Biochimica et Biophysica Acta, 1152 (1993) 289–299

BBAMEM 76111

$Na^+/K^+/2Cl^-$ cotransport in medullary thick ascending limb cells: kinetics and bumetanide binding

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(Received 18 March 1993)
(Revised manuscript received 14 June 1993)

Key words: Bumetanide; Sodium ion, potassium ion–chloride cotransport; Anion dependent cation transport; (mTAL cell); (Mouse)

We examined the properties of $Na^+/K^+/2Cl^-$ cotransport in cultured mouse mTAL cells with respect to its kinetics, the contribution of K/K exchange to K fluxes mediated by the cotransporter, and [^{3}H]bumetanide binding and turnover numbers in media with varying osmolality. The addition of bumetanide, the replacement of external Na^+ or the replacement of external Cl^- resulted in an almost identical (approx. 50%) decrease in K^+ influx, suggesting that Na^+-dependent, Cl^--dependent, BS K^+ influx was a measure of $Na^+/K^+/2Cl^-$ cotransport. The kinetics of the BS K^+ influx revealed a high affinity for external Na^+ (apparent K_m 7 mM) and external K^+ (apparent K_m 1.3 mM), but a very low affinity for external Cl^- (apparent K_m 67 mM with a two-site model). Of interest was the finding that none of the K^+ ($^{86}Rb^+$) efflux was sensitive to bumetanide, suggesting the absence of cotransport mediated K/K exchange in this cell type. Specific [^{3}H]bumetanide binding was a saturable function of free bumetanide concentration with a K_d of 0.20 μM and maximum binding (B_{max}) of 0.63 pmol/mg, or about 53 000 sites per cell. Simultaneous transport and bumetanide binding assays yielded a turnover number of 255 min^{-1}. The omission of external Na^+, K^+ or Cl^- reduced specific [^{3}H]bumetanide binding to values indistinguishable from zero. Changing medium osmolarity resulted in a co-ordinate change in BS K^+ influx and bumetanide binding, with a monotonic increase in both transport and bumetanide binding with increase in osmolality from 200 to 400 mosmol/kg. About 85% of the cotransporter sites were located on the apical side, as in the intact mTAL tubule. The simultaneous measurement of BS ion transport and [^{3}H]bumetanide binding in the mTAL cell may provide valuable insights into the regulation of $Na^+/K^+/2Cl^-$ cotransport in this nephron segment.

Introduction

The $Na^+/K^+/2Cl^-$ cotransport is the major route of NaCl entry from the lumen into the medullary thick ascending limb (mTAL) cell [1]. Salt reabsorption in the loop of Henle accounts for about 25–40% of the filtered load [1]. The importance of salt reabsorption in this nephron segment in general, and of $Na^+/K^+/2Cl^-$ cotransport in particular is underscored by the observation that administration of loop diuretics such as furosemide or bumetanide results in a significant natriuresis and diuresis. Despite its importance, many features of this cotransport pathway in the mTAL remain controversial or unknown.

The kinetic properties of the cotransporter have not been examined directly by transport studies in intact mTAL cells. The interest in the kinetic properties of $Na^+/K^+/2Cl^-$ cotransporter, and in particular the Cl^- dependence stems from the fact that luminal Cl^- concentrations in the mTAL are believed to be about 50 mM [1]. Thus, luminal Cl^- would not be rate limiting for transepithelial Cl^- reabsorption if the affinity for external Cl^- was high, but would be limiting if the affinity was low (i.e., $K_{1/2}$ for Cl^- was 50 mM or higher). Greger et al. have examined the kinetics of the equivalent short circuit current (I_{SC}) in the cortical (but not the medullary) TAL [2,3]. Since I_{SC} approximates the transepithelial Cl^- reabsorption [2], others have assumed that the kinetics of the I_{SC} reflect the kinetics of the apical $Na^+/K^+/2Cl^-$ cotransport in the cTAL [1]. However, this treatment assumes that the events at the apical membrane are rate limiting, an assumption that has not been tested rigorously. For this and other reasons, Greger et al. have cautioned that their description of kinetics of I_{SC} may not necessarily reflect the kinetics of $Na^+/K^+/2Cl^-$ cotransport

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[2]. Kinetics of the $Na^+/K^+/2Cl^-$ cotransport have also been examined in medullary vesicles [4,5], but not in intact cells. Conclusions regarding kinetics of the cotransport derived from the I_{SC} studies in cTAL and from medullary vesicle studies are widely discrepant. The I_{SC} studies in cTAL suggest a low affinity for Cl, with a K_m of about 55 mM [2], whereas the medullary vesicle studies suggest a relatively high Cl^- affinity (apparent K_m 15 mM [4–6]). Thus, it is not clear whether luminal Cl^- concentrations, which may be about 50 mM, are limiting for transepithelial NaCl reabsorption in the TAL.

The technique of [^{3}H]bumetanide binding has added considerably to our understanding of $Na^+/K^+/2Cl^-$ cotransport [7–16]. [^{3}H]Bumetanide binding has been measured in dog kidney medulla [7], mouse kidney membranes [12] and more recently in membranes from thick ascending limb [15], but [^{3}H]bumetanide binding and transport assays have not been performed in the same study. While earlier studies have shown a strictly co-ordinate correlation between the bumetanide-sensitive (BS) ion transport and [^{3}H]bumetanide binding sites, two recent studies suggest that [^{3}H]bumetanide binding alone may not always provide an accurate measure of the activity of the cotransporter. In endothelial cells, changes in BS K^+ influx with bradykinin were not accompanied by similar quantitative changes in [^{3}H]bumetanide binding, and a marked change in the turnover number per transporter site was observed [10]. Even more striking is the observation in human adenocarcinoma (HT-29) cells that chronic exposure to cAMP more than doubled BS K^+ influx without any change in the number of cotransporter sites [16]. These studies have underscored the need to measure both transport and binding at the same time.

The aim of the present study was (a), to define the kinetics and in particular, the Cl^- dependence of $Na^+/K^+/2Cl^-$ cotransport in mTAL cell; (b), to investigate whether the $Na^+/K^+/2Cl^-$ cotransporter in mTAL mediates a bidirectional K/K exchange, similar to the K/K exchange in almost every cell type studied so far, and if this K/K exchange (in addition to the K^+ efflux mediated by the K^+ channel) is in part responsible for the K^+ recycling across the apical membrane of the mTAL and (c), to examine the effect of medium osmolality on cotransport in this cell by simultaneously measuring transport and [^{3}H]bumetanide binding.

Detailed kinetic studies of Na^+, K^+ and Cl^- dependence and simultaneous [^{3}H]bumetanide binding isotherms and flux studies require quantities of tissues not easily obtainable from freshly isolated tubules or cells. Therefore, we used cells cultured from mTAL of the mouse kidney, and used between passage 7–25. These cells have the advantage in that they were derived from a known, specific kidney segment and have been well characterized [17,18].

Materials and Methods

Cell culture

Mouse mTAL cells, obtained by free-hand dissection of mTAL tubules [17,18] were a gift from Dr. John D. Valentich (Galveston, TX, USA). They were used between passage 7–25. These cells were maintained in stock cultures in 25-cm^2 flasks in a complete medium (CM) consisting of a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 medium, supplemented with 5% fetal bovine serum, 2 mM glutamine and ITS pre-mix (Collaborative Research, MA, USA) to provide final concentrations of insulin 5 mg/l, transferrin 5 mg/l and Na_2Se (5 μg/l) at 37°C in 5% CO_2 atmosphere. For transport and binding assays, cells were seeded in Falcon 6-well flat-bottom plates in 2 ml of complete medium per well and incubated at 37°C in 5% CO_2 until confluence. Medium was changed every 2–3 days as required. Transport studies were performed when cells were about 90% confluent.

K^+ influx assay

Washed cell monolayers incubated at 37°C for 15 min in transport assay buffer (TAB) containing (in mM) NaCl 145, KCl 5, glucose 5, Na phosphate 2.5, D-glucose 5 (pH 7.4) at 37°C. In ion substitution and kinetic studies, Na^+, K^+ or Cl^- was replaced by the indicated cation or anion, but the composition of media was otherwise identical to that of the TAB. $^{86}Rb^+$ (0.2 μCi/ml) was added at zero time and the transport was continued in the absence or presence of 20 μM bumetanide. K^+ uptake was terminated at 3 min by washing the cells 4-times with ice-cold 0.115 M $MgCl_2$. Preliminary results revealed that uptake was linear for at least 5 min. The cells were solubilized with 2 ml of 1% SDS with 0.1 N NaOH. Cell extracts (0.5-ml aliquots in duplicate) were counted for β-radioactivity. K^+ (Rb^+) uptake was expressed as nmol/mg protein per min. Protein content of cell extracts was measured by the method of Avruch and Wallach [19] which utilizes the intrinsic fluorescence of tryptophan, using bovine serum albumin as the standard. This method gave results which were nearly identical to those obtained by using the bicinchoninic acid assay (BCA protein kit, Pierce, Rockford, IL, USA).

K^+ efflux assay

Cells were loaded with isotope by adding $^{86}Rb^+$ (1 μCi/ml) to the complete culture medium and incubating cells for 3 h at 37°C in this medium. Cells were then washed 5-times with ice-cold TAB or $MgCl_2$ (0.115 M) to remove supernatant radioactivity and then incubated in 1 ml of prewarmed TAB (see above for composition) at 37°C. Final results were identical whether cells were washed with TAB or $MgCl_2$. At time intervals described in figure legends, 0.5-ml

aliquots were taken for counting radioactivity. The remaining medium was quickly aspirated and replaced with 1 ml of fresh TAB without isotope. At the end of efflux period, cells were solubilized with SDS-NaOH as described above. Samples were taken for measurement of radioactivity and proteins as described above. The total amount of $^{86}Rb^+$ in the cells at zero time was calculated as the sum of radioactivity in the supernatants at various time points and the cell radioactivity at the end of the efflux period.

^{3}[H]Bumetanide binding

Cell monolayers (approx. 1 mg protein per well) were incubated in 1 ml of TAB (transport assay buffer, for full composition see above) with 31 nM [^{3}H]bumetanide (spec. act. 80 Ci/mmol) and various concentrations of unlabelled bumetanide (0–2 μM) for 30 min at 37°C. In preliminary studies, we determined that steady state binding was complete by 15 min. Cells were washed 4-times rapidly with ice-cold 115 mM $MgCl_2$. Cell extracts were prepared with SDS-NaOH and protein was measured as described above. Radioactivity of cell extract (1 ml) was counted with 10 ml of scintillation cocktail (Formula 989) in a scintillation counter (LS 7000, Beckman Instruments, Fullerton, CA, USA). Nonspecific binding was measured in the presence of 100 μM unlabeled bumetanide and subtracted from total bumetanide binding to obtain specific bumetanide binding. Nonspecific binding was about 50% of the total binding. It was essential to repurify the radioligand frequently (about 3–4 months) by HPLC as described previously [7]. Failure to repurify the radioligand resulted in an increase in nonspecific binding to unacceptably high levels (> 50%), presumably because of breakdown of bumetanide into degradation products.

Cell counts

Washed cell monolayers were incubated with 2 ml of saline/EDTA (1:1 mixture) until the cells detached completely and appeared as single unclumped cells. An aliquot of the cell suspension was diluted for cell counting by the Coulter counter. Another aliquot was mixed with SDS for determination of protein content.

Water content of mTAL cells

Water content was measured by the method of Kletzein et al. [20] from the distribution of the nonmetabolized hexose 3-*O*-methyl D-[^{14}C]glucose. Briefly, monolayers of cells grown on plastic Falcon six well plates were incubated in 2 ml of DMEM containing 1, 2, 5 or 10 mM 3-*O*-methyl D-[^{14}C]glucose for 30 min at 37°C. The medium was removed and cells were washed 4-times with ice-cold phosphate-buffered saline containing 1 mM phloretin, which prevented the efflux of hexose from the cells during washing. The cells were solubilized with 0.2% SDS and cell extracts were counted for ^{14}C radioactivity. Since hexose concentration equalizes in the intracellular and extracellular compartments, the uptake of 3-*O*-methyl D-glucose gives the intracellular water content, which was expressed as μl/mg protein.

Solutions, chemicals and drugs

[^{3}H]Bumetanide was custom synthesized by Amersham at a spec. act. of 80 Ci/mmol. $^{42}K^+$ and $^{86}Rb^+$ were purchased from Amersham (Arlington Heights, IL, USA). Formula 989 was obtained from Dupont-NEN (Boston, MA, USA). Unlabeled bumetanide was a gift from Dr. Peter Sorter, Hoffman La Roche (Nutley, NJ, USA). Culture media were obtained from Gibco/BRL (Grand Island, NY, USA). ITS pre-mix was from Collaborative Research (Bedford, MA, USA). Flasks, petri dishes, multiwell plates and filter supports were from Falcon Labware (Becton Dickinson, Lincoln Park, NJ, USA). All other reagents were from Sigma (St. Louis, MO, USA), Alfa Products (Ward Hill, MA, USA), or J.T. Baker (through VWR Scientific, Piscataway, NJ, USA).

Presentation of data

Results are presented as means ± S.D. (rather than S.E.) unless stated otherwise. The differences in results between groups (Tables II–IV, Fig. 8) were analyzed by Student's *t*-test, and the null hypothesis was rejected when $P < 0.05$. n.s., not significant. Experimental data points were fitted to equations using non-linear regression with successive iteration using commercial software packages (Enzfitter, Elsevier-Biosoft, New York, NY, USA; Sigmaplot, Jandel Scientific, Cortes Madera, CA, USA). The lines accompanying the data points represent nonlinear (or linear) least square curve fits of the data to the equation noted in the figure legends or Results.

Results

Comparison of $^{42}K^+$ and $^{86}Rb^+$ fluxes

To examine the suitability of Rb^+ as an analog for K^+ for the pathway in mTAL cells, we compared K^+ influx in 5 mM K^+ media with either $^{42}K^+$ and $^{86}Rb^+$ added. There was no statistically significant difference between K^+ and Rb^+ influx in the absence and presence of 10 μM bumetanide (Table I). The rate constants for K^+ and Rb^+ efflux were also similar (0.040 ± 0.003 vs. 0.039 ± 0.003, respectively, n.s., not shown). In all subsequent studies, $^{86}Rb^+$ was used in lieu of $^{42}K^+$ to measure K^+ fluxes, because $^{86}Rb^+$, by virtue of its somewhat longer half life (18 vs. 0.5 days) was a more convenient isotope compared to $^{42}K^+$.

TABLE I

Comparison of K^+ influx using $^{42}K^+$ or $^{86}Rb^+$ in mTAL cells

Cell monolayers were incubated with transport assay buffer containing 5 mM KCl (see Materials and Methods for composition), pH 7.4 at 37°C for 15 min. $^{42}K^+$ or $^{86}Rb^+$ was added at zero time and influx was performed for 5 min in the presence and absence of bumetanide. K^+ influx was expressed as nmol/mg per min. Data represent mean ± S.D. for four determinations. n.s. = not significant.

	$^{42}K^+$	$^{86}Rb^+$	P
Total	13.44 ± 0.44	14.99 ± 0.35	n.s.
Bumetanide (10 μM)	7.14 ± 0.13	7.90 ± 0.42	n.s.
Bumetanide sensitive	6.30 ± 0.47	7.09 ± 0.42	n.s.

K^+ influx

The time-course of K^+ influx into normal mTAL cells, and the effect of bumetanide (10 μM) on K^+ influx from a 5 mM K^+ assay buffer is shown in Fig. 1. The curve for total K^+ influx, i.e., in the absence of bumetanide, corrected for zero time values (Fig. 1, open circles) was fitted according to the first-order equation $C_t = C_f(1 - e^{-kt})$, where C_t and C_f are the cell labels at time t and infinity respectively, and k (min^{-1}) is the rate constant for influx. Total K^+ influx, computed from Fig. 1 and expressed as an initial flux

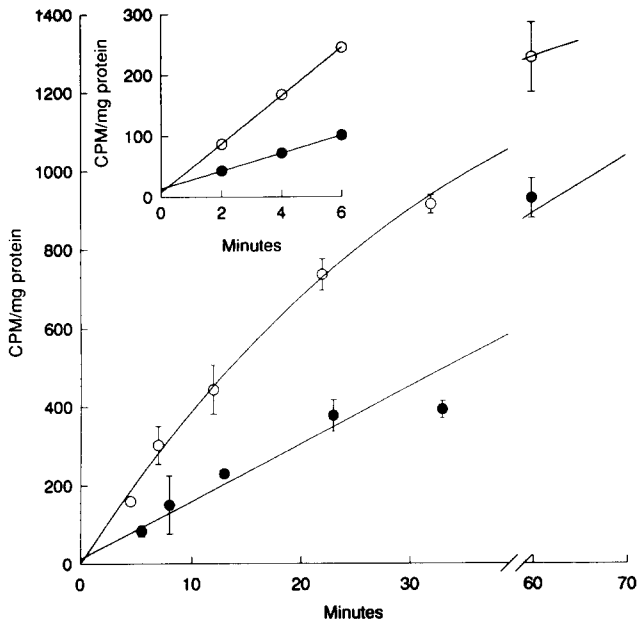

Fig. 1. Effect of bumetanide on potassium influx. Cells were incubated at 37°C with and without 10 μM bumetanide in media containing 145 mM NaCl and 5 mM KCl. At zero time, $^{86}Rb^+$ was added and the cells were assayed for radioactivity at various times as shown. The solid line for the total K^+ uptake (in the absence of bumetanide, (○) represents a theoretical line derived from a nonlinear regression ($C_t = C_f(1 - e^{-kt})$, whereas the solid line for bumetanide resistant uptake (●) represents a theoretical line from a linear regression. The inset shows that both total and bumetanide resistant uptakes were linear for the initial 6 min. Average of three different experiments, each in duplicate, is shown.

TABLE II

Effect of removing external Na^+, external Cl^- or adding bumetanide on total K^+ influx

BS K^+ influx was measured in transport assay buffer (145 mM NaCl, 5 mM KCl, see Materials and Methods for full composition), in media with nitrate substituting for Cl^- (zero Cl^-), in media with *N*-methyl glucamine Cl substituting for Na^+ (zero Na^+) or in transport assay buffer (145 mM NaCl, 5 mM KCl) with 10 μM bumetanide added. Average of three different experiments. * denotes $P < 0.001$ compared to Total.

	K^+ influx (nmol/mg per min)
Total	15.21 ± 0.48
Zero Cl^-	6.74 ± 0.22 *
Zero Na^+	5.12 ± 0.34 *
Bumetanide	7.65 ± 2.32 *

rate was 16.2 mmol/mg protein per min. As shown in the inset, both the total K^+ influx and the K^+ influx in the presence of bumetanide were linear for a period of at least 5 min at 37°C. Calculation of the initial rate from linear data obtained over the first 5 min (Fig. 1, inset) gave the same result as the initial rate computed from the first-order rate constant derived from the data points over 60 min. bumetanide reduced the initial flux rate by 55% to 7.3 nmol/mg protein per min. In various studies, bumetanide inhibited between 50% to 60% of the total K^+ influx.

Effect of removal of Na^+ or Cl^- on K^+ influx

The removal of external Na^+ (*N*-methylglucamine replacement), the removal of external Cl^- (nitrate replacement) and the addition of 10 μM bumetanide resulted in a significant decrease in K^+ influx compared to control condition ('Total' in Table II, $n = 6$ and $P < 0.001$ for each of the three experimental conditions). The portion of K^+ influx that was sensitive to external Cl^- removal (8.47 ± 0.53), to external Na^+ removal (10.09 ± 0.59) and to bumetanide addition (7.56 ± 2.37) was not statistically different. This finding strongly suggests that the bumetanide sensitive K^+ influx represents $Na^+/K^+/2Cl^-$ cotransport.

Anion specificity of cotransport

Table III shows the K^+ influx in cells from media with Cl^- replaced by various monovalent permeant anions. For comparison, the K^+ influx in Cl^- media with bumetanide is shown in the bottom row. The difference with and without bumetanide represents the BS K^+ influx. With 150 mM Br^-, K^+ influx was unchanged. K^+ influx was inhibited by all other substitute anions ($P < 0.01$ compared to Cl^- alone). Cl^- substitution with sulfamate (or methyl sulfate, not shown) resulted in greater inhibition of total K^+ influx than did substitution with NO_3^-, I^- or SCN^-, or addi-

TABLE III

Effect of various anions on K^+ influx

The effect on K^+ influx of complete replacement of external Cl^- with various monovalent anions (all at 150 mM). Cells were incubated in media with various substitute anions for 15 min at 37°C before addition of $^{86}Rb^+$. For comparison, K^+ influx in Cl^- media with 10 μM bumetanide is shown in the last row. $n = 8$, * denotes $P < 0.01$.

	K^+ influx (nmol/mg per min)
Chloride	16.6 ±1.44
Bromide	17.2 ±2.73
Thiocyanate	13.8 ±0.60 *
Iodide	9.94±2.16 *
Nitrate	8.61±0.44 *
Sulfamate	3.93±0.64 *
Chloride ± bumetanide	9.82±1.20 *

tion of bumetanide (Table III), suggesting that sulfamate may have an inhibitory effect on the bumetanide resistant component of K^+ influx. To ascertain whether SCN^- supported $Na^+/K^+/2Cl^-$ cotransport partially, we added bumetanide (10 μM) to 150 mM SCN^- media. In SCN^- media, addition of bumetanide did not inhibit K^+ influx further (13.55 ± 1.25 vs. 13.89 ± 1.89, n.s., $n = 4$, not shown), suggesting that bumetanide-resistant K^+ influx was higher with SCN^-. These results suggest that $Na^+/K^+/2Cl^-$ cotransport was highly selective in its requirement for Cl^- or Br^-.

Concentration dependence of bumetanide inhibition

Fig. 2 represents the concentration dependence of bumetanide inhibition. Inhibition of 50% of cotransport (defined as the Cl^--dependent K^+ influx) was achieved at 180 ± 31 nM (0.18 μM). Inhibition was nearly complete (> 95%) at 10 μM bumetanide.

Kinetics of $Na^+/K^+/2Cl^-$ cotransport

External Na^+ dependence of BS K^+ influx was investigated at physiologic external K^+ (5 mM) by replacing Na^+ with *N*-methylglucamine. Replacing Na^+ with tetramethylammonium in one experiment gave similar results. By nonlinear curve fitting, the apparent K_m was 7.3 ± 2.1 mM and the apparent V_{max} was 8.4 nmol/mg per min (Fig. 3). The Eadie-Hofstee plot (inset) was linear, and kinetic constants derived from that plot did not differ from those derived from nonlinear curve fitting.

External K^+ dependence was similarly investigated by replacing external K^+ with *N*-methyl glucamine. The affinity for external K^+ was high, with apparent K_m of 1.3 ± 0.4 mM (Fig. 4), and an apparent V_{max} of 8.9 nmol/mg per min.

External Cl^- dependence of BS K^+ influx was complex and the results varied with the nature of the

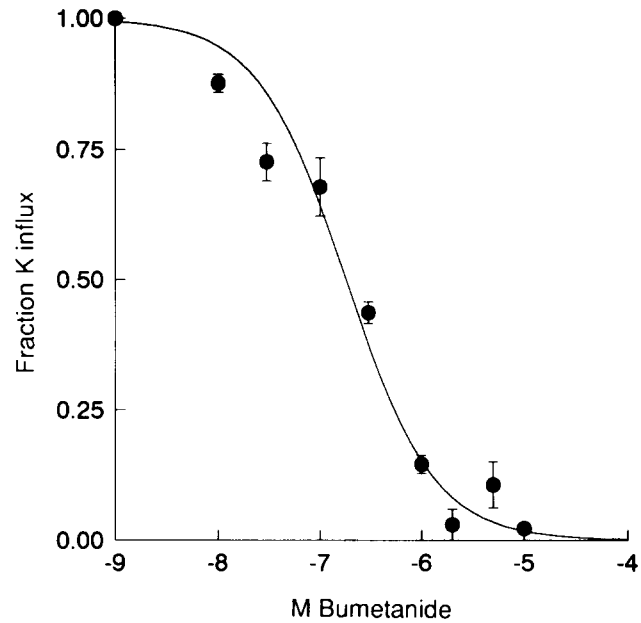

Fig. 2. The fraction of cotransport mediated K^+ influx remaining as a function of bumetanide concentration plotted on a logarithmic scale. The Cl^--dependent K^+ influx, i.e., the difference in flux in 150 mM Cl^- and 150 mM NO_3^- media was taken as the measure of cotransport. The solid line is the theoretical line derived from nonlinear regression equation $J_B/J_T = K_d/(K_d + B)$, where J_B and J_T represent the K^+ influx in the presence and absence of bumetanide respectively and K_d is the bumetanide concentration required for 50% inhibition of cotransport. The calculated K_d was 0.180±0.031 μM. Average ± S.D. of four separate experiments, each in duplicate, is shown.

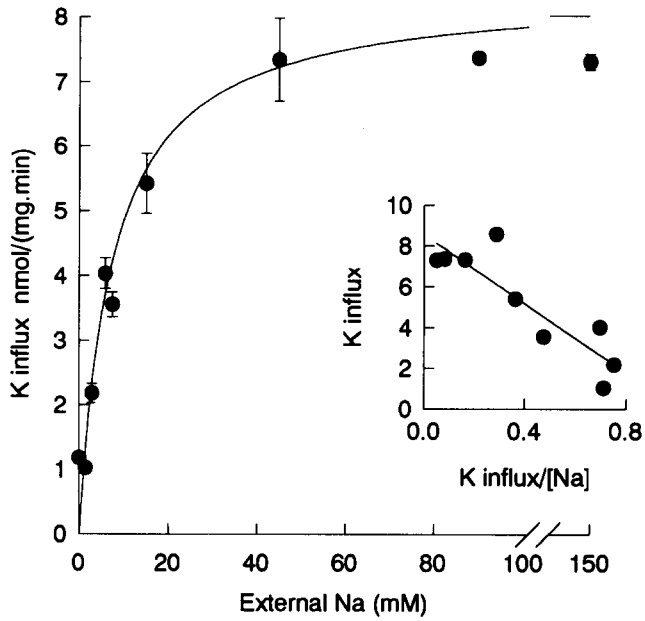

Fig. 3. Dependence of BS K^+ influx on external Na. Cells were incubated at 37°C in media containing Cl^- salts of (Na + *N*-methylglucamine) 145 mM, K^+ 5 mM, with $^{86}Rb^+$, with or without 10 μM bumetanide. The solid line represents the curve fitted with nonlinear regression using experimental data points. Error bars were omitted when smaller than symbols. Average ± S.D. of five experiments, each in duplicate. Inset shows Eadie-Hofstee plot.

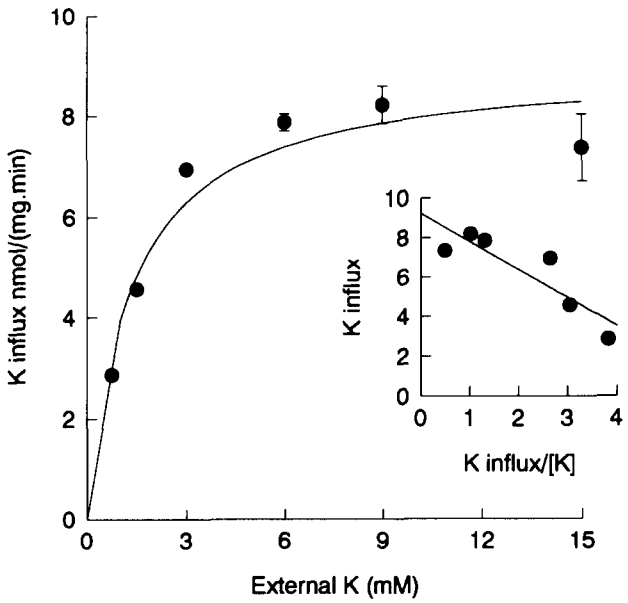

Fig. 4. Dependence of BS K^+ influx on external K^+. Cells were incubated in media with 150 mM Cl^- salts of Na, K or *N*-methylglucamine with or without 10 μM bumetanide. K^+ was replaced by *N*-methylglucamine. Inset shows Eadie-Hofstee plot. Average ± S.D. of six experiments, each in duplicate.

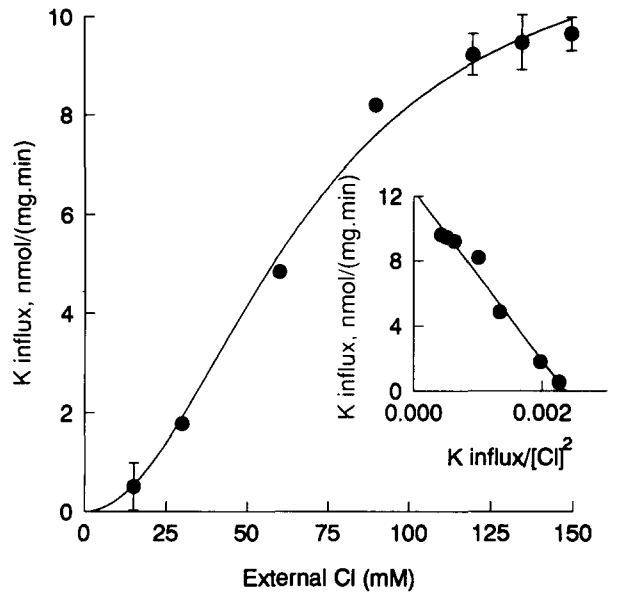

Fig. 5. bumetanide sensitive K^+ influx as a function of external Cl^- concentration. Cells were incubated in 150 mM (Cl + gluconate) salts of Na^+ (145 mM) and K^+ (5 mM) with $^{86}Rb^+$, with or without bumetanide. Cl^- was replaced by gluconate. The equation for non-linear regression was $v = V_{max}(1 + (K_m/s)^2)$, where v is the BS K^+ influx and V_{max} and K_m represent the apparent V_{max} and K_m values. Inset shows an Eadie-Hofstee plot of the same data using the two site model. Average ± S.D. of five experiments, each in duplicate.

replacing anion. The curve of K^+ influx as a function of external Cl^- concentration ($[Cl^-]_o$) was linear with nitrate and concave upward with methylsulfate or sulfamate (not shown). With gluconate as the replacement anion, a sigmoidal dependence was obtained (Fig. 5). Cell water content was not different in 150 mM Cl^- and 150 mM gluconate media (4.05 ± 0.25 vs. 3.99 ± 0.36, n.s.), suggesting that the cells did not shrink in the presence of impermeant gluconate. The Eadie-Hofstee plot was linear only if two binding sites were assumed (inset). Attempts to analyze the data using two Cl^- sites of different affinity failed; plot of v/s^2 vs. v did not show any break from linearity. Assuming two Cl^- binding sites with equal affinities, nonlinear iterative curve fitting gave an apparent $K_{0.5}$ of 68 ± 7 mM and an apparent V_{max} of 11.9 nmol/mg per min. Transformation of the above kinetic data yielded Hill coefficients of 0.8, 0.8 and 2.2 for Na^+, K^+ and Cl^-, respectively (Hill plots not shown), consistent with a 1:1:2 stoichiometry for $Na^+/K^+/Cl^-$.

$^{86}Rb^+$ efflux

The fraction of $^{86}Rb^+$ remaining in the cells decreased in a exponential fashion (Fig. 6). The fraction of cell K^+ remaining (y) was computed as $y = a \cdot e^{-kt}$, where a is the fraction at $t = 0$, k is the efflux rate constant and t is the time in min. The rate constant of efflux without the addition of inhibitors was 0.039 ± 0.003 min^{-1} (open circles). Somewhat surprisingly, bumetanide failed to inhibit K^+ efflux, ($k = 0.044 \pm$

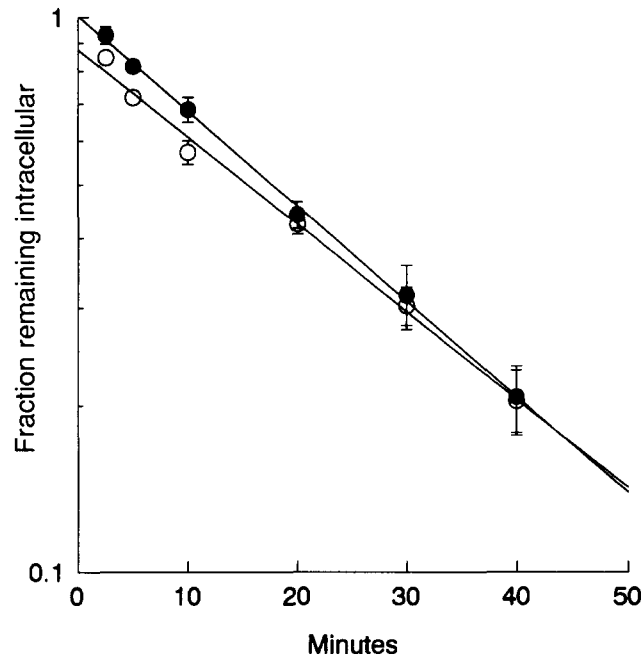

Fig. 6. Lack of effect of bumetanide (10 μM) on $^{86}Rb^+$ efflux. Cells were loaded with $^{86}Rb^+$ for 3 h, washed and incubated in isotope-free media with (•) and without (○) bumetanide (10 μM). The media contained NaCl (145 mM) and KCl (5 mM). The fraction of radioactivity remaining in cells was plotted on a logarithmic scale as a function of time. Average ± S.D. of six experiments, each in duplicate.

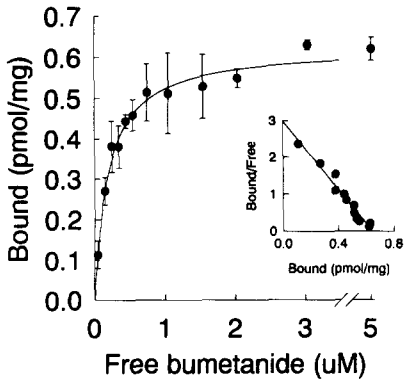

Fig. 7. The specific [^{3}H]bumetanide bound to intact mTAL cells as a function of free bumetanide concentration. Cells were incubated for 30 min in media contaning NaCl 145 mM and KCl 5 mM, glucose 5 mM (pH 7.4) at 37°C. Nonspecific binding (i.e., binding in the presence of 100 μM unlabeled bumetanide) was about 50% of the total binding and was subtracted from total binding to yield the specific bumetanide binding plotted here. The solid line represents the theoretical curve given by fitting the experimental data points to the equation $B = B_{max} \cdot F/(K_d + F)$, where B is the amount of bound bumetanide, F is the free bumetanide concentration and B_{max} is the maximum binding. Inset: Scatchard plot of the same data, showing a linear fit. Average ± S.D. of eight experiments, each in duplicate.

0.003), indicating an absence of any appreciable bumetanide-sensitive K/K exchange in this cell (Fig. 6).

Bumetanide binding

Specific [^{3}H]bumetanide binding (i.e., binding inhibited by excess unlabeled bumetanide) was a saturable function of free bumetanide concentration (Fig. 7). The best fit for the Scatchard plot was linear (Fig. 7, inset), suggesting a single binding site for bumetanide. Half-maximum binding was observed at 0.199 ± 0.039 μM, a value that agreed well with the bumetanide concentration required for 50% of Cl^- dependent K^+ influx (0.180 μM, Fig. 2). The maximum specific [^{3}H]bumetanide binding was 0.631 pmol/mg, corresponding to approx. 53 000 sites per cell.

Total [^{3}H]bumetanide binding, measured at a near saturating concentration of 1 μM, was significantly decreased by the addition of 100 μM unlabeled bumetanide, and the removal of external Na^+, K^+ or Cl^- ($P < 0.001$ compared to total binding, $n = 6$, Table IV). In contrast to results obtained with dog medullary membranes [7], NO_3^- did not support bumetanide binding. Specific [^{3}H]bumetanide binding, i.e., binding that was inhibited by the presence of 100-fold excess of unlabeled bumetanide, represented 55% of the total bumetanide binding. The dependence of specific [^{3}H]bumetanide binding on Na^+ and Cl^- provides strong support for the suggestion that specific bumetanide binding to mTAL cells represents binding almost exclusively to the $Na^+/K^+/2Cl^-$ cotransporter molecule.

TABLE IV

Effect of substrate removal on total bumetanide binding

The effect of adding excess unlabeled bumetanide (nonspecific), removing external Na^+ (with *N*-methylglucamine replacement), removing external K^+ (*N*-methylglucamine replacement) and removing external Cl^- (NO_3^- replacement) on total [^{3}H]Bumetanide binding. bumetanide concentration was 1 μM under all conditions, except when nonspecific binding was measured, then the concentration was 100 μM. For total and nonspecific binding, the medium contained NaCl 145 mM, KCl 5 mM, glucose 5 mM (pH 7.4) as described in the legend to Fig. 7. * denotes $P < 0.001$, $n = 6$.

	[^{3}H]Bumetanide binding (pmol/mg protein)
Total	1.02 ± 0.11
Excess unlabeled bumetanide	0.47 ± 0.09 *
Zero external Na^+	0.53 ± 0.12 *
Zero external K^+	0.61 ± 0.19 *
Zero external Cl^-	0.52 ± 0.10 *

We next examined the effect of medium osmolality on K^+ influx and specific [^{3}H]bumetanide binding. Cells were exposed for 30 min to media with varying osmolalities by addition of increasing sucrose concen-

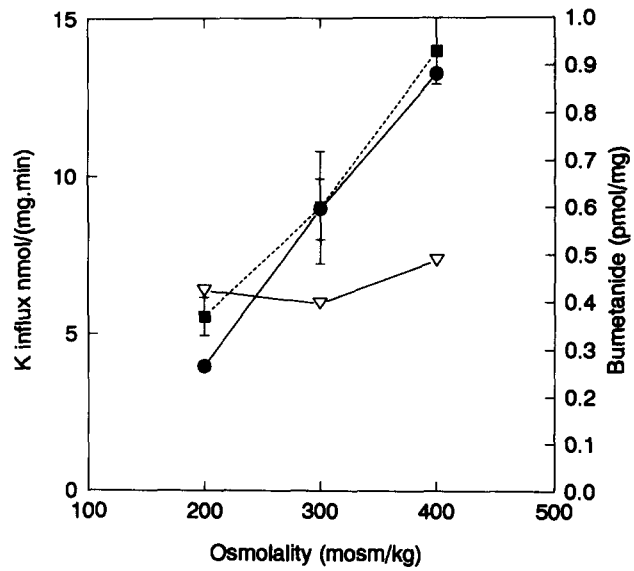

Fig. 8. Effect of varying osmolality on K^+ influx and bumetanide binding. For bumetanide binding, cells were incubated for 30 min in media containing NaCl 95 mM, KCl 5 mM, with 0, 100 or 200 mM sucrose, glucose 5 mM (pH 7.4). Radiolabeled bumetanide was added at zero time. Total and nonspecific bumetanide binding were performed at bumetanide concentrations of 1 and 100 μM, respectively. For K^+ influx, cells were preincubated in the same media for 26 min at 37°C. $^{86}Rb^+$ was then added and influx was allowed to continue for 4 min. bumetanide sensitive K^+ influx (●) and bumetanide binding (■) increased in a co-ordinate fashion with increasing osmolality, whereas bumetanide-resistant K^+ influx (▽) did not change significantly with increasing medium osmolality. Error bars were omitted when smaller than symbols. Average ± S.D. of six experiments, each in duplicate.

TABLE V

Simultaneous transport and bumetanide binding in mTAL cells

BS K^+ influx and bumetanide binding measured from different wells of a 12-well cluster plated on the same day with the same number of cells. The cell counts and proteins were measured in the same well as described in Materials and Methods. $(6.87 \pm 0.61) \cdot 10^6$ cells correspond to 1 mg protein. The [^{3}H]Bumetanide binding sites per cell were calculated using the Avogadro number, assuming that one transporter molecule binds to one molecule of bumetanide. The turnover number was calculated by assuming that one cotransporter molecule transports one K^+ ion per transport cycle. ** $P < 0.001$ and ** $P < 0.05$ from results in medium osmolality of 300 mosmol/kg.

Medium osmolality (mosmol/kg)	BS K^+ influx (nmol/10^9 cells per min)	[^{3}H]Bumetanide binding (sites per cel)	Turnover number (s^{-1})
200	581 ± 16*	31556 ± 3492*	187 ± 17 **
300	1302 ± 140	52573 ± 10779	255 ± 68
400	1927 ± 28 *	82381 ± 6218 *	237 ± 20

trations to a solution containing 95 mM NaCl and 5 mM KCl (pH 7.4). Because of the low water permeability in this cell, we used a long incubation period to achieve changes in cell water content. Bumetanide-resistant K^+ influx (inverted open triangles, Fig. 8) did not change with changes in medium osmolality. BS K^+ influx (filled circles) was 8.99 ± 0.97 nmol/mg per min in 300 mosM media and the corresponding bumetanide binding was 0.61 ± 0.12 pmol/mg, or 52573 sites per cell (Table V). The calculated turnover number was 255 per second (Table V) In hypotonic media, BS K^+ influx was reduced to 3.98 ± 0.11 nmol/mg per min, a 56% decrease. compared to 300 mosM media ($n = 6$, $P < 0.001$). Specific bumetanide binding also decreased from 0.61 ± 0.12 to 0.36 ± 0.04 pmol/mg, a 40% decrease ($P < 0.001$, $n = 6$). The calculated turnover number was 185 ± 21 per second (Table V). Thus, most of the decrease in BS K^+ influx was explained by a corresponding decrease in bumetanide binding, but a small ancillary contribution of other factors can not be ruled out. In hypertonic media, BS K^+ influx increased 47%, from 8.99 ± 0.97 to 13.22 ± 0.19 pmol/mg per min ($P < 0.001$). Specific [^{3}H]bumetanide binding (filled squares, Fig 8, Table V) also increased, from 0.61 ± 0.12 pmol/mg in hypotonic media to 0.93 ± 0.07 pmol/mg, a 5.6% increase ($P < 0.001$). Thus, despite a major increase in BS K^+ influx in hypertonic media, the turnover number was virtually unchanged at 237 per second (Table V).

Polarity of mTAL cells

Valentich and Stokols [16] noted that mouse mTAL cells in culture exhibited (a), structural polarity, since dense villi were noted only on the apical surface (facing up) and (b), functional polarity, since mouse mTAL cells grown on plastic dishes exhibited dome formation. We confirmed these findings and in addition examined whether $Na^+/K^+/2Cl^-$ cotransporters were sorted to the apical surface, as in intact mTAL tubules. When grown to confluence on permeable filter supports (25-mm^2 surface area, 0.45 μm pore size, high pore density Cyclopore filters, Falcon, Livingston, NJ, USA), the BS K^+ influx from the apical (facing up) and the basolateral surface measured 7.93 ± 0.89 and 1.12 ± 0.23 nmol/mg per min respectively ($n = 6$). [^{3}H]Bumetanide binding, performed at a ligand concentration of 1 μM, averaged 0.65 ± 0.15 and 0.11 ± 0.06 pmol/mg on the apical and the basolateral membrane. Thus, $Na^+/K^+/2Cl^-$ cotransport (as defined by BS K^+ influx and bumetanide binding) was predominantly (85%) apical in the mTAL cells. Furthermore, we found no decay of BS K^+ influx with repeated passages (not shown). These findings suggest that the cultured mouse mTAL cells may be a good model for the study of $Na^+/K^+/2Cl^-$ cotransport in mTAL.

Discussion

Our results suggest that the cultured mouse mTAL cells used in the present study provide a good model for the study of $Na^+/K^+/2Cl^-$ cotransport. Structural and functional polarity was established in these cells, as shown by the presence of microvilli on the apical surface, the presence of dome formation on plastic surfaces and the sorting of $Na^+/K^+/2Cl^-$ cotransporters to the apical surface on filters. In comparison with total K^+ influx, the fraction of BS K^+ influx was large, and no decay in cotransport activity was discernible with repeated cell passages. While further studies are required to establish the extent to which these cells preserve the phenotypic expression of mTAL tubules, the cells appear suitable for examining $Na^+/K^+/2Cl^-$ cotransport in mTAL.

Kinetics

The kinetics of the $Na^+/K^+/2Cl^-$ cotransport in the TAL remain controversial. In isolated medullary plasma membrane vesicles, a relatively high affinity for Cl^- (low apparent K_m) was reported by two different groups [4,6]. Thus, Koenig et al. reported an apparent K_m of 15 mM for external Cl^-, and Burnham et al. reported maximum cotransport at 50 mM, a finding that can be interpreted to support the idea that Cl^- affinity in medullary vesicles is high [4,6]. In contrast, in the cortical TAL, kinetics of equivalent I_{SC} revealed a low affinity for Cl^- (apparent $K_m = 50$ mM [3]). A low affinity for Cl^- (apparent $K_m = 41$ mM) was also inferred from the kinetics of CO_2 production from [^{14}C]lactate in cortical TAL tubules [21], and from O_2 consumption from mTAL (K_m 79 mM [22]). In a recent review, Reeves and Andreoli attributed these differences in apparent K_m values to axial heterogene-

ity (true differences in affinity between the cortical and medullary TAL [1]). However, if axial heterogeneity accounted for the differences in affinities reported previously, we would expect high Cl^- affinity in mTAL cells. Instead, our findings suggest that the cotransporter has a low affinity for Cl^- in the mTAL, as others have described in the cortical TAL [3,21]. Thus, it appears more likely that the differences in results stem from some other difference in experimental conditions.

The differences in Cl^- affinity between our studies on one hand and the studies of Koenig et al. [4] and Burnham et al. [6] in mTAL vesicles on the other hand, may, at least in part, be explained by the unphysiological low intravesicular substrate concentrations used by these workers [4,6]. Both groups used vesicles with sucrose and Tris but no Na^+, K^+ or Cl^- [4,6]. A decrease in *trans* concentration of the substrate (Cl^-) may result not only in a decrease in rate of transport (absence of *trans* acceleration), but also in a decrease in the apparent K_m [23,24]. The lower apparent K_m with unphysiological, low *trans* (intravesicular) and high *cis* (external) substrate concentrations in the vesicle studies [4,6] may reflect a greater prevalence of transporters in the inward facing conformation and a corrosponding decrease in the fraction of transporters in the outward facing conformation. Because there are less transporter sites to saturate on the external surface, a smaller substrate concentration is required to produce half-maximum stimulation of flux, resulting in a lower apparent K_m for influx in the vesicle studies, even if the intrinsic binding constants are identical. These changes in apparent or observed K_m values secondary to changes in *trans* substrate concentrations are well known to kineticists [23,24]. Thus, the present data are consistent with the postulate that low Cl^- concentrations in the lumen may be rate limiting for transepithelial Cl^- reabsorption in the mTAL. At physiological, high *trans* (intracellular) ion concentrations, the apparent K_m for Cl^- may be quite high.

We observed a high cotransporter affinity for Na^+ and K^+ with apparent K_m of 7.3 and 1.3 for Na^+ and K^+, respectively. While some estimates of lower Na^+ and K^+ affinities have been reported in transport studies in vesicles (see above and Ref. 4), and in indirect studies which measured O_2 consumption [22], our data are consistent with relatively high Na^+ and K^+ affinity observed by Greger in intact cortical TAL [2] and with data obtained for the cotransporter in other cell types [25,26]. Taken together, the data suggest that transepithelial salt reabsorption from the lumen may be limited by Cl^-, but not by Na^+ or K^+.

Absence of K/K exchange

In most cells, including renal mesangial and epithelial (MDCK) cells, the $Na^+/K^+/2Cl^-$ cotransport mediates both unidirectional K^+ influx and efflux [25–32]. Thus, the cotransport performs a sizable obligatory 1:1 K/K exchange [25–32], either exclusively or in addition to performing a net cation and Cl^- influx. The K/K exchange is attributed to a partial reaction of the carrier [25,27]. In contrast, BS K^+ efflux was absent in the mTAL cell. The latter finding suggests that the $Na^+/K^+/2Cl^-$ cotransporter performs net cation flux exclusively and is, thus, asymmetric in the mTAL cell. Asymmetry of transport proteins in general and of $Na^+/K^+/2Cl^-$ cotransport in particular has been described previously [23–25,33–35]. We propose that in the mTAL, the cotransporter is loaded at the external surface with Na^+, K^+ and 2 Cl^- ions, undergoes a conformational change so that the binding sites face inward and then debinds cations and Cl^-. Based on the absence of K/K exchange, we postulate that the cotransporter then returns to the external surface empty, completing the transport cycle. The reason why most of the cotransporter molecules return empty rather than in a fully loaded state in the mTAL cell is not clear. Cl^- is believed to be the first ion to bind on the cotransporter at the internal surface [27]. A very low intracellular Cl^- concentration, or low cotransporter affinity for Cl^- at the internal aspect may result in lack of occupation of ion-binding sites on the cotransporter, return of the empty cotransporter to the external aspect and thus the lack of BS K^+ efflux. The finding that the cotransport performs only net influx in the mTAL cell may be physiologically important; since K/K exchange represents a futile cycle in terms of net transport, the absence of K/K exchange confers increased efficiency on the mTAL cell for transepithelial NaCl reabsorption.

[^{3}H]Bumetanide binding

The characteristics of [^{3}H]bumetanide binding to mTAL cells are consistent with the view that the bumetanide binding represented the number of functioning sites. First, specific bumetanide binding was dependent on the simultaneous presence of Na^+ and Cl^- (Table IV). Second, the K_d for bumetanide binding (199 nM) agreed well with the IC_{50} for bumetanide inhibition of cotransport-mediated K^+ influx (180 nM), and a tight correlation was observed between inhibition of K^+ influx and [^{3}H]bumetanide binding (not shown). Third, cell shrinkage, a maneuver that increased the activity of cotransport, also increased bumetanide binding (Fig. 8, Table V). Taken together, these findings provide strong support to the suggestion [7–15] that specific [^{3}H]bumetanide binding is a measure of the number of functioning $Na^+/K^+/2Cl^-$ cotransporter sites.

We found evidence for a single bumetanide binding site with a high affinity for bumetanide (K_d 0.2 μM) Calculation of bumetanide binding revealed a B_{max} of

approx. 53 000 sites per cell, a value in the same range as that found in vascular smooth muscle [9], endothelial [10] and HeLa cells [36]. From simultaneous measurements of BS K^+ influx and bumetanide binding under identical conditions, we calculated that the turnover number for the cotransporter was 248 ± 16 s^{-1}. Various workers have reported turnover numbers from 60 s^{-1} in HT29 colonic carcinoma cells [37] to 4000 s^{-1} in avian erythrocytes [8]. While other workers have provided estimates for turnover number in a variety of cell types, few investigators have measured both flux and binding simultaneously and under identical conditions. Our value of 248 s^{-1} agrees quite well with the value of 293 s^{-1} in aortic endothelial cells where ion fluxes and bumetanide binding were measured simultaneously and under identical conditions [10]. Changes in BS K^+ influx (with changes in medium osmolality) were accompanied by coordinate changes in the bumetanide binding sites. The proportional rise in BS K^+ influx and [^{3}H]bumetanide binding in hypertonic media suggests that the increase in ion flux was most likely consequent to an increase in the number of cotransporter sites. Our studies do not allow us to decide whether the increase in the number of cotransporter sites reflects increased synthesis of sites, decreased degradation, recruitment of sites from a cytoplasmic pool or simply a change in transporter conformation such that more sites are available for binding to the external ligand.

It is worth noting that our results are not in accord with the results obtained recently in the shark rectal gland. In the shark rectal gland, $Na^+/K^+/2Cl^-$ cotransport was stimulated not only with cell shrinkage but also with cell swelling [13,14]. As osmolality was increased from hypotonic to hypertonic media, bumetanide binding decreased to a minimum value at isotonicity (approx. 900 mosmol/kg for the shark) and then increased again in hypertonic media. In contrast, we observed a monotonic increase in [^{3}H]bumetanide binding and BS K^+ influx in mouse mTAL cells with the increase in medium osmolality from 200–400 mosmol/kg. Earlier workers also observed an increase in cotransport in isolated mTAL cells with cell shrinkage [38,39]. The reason(s) for the difference in results is not clear, but is likely related to species difference. Marine animals living in media that would be hypertonic for land mammals may have a different intracellular Cl^- concentration ($[Cl^-]_i$) as compared to other mammals. If $[Cl^-]_i$ is a regulator of the activity of $Na^+/K^+/2Cl^-$ cotransport, as postulated [13,14], the differences in resting $[Cl^-]_i$ between the mouse and the shark may explain why the cotransporter in the shark rectal gland behaves differently from the mouse. In the shark rectal gland, $[Cl^-]_i$ may be a more important regulator of cotransport than cell volume, whereas cell volume may be more important than $[Cl^-]$ as a regulator of cotransport in mTAL cells of mouse and other land mammals.

In summary, the present studies show that the mouse mTAL cells in culture show a robust expression of $Na^+/K^+/2Cl^-$ cotransport. $Na^+/K^+/2Cl^-$ cotransporters are localized to the apical membrane of cultured mouse mTAL cells, as in the freshly dissected, microperfused mTAL tubules. The Cl^- affinity of intact cultured mTAL cells is low (apparent K_m 68 mM). The $Na^+/K^+/2Cl^-$ cotransporter in mTAL cells mediates only unidirectional net influx, and is therefore asymmetric. Bumetanide binding reveals a single class of high-affinity binding sites (K_d 0.2 μM) with approx. 53 000 sites per cell. Simultaneous mesurements of transport and bumetanide binding yield a turnover number of 248 s^{-1}. Changes in medium osmolality result in coordinate changes in BS K^+ influx and [^{3}H]bumetanide binding sites. Furthermore, both BS K^+ influx and bumetanide binding show a monotonic increase as osmolality is increased from hypotonic to hypertonic values.

Acknowledgements

This work was supported by the Merit Review Grant, Veterans Affairs, Washington, DC. I thank Ms. J Diaz for excellent technical assistance and Dr. Thomas Kahn for reading the paper.

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