Functional Properties of the Apical Na-K-2Cl Cotransporter

Isoforms

by

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Abstract

The bumetanide-sensitive Na\(^+\):K\(^+\):2Cl\(^-\) cotransporter (BSC1) is the major pathway for salt reabsorption in the apical membrane of the mammalian thick ascending limb of Henle. Three isoforms of the cotransporter, known as A, B, and F, exhibit axial expression along the thick ascending limb. We report here a functional comparison of the three isoforms from mouse kidney. When expressed in *Xenopus* oocytes the mBSC1-A isoform showed higher capacity of transport, with no difference in the amount of surface expression. Kinetic characterization revealed divergent affinities for the three cotransported ions. The observed EC50s for Na\(^+\), K\(^+\), and Cl\(^-\) were 5.0 ± 3.9, 0.96 ± 0.16, and 22.2 ± 4.8 mM for mBSC1-A, 3.0 ± 0.6, 0.76 ± 0.07, and 11.6 ± 0.7 mM for mBSC1-B, and 20.6 ± 7.2, 1.54 ± 0.16, and 29.2 ± 2.1 mM for mBSC1-F, respectively. Bumetanide sensitivity was higher in mBSC1-B, as compared with the mBSC1-A and mBSC1-F isoforms. All three transporters were partially inhibited by hypotonicity, but to different extent. The cell swelling-induced inhibition profile was mBSC1-F > mBSC1-B > mBSC1-A. The function of the Na\(^+\):K\(^+\):2Cl\(^-\) cotransporter was not affected by extracellular pH or by addition of metolazone, DIDS or DIOA to the extracellular medium. In contrast, exposure of oocytes to HgCl\(_2\) before the uptake period reduced the activity of the cotransporter. The effect of HgCl\(_2\) was dose-dependent and mBSC1-A and mBSC1-B exhibited higher affinity than mBSC1-F. Overall, the functional comparison of the murine apical renal-specific Na\(^+\):K\(^+\):2Cl\(^-\) cotransporter isoforms A, B, and F reveals important functional, pharmacological, and kinetic differences, with both physiological and structural implications.

**Key words:** bumetanide, thick ascending limb, isoforms, salt reabsorption
Introduction

The bumetanide-sensitive Na\(^{+}\):K\(^{+}\):2Cl\(^{-}\) cotransporter is the major salt transport pathway in the apical membrane of the mammalian thick ascending limb of Henle’s loop (TALH). The function of this cotransporter in the TALH is critical for salt reabsorption, for the production and maintenance of the countercurrent multiplication mechanism, and is also involved in the regulation of the acid-base and divalent mineral cation metabolism (1). The disruption of the Na\(^{+}\):K\(^{+}\):2Cl\(^{-}\) cotransporter gene in humans (2) and mice (3) produce Bartter’s syndrome, an autosomal recessive disease characterized by metabolic alkalosis, hypokalemia, hypercalciuria and severe volume depletion, accompanied by reduction in arterial blood pressure. In addition, the Na\(^{+}\):K\(^{+}\):2Cl\(^{-}\) cotransporter protein in the TALH is the main pharmacological target of loop diuretics (4), which are extensively used in the treatment of edematous states.

The primary structure of the kidney-specific, bumetanide-sensitive Na\(^{+}\):K\(^{+}\):2Cl\(^{-}\) cotransporter (BSC1 or NKCC2) has been elucidated by cloning cDNA from rat (5), rabbit (6), mouse (7), and human kidney (2). BSC1 belongs to the superfamily of electroneutral cation-coupled chloride cotransporters for which 8 genes have been identified (8). Two of these genes encode for Na\(^{+}\):K\(^{+}\):2Cl\(^{-}\) cotransporters: BSC1, a kidney specific cotransporter expressed only at the apical membrane of the TALH, and BSC2 (also known as NKCC1), a ubiquitously expressed gene at the basolateral membrane of epithelial cells, which is also expressed in several non-epithelial cells. The degree of identity between these proteins is ~60%, and in humans, the BSC1 and BSC2 genes are localized in chromosomes 15 and 5, respectively. The murine BSC1 gene gives rise to six alternatively spliced isoforms due to the combination of two splicing mechanisms. One is due to the existence of three mutually exclusive cassette exons of 96 bp named A, B and F, that encode 31 amino acid residues that are part of the putative
transmembrane segment 2 and the connecting segment between transmembrane segments 2 and 3 (6,7). The other splicing mechanism is a polyadenylation signal in the intron between exons 16 and 17 producing a COOH-terminal truncated isoform that lacks the last 327 amino acid residues, but contains 55 residues at the end that are not present in the longer isoforms (9). Because the two splicing mechanisms are independent of each other, six isoforms are present in the TALH cells. That is, three isoforms with a long COOH-terminal domain (A, B or F) and three with a short COOH-terminal domain (A, B or F) (9,10).

The splicing at the COOH-terminal domain in mouse BSC1 has remarkable effects on the cotransporter properties. While the three longer isoforms (A, B or F) function as bumetanide-sensitive Na⁺:K⁺:2Cl⁻ cotransporters, that are partially inhibited by hypotonicity (5,11), the shorter isoform operates as a K⁺-independent, but nevertheless bumetanide-sensitive Na⁺:Cl⁻ cotransporter that is activated by hypotonicity (12). Both transporters are equally sensitive to loop diuretics. In addition, the shorter isoform is sensitive to cAMP and exerts a dominant-negative effect upon the Na⁺:K⁺:2Cl⁻ cotransporter that can be abrogated by camp (11). Thus, splicing of the COOH-terminal domain changes the type and stoichiometry of the cotransported ions, the response to cell swelling, and provides a potential regulatory mechanism of the Na⁺:K⁺:2Cl⁻ cotransporter activity.

The functional effect of splicing of the mutually exclusive cassette exons A, B, and F, encoding part of the transmembrane segment 2, is still unknown, but it has been suggested that the exons could affect the transport properties of the cotransporter. Early studies on isolated cortical TALH (cTALH) segments by Burg (13) and medullary TALH (mTALH) segments by Rocha and Kokko (14) indicated that mTALH transports NaCl more rapidly than the cTALH, but with greater diluting power in the cTALH (15), suggesting heterogeneity of the transport
properties along the TALH. Supporting this possibility, the apparent affinity for Cl⁻ observed by Greger (16), Hus-Citharel and Morel (17), and Eveloff (18), when cTALH was used as a source of the plasma membrane vesicles, was different to the apparent affinity obtained by Koenig (19) and Burnham (20) when mTALH was used. In this regard, it has been shown that the splicing isoforms A, B, and F exhibit axial distribution along the TALH. The F isoform is absent in the cTALH and present in the mTALH, with higher expression in the inner stripe of the outer medulla. The A isoform is present in both cTALH and mTALH, with higher expression in the outer stripe of the outer medulla, and the B isoform is present only in the cTALH (6,7,21). Thus heterogeneity in the salt transport along the TALH could be due to the axial distribution of the three isoforms A, B, and F of the Na⁺:K⁺:2Cl⁻ cotransporter. However, the functional characterization of these isoforms has not been addressed.

In the present study, we show a functional characterization of the longer isoforms A, B, and F of the murine Na⁺:K⁺:2Cl⁻ cotransporter using the *Xenopus laevis* oocytes as an heterologous expression system. Our data revealed significant differences in the affinity for Na⁺, K⁺, and Cl⁻ between isoforms, as well as in the sensitivity to bumetanide and response to hypotonicity.
Methods

Xenopus laevis oocytes preparation

Adult female *Xenopus laevis* frogs were obtained from Nasco (Fort Atkinson, MI). Oocytes were harvested by surgery under tricaine (0.17%) and incubated for 1 h in the frog Ringer ND96 (in mM: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl, and 5 HEPES/Tris pH 7.4) in the presence of 2 mg/ml of collagenase B. Then, oocytes were washed four times in ND96, manually defolliculated, and incubated overnight in the same media at 18°C supplemented with 2.5 mM sodium pyruvate and 5 mg/100 ml of gentamicin. Next day, stage V-VI oocytes (22) were injected with 50 nl of water or cRNA at a concentration of 0.5 µg/µl (25 ng cRNA per oocytes). After injection, oocytes were incubated during 3 to 4 days in ND96 with sodium pyruvate and gentamicin. The incubation medium was changed every 24 hours. The night before the uptake experiments were performed, oocytes were incubated in Cl⁻-free ND96 (in mM: 96 Na⁺ isethionate, 2 K⁺-gluconate, 1.8 Ca²⁺ gluconate, 1.0 Mg²⁺-gluconate, 5 mM Hepes, 2.5 sodium pyruvate, 5 mg% gentamicin, pH 7.4) (23).

In vitro mBSC1 cRNA translation

The cloning and preparation of mouse mBSC1 cDNA used in the study has been previously reported (9). In brief, mBSC1-F and mBSC1-A isoforms were cloned by homology from a mouse outer medulla cDNA library, using the flounder thiazide-sensitive Na⁺:Cl⁻ cotransporter cDNA as a probe (5,9). The short B cassette cDNA was lengthened by PCR and ligated into the *BsmI* and *NsiI* sites of mBSC1-F isoform (9). All the mBSC1 isoforms used in the present study are inserted in the plasmid pSPORT1 (Gibco-BRL). To prepare cRNA, each isoform cDNA was linearized at the 3’ end using *Not I* from Boehringer (Mannheim, Germany) and cRNA was transcribed *in vitro*, using the T7 RNA polymerase mMESSAGE kit (Ambion).
Transcription product integrity was confirmed on agarose gels and concentration was determined by absorbance reading at 260 nm (DU 640, Beckman, Fullerton, CA). cRNA was stored frozen in aliquots at -80°C until used.

Assessment of the Na⁺:K⁺:2Cl⁻ cotransporter function

The function of the Na⁺:K⁺:2Cl⁻ cotransporter was assessed by measuring tracer ⁸⁶Rb⁺ uptake (New England Nuclear) in groups of at least 15 oocytes following this general protocol: 30 minute incubation in isotonic K⁺ and Cl⁻-free medium (mM: 96 Na⁺-gluconate, 6.0 Ca²⁺-gluconate, 1.0 Mg²⁺-gluconate, 5 Hepes/Tris, pH 7.4) with 1 mM ouabain, followed by 60 minutes uptake period in the presence of Na⁺, K⁺ and Cl⁻. For most experiments the isotonic medium contained (mM): 96 NaCl, 10 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, pH7.4, supplemented with 1 mM ouabain, and 2.0 µCi of ⁸⁶Rb⁺. Because *Xenopus laevis* oocytes express an endogenous Na⁺:K⁺:2Cl⁻ cotransporter (5) every single experiment included the appropriate groups of water-injected oocytes.

To analyze the ion transport kinetics of the Na⁺:K⁺:2Cl⁻ cotransporter isoforms, experiments were performed varying the concentrations of Na⁺, K⁺ and Cl⁻. For Na⁺ kinetics, the extracellular K⁺ and Cl⁻ concentrations were fixed at 10 and 90 mM, respectively. For K⁺ kinetics, Na⁺ and Cl⁻ were fixed at 90 mM, and for Cl⁻ kinetics the Na⁺ and K⁺ concentrations were fixed at 90 and 10 mM, respectively. To maintain osmolarity and ionic strength, NMDG was used as Na⁺ and K⁺ substitute, and gluconate was used as Cl⁻ substitute. The transport kinetics for a single ion (Na⁺, K⁺, or Cl⁻) was assessed for the three-mBSC1 isoforms at the same time, with the same batch of oocytes and solutions. In the same experiment uptake was also measured for each point in water injected oocytes (data not shown) and the mean values for water groups were subtracted in corresponding mBSC1 groups to analyze only the ⁸⁶Rb⁺ uptake due to
the injected mBSC1 isoform. Kinetic analysis was performed by estimating the EC50 values for each ion. The Ec50 values were calculated from Log[ion conc] vs. V/Vmax plots using GraphPad Prism software and an Uphill Dose Response equation with variable slope (the latter allows the Hill slope to vary from unity). The sensitivity and kinetics for bumetanide was assessed by exposing groups of mBSC1 cRNA injected oocytes to bumetanide at concentrations varying from $10^{-9}$ to $10^{-4}$ M. The desired concentration of the loop diuretic was present in both, the incubation and uptake periods. Finally, we also assessed the effect of osmolarity upon the function of mBSC1 isoforms using the following conditions during uptake: hypotonicity (160 mOsm/kg), isotonicity (210 mOms/kg), and hypertonicity (260 mOsm/kg). For these experiments the three mBSC1 isoforms were also analyzed at the same time and all solutions contained 65 mM NaCl and 5 mM KCl, which resulted in an osmolarity of ~ 160 mOsm/kg. To prepare the solutions with 210 and 260 mOsm/kg we added 45 mM and 90 mM of sucrose, respectively.

All uptakes were performed at 30°C. At the end of the uptake period, oocytes were washed five times in ice-cold uptake solution without isotope to remove extracellular fluid tracer. After the oocytes were dissolved in 10% sodium dodecyl sulfate, tracer activity was determined for each oocyte by β-scintillation counting.

Assessment of the mBSC1 isoforms expression in the oocytes plasma membrane

The surface expression of each mBSC1 isoform in the oocyte plasma membrane was measured by fluorescence using enhanced green fluorescent protein-mBSC1 fusion constructs. To make the GFP-mBSC1 fusion constructs, the fragment containing the full-length mBSC1-A cDNA was removed from pSPORT1-BSC1, with the restriction enzymes Sal I and Not I, gel isolated and ligated into pEGFP-C1 (Clontech, Palo Alto, CA), resulting in the plasmid pEGFP-C1/BSC1, which contains an in-frame fusion of the mBSC1-A ligated into the COOH-terminus.
of GFP. Then, the cDNA fragment containing the GFP-mBSC1-A was removed from pEGFP-C1/BSC1 by restriction enzyme digestion with *Age* I and *Not* I, and ligated into pSPORT1. To obtain GFP-mBSC1-B and GFP-mBSC1-F, the fragment *Sal* I to *Nsi* I of GFP-mBSC1-A, which contains the entire GFP sequence and part of mBSC1 sequence before the second transmembrane domain, was ligated into mBSC1-B and mBSC1-F, which were already in pSPORT1 (9). GFP-mBSC1-A, GFP-mBSC1-B, and GFP-mBSC1-F cRNA was transcribed *in vitro* and microinjected into *X. laevis* oocytes (25 ng/oocyte). Water and non-GFP mBSC1-F injected oocytes were used as control. After four days of incubation in regular ND96, oocytes were monitored for GFP fluorescence using a Zeiss laser scanning confocal microscope (objective lens x10, Nikon). Light of excitation wavelength 488 nm and emission 515-565 nm was used to visualize GFP fluorescence. Plasma membrane fluorescence was quantified by determining the pixel intensity around the entire oocyte circumference using SigmaScan Pro image analysis software.

**Statistical analysis**

The significance of the differences between groups was tested by one way ANOVA with multiple comparison using Bonferroni correction or by the Kruskal-Wallis one way analysis of variance on ranks with the Dunn method for multiple comparison procedures, as needed. The results are presented as mean ± SEM.
Results

Expression of mBSC1 isoforms in Xenopus oocytes

We and others (5,24-26) have previously shown that Xenopus oocytes exhibit an endogenous expression of the bumetanide-sensitive Na⁺:K⁺:2Cl⁻ cotransporter. As shown in Fig. 1, ⁸⁶Rb⁺ uptake in H₂O-injected oocytes was 2113 ± 346 pmol·oocyte⁻¹·h⁻¹ in control conditions and 417 ± 202 pmol·oocyte⁻¹·h⁻¹ in the presence of 10⁻⁴ M concentration of the loop diuretic bumetanide. Background ⁸⁶Rb⁺ uptake was, however, increased by microinjection of X. laevis oocytes with mBSC1-A, mBSC1-B, or mBSC1-F cRNA. The uptake was significantly reduced in all groups in the presence of bumetanide. Thus, in order to analyze the ⁸⁶Rb⁺ uptake induced only by each mBSC1 isoform, in all experiments performed for this study, ⁸⁶Rb⁺ uptake was simultaneously measured in water injected oocytes and the mean values for water groups were subtracted in corresponding mBSC1 groups.

As shown in Fig. 1, ⁸⁶Rb⁺ uptake in mBSC1-A injected oocytes was 19395 ± 1997 pmol·oocyte⁻¹·h⁻¹, whereas in mBSC1-B oocytes was 13229 ± 1640 pmol·oocyte⁻¹·h⁻¹ and in mBSC1-F was 12088 ± 1561 pmol·oocyte⁻¹·h⁻¹. Thus ⁸⁶Rb⁺ uptake in the mBSC1-A isoform is significantly higher than in mBSC1-B or mBSC1-F isoforms (p<0.001). The results shown in Fig. 1 are the pooled data from 11 different experiments, using oocytes from different frogs, with an average of 18 oocytes per group in each experiment. The cRNA used was obtained from three different batches and every time oocytes were injected with the same amount of cRNA (25 ng/oocyte). The cDNA of the three isoforms used are inserted in the same vector (pSPORT1), contains the same 5’ and 3’ untranslated regions, and cRNA was transcribed in vitro for the three isoforms simultaneously, using the same T7 RNA polymerase. Thus, differences between isoforms in Fig. 1 are unlikely to be result of injecting mBSC1-A oocytes with a better quality...
cRNA, with higher concentration of cRNA per oocyte or that mBSC1-A cRNA was better translated than the other two. Instead, these results suggest that the mBSC1-A isoform exhibits either higher surface expression or higher capacity of transport than mBSC1-B and mBSC1-F isoforms. In order to determine if the differences in functional expression were due to variation in the surface expression of the Na⁺:K⁺:2Cl⁻ cotransporter isoforms, *X. laevis* oocytes injected with GFP-mBSC1-A, GFP-mBSC1-B or GFP-mBSC1-F cRNA isoforms were analyzed by confocal fluorescence microscopy. Figures 2A to 2D depict a representative picture of oocytes injected with each isoform and Figure 2E shows the result of these experiments in which at least 40 oocytes per isoform were evaluated. As shown in Fig. 2E, although numbers were smaller on mBSC1-F (31212 ± 4165 [N=48]), than in mBSC1-A (48888 ± 8042 [N=50]) or mBSC1-B-injected oocytes (43995 ± 8495 [N=40]), ANOVA analysis showed no significant differences in surface expression between the three isoforms. Thus, under our experimental conditions it is unlikely that the type of mutually exclusive cassette exon affects the surface expression of the cotransporter in oocytes. This observation supports the hypothesis from Fig. 1 that mBSC1-A might be the isoform with the higher capacity of transport.

*Transport Kinetics of mBSC1 isoforms*

The kinetic transport properties for each ion were assessed for the three isoforms simultaneously, in the same batch of injected oocytes. Figure 3A shows the Na⁺-transport kinetics of each isoform and Figures 3B, 3C, and 3D depicts the Hill coefficient plots for Na⁺ in mBSC1-B, mBSC1-A, and mBSC1-F, respectively. The Na⁺-dependency of ⁸⁶Rb⁺ uptake was performed with fixed concentrations of K⁺ and Cl⁻ at 10 and 96 mM, respectively, with changing concentrations of Na⁺ from 0 to 80 mM. ⁸⁶Rb⁺ uptake increased as the Na⁺ concentration was increased until a plateau phase was reached, compatible with Michaelis-Menten behavior. Table
show the EC50 and Hill coefficient values. The EC50 values for Na⁺ were similar between mBSC1-A and mBSC1-B isoforms, but different to the values observed for mBSC1-F isoform. Figure 4A shows the K⁺-transport kinetics of each isoform and Figures 4B, 4C, and 4D depicts the Hill coefficient plots for K⁺ in mBSC1-B, mBSC1-A, and mBSC1-F, respectively. The experiments were performed with fixed concentrations of Na⁺ and Cl⁻ at 96 mM, with increased concentration of K⁺ from 0 to 10 mM. The ⁸⁶Rb⁺ uptake increased as K⁺ concentration in the extracellular medium until a plateau phase was reached. EC50 and Hill coefficients are shown on Table 1. As with Na⁺ transport kinetics, the EC50 values observed in mBSC1-A and mBSC1-B were similar, whereas EC50 for K⁺ in mBSC1-F isoform was higher. Figure 5A depicts the Cl⁻-transport kinetics for each mBSC1 isoform and Figures 5B, 5C, and 5D show the Hill plots for Cl⁻. These experiments were carried out with Na⁺ and K⁺ concentrations at 96 and 10 mM, respectively, with increased Cl⁻ concentrations from 0 to 96 mM. ⁸⁶Rb⁺ uptake increased as a function of the Cl⁻ concentration. The plateau phase was reached in mBSC1-A and mBSC1-B, but not in mBSC1-F. As shown in Table 1, the EC50 value for Cl⁻ was higher in mBSC1-F, than values observed in mBSC1-A or mBSC1-B. Hill coefficients for Na⁺ and K⁺ in the three isoforms were close to unity, while Hill coefficients for Cl⁻ were above unity, consistent with the 1Na⁺, 1K⁺, and 2Cl⁻ stoichiometry. As figures 3, 4 and 5 show, in general mBSC1-A and mBSC1-B exhibit very similar kinetic properties for the three cotransported ions, suggesting that affinity for each ion is similar between these two isoforms. In contrast, the EC50 values for Na⁺, K⁺, and Cl⁻ in mBSC1-F-injected oocytes were higher, suggesting that this is the isoform with the lowest affinity for the cotransported ions.

*Kinetics of bumetanide inhibition of mBSC1 isoforms*
Bumetanide-induced inhibition of cotransport activity is one of the hallmarks of the Na⁺:K⁺:2Cl⁻ cotransporter. Thus, we analyzed the inhibitory kinetics of bumetanide on mBSC1-A-, mBSC1-B-, and mBSC1-F transport in oocytes. As shown in Fig. 6, all three isoforms were inhibited by the loop diuretic in a dose-dependent manner. However, the IC₅₀ for bumetanide inhibition of ⁸⁶Rb⁺ uptake was lower in mBSC1-B (600 nM) than in mBSC1-A (2 µM) or mBSC1-F (3.4 µM). In addition, the percentage of inhibition of the Na⁺:K⁺:2Cl⁻ cotransporter function from 10⁻⁷ to 10⁻⁵ M concentration was significantly higher in mBSC1-B than in mBSC1-F and mBSC1-A. Thus, the mBSC1-B isoform exhibited higher affinity for bumetanide than the other two isoforms.

**Regulation of mBSC1 isoforms by osmolarity**

As all members of the electroneutral cation coupled chloride cotransporter family, the Na⁺:K⁺:2Cl⁻ cotransporter is a cell volume regulated protein. We have shown before (5) a significant reduction of the rat BSC1-F cotransporter function when oocytes were incubated in hypotonic medium (~160 mOsm/kg), compared to isotonic frog Ringer (~210 mOsm/kg). We also observed in hypotonic medium that the reduction of the endogenously expressed Na⁺:K⁺:2Cl⁻ cotransporter in oocytes was significantly higher than the inhibition observed in rat BSC1-F, suggesting that sensitivity to cell volume might be different between Na⁺:K⁺:2Cl⁻ cotransporter isoforms. Accordingly, we assessed the bumetanide-sensitive ⁸⁶Rb⁺ uptake in mBSC1-A-, mBSC1-B-, and mBSC1-F cRNA injected oocytes exposed to an uptake medium containing 65 mM NaCl at three different osmolarities: hypotonic (~ 160 mOms/kg, the osmolarity obtained by the 65 mM NaCl concentration in the uptake medium), isotonic (~ 210 mOsm/kg), or hypertonic (~ 260 mOsm/kg) with sucrose added to the 65 mM NaCl uptake medium to adjust osmolarity. Therefore, ⁸⁶Rb⁺ uptake was assessed in three osmolar conditions,
without differences in extracellular NaCl concentration or ionic strength. The uptake in isotonic medium was taken as 100% activity. As shown in Fig. 7, incubation of oocytes in 260 mOsm/kg resulted in a significant increase in the activity of the endogenously expressed oocyte Na⁺:K⁺:2Cl⁻, while the activity of the mBSC1 isoforms was unchanged. When ⁸⁶Rb⁺ uptake was performed in 160 mOms/kg, the endogenous oocyte Na⁺:K⁺:2Cl⁻ cotransporter activity was completely inhibited (5.1 ± 1.0% of the function observed in isotonicity), whereas the activity of mBSC1 isoforms was only partially reduced, but to a different extent among the isoforms. Comparing with uptake assessed in isotonicity, the ⁸⁶Rb⁺ uptake in 160 mOsm/kg in mBSC1-A was 74 ± 3.3%, in mBSC1-B was 57 ± 3.2%, and in mBSC1-F was 46 ± 2.9% (p<0.01). Thus, the cell swelling induced inhibition profile of the Na⁺:K⁺:2Cl⁻ cotransporter isoforms was mBSC1-F > mBSC1-B > mBSC1-A.

**Effect of pH on rBSC1 function and bumetanide inhibition**

Figure 8A shows the ⁸⁶Rb⁺ uptake in *X. laevis* oocytes injected with each of the mBSC1 isoforms and exposed to extracellular pH from 6.0 to 8.0. Fig. 8B shows the percentage of bumetanide inhibition of each isoform. Uptake experiments were performed in solutions containing 96 mM NaCl and 10 mM KCl, with pH of 6.0, 6.5, 7.0, 7.5, and 8.0. Bumetanide was used at 5 x 10⁻⁷ M. As shown in Fig. 8A, ⁸⁶Rb⁺ uptake was similar from 6.0 to 8.0 for each isoform. Thus, we observed no difference in the Na⁺:K⁺:2Cl⁻ cotransporter activity at different pH values. Also, as shown in Fig. 8B, no significant difference was observed in the degree of bumetanide inhibition of each isoform at pH from 6.0 to 8.0. Note, however, that at most of the studied pH values, the degree of inhibition by 5x10⁻⁷ M bumetanide was significantly lower in mBSC1-F isoform, except when uptake was performed at 7.5, suggesting that lower or higher pH
magnified the difference in bumetanide sensitivity between mBSC1 isoforms, making mBSC1-B and mBSC1-A more sensitive to the effect of loop diuretics than mBSC1-F.

*Effect of inhibitors and mercury*

The electroneutral cation-coupled chloride cotransporters are defined in part due to their sensitivity to several diuretics and inhibitors. For instance, thiazide-type diuretics are specific inhibitors of the Na⁺:Cl⁻ cotransporter (5) and the alkaloid compound DIOA has been proposed as a specific inhibitor of the K⁺:Cl⁻ cotransporter (27). In addition, other drugs such as the stilbene compounds exhibit inhibitory properties upon Cl⁻ transporters, including the Cl⁻-HCO₃⁻ exchanger (28), the K⁺:Cl⁻ cotransporter (29), and the thiazide-sensitive cotransporter (5). Thus, we assessed the effect of metolozone, DIOA or DIDS upon ⁸⁶Rb⁺ uptake in mBSC1-F injected oocytes. As shown in Fig. 9, the thiazide-like diuretic metolozone, the alkaloid DIOA and the stilbene DIDS had no inhibitory properties upon the Na⁺:K⁺:2Cl⁻ cotransporter. As expected, 10⁻⁴ M concentration of bumetanide resulted in complete inhibition of the cotransporter activity. In addition to the specific inhibitors, it is well known that many ion transporters are affected by exposure to HgCl₂. In the electroneutral cotransporter family, Mercado et al. (30) have shown that the *X. laevis* K⁺:Cl⁻ cotransporter in oocytes is activated by HgCl₂, while Jacoby et al. (31) found that the basolateral isoform of the Na-K-2Cl cotransporter is inhibited by HgCl₂, and we have also evidence that HgCl₂ reduces the function of the thiazide-sensitive Na⁺:Cl⁻ cotransporter (32). As shown in Fig. 9, we also analyzed the effect of 50 µM of HgCl₂ upon the ⁸⁶Rb⁺ uptake induced by mBSC1-F. A significant inhibitory effect of HgCl₂ on the function of the apical Na⁺:K⁺:2Cl⁻ cotransporter was observed. Then, in order to assess the effects of HgCl₂ on the three isoforms, *X. laevis* oocytes injected with mBSC1-A, mBSC1-B, or mBSC1-F cRNA were exposed to increased concentration of extracellular HgCl₂ from 1 to 75 µM. Higher
concentrations were not used because we have observed a dramatic increase in $^{86}\text{Rb}^+$ uptake in oocytes when HgCl$_2$ is used at 100 µM or above (30). As shown in Fig. 10, the exposure of mBSC1 isoforms to HgCl$_2$ resulted in significant and dose-dependent inhibition of the cotransporter function. In addition, mBSC1-A and mBSC1-B exhibited a similar pattern of inhibition, whereas the percentage of reduction in the function of mBSC1-F was significantly lower than in the other isoforms.
Discussion

The gene encoding for the apical Na⁺:K⁺:2Cl⁻ cotransporter in mouse gives rise to six alternatively spliced isoforms, that are exclusively expressed in the apical membrane of the TALH (9). On one hand, two isoforms are produced after truncation of the COOH-terminal domain. The longer isoform is made up by 1095 amino acid residues and the shorter isoform by 770 residues. On the other hand, three isoforms are produced due to the existence of three 96 bp mutually exclusive cassette exons denominated A, B, and F, which encode part of the transmembrane domain 2 and the connecting segment between transmembrane domains 2 and 3 (7). Because this splicing mechanism can be combined with the COOH-terminal domain splicing, then, six isoforms are produced: three with long COOH-terminal domain and three with short COOH-terminal domain (1). We have shown that the three long COOH-terminal domain isoforms encode for the bumetanide-sensitive Na⁺:K⁺:2Cl⁻ cotransporter (11) and that the short isoforms exerts a dominant-negative effect upon the Na⁺:K⁺:2Cl⁻ cotransporter that can be abrogated by camp (11). In addition, we have also demonstrated that the shorter isoforms work as hypotonically activated, bumetanide-sensitive, K⁺-independent, Na⁺:Cl⁻ cotransporter, which is inhibited by activation of protein kinase A with camp (12).

In the present study we have established the major properties of the three long isoforms A, B, and F of the murine apical Na⁺:K⁺:2Cl⁻ cotransporter. The long isoforms mBSC1-A and mBSC1-B exhibit transport kinetic properties for Na⁺, K⁺, and Cl⁻ that are similar between each other, but different from the transport kinetic properties observed in the long isoform mBSC1-F. Our data show that this last isoform possess the lowest affinity for the cotransported ions. In addition, although surface expression of the three isoforms in the oocytes plasma membrane is similar (Fig. 2), ⁸⁶Rb⁺ uptake was significantly higher in mBSC1-A-injected oocytes, even after
11 experiments were pooled together (Fig. 1), suggesting that this isoform could have a higher transport capacity. Taking all these data together, we propose that mBSC1-A is the high affinity, high capacity transporter; mBSC1-B is the high affinity, low capacity isoform, and mBSC1-F the low affinity, low capacity isoform. These transport kinetics properties are in accordance with the localization of the isoforms along the TALH. It has been shown that mTLAH possess higher capacity for NaCl transport than cTALH, but that cTALH possess a higher capacity for ion dilution (13-15). At the beginning of the TALH, ion concentrations in the tubular fluid that comes from the inner medulla is very high, whereas as TALH reaches the cortex, the concentration of ions is reduced, due to the combination of intense salt reabsorption and low water permeability. In fact, at the end of the cTALH the tubular fluid is more diluted than plasma. Accordingly, the mBSC1-A isoform, which exhibits the higher capacity of transport, is present all along TALH, but with higher expression levels in the outer medulla. In addition, mBSC1-F, the isoform with the lower affinity for the cotransported ions, has been localized only in the mTALH, with predominant expression at the inner stripe of the outer medulla where ion concentration is very high (7,21). Thus, the higher capacity of transport in mTALH can be due to the higher expression of the mBSC1-A cotransporter. In contrast, in cTALH mBSC1-B is the predominant isoform, with some expression of mBSC1-A. These two isoforms exhibit high affinity for the cotransported ions, with EC50 values for Na⁺ (~3 mM), K⁺ (~ 1 mM), and Cl⁻ (11 to 20 mM) that are clearly below the concentration of these ions in tubular fluid, allowing the reabsorption of salt to take place, even when tubular fluid is more diluted than plasma. Thus the expression of the high affinity isoforms mBSC1-B and mBSC1-A in cTALH can be the reason behind the greater dilution power of cTLAH, as compare with mTALH. Insering et al (33-35) performed a series of chimera clones and point mutations between the human and shark
basolateral isoform of the Na\(^+\):K\(^+\):2Cl\(^-\) cotransporter, known as NKCC1 or BSC2, and concluded that the transmembrane domains important to define kinetic properties are No. 2 and 4 for Na\(^+\) affinity, 2, 4 and 7 for K\(^+\) affinity and only 4 and 7 for Cl\(^-\) affinity. Here we show that mutually exclusive cassette exons A, B, and F in mBSC1 are critical to define the affinity for the three cotransported ions. We cannot verify the role of other membrane spanning domains in ion affinities because, with exception of the exon cassettes, the rest of the mBSC1 isoforms are identical. However, the fact that the only difference between mBSC1-A, mBSC1-B, and mBSC1-F is the exon cassette, indicates that in the apical Na\(^+\):K\(^+\):2Cl\(^-\) cotransporter, this is the region that defines differences in affinities for Na\(^+\), K\(^+\), as well as for Cl\(^-\).

We observed some correlation between the affinity for ions and for bumetanide. mBSC1-F exhibits the lower affinity for ions and also for bumetanide, whereas mBSC1-B behaves as the isoform with the higher affinity for Cl\(^-\) and also for bumetanide. In this regard, Isenring and Forbush (36) showed that affinity for Na\(^+\), K\(^+\), Cl\(^-\), and bumetanide of the human basolateral Na\(^+\):K\(^+\):2Cl\(^-\) cotransporter is higher than the shark ortholog and we have made a similar observation in the thiazide-sensitive Na\(^+\):Cl\(^-\) cotransporter: the rat cotransporter exhibits higher affinity for Na\(^+\), Cl\(^-\), and also for thiazides, than the winter flounder’s urinary bladder ortholog (23,37), indicating that in members of the electroneutral cotransporter family, the higher affinity for the cotransported ions is accompanied by higher affinity for inhibitors. These observations support the hypothesis that inhibition of the cotransporter activity by bumetanide probably involves competence between ions (particularly Cl\(^-\)) and the loop diuretic for the same site on the protein (38).

In the present study we observed a significant difference in the response to changes in cell volume by mBSC1 isoforms. When oocytes were exposed to variations in extracellular
osmolarity, the change in mBSC1 function was different between the three isoforms. During cell swelling, the decrease in cotransporter function was 54% in mBSC1-F, 43% in mBSC1-B, and 26% in mBSC1-A, and during cell shrinkage the increase in cotransporter activity was 24%, 9%, and 1%, respectively. Thus mBSC1-F is the isoform with the higher sensitivity to changes in cell volume. We also observed that endogenous Na⁺:K⁺:2Cl⁻ cotransporter in oocytes exhibited an even higher sensitivity to cell volume because the function of this cotransporter was inhibited by 95% during cell swelling and activated by 44% in hypertonicity. The reduction in cotransporter activity in our experiments was observed by changing the normal osmolarity for oocytes from ~210 mOsm/Kg to 160 mOsm/kg; i.e., about 25% change. This osmolarity (160 mOms/kg) is unlikely to be present in mammalian renal medulla. However, similar and even higher percentages of reduction in renal medulla osmolarity can occur as a consequence of water loading. Under these conditions, interstitial NaCl and urea concentration drops rapidly and renal medulla tonicity is reduced, but due to the high contents of osmolytes, such as betaine, inositol, or sorbitol within the mTALH cells, when extracellular osmolarity is reduced, cells take up water and swell (9). Along the TALH, this phenomenon occurs with more intensity in the inner stripe of the outer medulla, where the mBSC1-F isoform is mainly expressed. Thus, our observation of mBSC1-F as the isoform with the higher sensitivity for changes in cell volume agrees with its proposed localization. The present study, however, does not elucidate the mechanisms by which hypotonicity reduces the function of the mBSC1 isoforms to different extent.

During the first half of the XX century, mercurials were used as the first potent diuretic agents (39) that were later discontinued because of the high toxicity and the tendency toward tachyphylaxis, in addition to the concomitant development of better diuretic agents such as loop diuretics and thiazides. The site of action in the nephron was localized at the thick ascending
limb and distal nephron, where mercury inhibited net Cl⁻ reabsorption (40). However, the mechanism of action was never determined. We have recently observed that mercury reduces the function of both the rat and the flounder thiazide-sensitive Na⁺:Cl⁻ cotransporter (32). In addition, Jacoby et al. (31) have shown that the basolateral isoform of the Na⁺:K⁺:2Cl⁻ cotransporter can also be inhibited by mercury. In the present study we show that exposure of *X. laevis* oocytes to HgCl₂ few minutes before the beginning of the uptake period, resulted in a significant and dose-dependent reduction of mBSC1 activity. Thus, the diuretic effect of mercury could be due to direct inhibition of both the Na⁺:K⁺:2Cl⁻ and the Na⁺:Cl⁻ cotransporters located at the apical membrane of the TALH and the distal tubule, respectively.

Depicted in Fig. 11 are the amino acid sequences of the mutually exclusive cassette exons from mouse kidney (7,9). Although the exons expand 31 amino acid residues, differences between isoforms are small. There are only three amino acid residues that are completely different in the three isoforms. In addition to these three residues, some amino acids are different in one isoform, as compare with the other two. For instance, the leucine, isoleucine, methionine and cysteine marked on mBSC1-F are different in mBSC1-A and mBSC1-B, but these residues are identical in isoforms A and B, suggesting that these four amino acid residues could be responsible for kinetic differences between mBSC1-A and mBSC-B, with mBSC1-F isoforms. Particularly interesting is the presence of one methionine and cysteine in mBSC1-F that could confer different tertiary structure to this isoform.

In summary, our data revealed significant kinetic, pharmacological, and regulatory differences between the isoforms A, B, and F of the murine Na⁺:K⁺:2Cl⁻ cotransporter. Because the only structural variation between these three isoforms is the mutually exclusive cassette exon, then, some amino acid residues within these exons must be responsible for the observed
differences in functional properties. Further studies will be necessary to elucidate the role of each different amino acid residue of the exon cassettes upon the functional properties of mBSC1 isoforms shown in the present study.
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FOOTNOTES

1. The abbreviations used are: $^{86}\text{Rb}^+$, tracer rubidium; mBSC1, mouse bumetanide-sensitive cotransporter 1 (also known as NKCC2); BSC2, Bumetanide-sensitive Na-K-2Cl cotransporter 2 (also known as NKCC1); TSC, thiazide-sensitive cotransporter; DIDS, 4,4-diisothiocyanostilbene-2,2disulfonic acid; DIOA, R(+)-[2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1-$H$-indenyl-5-yl]-oxy]acetic acid; MTZ, metolazone.
FIGURES

Figure 1. Functional expression of mBSC1 isoforms in *Xenopus laevis* oocytes that were injected with water or with 25 ng of cRNA from mBSC1-A, mBSC1-B, or mBSC1-F, as indicated. $^{86}$Rb$^+$ uptake was assessed in control conditions (Open bars) or in the presence of $10^{-4}$ bumetanide (closed bars). Each bar represents a mean ± SE of eleven experiments from different frogs. *Significantly different from uptake in control group (p<0.001). † Significantly different from uptake in mBSC1-B and mBSC1-F groups (p<0.001).

Figure 2. Plasma membrane fluorescence of green fluorescent protein-mBSC1 fusion constructs expressed in *X. laevis* oocytes. Oocytes were injected with water or with 25 ng of cRNA from mBSC1-A-GFP, mBSC1-B-GFP, or mBSC1-F-GFP constructs, as indicated. 2A-D) Confocal micrographs showing representative examples of *X. laevis* oocytes injected with water or with mBSC1-GFP constructs. 2A) water-injected oocytes showed no plasma membrane associated fluorescence. Oocytes injected with mBSC1-A-GFP (2B), mBSC1-B-GFP (2C), and mBSC1-F-GFP (2D) cRNA exhibit a distinct plasma membrane-associated fluorescence, which is similar in the three isoforms. 2E) Each bar represents a mean ± SE of at least 40 oocytes from three different frogs. mBSC1 groups were not statistically different according to Kruskal-Wallis One Way Analysis of Variance.

Figure 3. Kinetic transport analysis for Na$^+$ in mBSC1 isoforms. 3A) Na$^+$-dependent $^{86}$Rb$^+$ uptake in *X. laevis* oocytes injected with mBSC1-A (circles), mBSC1-B (boxes), and mBSC1-F (triangles) cRNA. Experiment was performed with increasing Na$^+$ concentrations of 0.5, 1, 2, 3.5, 5, 10, 20, 40, and 80 mM, with fixed concentration of K$^+$ and Cl$^-$ at 10 and 96 mM,
respectively. Lines were fit using the Michaelis-Menten equation. Each point represents the mean ± SE of 15 oocytes. 3B, 3C, and 3D show the Hill plots for Na⁺ in mBSC1-B, mBSC1-A, and mBSC1-F, respectively.

**Figure 4.** Kinetic transport analysis for K⁺ in mBSC1 isoforms. 4A) K⁺-dependent ⁸⁶Rb⁺ uptake in oocytes injected with mBSC1-A (circles), mBSC1-B (boxes), and mBSC1-F (triangles) cRNA. Uptake was assessed in the presence of increasing K⁺ concentration of 0.1, 0.25, 0.4, 0.6, 1.0, 2, 5, and 10 mM. For the K⁺ kinetics analysis Na⁺ and Cl⁻ concentration was fixed at 96 mM. Lines were fit using the Michaelis-Menten equation. Each point represents the mean ± SE of 15 oocytes. 4B, 4C, and 4D show the Hill plots for K⁺ in mBSC1-B, mBSC1-A, and mBSC1-F, respectively.

**Figure 5.** Kinetic transport analysis for Cl⁻ in mBSC1 isoforms. 5A) Cl⁻-dependent ⁸⁶Rb⁺ uptake in oocytes injected with mBSC1-A (circles), mBSC1-B (boxes), and mBSC1-F (triangles) cRNA. Uptake was assessed in the presence of increased concentration of extracellular Cl⁻ of 2.5, 5, 12, 20, 40, 60, 80, and 100 mM, with fixed concentration of Na⁺ and K⁺ in 96 and 10 mM, respectively. Lines were fit using the Michaelis-Menten equation. Each point represents the mean ± SE of 15 oocytes. 5B, 5C, and 5D show the Hill plots for K⁺ in mBSC1-B, mBSC1-A, and mBSC1-F, respectively.

**Figure 6.** Kinetic analysis of the Na⁺:K⁺:2Cl⁻ cotransporter isoforms inhibition by bumetanide. Oocytes were microinjected with mBSC1-A (circles), mBSC1-B (boxes), and mBSC1-F (triangles) cRNA, and four days later ⁸⁶Rb⁺ uptake was assessed under control conditions or in
the presence of increased concentration of bumetanide from $10^{-8}$ to $10^{-4}$ M. Uptakes were performed during 60 min. in uptake solution containing 96 mM Na$^+$ and Cl$^-$, and 10 mM K$^+$. IC$_{50}$s for bumetanide inhibition were 600 nm, 2 µM, and 3.4 µM for mBSC1-B, mBSC1-A, and mBSC1-F isoforms, respectively. Each point represents the mean ± SE of 15 oocytes. *p<0.05 vs uptake in mBSC1-A and mBSC1-F. †p<0.05 vs uptake in mBSC1-A.

**Figure 7.** Effect of osmolarity in H$_2$O (hatched bars), mBSC1-A (open bars), mBSC1-B (black bars), and mBSC1-F (gray bars) injected *X. laevis* oocytes. Uptake was assessed in the absence and presence of $10^{-4}$ M bumetanide and the mean value of the bumetanide groups was subtracted in the corresponding control group to show the bumetanide-sensitive portion of the uptake. Oocytes were exposed to uptake media with osmolarities of 160 mOsm/kg, 210 mOsm/kg, or 260 mOsm/kg. *p<0.05 vs uptake in isotonicity. † p<0.01 vs all other groups in 160 mOsm/kg. Each point represents the mean ± SE of 40 oocytes from two different frogs.

**Figure 8.** Effect of extracellular pH upon the function and bumetanide sensitivity of mBSC1-A (circles), mBSC1-B (boxes), and mBSC1-F (triangles). 8A) $^{86}$Rb$^+$ uptake in control conditions. 8B) Percentage of inhibition by 5x$10^{-7}$ M of bumetanide. * p<0.05 mBSC1-F vs mBSC1-A or mBSC1-B. Each point represents the mean ± SE of 15 oocytes.

**Figure 9.** $^{86}$Rb$^+$ uptake in mBSC1-F cRNA injected *X. laevis* oocytes under control conditions or in the presence of $10^{-4}$ M concentration of metolazone (MTZ), DIOA, DIDS, bumetanide or 50 µM HgCl$_2$, as stated. *p<0.05 vs control. Each point represents the mean ± SE of 15 oocytes.
Figure 10. Dose-dependent inhibition of mBSC1 isoforms by HgCl$_2$. *X. laevis* injected with mBSC1-A (circles), mBSC1-B (boxes), and mBSC1-F (triangles) cRNA were exposed to increased concentration of extracellular HgCl$_2$ in the last 15 minutes before uptake period.

* p<0.05 vs control in the same isoform in the absence of HgCl$_2$. † p<0.05 vs same point in mBSC1-A and mBSC1-B. Each point represents the mean ± SE of 12 oocytes.

Figure 11. Amino acid sequence of the murine mutually exclusive cassette exons A, B, and F. Gray boxes depict amino acid residues that are different in the three exons. Residues in black boxes are those different in one of the three exons.
Table 1. EC50 values and Hill coefficient for Na\(^+\), K\(^+\), and Cl\(^-\) transport in mBSC1 isoforms

|         | Sodium |                  | Potassium |                  | Chloride |                  |
|---------|--------|------------------|-----------|------------------|----------|------------------|
|         |        | EC50             | Hill      | EC50             | Hill     | EC50             | Hill     |
| mBSC1-B | 3.0 ± 0.6 | 1.09 ± 0.1 | 0.76 ± 0.07 | 1.00 ± 0.1 | 11.6 ± 0.7 | 1.53 ± 0.06 |
| mBSC1-A | 5.0 ± 3.9 | 1.16 ± 0.1 | 0.96 ± 0.16 | 0.83 ± 0.09 | 22.2 ± 4.8 | 1.93 ± 0.31 |
| mBSC1-F | 20.6 ± 7.2 | 0.78 ± 0.1 | 1.54 ± 0.16 | 0.95 ± 0.05 | 29.2 ± 2.1 | 2.85 ± 0.25 |
mBSC1-A LGVIIIILYSTMVTSITGLSTSAIAATNGFV
mBSC1-B LGVIIIIGLAVTVTAITGLSTSAIAATNGV
mBSC1-F LGVIIIIGLSVVVTTLTGTSMSAICTNGVV

Transmembrane Domain 2
Functional properties of the apical Na-K-2Cl cotransporter isoforms
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