -17 ± 5 and $-13 \pm 4\%$ ($n = 5,2$; measured 5 min after Na^+ or Cl^- removal), respectively. If it is assumed *1*) that cell shrinkage reflects complete loss of these ions from the cytoplasm, *2*) that only monovalent counterions accompany Na^+ and Cl^- efflux, and *3*) that cell water content is 75% of the total cell volume, which is typical for most cells (9), the minimal estimates for intracellular Na^+ and Cl^- concentrations are 32.5 and 26 mM, respectively.

The direction of the driving force for the cotransporter is dependent on the ratio of the extracellular and intracellular concentrations of Na^+, K^+, and Cl^-

$$[Na]_o \times [K]_o \times [Cl]_o^2 / [Na]_i \times [K]_i \times [Cl]_i^2$$

where i and o denote the concentrations of a given ion inside and outside the cell, respectively. Using the intracellular Na^+ and Cl^- concentrations estimated from the cell volume changes and the extracellular Na^+, K^+, and Cl^- concentrations shown in Table 1 and assuming that intracellular K^+ concentration is ~120 mM, we calculate that the driving force for the cotransporter favors net CSF-to-cell solute uptake by a factor of 2.7:1.

K^+ efflux from CP cells is mediated in part by Ba^{2+}-sensitive K^+ channels. If the Na^+-K^+-$2Cl^-$ cotransporter is constitutively active and moving K^+ into the cell, then K^+ must exit via other transport pathways for cell volume to remain stable. In certain epithelia, there is a tight coupling between the activity of K^+ channels and the Na^+-K^+-$2Cl^-$ cotransporter (37). We assessed the role of K^+ channels in CP K^+ transport by exposing cells to 5 mM $BaCl_2$. As shown in Fig. 4*A*, exposure to $BaCl_2$ caused CP cells to swell $35 \pm 9\%$ (measured 5 min after Ba^{2+} addition) at an initial rate of $7.3 \pm 2 \%/min$ ($n = 13,6$). Removal of $BaCl_2$ caused the cells to shrink back toward their original volume (Fig. 4*A*). These findings indicate that K^+ channels mediate net K^+ efflux from CP cells.

Figure 4*B* shows the effects of 100 μM bumetanide on $BaCl_2$-induced cell swelling. Bumetanide reduced the amount and initial rate of swelling ~65%, to $12 \pm 5\%$ and $2.6 \pm 1 \%/min$ ($n = 6,2$), respectively. Both these values were significantly different ($P < 0.04$) from those observed in the presence of $BaCl_2$ alone. Exposure of cells to 5 mM ouabain in the presence of $BaCl_2$ completely blocked cell swelling (Fig. 4*B*). Results shown in Figs. 3 and 4 indicate that K^+ channels mediate the efflux from CP cells of K^+ taken up by the Na^+-K^+-$2Cl^-$ cotransporter and the Na^+-K^+-ATPase.

DISCUSSION

Reabsorption of NaCl and KCl from the CSF by the apical Na^+-K^+-$2Cl^-$ cotransporter. A number of in vivo and in vitro studies have suggested an important role for the Na^+-K^+-$2Cl^-$ cotransporter in CP function. Most models of CP transport have localized the cotransporter to the basolateral membrane and have proposed that it plays a central role in the secretion of NaCl and water into the brain interstitium (12, 13, 15). The postulated basolateral localization is consistent with the function
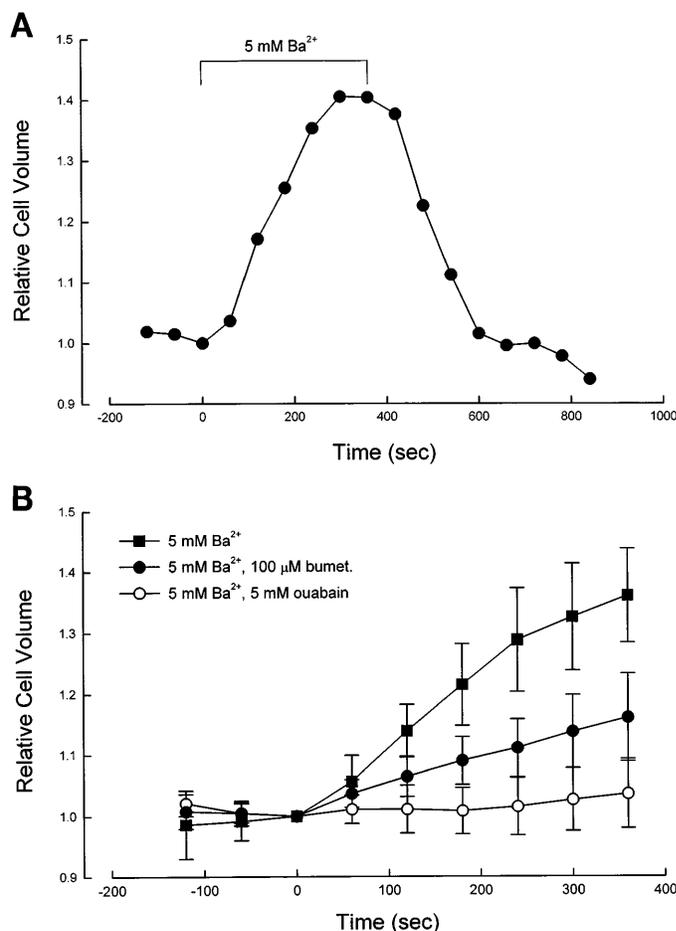
Fig. 4. Ba^{2+}-inhibitable K^+ channels mediate efflux of K^+ taken up into CP cells by cotransporter. *A*: exposure of CP cells at *time 0* to 5 mM $BaCl_2$ induces rapid and reversible cell swelling. Cell swelling indicates that K^+ taken up into CP cells by other transport pathways exits via Ba^{2+}-inhibitable K^+ channels. Data shown are from a single cell. *B*: Ba^{2+}-induced cell swelling is inhibited by bumetanide or ouabain. Cells were exposed to 5 mM $BaCl_2$, 5 mM $BaCl_2$ + 100 μM bumetanide, or 5 mM $BaCl_2$ + 5 mM ouabain at *time 0*. Bumetanide inhibition of Ba^{2+}-induced swelling indicates that K^+ channels mediate, at least in part, efflux of K^+ taken up by Na^+-K^+-$2Cl^-$ cotransporter (see RESULTS and DISCUSSION). Values are means $\pm$ SE ($n = 6$–$13, 2$–6).

and localization of the cotransporter in a variety of other secretory epithelia (8).

An apical localization of the cotransporter was proposed by Keep et al. (20). This hypothesis was subsequently confirmed by Plotkin et al. (29) using immunocytochemistry and radioisotope flux measurements in cultured CP cells. Based on unidirectional flux measurements and assumptions of intracellular Na^+ and Cl^- concentrations, Keep et al. (20) postulated that the cotransporter operates in a secretory mode, transporting Na^+, K^+, and Cl^- from the cytoplasm to the CSF. The postulated operation of the cotransporter in an efflux mode is opposite to that observed in most other cell types and is inconsistent with the results of this study. As shown in Fig. 2, exposure of CP cells to 100 μM bumetanide induces a rapid cell shrinkage. This shrinkage is due to inhibition of NaCl and KCl reabsorption through the cotransporter in the presence of

continued solute and osmotically obliged water loss from the cell via other transport pathways. Our findings are consistent with studies of Johanson and co-workers (2, 17), which have shown that exposure of isolated, intact CP to loop diuretics induces net water loss from the tissue.

In rat CP cells, the driving force on the cotransporter favors reabsorption of NaCl and KCl from CSF to cell by a factor of 2.7:1. With the assumption that intracellular Na^+ and Cl^- concentrations in rat CP were 30 and 50 mM, respectively, Keep et al. (20) concluded that the ion gradients favored reverse operation of the cotransporter. In rat CP, we estimated intracellular Na^+ and Cl^- concentrations using cell volume measurements. The minimal intracellular Na^+ concentration is 32.5 mM, which is similar to that assumed by Keep et al. (19). However, intracellular Cl^- concentration in the rat CP is significantly lower (26 mM) than was assumed previously. If Cl^- concentration was indeed 50 mM, then there would be a small driving force favoring reverse operation of the cotransporter.

It is possible that the driving force on the cotransporter in the intact CP is different from that in isolated cells. In addition, it is possible and likely that the driving force is altered by the physiological state of the animal. Thus it is conceivable that the cotransporter could operate in the reverse direction in vivo under certain physiological conditions. Additional studies are clearly needed to assess the function of the cotransporter in the intact CP.

Role of K^+ channels in CP K^+ transport. If K^+ is taken up into CP cells via the Na^+-K^+-$2Cl^-$ cotransporter, it must also exit at a similar rate for cell volume to remain stable. Under normal conditions, K^+ typically exits cells via K^+ channels and/or the K^+-Cl^- cotransporter. We assessed the role of channels in K^+ efflux by exposing CP cells to 5 mM $BaCl_2$, a selective K^+ channel blocker. Ba^{2+} caused CP cells to swell rapidly (Fig. 4). Both the initial rate and maximal swelling were inhibited ~65% by 100 μM bumetanide (Fig. 4*B*), indicating that K^+ taken up by the cotransporter exits the cell, at least in part, via K^+ channels. Ouabain completely inhibited Ba^{2+}-induced swelling (Fig. 4*B*). This inhibition most likely reflects inhibition of pump-mediated K^+ uptake, as well as inhibition of the Na^+-K^+-$2Cl^-$ cotransporter, which is expected to occur as intracellular Na^+ concentration rises during exposure of CP cells to ouabain.

Results shown in Figs. 2–4 raise important questions regarding the role of the Na^+-K^+-$2Cl^-$ cotransporter in mediating net transepithelial salt and water transport in the CP. We propose that the cotransporter plays a central role in the reabsorption of K^+ from the CSF to the blood and in the buffering and regulation of brain interstitial K^+ concentration. Figure 5 shows a hypothetical model of these functions. A key test of this model requires localization of the Ba^{2+}-inhibitable K^+ conductance. Patch-clamp studies on mammalian CP have demonstrated the presence of K^+ channels on the apical cell membrane (10). However, such studies do not provide insight into the relative K^+ conductance of
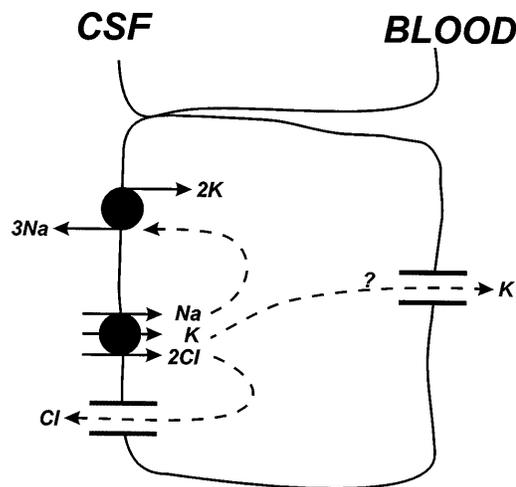

Fig. 5. Model of Na^+-K^+-$2Cl^-$ cotransporter function in CP epithelial cells. Presence of K^+ channel in basolateral membrane is postulated.

the apical and basolateral membranes, nor do they provide information on the net driving forces for passive K^+ transport at the two cell poles. Detailed microelectrode and patch-clamp studies are required to address this issue. If the cotransporter is indeed involved in reabsorption of K^+ from the CSF, we anticipate that the cellular K^+ conductance and passive driving forces will favor apical-to-basolateral K^+ transport.

In addition to K^+ channels, other transport pathways may mediate K^+ efflux from CP cells. Importantly, a KCl cotransporter has been postulated to play a role in the function of the rat (16) and amphibian (38) CP. It may be possible to assess the role of a K^+-Cl^- cotransporter in CSF formation using isolated, polarized CP cells and optical techniques.

The Na^+-K^+-ATPase also mediates K^+ reabsorption from the CSF, which raises the question of what additional role the cotransporter plays. Because the K^+ affinity of the cotransporter is relatively low (28) and because changes in CSF K^+ levels (see RESULTS) alter the driving force on the cotransporter, even small increases in K^+ concentration will result in immediate changes in K^+ uptake. In contrast, the pump has a K^+ affinity of 0.5–1.5 mM (4). Thus increases in extracellular K^+ concentration will almost certainly have little direct effect on the rate of pump transport. Furthermore, the pump plays a central role in net water and salt secretion into the CSF (13). Alterations in the rate of pump turnover as a primary response to changes in CSF K^+ concentration would therefore likely alter the rate of CSF secretion.

We propose that the cotransporter provides a mechanism for dissociating the critical functions of CSF secretion and K^+ reabsorption/buffering. This hypothesis is supported by studies of the regulation of CSF K^+ concentration in cat CP carried out by Husted and Reed (11). These investigators demonstrated that experimental changes in CSF K^+ concentration induced alterations in net K^+ transport across the CP. Changes in K^+ transport functioned to return CSF K^+ concentration to

its original level and apparently occurred without changes in the rate of CSF secretion. Husted and Reed (11) proposed that an active K^+ transport process was present in the CP and that it "may be under the control of a mechanism that senses c.s.f. potassium concentration." This putative sensing mechanism may simply be the driving force on the Na^+-K^+-$2Cl^-$ cotransporter.

Clinical implications of CSF solute reabsorption through the apical Na^+-K^+-$2Cl^-$ cotransporter. Intracranial hypertension is a serious and life-threatening consequence of traumatic brain injury and a variety of disease states. Systemic administration of furosemide is commonly used to treat intracranial hypertension. The effect of furosemide is widely believed to be due to inhibition of CSF secretion via inhibition of a basolateral NaCl transporter in the CP, as well as to a furosemide-induced diuresis and increased whole body fluid loss (12). Experimental studies in a variety of animal models, however, have failed to show any effect of furosemide on CSF secretion (12). Furthermore, the recent studies of Plotkin et al. (29) demonstrating an apical localization of the Na^+-K^+-$2Cl^-$ cotransporter, as well as the findings of this investigation, indicate that there is no sound rationale for attempting to directly inhibit CP CSF secretion via systemic administration of furosemide. We propose that if furosemide inhibits CSF secretion in patients with intracranial hypertension, it does so by indirect and nonspecific mechanisms.

The findings of our studies raise some interesting and important considerations for developing novel approaches to treating intracranial hypertension. Rather than attempting to inhibit CSF secretion, it may be more beneficial to stimulate CSF reabsorption via stimulation of the apical Na^+-K^+-$2Cl^-$ and associated basolateral solute efflux pathways. Such an approach would have the added benefit of reducing brain interstitial K^+ levels. Extracellular K^+ concentration is tightly regulated in the central nervous system. However, during brain injury, extracellular K^+ levels rise, which in turn can alter neural activity and lead to cell swelling and further injury (21, 23, 36). Clearly, it is of major clinical importance to define the mechanisms and regulation of solute and fluid transport in the CP at the cellular and molecular level. The isolated, polarized CP cell preparation we have developed, combined with optical and electrophysiological methods, provides a novel and powerful approach for defining in detail the mechanisms of CSF secretion and the control of CSF composition.

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