Transport and regulatory properties of the apical Na-K-2Cl cotransporter of macula densa cells

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1Groupe de Recherche en Transport Membranaire, Universite´ de Montr´eal, Montreal, Quebec, Canada H3C 3J7; and 2Nephrology Research and Training Center, Division of Nephrology and Departments of Medicine and Physiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Laamarti, M. Anuar, P. Darwin Bell, and Jean-Yves Lapointe. Transport and regulatory properties of the apical Na-K-2Cl cotransporter of macula densa cells. Am. J. Physiol. 275 (Renal Physiol. 44): F703–F709, 1998. NH4+ is used to probe apical Na-K-2Cl transport activity of macula densa (MD) cells from rabbit kidney. In the presence of 25 mM NaCl and 5 mM Ba2+, addition of 20 mM NH4+ to the lumen produced a profound intracellular acidification, and ~80% of the initial acidification rate was bumetanide sensitive. The NH4+-induced acidification rate was dependent on luminal Cl− and Na+ with apparent affinities of 17 ± 4 mM (Hill number 1.45) and 1.0 ± 0.3 mM, respectively. In the presence of saturating luminal NaCl concentration ([NaCl]L), blockage of basolateral Cl− efflux with 10 μM 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) reduced the NH4+-induced acidification rate by 51 ± 6% (P > 0.01, n = 5). Under similar conditions, dibutyryl-cAMP (DBcAMP) + forskolin increased the NH4+-induced acidification rate by 27%, whereas it produced no detectable effect at low luminal NaCl concentration. Most of the observed DBcAMP + forskolin effect was probably due to the stimulation of the basolateral Cl− conductance, since, in the presence of basolateral NPPB, this activation was changed to a 17.1% and 16.6% inhibition of the NH4+-induced acidification rate observed at high or low [NaCl]L, respectively. We conclude that the cotransporter found in MD cells displays, with respect to other Na-K-2Cl cotransporters, a relatively high affinity for luminal Na+ and luminal Cl− and can be specifically inhibited by increases in intracellular Cl− and cAMP concentrations.

Sodium-potassium-chloride cotransport; adenosine 3',5'-cyclic monophosphate; forskolin; protein kinase A; bumetanide; kinetics

MACULA DENSA (MD) cells are thought to function as sensor devices detecting increases in luminal NaCl concentration ([NaCl]L) and initiating signals controlling renin secretion and tubuloglomerular feedback (TGF). Since both renin secretion and TGF are sensitive to bumetanide (or furosemide) (19, 46), the apical Na-K-2Cl cotransporter found in MD cells (28, 40) is very likely to be responsible for detecting changes in [NaCl]L. After many years of investigation, the exact nature of the signal(s) transmitted to smooth muscle and granular cells remains elusive, although a number of factors capable of modulating signal transmission have been identified (42). These include angiotensin II (20), adenosine (9, 41), arachidonic acid metabolites (3, 8, 50), cAMP (2), Ca2+ (3), and nitric oxide (45). Some of these factors are probably involved in the adjustment of TGF amplitude and sensitivity, which are expected in different physiological conditions (5), but the mechanism of action and, sometimes, even the cell type affected remain uncertain.

In terms of the MD cells, nothing is known about the effect of any of the factors mentioned above on the different transport pathways already identified in these cells (4, 7, 21, 27–29, 40). A central mechanism in the function of MD cells is the apical Na-K-2Cl cotransporter, which, under the ionic conditions prevailing ([NaCl]L = 20–60 mM) at the end of the thick ascending limbs (TAL), mediates NaCl reabsorption and is exquisitely sensitive to changes in [NaCl]L (30). Following the cloning of the thiazide-sensitive Na-Cl cotransporter (11) and the cloning of the secretory form of the Na-K-2Cl cotransporter (47), investigators have identified specific isoforms of the Na-K-2Cl cotransporters involved in renal reabsorption (NKCC2 or rBSC1) in rabbit (36), rat (10), and mouse (22). Mouse NKCC2 and rat BSC1 are ~97% identical and are localized to the apical membrane of medullary and cortical TAL (MTAL and CTAL, respectively) (24, 32). In the case of MD cells, anti-rBSC1 antibody failed to detect a significant signal in rat MD cells, but, after denaturation with SDS and 2-mercaptoethanol, a weak signal could be found (24). Using an antisense probe for the apical form of the Na-K-2Cl cotransporter, Obermuller et al. (33) found mRNA in both TAL and MD cells from rat and rabbit kidney. More specifically, the “B” isoform of the NKCC2 cotransporter was detected in rat MD-containing tubule segments using polymerase chain reaction with isoform-specific primers (48).

In this study, we proceed on the previously established fact that, in the presence of Ba2+, more than 80% of the luminal NH4+-induced acidification rate is bumetanide sensitive. As discussed earlier (27), this indicates that NH4+ is taken up by the Na-K-2Cl cotransporter and dissociates within the cell to H+ + NH3. This provides a sensitive method with which to measure the NH4+ influx rate, obtain apparent affinity constants for luminal Na+ and Cl−, and identify some of the intracellular factors capable of modulating the activity of MD apical Na-K-2Cl cotransporter.

MATERIALS AND METHODS

Micropерfusion and fluorescence measurements. Micropерfusion of rabbit CTALs dissected with their attached glomeruli was performed exactly as described in a recent study from this laboratory (27). Tubules were bathed with a bicarbonate-free solution containing (in mM) 146 NaCl, 5 potassium gluconate, 1 MgCl2, 1 CaCl2, 5 glucose, 10 HEPES, and 7.2 Tris. Luminal solutions were identical to the bathing solution in the microperfusion system.
solution with the exception that the [NaCl], was maintained at 25 mM by isosmotically replacing Na+ with N-methyl-


glucamine (NMDG) and Cl− with cyclamate. Addition of luminal NH4+ (20 mM) and/or Ba2+ (5 mM) was accomplished by isosmotically replacing NMDG-cyclamate with either ammonium acetate or barium acetate at constant luminal Na+ concentration ([Na+]L) and [Cl−]L. All solutions were adjusted to a pH of 7.4 and all experiments were performed at 39°C.

Intracellular pH (pHi) was measured using the fluorescent probe 2−,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) as previously described (7, 27, 30). Intracellular dye was alternately excited at 500- and 450-nm wavelengths (model CM-III; Spex Industries, Edison, NJ), and fluorescence emission was monitored at 530 nm using a photomultiplier tube and a band-pass filter. The acetoxymethyl ester of BCECF was perfused through the lumen, and loading was continued until the fluorescence measured for both excitation wavelengths had increased by a least one order of magnitude with respect to background fluorescence. Calibration curves for BCECF fluorescence were obtained using the high K+-nigericin technique (43) as described previously (27). Since a complete calibration curve could not be performed in each experiment, we obtained one or two calibration points (including pH of ~7.2–7.4) at the end of each experiment and used previously obtained calibration curves to convert fluorescence ratios to pH measurements.

Determination of apical NH4+ transport rates. Initial acidification rates were obtained from fitting exponential or linear equations to the recorded pH time courses after replacing 20 mM of luminal NMDG-Cl with 20 mM NH4Cl. It was quantitatively shown that the resulting acidification rate multiplied by the MD cell buffering power was proportional to the apical NH4+ influx rate (27). The buffering power was previously measured for MD cells and was found to double when the cell acidified from 7.5 to 6.7 (27). Under the experimental conditions of the present studies, no systematic changes in the initial pH, before adding luminal NH4+, were found in any experimental group. For example, even if an abrupt increase in luminal Cl− concentration was observed to acidify MD cells as previously reported (30), the pH, obtained after luminal NH4+ addition (2–3 min) and washout (5–7 min) in the presence of a different luminal Cl− concentration was not statistically different from the initial pH. This allows us to use NH4+-induced acidification rate without having to add a correction factor based on the average buffering power previously measured for a different series of experiments.

Statistics. Data are presented as mean ± SE, and n is the number of MD plaques studied. In some experiments, data were fitted to theoretical equations using commercial software, and the uncertainty of fitted parameters is the standard error of the fit (Fig 6.0; Biosoft, Milltown, NJ). Statistical significance of the difference between two means was assessed using Student’s t-test for paired samples. P < 0.05 was considered significant.

RESULTS

NH4+-induced acidification. As previously shown, NH4+ is mainly transported across the apical membrane of MD cells through a bumetanide-sensitive pathway and a Ba2+-sensitive pathway in an additive manner (57% and 35% of the initial NH4+-induced acidification rate were sensitive to bumetanide and Ba2+, respectively; Ref. 27). In the present series of experiments where 5 mM Ba2+ was constantly present in the lumen, we confirmed that NH4+-induced acidification was largely mediated by the Na-K-2Cl cotransporter: 89 ± 14% of the maximal acidification rate observed was Cl− dependent (n = 6) and 84 ± 7% was Na+ dependent (n = 6) (see below). This is illustrated in Fig. 1, where we compare, in the same experiment, the NH4+-induced acidification obtained in the presence of 25 mM [NaCl], with that obtained in the absence of functional Na-K-2Cl cotransport (0 mM Na+ + 5 µM bumetanide). In these experiments, 1 mM amiloride was also present in the lumen to block the effect of changing [Na+] on the apical Na+↔H+ exchanger (7).

Cl− and Na+ affinity. In the presence of 25 mM [Na+]L, NH4+-induced acidification rate was measured with [Cl−]L varying from 1 to 100 mM. At high [Cl−]L, the initial dpH/dt averaged 0.095 ± 0.019 pH units/s (n = 6). Individual measurements were normalized using the value measured at 100 mM [Cl−]L, and averaged data at each [Cl−]L were fitted (see Fig. 2) using the following expression

\[
\frac{dpH}{dt} = \frac{V_{\text{max}} \times ([Cl]_L)^h}{(K_{m}^C)^h + ([Cl]_L)^h}
\]

where h is the Hill number, K_{m}^{C} is the luminal Cl−-apparent affinity constant, C is the remaining dpH/dt at zero [Cl−]L, and V_{\text{max}} + C is the maximal dpH/dt at infinite [Cl−]L. The Cl− affinity constant was found to be 17 ± 4 mM, and the Hill number was significantly larger than 1 (1.45 ± 0.45), which is consistent with the expected stoichiometry of the cotransporter.

Fig. 1. Example of NH4+-induced acidification in the presence of Ba2+. A: in presence of 25 mM NaCl in the lumen and 1 mM amiloride, addition of 20 mM NH4+ produced cell acidification at an initial rate of 0.031 pH units/s. B: in contrast, when the cotransporter is blocked with 5 µM bumetanide (BUM; 0 mM Na+, 25 mM Cl−, 1 mM amiloride), NH4+-induced acidification was reduced to 0.007 pH units/s. An exponential fit was used to estimate initial acidification rate.
In obtaining the apparent cotransporter affinity for luminal Na⁺, care must be taken to avoid systematic pH_i variations due to the activity of the recently described luminal Na⁺/H⁺ exchanger (7). Thus these experiments were performed in the presence of 1 mM luminal amiloride while [Na⁺], was varied from 0 to 60 mM. The final [Na⁺], values for solutions nominally containing 0, 0.5, 1, and 2 mM Na⁺ were measured using flame photometry (model IL943; Instrumentation Laboratory, Milan, Italy) and found to be 0.47, 0.98, 1.47, and 2.67 mM, respectively. On the basis of preliminary experiments, we found the acidification rates obtained with 0.47 and 0.98 mM Na⁺ to be very weak, and we chose to add 5 μM bumetanide to these solutions to obtain a better estimation of the baseline acidification rate (i.e., bumetanide insensitive). The data were normalized using the measured acidification rate obtained at 60 mM [Na⁺], and 25 mM [Cl⁻], which averaged 0.046 ± 0.006 pH units/s (n = 6). After normalization, the data (see Fig. 3) could be fitted to a modified Michaelis-Menten equation like the one given above in the case of Cl⁻. The Na⁺ apparent affinity constant was 1.0 ± 0.3 mM (n = 6).

Effects of intracellular Cl⁻ concentration. Under normal conditions, the apical Na-K-2Cl cotransporter mediates NaCl entry in MD cells (30) and maintains intracellular Cl⁻ above its electrochemical equilibrium. This provides the driving force for Cl⁻ exit across the basolateral membrane Cl⁻ conductance (29). Intracellular Cl⁻ could interfere with the apical cotransporter activity by either reducing the chemical gradient for Cl⁻ entry across the apical membrane or by changing the phosphorylation state of the cotransporter as was shown for the secretory form of the cotransporter (18). The level of intracellular Cl⁻ was modulated by blocking the basolateral Cl⁻ conductance with 10 μM 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (40). In the presence of saturating [Na⁺], and [Cl⁻], (25 mM Na⁺ and 60 mM Cl⁻), basolateral addition of NPPB reduced the NH₄⁺-induced acidification rate from 0.075 ± 0.010 to 0.035 ± 0.004 (P < 0.01, n = 5), an average inhibition of 51 ± 6%. In the presence of low [Na⁺], and [Cl⁻], (1 mM Na⁺ and 5 mM Cl⁻), basolateral NPPB did not significantly affect NH₄⁺-induced acidification [0.023 ± 0.003 to 0.017 ± 0.002; not significant (NS), n = 5] (Fig. 4). Interestingly, under these conditions, the apical cotransporter is expected to mediate a minimal NaCl net influx (30), and consequently, basolateral NPPB application is not expected to produce any significant increase in intracellular Cl⁻ concentration ([Cl⁻]ᵢ).

Effects of cAMP. TGF was previously shown to be partially inhibited by cAMP (2). In a variety of tissues, maneuvers that increase intracellular cAMP levels and protein kinase A activity were found to stimulate (16, 17, 34) or inhibit the Na-K-2Cl cotransporter (16, 31, 34, 37). The effects of 0.1 mM luminal dibutyryl-cAMP (DBcAMP) and 1 μM forskolin on NH₄⁺-induced acidification rate are shown in Fig. 5. In the presence of 25 mM [Na⁺], and 60 mM [Cl⁻], NH₄⁺-induced acidification rate was 0.079 ± 0.010 pH units/s and increased to 0.100 ± 0.010 pH units/s in the presence of DBcAMP and forskolin (a significant stimulation by 26.6%; P < 0.05, n = 11). This effect appeared to require a high apical Na-K-2Cl cotransporter rate since, in the presence of 1 mM [Na⁺], and 5 mM [Cl⁻], DBcAMP and forskolin did not stimulate NH₄⁺-induced acidification rate (0.033 ± 0.008 vs. 0.032 ± 0.008 pH units/s, n = 11).

As previously shown for the secretory form of the cotransporter, [Cl⁻]ᵢ affects the apical cotransporter...
phosphorylation state and activity (18). This raises the question of whether DBcAMP modulates the apical Na-K-2Cl cotransporter in a direct or indirect manner. Indeed, the basolateral Cl⁻ conductance (29) may also be affected by cAMP, which in turn would provide a secondary stimulation of the apical cotransporter. For example, in TAL, the basolateral Cl⁻ channel was reported to be activated by a rise in intracellular cAMP (35, 39). This would tend to decrease [Cl⁻], a situation that was shown to stimulate the apical cotransporter. Therefore the effects of luminal DBcAMP and forskolin were further measured in the presence of 10 µM basolateral NPPB. Under these conditions and in the presence of saturating [Na⁺]ₗ and [Cl⁻]ₗ (25 and 60 mM, respectively), the NH₄⁺-induced acidification rate averaged 0.035 pH units/s and was reduced to 0.029 ± 0.004 pH units/s in the presence of DBcAMP and forskolin (P < 0.05, n = 5, see Fig. 6). This significant inhibition by 17.1% was paralleled by a reduction of 16.6% when the effects of DBcAMP and forskolin were tested in the presence of low [Na⁺]ₗ and [Cl⁻]ₗ (1 mM Na⁺ and 5 mM Cl⁻). Under these conditions, the NH₄⁺-induced acidification rate was reduced from 0.017 ± 0.002 to 0.014 ± 0.003 pH units/s (P < 0.002, n = 5). This reduction cannot be accounted for by a putative cAMP inhibition of the Na⁺/H⁺ exchanger, as the alkalination rate after NH₄⁺ washout was not affected by the cAMP/forskolin treatment (0.0136 ± 0.0025 before vs. 0.0133 ± 0.0022 pH units/s after the treatment; NS, n = 9). This rate of pHᵢ recovery was previously shown to be inhibited by 71% through luminal addition of 1 mM amiloride (27).

**DISCUSSION**

Utilization of the NH₄⁺/NH₃ technique was proved useful in obtaining an estimate for NaCl transport rate mediated by MD cells and in the detection of new transport mechanisms in MTAL (1, 27). In the present study, it was shown that this method is also sufficiently sensitive to detect small variations in transport rates and to provide data on both kinetic parameters and regulatory mechanisms for the Na-K-2Cl cotransporter.

Kinetic parameters. The apparent affinity of the cotransporter for luminal Cl⁻ was found to be 17 mM in the presence of 25 mM [Na⁺]ₗ, 5 mM luminal K⁺, and 20 mM luminal NH₄⁺. We had previously obtained an initial estimation for Cl⁻ affinity of 32.5 mM using a less direct method (30) which was primarily used to determine the direction of Na-K-2Cl flux. In that previous study, Na-K-2Cl activity was estimated on the basis of intracellular Na⁺-induced changes in pHᵢ occurring through the activity of the apical Na⁺/H⁺ exchanger. To obtain an affinity for luminal Cl⁻ in that study, the assumptions were 1) stimulation of the cotransporter would produce a proportional increase in intracellular Na⁺ concentration ([Na⁺]ᵢ), 2) the apical Na⁺/H⁺ exchanger is sensitive to changes in [Na⁺]ᵢ over a wide range, and 3) changes in the steady-state level of pHᵢ are proportional to Na⁺/H⁺ exchanger activity. It is likely that at least some of these conditions would not be fully satisfied, thereby introducing a degree of uncertainty regarding the estimation of Cl⁻ cotransporter affinity. The method we used in the present study is more direct, because it is based on the initial acidification rate (instead of steady-state pHᵢ levels) directly resulting from NH₄⁺ influx. The complete time course of NH₄⁺-induced acidification and recovery was analyzed in a previous study (27), in which it was shown that the initial acidification rate times the buffering power was proportional to the absolute value of apical NH₄⁺ influx. In the present studies, baseline pHᵢ for a given series of experiments (for example, the effect of changes in [Na⁺]ᵢ in the presence of amiloride) were similar, so that buffering power can be assumed to be constant and need not to be considered here.

An apparent affinity constant for Cl⁻ of 17 mM appears to be significantly lower than the value of 49 mM reported for the rabbit CTAL cotransporter (15) and the value of 67 mM for mouse TAL in culture (23). Note that, in the first case, the electrophysiological method used was quite indirect, and a large uncer-
tainty affected the reported $K_{v}$ for $Cl^-$. In the second case, the type of cotransporter (BSC1 or BSC2) expressed by mouse TAL in culture was not identified. Interestingly, with the plasma membrane vesicle from rabbit TAL, an apparent affinity constant for $[Cl^-]_{l}$ of 15.3 mM was found (26), which is almost identical to the affinity constant reported here for MD cells. For a variety of different epithelial tissues expressing the Na-K-2Cl cotransporter, apparent $Cl^-$ affinities ranging from 20 to 75 mM have been reported (34). Thus MD cells express a cotransporter with a relatively high $Cl^-$ affinity.

The apparent affinity constant of the MD apical cotransporter for $[Na^+]_l$ was found to be 1 mM, which is slightly lower than the 3.8 mM $Na^+$ affinity constant reported for the rabbit CTAL cotransporter (13). In the case of mouse MTAL cells in culture, a $Na^+$ affinity constant of 7 mM was reported (23). In other epithelial tissues, $Na^+$ affinity constants vary between 0.42 mM for LLC-PK$_1$ cells to 15 mM for human fibroblasts (34). Therefore, the MD cell cotransporter displays a high affinity for $Na^+$, which is also the case for the cotransporter of TAL cells.

The affinity for luminal $K^+$ was not determined in the present study, because 20 mM NH$_4^+$ was present and should effectively compete for the cotransporter site with the 5 mM luminal $K^+$. If NH$_4^+$ affinity for the MD cotransporter is similar to the one reported for MTAL ($K_{v} = 0.5$ mM NH$_4^+$, Ref. 25), then 20 mM is well above the $K_{m}$ value and should completely displace $K^+$ from its site on the cotransporter.

Regulation of cotransporter activity. As expected from previous experiments on the Na-K-2Cl cotransporter (17, 18), intracellular $Cl^-$ was shown to play an important role in modulation of apical cotransporter activity. In agreement with these observations, increasing $[Cl^-]_{l}$ by blocking basolateral $Cl^-$ channels with NPPB inhibited NH$_4^+$-induced acidification rate by 51%. These results do not discriminate between an inhibition due to a diminished $Cl^-$ chemical gradient across the apical membrane or through a change in the phosphorylation state of the cotransporter as directly shown for the secretory form of the cotransporter in dog tracheal cells (18). Nevertheless, MD intracellular $Cl^-$ does appear to be an important regulator of the apical cotransporter. Any maneuver that affects basolateral electrogenic $Cl^-$ efflux (e.g., blockade of apical $K^+$ channels, inhibition of basolateral Na-K-ATPase, inhibition/stimulation of basolateral $Cl^-$ channels, hormonal regulation) should alter cotransporter activity through changes in $[Cl^-]_{l}$.

Interestingly, stimulation by cAMP of the NH$_4^+$-induced acidification rate was clearly shown to include an effect of cAMP on basolateral $Cl^-$ channels. Indeed, a stimulation of cotransporter activity by 26.6% with cAMP was reversed to an inhibition by 17.1% when basolateral $Cl^-$ channels were inhibited by NPPB. Therefore, the specific effect of cAMP on the apical Na-K-2Cl cotransporter is, most likely, an inhibition that can be detected both at saturating and nonsaturating $[Na^+]_l$ and $[Cl^-]_l$. The similarity of the level of inhibition at low and high apical $[Na^+]_l$ and $[Cl^-]_l$ suggests that the effect of cAMP occurs exclusively on the cotransporter $V_{max}$. However, the percent inhibition is likely to be much higher at low $[Na^+]_l$ and $[Cl^-]_l$, if one corrects for the bumetanide-insensitive component, which would suggest that cAMP has also increased $Na^+$ and/or $Cl^-$ $K_m$. In other cell types, regulation in Na-K-2Cl cotransport activity is often, but not always (6), accompanied by a parallel change in the number of bumetanide binding sites, suggesting an effect through addition or removal of transporter units in the membrane (16, 17). Also, cAMP has been shown to both stimulate and inhibit the Na-K-2Cl cotransporter depending on the cell type studied (16, 17). In the case of the TAL, vasopressin stimulates cAMP production, but the amplitude of its stimulatory effects on transepithelial NaCl fluxes ($J_{NaCl}$) are both species dependent and heterogeneous (cortical vs. medullary) (38). Similar to what we have found in MD cells, cAMP was shown to stimulate the basolateral $Cl^-$ conductance in TAL (12, 14, 35, 39). In a mouse MTAL cell line, the basolateral cAMP-dependent $Cl^-$ channel has recently been identified as rdlClC-Ka, a member of the ClC family (49). In addition, cAMP was also shown to stimulate the TAL apical Na-K-2Cl cotransporter independently of its effect on the basolateral $Cl^-$ conductance (i.e., the presence of basolateral $Cl^-$ channel blockers) (44). Thus the specific effect of cAMP on the MD apical Na-K-2Cl cotransporter appears to be different from the effects reported in the case of TAL.

In conclusion, we have shown that the NH$_4^+/NH_3$ method, which has been successfully used for monitoring apical ionic flux through at least two types of pathways (27), can also be used to determine affinity constants and identify regulatory mechanisms of the apical Na-K-2Cl cotransporter activity. With respect to Na-K-2Cl cotransporters of other tissues, the MD cell cotransporter has a relatively high affinity for luminal $Na^+$ and $Cl^-$. In addition, intracellular $Cl^-$ is a potent regulator of cotransporter activity in MD cells, and cAMP directly inhibits the cotransporter activity independently of its effect on basolateral $Cl^-$ channels. These new properties of the MD Na-K-2Cl cotransporter will be helpful in understanding the role of MD cells in TGF and the alteration in sensitivity and amplitude of feedback responses in different experimental or physiological conditions.

This work was supported by funds from the Kidney Foundation of Canada (to J.-Y. Lapointe) and by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-32032 (to P. D. Bell). Address for reprint requests: J.-Y. Lapointe, Groupe de Recherche en Transport Membranaire, Université de Montréal, PO Box 6128, Succurselles Centre-ville, Montreal, Quebec, Canada H3C 3J7.

Received 3 November 1997; accepted in final form 13 August 1998.

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