J. Membrane Biol. 153, 125–135 (1996)

The Journal of
Membrane
Biology

Bumetanide-sensitive Ion Fluxes in Vascular Smooth Muscle Cells: Lack of Functional Na^+, K^+, 2 Cl^- Cotransport

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Received: 30 January 1996/Revised: 20 May 1996

Abstract. To examine the involvement of Na^+,K^+,2Cl^- cotransport in monovalent ion fluxes in vascular smooth muscle cells (VSMC), we compared the effect of bumetanide on ^{86}Rb, ^{36}Cl and ^{22}Na uptake by quiescent cultures of VSMC from rat aorta. Under basal conditions, the values of bumetanide-sensitive (BS) inward and outward ^{86}Rb fluxes were not different. Bumetanide decreased basal ^{86}Rb uptake by 70–75% with a K_i of ~0.2–0.3 μM. At concentrations ranging up to 1 μM, bumetanide did not affect ^{36}Cl influx and reduced it by 20–30% in the range from 3 to 100 μM. In contrast to ^{86}Rb and ^{36}Cl influx, bumetanide did not inhibit ^{22}Na uptake by VSMC. BS ^{86}Rb uptake was completely abolished in Na^+- or Cl^--free media. In contrast to ^{86}Rb, basal BS ^{36}Cl influx was not affected by Na^+_o and K^+_o. Hyperosmotic and isosmotic shrinkage of VSMC increased ^{86}Rb and ^{36}Cl influx to the same extent. Shrinkage-induced increments of ^{86}Rb and ^{36}Cl uptake were completely abolished by bumetanide with a K_i or ~0.3 μM. Shrinkage did not induce BS ^{86}Rb and ^{36}Cl influx in (Na^+ or Cl^-)- and (Na^+ or K^+)-depleted media, respectively. In the presence of an inhibitor of Na^+/H^+ exchange (EIPA), neither hyperosmotic nor isosmotic shrinkage activated ^{22}Na influx. Bumetanide (1 μM) did not modify basal VSMC volume and intracellular content of sodium, potassium and chloride but abolished the regulatory volume increase in isosmotically-shrunken VSMC. These data demonstrate the absence of the functional Na^+,K^+,2Cl^- cotransporter in VSMC and suggest that in these cells basal and shrinkage-induced BS K^+ influx is mediated by (Na^+_o + Cl^-_o)-dependent K^+/K^+ exchange and Na^+_o-dependent K^+,Cl^- cotransport, respectively.

Key words: Smooth muscle — ^{86}Rb — ^{36}Cl and ^{22}Na influx — Bumetanide — Na^+,K^+,2Cl^- Cotransport — K^+/K^+ exchange — K^+,Cl^- cotransport

Introduction

In the early 1970s, Burg et al. (1973) reported that a p-sulfamoylbenzoic acid (SBA)[1] derivative, furosemide, inhibited NaCl absorption in the thick ascending limb of Henle's loop of the mammalian kidney (Burg et al., 1973). During the last two decades, it was shown that SBA-sensitive transporters are involved in transcellular salt transport in different types of absorptive and secretory epithelia (for review *see* (Greger, 1985; Molony et al., 1989; Haas, 1989). Based on the relative values of net furosemide-sensitive ion fluxes in Ehrlich ascites tumor cells with an inverse electrochemical gradient of monovalent cations ($[Na^+]_i$ = 200 mM; $[K^+]_i$ = 10 mM) (Geck et al., 1980), it was proposed that SBA-sensitive transporters are operated as Na^+,K^+,2Cl^- cotransport (Greger, 1985). Indeed, it was shown that in Na^+-depleted epithelial cells derived from the collecting duct and thick ascending limb of Henle's loop of the mammalian kidney, the stoichiometry of bumetanide-sensitive (BS) unidirectional K^+ (^{86}Rb), Na^+ and Cl^- fluxes is close to 1Na^+:1K^+:2Cl^- (McRoberts et al., 1982; Vuillemin et al., 1992).

It should be underlined that despite 20 years of successfully using SBA derivatives in the therapy of edema

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[1] Abbreviations: BS—bumetanide-sensitive; EIPA—ethylisopropyl amiloride; RVI—regulatory volume increase; SBA—sulfamoylbenzoic acid; VSMC—vascular smooth muscle cells.

of different origins and impressive recent progress in the molecular biology of SBA-sensitive transporters (Haas, 1994; Payne et al., 1995), the mechanism of the highly selective action of these compounds in epithelial cells is still unexplained. Indeed, it was shown that apart from epithelia, furosemide, bumetanide and other SBA derivatives inhibit ion fluxes in all types of nonepithelial cells studied so far (Haas, 1989; Haas, 1994) with the exception of erythrocytes from several species (Orlov et al., 1992*a;* Orlov et al., 1994). In electrically excitable tissues and in vascular smooth muscle cells (VSMC) in particular, both bumetanide and furosemide decrease ouabain-resistant K^+ (^{86}Rb) influx up to 70% (Owen, 1984; Smith et al., 1987). Based on these results, it may be assumed that inhibition of $Na^+,K^+,2Cl^-$ cotransport in nonepithelial cells during long term treatment with SBA derivatives should lead to widespread alteration of intracellular ion concentration and related cellular functions. However, clinical studies did not reveal any side-effect of loop diuretics on nonepithelial tissues (Unwin et al., 1995). To clarify this discrepancy, it should be pointed out that in contrast to monolayers of epithelial cells (O'Grady et al., 1986) and the cell cultures mentioned above, another mode of operation of SBA-sensitive transporter ($2Na^+:1K^+:3Cl^-$) was found in ferret erythrocytes (Hall et al., 1985) and squid axons (Russell, 1983). In bovine tracheal epithelia, this carrier operates as K^+-dependent Na^+,Cl^- cotransport (Musch et al., 1989). Moreover, it should be underlined that for the majority of nonepithelial cells the stoichiometry of SBA-sensitive ion fluxes has not been studied, and the hypothesis of $Na^+,K^+,2Cl^-$ cotransport involvement in the regulation of net ion fluxes is mainly based on the typical rank order of potency of SBA derivatives to inhibit ^{86}Rb influx (benzmetamide>bumetanide>piretanide>furosemide) and on obligatory dependence on SBA-inhibited K^+ (^{86}Rb) influx on extracellular Na^+ and Cl^-. Keeping this in mind, we investigated the stoichiometry of basal and shrinkage-induced BS Na^+, K^+ and Cl^- fluxes as well as the effect of bumetanide on the intracellular content of monovalent cations and VSMC volume. Our results demonstrate the absence of a functional $Na^+,K^+,2Cl^-$ cotransporter in VSMC and suggest that in these cells, basal and shrinkage-induced BS K^+ inward fluxes are mediated by (Na^+_o + Cl^-_o)-dependent K^+/K^+ exchange and Na^+_o-dependent K^+,Cl cotransport, respectively. The results of this study were reported in part at the XIII International Congress of Nephrology (Orlov et al., 1995).

Materials and Methods

Cell Culture

VSMC were obtained by the explant method from aortas of 10- to 13-week-old male Brown Norway (BN.1x) rats (Institute of Biology, Charles University, Prague, Czech Republic). They were seeded and grown in Dulbeco's modified Eagle medium (DMEM) with 10% calf serum (Gibco Laboratories, Burlington, Ontario, Canada), 100 U/ml penicillin and 100 μg/ml streptomycin, as described previously in detail (Franks et al., 1984). When the cells reached confluency in 7–10 days, they exhibited a hill-and-valley pattern which is typical of smooth muscle cells in culture. Under these conditions, the VSMC reacted positively to specific smooth muscle myosin antibodies, as examined by fluorescence microscopy (Hadrava et al., 1989). They were then passaged by treatment with 0.05% trypsin (Gibco) in Ca^{2+}- and Mg^{2+}-free Dulbeco's phosphate-buffered saline (PBS) and incubated in 80 cm^2 tissue culture flasks at a density of 10^5 cells/ml. This study was performed on VSMC between 10–16 passages. Before experimentation, the cells were plated in 12-well (cell volume measurement) or 24-well dishes (^{86}Rb, ^{22}Na and ^{36}Cl flux measurement) and allowed to grow in DMEM containing 10% calf serum for 20–24 hr. To establish quiescence, this medium was replaced for 48–72 hr by synchronization medium (DMEM containing 0.2% calf serum). Cell protein content was determined by modified Lowry methods (Hartee, 1972).

^{86}Rb, ^{22}Na and ^{36}Cl Influx

VSMC were washed with 2 × 2 ml of medium A containing (in mM): 150 NaCl and 10 HEPES-tris buffer (pH 7.4), then preincubated for 30–60 min at 37°C in 0.5 ml of isosmotic (293 mosm) medium B containing 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 D-glucose and 20 HEPES-tris buffer (pH 7.4, 37 °C). To induce isosmotic shrinkage, the cells were preincubated in isosmotic monovalent ion-depleted medium contained (in mM) 300 sucrose, 1 $MgSO_4$, 1 $CaCl_2$, 5 glucose and 20 HEPES-tris (pH 7.4). Then, the preincubation medium was replaced by 0.25 ml of medium B containing 1 mM ouabain with or without bumetanide and 1 μCi/ml $^{86}RbCl$, 2 μCi/ml $H^{36}Cl$ or 4 μCi/ml $^{22}NaCl$. To induce hyperosmotic shrinkage, this medium was also supplied with sucrose up to a final osmolality of 392 mosm. Medium osmolality was measured with a Knauer milliosmometer (Berlin, Germany). Isotope uptake was terminated by the addition of 2 ml of ice-cold medium C containing 100 $MgCl_2$ and 10 HEPES-tris buffer (pH 7.4). The cells were washed with 4 × 2 ml of ice-cold medium C and lysed with 1 ml of a 1% SDS/4 mM EDTA mixture. The radioactivity of the cell lysate (R − cpm) and incubation medium was measured with a liquid scintillation analyzer (TR-1600, Canberra-Packard Canada Ltd., Mississauga, Ont., Canada). Monovalent ion influx (I_{inf}) was determined as $I_{inf} = R/a_o mt$, where a_o is the specific radioactivity of ^{22}Na, ^{86}Rb (K^+) and ^{36}Cl in the incubation medium (cpm/nmol), and m is protein content in the cell lysate (mg) and t is the time of incubation with isotopes (min). In the absence of ion transport inhibitors, the kinetics of ^{22}Na, ^{86}Rb and ^{36}Cl uptake were linear up to 5, 15 and 10 min, respectively (*data not shown*). To determine the initial rate of isotope influx, incubation time was limited by 5 min. In all type of cells studied so far, half-maximal inhibition of ^{86}Rb uptake by bumetanide was observed with concentrations less than 1 μM (Haas et al., 1988; Haas, 1994). In VSMC, an increase of bumetanide concentration from 3 to 100 μM did not cause further inhibition of ^{86}Rb fluxes (Smith et al., 1987). Based on these results, we used 10 μM bumetanide for most of our study. In the experiments on dose-dependencies of ^{86}Rb and ^{36}Cl uptake, bumetanide concentration was varied in the range from 0.1 to 100 μM. To determine the apparent affinity constants of monovalent influx pathways for extracellular K^+, Na^+ and Cl^-, VSMC were washed with 2 ml of media containing 150 mM choline chloride (medium D) or 150 mM $NaNO_3$ (medium E) and 10 mM HEPES-tris buffer (pH 7.4). The concentrations of potassium, sodium and chloride in the incubation medium were varied as mentioned in the legends to Figs. 2, 3 and 4, respectively.

^{86}Rb Efflux

To load with ^{86}Rb, VSMC were preincubated for 3 hr at 37°C in synchronization medium containing 1 μCi/ml ^{86}RbCl. A further increase of preincubation time with ^{86}Rb did not significantly modify intracellular isotope content (*data not shown*), indicating the establishment of isotope equilibrium under steady-state conditions. This enabled us to determine intracellular potassium content and the specific radioactivity of intracellular ^{86}Rb for the calculation of absolute values of the ^{86}Rb efflux rate (*see below*). After preincubation with ^{86}Rb, the dishes were transferred onto ice, the radioactive medium was aspirated and the cells were washed with 5 × 2 ml aliquots of ice-cold medium A. To initiate ^{86}Rb efflux, 0.5 ml of medium B, prewarmed at 37°C and containing 1 mM ouabain, was added. In part of the samples, this medium also contained 10 μM bumetanide. Isotope efflux was terminated by transfer of the incubation medium onto scintillation vials and the cells were lysed with SDS/EDTA mixture. Radioactivity of the incubation medium (R_1 − cpm per well) and radioactivity remaining in cells (R_2 − cpm per well) were determined as mentioned above. ^{86}Rb efflux was calculated as $I_{eff} = R_1/a_i m$, where a_i is the specific radioactivity of intracellular potassium (cpm/nmol) and m is protein content in the cell lysate (mg). To calculate the specific radioactivity of intracellular potassium (a_i), intracellular potassium concentration (nmol per well) was determined based on the initial values of R_2 and specific radioactivity of the preincubation medium. Previously, it was shown that the kinetics of ^{86}Rb efflux are linear for up to 5–8 min, and initial isotope content in VSMC decreased by 50% after 15–20 min of incubation (Orlov et al., 1992*b*). In the present experiments, incubation time was limited to 5 min.

Volume of Intracellular Water

The equilibrium distribution of [^{14}C]-urea was used to measure intracellular water space. VSMC were washed twice with 2-ml aliquots of medium A and preincubated in 1 ml of medium B or in monovalent ion-depleted medium containing (in mM): 300 sucrose, 1 $MgSO_4$, 1 $CaCl_2$, 5 glucose and 20 HEPES-tris buffer (pH 7.4). After 30 min, the preincubation medium was aspirated and replaced by 0.5 ml of medium B containing 1 mM ouabain and 2 μCi/ml [^{14}C]-urea with or without 10 μM bumetanide. The kinetics of [^{14}C]-urea uptake plateau at 8–10 min (*data not shown*). In experiments presented in this paper, [^{14}C]-urea uptake was terminated at 10 and 45 min by the addition of 3 ml of ice-cold medium C. The cells were washed with 4 × 3 ml of ice-cold medium C and lysed with 1 ml of a 1% SDS/4 mM EDTA mixture. The volume of intracellular water (V_i − μl/mg protein) was calculated as $V_i = V_o R_i / R_o m$ where R_i and R_o are the radioactivity of [^{14}C]-urea in the cell lysate and incubation medium, respectively (cpm), m is protein content in the cell lysate (mg), and V_o is the volume of incubation medium (ml) used for R_o determination.

Intracellular Monovalent Ion Content

The intracellular content of Na, K and Cl was determined based on the distribution values of isotopes between cells and extracellular medium under steady-state conditions. To adjust isotope equilibrium under steady-state conditions, VSMC were preincubated for 3 hr in synchronization medium containing 0.5 μCi/ml ^{86}Rb, 1 μCi/ml ^{36}Cl or 2 μCi/ml ^{22}Na and for 2 hr in medium B containing a radioactive compound with the same specific activity with or without bumetanide. Aliquots of incubation medium were then transferred into scintillation vials and the VSMC were washed with 5 × 2 ml of ice-cold medium C and lysed with SDS/EDTA mixture, as mentioned above. Intracellular ion content (nmol/mg prot) was determined as A/am where A is radioactivity of the cell lysate, m is mg protein and a is specific radioactivity of the incubation medium. The intracellular concentration of ions (mM) was calculated based on the values of intracellular water content (μl/mg protein) measured in parallel experiments, as mentioned above.

Chemicals

^{86}RbCl, ^{22}NaCl, [^{14}C]-urea—Amersham International (Buckinghamshire, UK); H^{36}Cl—NEN Research Products (Mississauga, Ont., Canada); bumetanide—Sigma (St. Louis, MO); ouabain—Aldrich Chemical (Milwaukee, WI); EIPA (amiloride, 5-(N-ethyl-N-isopropyl))—Research Biochemical International (Natick, MA); D-glucose, salts and buffers—Sigma, Gibco (Gaithersburg, MO) and Anachemia (Montreal, Que., Canada).

Results

Effect of Bumetanide on ^{86}Rb, ^{22}Na and ^{36}Cl Influx

In the presence of ouabain, bumetanide decreased the rate of ^{86}Rb influx from 28.5 ± 3.3 to 7.8 ± 0.9 nmol · mg prot^{-1} · 5 min^{-1} (Fig. 1*a*), which is in accordance with previously reported data (Owen, 1984; Smith et al., 1987; Orlov et al., 1992*b*). In the absence of bumetanide, the rate of ^{36}Cl influx was 2- to 3-fold higher compared with ouabain-resistant ^{86}Rb influx, and bumetanide decreased it by 20–25% (Fig. 1*b*). The value of the BS component of ^{86}Rb influx was 30–35% higher than the same component of ^{36}Cl influx (20.7 ± 2.3 and 16.4 ± 1.6 nmol · mg prot^{-1} · 5 min^{-1}, respectively).

It is known that Na^+/H^+ exchange is the major pathway of Na^+ influx in vascular smooth muscles (Little et al., 1986; Orlov et al., 1992*b*). Indeed, EIPA, an inhibitor of Na^+/H^+ exchange, decreased ^{22}Na influx from 24.8 ± 2.4 to 8.3 ± 1.2 nmol mg prot^{-1} 5 min^{-1} (Fig. 1*c* and *d*). However, neither basal nor EIPA-resistant ^{22}Na uptake by VSMC was affected by bumetanide.

Dependence of Bumetanide-sensitive ^{86}Rb and ^{36}Cl Influx on Extracellular Concentration of Monovalent Ions

BS ^{86}Rb influx exhibited a hyperbolic dependence on external potassium and sodium concentrations (choline substitution) (Figs. 2*a* and 3*a*, curves 3). These curves were linearized in Eadie-Hofstee plots (*data not shown*), giving apparent affinity values for $[K^+]_o$ and $[Na^+]_o$ of 2.6 ± 0.4 and 29 ± 6 mM, respectively. In contrast to ^{86}Rb, neither total nor BS ^{36}Cl influx was dependent on external potassium or sodium concentration (Figs. 2*b* and 3*b*).

The dependence of BS ^{86}Rb influx on extracellular chloride concentration (NO_3^- substitution) was linear in

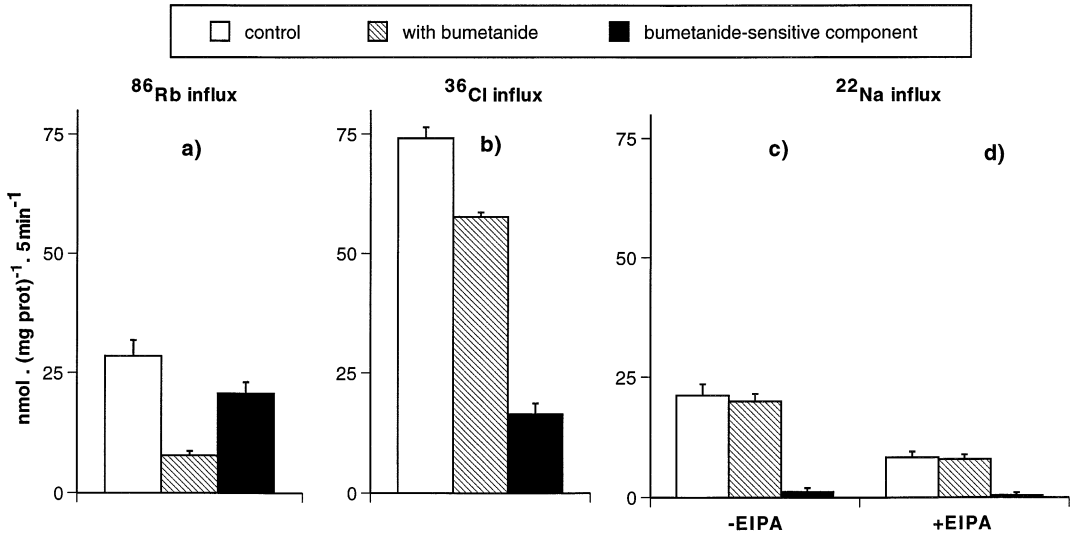

Fig. 1. ^{86}Rb (*a*), ^{36}Cl (*b*) and ^{22}Na (*c,d*) uptake by vascular smooth muscle cells. After 30 min of preincubation, medium B (*see* Materials and Methods) was replaced by the same medium containing 1 μCi/ml ^{86}Rb (*a*), 2 μCi/ml ^{36}Cl (*b*) or 4 μCi/ml ^{22}Na (*c,d*) and 1 mM ouabain with or without 10 μM bumetanide. In part of these experiments (*d*), this medium contained 10 μM EIPA. The means ± SE of 3 (*a* and *b*) and 4 (*c* and *d*) experiments performed in quadruplicate are given.

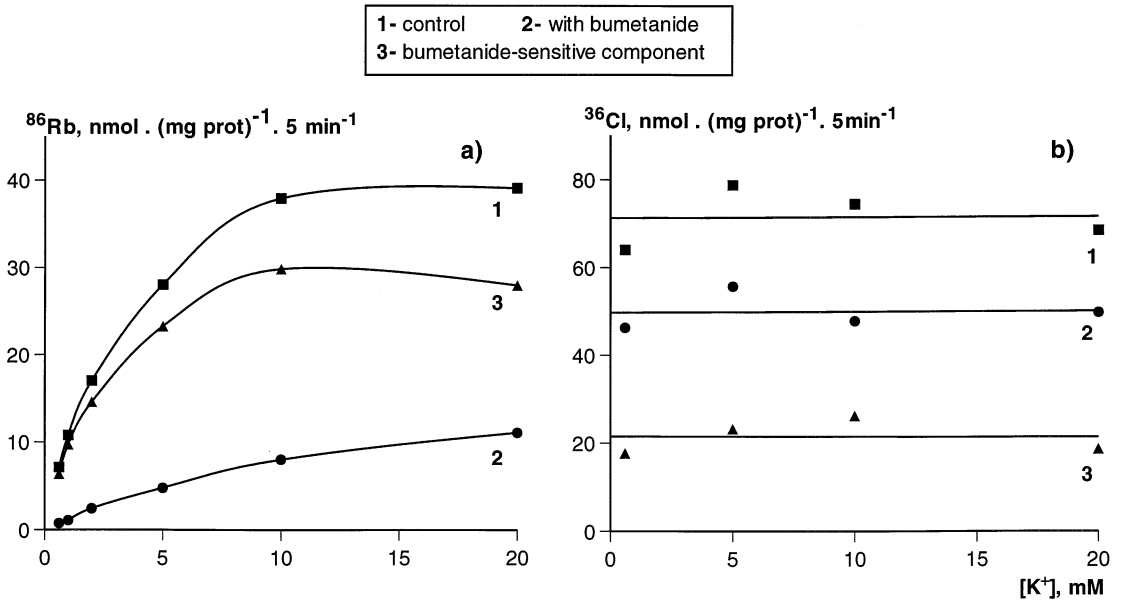

Fig. 2. Dependence of ouabain-resistant (1), (ouabain + bumetanide)-resistant (2) and ouabain-resistant bumetanide-sensitive (3) components of ^{86}Rb (*a*) and ^{36}Cl (*b*) uptake by cultured vascular smooth muscle cells (VSMC) on extracellular potassium concentration. VSMC were preincubated for 30 min in medium B and washed with 2 ml of medium D. Isotope uptake was initiated by the addition of 0.25 ml medium B containing 1 μCi/ml ^{86}Rb or 2 μCi/ml ^{36}Cl and 1 mM ouabain with or without bumetanide (10 μM). Extracellular potassium concentration was varied in the range of 0.6 to 20 mM by equimolar substitution of KCl with choline chloride in the presence of 120 mM NaCl. The means ± SE of 2 experiments performed in quadruplicate are given.

the range from 20 to 140 mM (Fig. 4*a*, curve 3). Extracellular chloride did not affect basal bumetanide-resistant ^{86}Rb influx (Fig. 4*a*, curve 2). The rate of ^{36}Cl influx was exponentially increased with a rise in $[Cl^-]_o$ (Fig. 4*b*, curve 1). In contrast to ^{86}Rb, bumetanide did not significantly affect basal ^{36}Cl influx at $[Cl^-]_o$ <80 mM. In the range of extracellular chloride concentration between 100 and 140 mM, the values of BS compounds

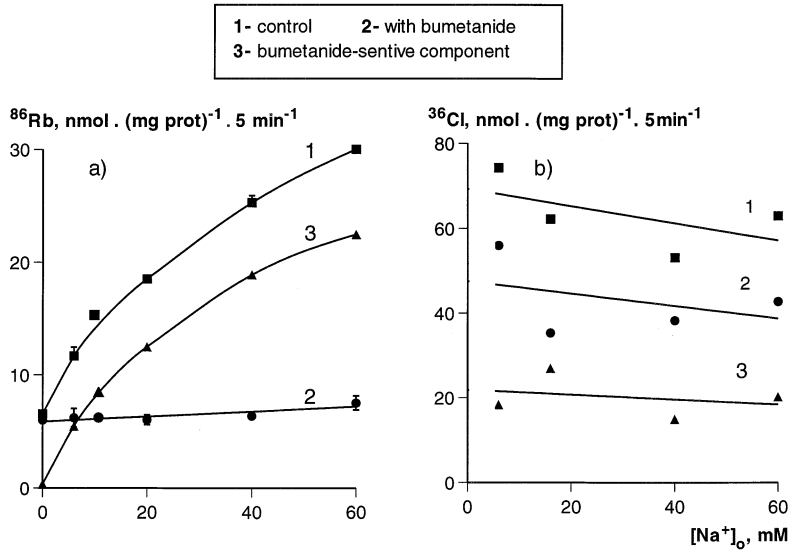

Fig. 3. Dependence of ouabain-resistant (1), (ouabain + bumetanide)-resistant (2) and ouabain-resistant bumetanide-sensitive (3) components of ^{86}Rb (*a*) and ^{36}Cl (*b*) uptake by cultured vascular smooth muscle cells (VSMC) on extracellular sodium concentration. VSMC were preincubated for 30 min in medium B and washed with 2 ml of medium D. ^{86}Rb and ^{36}Cl uptake was initiated by the addition of 0.25 ml medium B containing 1 μCi/ml ^{86}Rb or 2 μCi/ml ^{36}Cl and 1 mM ouabain with or without bumetanide (10 μM). Extracellular sodium concentrations were varied in the range of 0 to 60 mM (^{86}Rb influx) or 6 to 60 mM (^{36}Cl influx) by equimolar substitution of NaCl with choline chloride (140–80 mM) in the presence of 5 mM KCl. The means ± SE of two experiments performed in quadruplicate are given.

of ^{36}Cl influx were less than BS ^{86}Rb uptake by 30–50% (Fig. 4*a*, and *b*, curves 3).

Bumetanide-sensitive Inward and Outward ^{86}Rb Fluxes

Previously, it was shown that in the absence of ion transport inhibitors, the absolute values of ^{86}Rb (K^+) influx and efflux are about the same (70–80 nmol · (mg prot)$^{-1}$ · 5 min^{-1}) and ouabain inhibits the rate of ^{86}Rb influx by 40–60% (Orlov et al. 1992*b*). In the present study, we compared the absolute values of BS inward and outward fluxes. Table 1 shows that in the presence of ouabain, the rate of total ^{86}Rb efflux was 3-fold higher compared to ouabain-resistant ^{86}Rb influx.

Bumetanide decreased the rate of ^{86}Rb influx and efflux by 70 and 25%, respectively. However, despite different contribution of the BS components in inward and outward potassium movement, the absolute values of the BS components of ^{86}Rb influx and efflux were about the same (~18 nmol · mg prot^{-1} · 5 min^{-1}). The bumetanide-insensitive components of ^{86}Rb fluxes are probably mediated by K^+ channels and membrane leakage that is in accordance with higher values of this component of ^{86}Rb efflux as compared with those of influx (53 and 8 nmol · mg prot^{-1} · 5 min^{-1}, respectively, Table 1).

Effect of Hyperosmotic and Isosmotic Shrinkage on Bumetanide-sensitive Ion Fluxes

To induce isosmotic shrinkage, VSMC were preincubated for 30 min in isosmotic monovalent ion-depleted medium and then transferred to control isosmotic medium (medium B) (Orlov et al., 1996). Hyperosmotic shrinkage of VSMC was triggered by the addition of sucrose (Orlov et al., 1992*b*). Neither hyper- nor isosmotic shrinkage affected bumetanide-resistant ^{86}Rb and ^{36}Cl uptake (Table 2). In accordance with previously reported data (Orlov et al., 1992*b;* Orlov et al., 1996) hypertonic shrinkage of VSMC increased BS ^{86}Rb influx by 70–80% (Fig. 5*a*, column 2), whereas isosmotic shrinkage resulted in increased of BS ^{86}Rb uptake by 200–250% (Fig. 5*a*, column 3). Both hyperosmotic and isosmotic shrinkage augmented BS ^{36}Cl influx (Fig. 5*b*). The values of shrinkage-induced increment of BS ^{86}Rb and ^{36}Cl influx were not significantly different (Table 2). Neither hyperosmotic nor isosmotic shrinkage increased EIPA-resistant ^{22}Na influx in VSMC (Fig. 5*c*).

Table 3 shows that shrinkage-induced ^{86}Rb influx was abolished in sodium- or chloride-depleted media. In contrast to basal ^{36}Cl influx (Figs. 2*b* and 3*b*), shrinkage-induced BS ^{36}Cl influx was absent in media with equimolar substitution of K by Na or Na by choline.

Dose-dependency of Bumetanide

Half-maximal and full inhibition of ^{86}Rb influx by bumetanide in control (unshrunken) VSMC was observed at 0.2–0.3 μM and 1 μM, respectively. A further increase of bumetanide concentration up to 100 μM did not cause any significant inhibition of ^{86}Rb uptake (Fig. 6*a*, curve 1). These results are in accordance with data on VSMC (Smith et al., 1987) and other cells studied so far (Haas, 1989). Unlike ^{86}Rb, basal ^{36}Cl influx was unaffected by bumetanide in the range up to 1 μM and was inhibited by 20–30% with bumetanide concentrations from 3 to 100

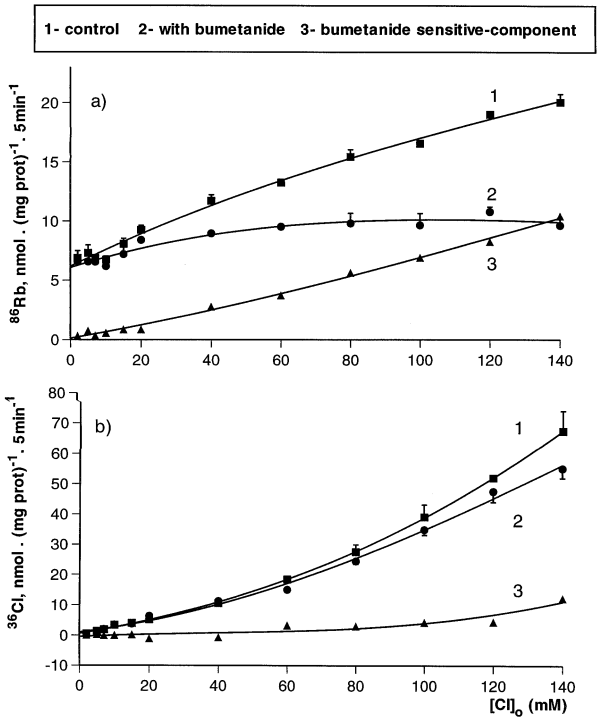

Fig. 4. Dependence of ouabain-resistant (1), (ouabain + bumetanide)-resistant (2) and ouabain-resistant bumetanide-sensitive (3) components of ^{86}Rb (*a*) and ^{36}Cl (*b*) uptake by cultured vascular smooth muscle cells (VSMC) on extracellular chloride concentration. VSMC were preincubated for 30 min in medium B and washed with 2 ml of medium E. Isotope uptake was initiated by the addition of 0.25 ml medium B containing 5 mM KNO_3 and 1 mM $MgSO_4$ instead of KCl and $MgCl_2$, respectively, 1 μCi/ml ^{86}Rb or 2 μCi/ml ^{36}Cl and 1 mM ouabain with or without bumetanide (10 μM). Extracellular chloride concentration was varied in the range of 2 to 140 mM by equimolar substitution of NaCl with $NaNO_3$. The means ± SE of three experiments performed in quadruplicate are given.

μM (Fig. 6*b*, curve 1). In contrast to control (unshrunken) cells, the dose-dependency of bumetanide for shrinkage-induced increments of ^{86}Rb and ^{36}Cl influx was not significantly different (K_i of ~0.3 μM) (Fig. 6, curves 2).

Intracellular Content of Monovalent Ions and Water

As shown above, 1 μM bumetanide caused maximal inhibition of ^{86}Rb influx whereas significant inhibition of ^{36}Cl influx in control (unshrunken) VSMC was observed in the range from 3 to 100 μM (Fig. 6). To examine the involvement of the transport pathway with high affinity for bumetanide in net fluxes of ions and osmotically obliged water, we compared the effect of bumetanide on volume and intracellular content of monovalent ions in VSMC. As can be seen from Fig. 7*a*, 45 min of preincubation with 1 μM bumetanide did not alter VSMC volume. The data presented in Table 4 show that 2-hr incubation of VSMC with 1 μM bumetanide also did not modify the intracellular content of monovalent cations. At 10 μM, bumetanide decreased intracellular chloride concentration by 20%. However, this effect was not statistically significant and was not accompanied by a significant decrease of cell volume (*data not shown*). Under protocol of isosmotic shrinkage intracellular Cl^- and Na^+ concentration was decreased by 4–5-fold whereas $[K^+]_i$ was decreased by 25–30% only.

Table 1. Effect of bumetanide on the rate of ^{86}Rb influx and efflux

Additions to the incubation medium	^{86}Rb influx, nmol · mg prot^{-1} · 5 min^{-1}	^{86}Rb efflux, nmol · mg prot^{-1} · 5 min^{-1}
1. Ouabain	26.5 ± 2.0	70.7 ± 4.8
2. Ouabain + bumetanide	8.0 ± 0.9	53.1 ± 4.0
3. Bumetanide-sensitive component	18.5 ± 1.7	17.6 ± 4.6
$P_{1,2}$	<0.001	<0.05

VSMC were preincubated for 3 hr in synchronization medium with or without 1 μCi/ml ^{86}Rb for influx and efflux experiments, respectively. They were then washed with 5 × 2 ml aliquots of ice-cold medium A and mixed with 0.5 ml of medium B containing 1 mM ouabain with or without 10 μM bumetanide. For influx measurement, this medium also contained 1 μCi/ml ^{86}RbCl. The means ± SE obtained for experiments performed in quadruplicate are given.

Effect of Bumetanide on Regulatory Volume Increase

Transfer from monovalent ion-depleted medium to control medium B led to isosmotic shrinkage of VSMC. After 10 min of incubation in medium B, cell volume was decreased by 20–22% compared with the controls (Fig. 7*b*). This isosmotic shrinkage followed a regulatory volume increase and in 45 min, the volume of control and shrunken VSMC was not significantly different. As can be seen from Fig. 7*b*, 1 μM bumetanide completely abolished the regulatory volume increase in isosmotically shrunken VSMC. In contrast to bumetanide, an inhibitor of Na^+/H^+ exchange EIPA did not affect regulatory volume increase in these cells (*data not shown*).

Discussion

The hypothesized involvement of Na^+,K^+,2Cl^- cotransport in monovalent ion fluxes in VSMC is based on: (i) the relative potency of SBA derivatives as inhibitors of ouabain-resistant ^{86}Rb influx; (ii) the complete inhibition of BS ^{86}Rb influx in Na^+ and Cl^--free media; and (iii) the values of Hill's coefficients of ^{86}Rb influx for extracel-

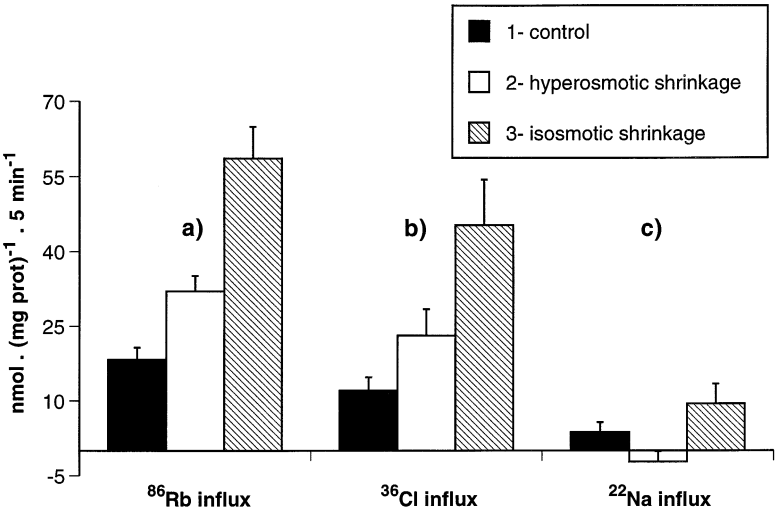

Fig. 5. Bumetanide-sensitive components of ^{86}Rb (*a*), ^{36}Cl (*b*) and ^{22}Na (*c*) influx in cultured vascular smooth muscle cells (VSMC) under basal conditions (1) or under hyperosmotic (2) and isosmotic (3) shrinkage. VSMC were preincubated in isosmotic control medium B (1 and 2) or in isosmotic monovalent ion-depleted medium (3–300 mM sucrose, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose and 20 mM HEPES-tris buffer, pH 7.4). After 30 min, these media were replaced by isosmotic medium B (1 and 3) or hyperosmotic medium B containing an additional 120 mM sucrose (2). These media were also supplied with 1 μCi/ml $^{86}RbCl$ (*a*), 2 μCi/ml ^{36}Cl (*b*) or 4 μCi/ml ^{22}Na (*c*), 1 mM ouabain (*a–c*), 10 μM EIPA (*c*) with or without 10 μM bumetanide. The means ± SE of 3 (*a* and *b*) and 5 (*c*) experiments performed in quadruplicate are given.

Table 2. Shrinkage-induced increments of bumetanide-sensitive ^{86}Rb and ^{36}Cl influx in VSMC

	^{86}Rb influx nmol. · mg prot^{-1} · 5 min^{-1}		^{36}Cl influx nmol. · mg prot^{-1} · 5 min^{-1}	
	Without bumetanide	With bumetanide	Without bumetanide	With bumetanide
1. Hyperosmotically shrunken VSMC	13.7 ± 2.0	0.2 ± 0.4	11.2 ± 5.0	1.3 ± 0.9
2. Isosmotically shrunken VSMC	40.3 ± 6.1	0.7 ± 0.4	33.3 ± 8.0	0.5 ± 1.6

VSMC were shrunken isosmotically or hyperosmotically by 30 min of preincubation in monovalent ion-depleted medium or by the addition of 120 mM sucrose in isotonic medium, respectively. For more details *see* Fig. 5. The means ± SE of three experiments performed in quadruplicate are given.

lular K^+, Na^+, and Cl^- (Owen, 1984). To further examine this hypothesis, we compared the effect of bumetanide on inward ^{86}Rb, ^{22}Na and ^{36}Cl fluxes in VSMC and their regulation by extracellular monovalent ions. BS ^{86}Rb uptake was absent in Na^+- or Cl^--free media. The $K_{0.5}$ values of BS ^{86}Rb influx for external K^+ and Na^+ obtained in our study (2.5 and 30 mM, respectively) are in the range reported by Smith & Smith, 1987 and O'Donnell & Owen, 1988. In these investigations, it was also shown that dependence of BS ^{86}Rb influx in VSMC on external $[Cl^-]_o$ is linearized in Eadie-Hofstee plots when Cl^- concentration (Smith & Smith, 1987) or the square of Cl^- concentration (O'Donnell & Owen, 1988) was used, given $K_{0.5}$ values for Cl^-_o in the range between 40 and 80 mM (O'Donnell & Owen, 1988; O'Donnell & Owen, 1994). The dependence of BS ^{86}Rb influx on $[Cl^-]_o$ in our study was well fitted by a straight line and not linearized in Eadie-Hofstee plots. It should be underlined, however, that we substituted Cl^- with NO_3^-, whereas gluconate substitution was used in the above-cited studies. These data suggest that in contrast to gluconate, inorganic anions such as NO_3^- and SO_4^{2-} compete with Cl^-_o and partly inhibit (Na^+_o + Cl^-_o)-dependent ^{86}Rb influx, thus affecting the apparent affinity of BS transporter for $[Cl^-]_o$. This suggestion is in accordance with data on the effect of anion substitution on Cl^- fluxes obtained in ascites tumor cells (Aull, 1972), human erythrocytes (Gunn et al., 1973) and on BS ^{86}Rb influx in

Table 3. Effect of extracellular ions on shrinkage-induced increment of bumetanide-sensitive ^{86}Rb and ^{36}Cl influx in VSMC

Concentration of monovalent ions in incubation medium, mM			Shrinkage-induced increment of bumetanide-sensitive influx, nmol · mg prot^{-1} · 5 min^{-1}	
Na^+	K^+	Cl^-	^{86}Rb influx	^{36}Cl influx
140	5	149	15.7 ± 1.9	16.5 ± 3.7
0	5	149	0.1 ± 0.4	0.9 ± 2.3
140	0	149	ND	1.5 ± 2.0
140	5	0	0.2 ± 0.6	ND

Shrinkage-induced ^{86}Rb and ^{36}Cl influx was determined as the difference between the rate of BS isotope uptake in hyperosmotic media containing an additional 120 mM sucrose and in isosmotic (sucrose-free) media. Na^+, K^+ and Cl^- were substituted with choline, Na and NO_3^-, respectively. Concentrations of monovalent ions in media are shown in the left column. For more details, *see* Materials and Methods. The means ± SE of two experiments performed in quadruplicate are given.

ND—ion fluxes in these media were not determined.

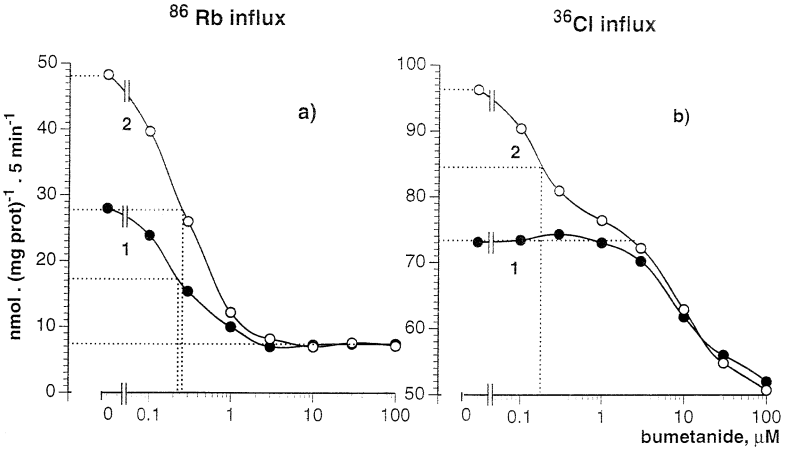

Fig. 6. Dose-dependence of the effect of bumetanide on basal (curves 1) and shrinkage-induced (curves 2) ^{86}Rb (*a*) and ^{36}Cl (*b*) influx in vascular smooth muscle cells preincubated in medium *B*. After 30 min, these media were replaced by isosmotic medium B (curves 1) or hyperosmotic medium (curves 2—medium B containing 120 mM sucrose). These media were also supplied with 1 μCi/ml ^{86}RbCl (*a*) or 2 μCi/ml ^{36}Cl (*b*), 1 mM ouabain and bumetanide. The means of experiments performed in quadruplicate are given.

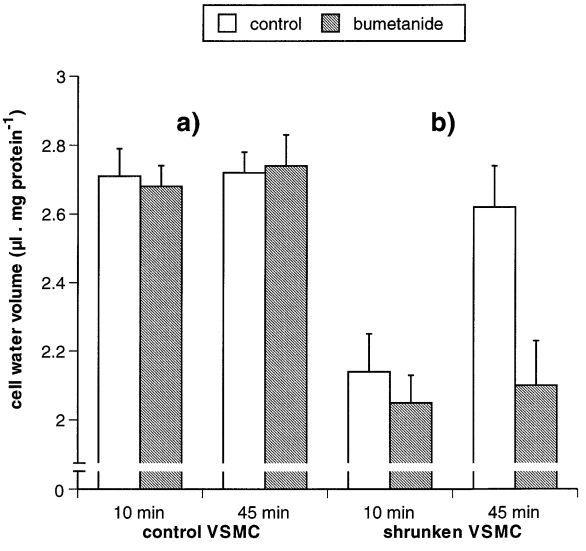

Fig. 7. Effect of bumetanide on cell volume adjustment in control (*a*) and isotonically shrunken (*b*) cultured vascular smooth muscle cells preincubated in isosmotic control medium B (*a*) or in isosmotic monovalent ion-depleted medium containing (in mM): 300 sucrose, 1 $MgSO_4$, 1 $CaCl_2$, 5 glucose and 20 HEPES-tris buffer (pH 7.4) (*b*). After 30 min, these media were replaced by medium B containing 2 μCi/ml [^{14}C]-urea, 1 mM ouabain with or without 1 μM bumetanide. [^{14}C]-urea uptake was terminated 10 or 45 min after incubation. The means ± SE of four experiments performed in triplicate are given.

Table 4. Intracellular concentration of potassium, sodium and chloride in control and isosmotically shrunken vascular smooth muscle cells (VSMC)

Type of VSMC	Bumetanide, μM	$[K^+]_i$, mM	$[Na^+]_i$, mM	$[Cl^-]_i$, mM
Control	0	137 ± 12	13.2 ± 0.8	48.8 ± 5.9
Control	1	130 ± 11	14.0 ± 1.6	52.4 ± 8.9
Control	10	126 ± 12	13.8 ± 1.4	39.6 ± 7.0
Isosmotic shrinkage	0	101 ± 14	2.7 ± 0.8	12.3 ± 3.4

VSMC were preincubated for 3 hr in synchronization medium containing 0.5 μCi/ml ^{86}Rb, 1 μCi/ml ^{36}Cl or 2 μCi/ml ^{22}Na and then for 2 hr in medium B (control cells) or 1.5 hr in medium B and then 0.5 hr in monovalent ion-depleted medium contained (in mM): 300 sucrose, 1 $MgSO_4$, 1 $CaCl_2$, 5 glucose and 20 HEPES-tris buffer (pH 7.4) (isosmotically shrunken VSMC) with the same specific activities and with 1 or 10 μM bumetanide where indicated. The means ± SE of two experiments performed in quadruplicate are given.

the PC12 pheochromocytoma cell line (Leung et al., 1994).

Data presented in Figs. 2*a*–4*a* demonstrate that BS ^{86}Rb influx in VSMC is mediated by ($[Na^+]_o$ + $[Cl^-]_o$)-activated transporter. To verify whether Na^+_o and Cl^-_o activate ^{86}Rb influx via interaction with regulatory or cotransporting sites in the carrier molecule, we compared the effect of bumetanide on ^{86}Rb, ^{22}Na and ^{36}Cl uptake. As seen in Fig. 1, the rate of total ^{22}Na influx was about 18–22 nmol · mg prot^{-1} · 5 min^{-1}. In accordance with the model of Na^+,K^+,2Cl^- cotransport and the value of basal BS ^{86}Rb influx (~20 nmol · mg prot^{-1} · 5 min^{-1}), ^{22}Na uptake should be completely blocked by bumetanide. However, we failed to detect any effect of this compound on ^{22}Na influx. In accordance with the same model, BS ^{36}Cl influx should be 2-fold higher than BS ^{86}Rb uptake. Actually, BS ^{36}Cl influx was ~30% lower than BS ^{86}Rb uptake. These results demonstrate the lack of functional Na^+,K^+,2Cl^- cotransport in VSMC and suggest that BS ^{86}Rb influx is mediated by (Na^+_o + Cl^-_o)-dependent K^+/K^+ exchange, Na^+_o-dependent K^+,Cl^- cotransport or (Na^+_o + Cl^-_o)-dependent K^+ uniporter. The data listed below show that *under basal conditions* the BS ion transporter is operated as (Na^+_o + Cl^-_o)-dependent K^+/K^+ exchanger.

(i) The apparent affinity of ^{86}Rb influx for bumetanide in control (*unshrunken*) VSMC is one order of magnitude higher than ^{36}Cl uptake (Fig. 6). Moreover,

unlike BS ^{86}Rb influx, neither Na^+_o nor K^+_o affected BS ^{36}Cl uptake (Figs. 2 and 3). These results indicate that the partial suppression of basal ^{36}Cl influx by bumetanide was not caused by inhibition of the ($Na^+ + K^+$)-coupled transport system and contradict the hypothesis of Na^+_o-dependent equimolar K^+,Cl^- cotransport.

(ii) Inhibition of electrogenic Na^+,K^+ pump with ouabain leads to depolarization of smooth muscle cells by 10–15mV (Fleming, 1980). Values of the BS component of ^{86}Rb influx in control and ouabain-treated VSMC were not different (*data not shown*). These results indicate that unidirectional fluxes mediated by channels or other hypothetical (Na^+_o + Cl^-_o)-dependent electrogenic K^+ transport pathways can not be involved in BS ^{86}Rb influx.

(iii) Neither cell volume (Fig. 7) nor intracellular concentration of monovalent ions (Table 4) was affected by 1 μM bumetanide. Recently, it was shown that 10 μM bumetanide causes a rapid and reversible 25% decrease of intracellular Cl^- content in saphenous artery smooth muscle (Davis et al., 1993). In our study, 10 μM bumetanide decreased $[Cl^-]_i$ influx by 15–25% (Table 4). However, as mentioned above, bumetanide inhibits ^{36}Cl influx at high concentrations through a mechanism which is unrelated to BS K^+ transporter. The absolute values of BS components of ^{86}Rb influx and efflux were the same (Table 1). These results support the hypothesis of (Na^+_o + Cl^-_o)-dependent K^+/K^+ exchange.

Equimolar K^+/K^+ exchange as a mode of operation of BS ion transporter in VSMC can not be involved in the regulation by SBA derivatives of intracellular concentrations of monovalent ions, membrane potential and related cellular functions. Thus, the prevalence of this mode of operation of the SBA-sensitive carrier in nonepithelial tissue can be viewed as a reason for the lack of side-effect of long-term treatment with furosemide and related compounds (Unwin et al., 1995). This hypothesis is supported by data obtained for other nonepithelial tissues. Thus, it was shown that in preimplantation mouse conceptuses, BS ^{86}Rb influx is mediated by Na^+_o-insensitive, Cl^-_o-dependent K^+/K^+ exchange (Van Winkle et al., 1992). In human erythrocytes, investigation of the mode of operation of BS transporter is complicated by extremely high permeability for Cl^- through band 3 protein. It has been established, however, that Na^+_o-insensitive K^+/K^+ exchange is at least partly involved in furosemide-sensitive ^{86}Rb fluxes (Canessa et al., 1986; Lauf et al., 1987). In rat erythrocytes (I.A. Kolosova, *personal communication*) and in L6 myoblasts derived from embryonic rat skeletal muscle (Sen et al., 1995), bumetanide inhibited ^{86}Rb influx but did not modify inward ^{22}Na transport. In NIH/3T3 fibroblasts, the values of BS ^{86}Rb and ^{36}Cl influx were about the same whereas basal ^{22}Na uptake was not identified (Mayer et al., 1994).

It has been reported that in the absence of arginine-vasopressin, furosemide-sensitive Cl^- entry in medullary thick ascending limbs of the mouse kidney requires Na^+ but not K^+, while in its presence this apical cotransporter operates with both Na^+ and K^+ (Sun et al., 1991). These findings suggest that the mode of operation of BS transporter in epithelial cells is under the control of intracellular signaling pathways. Data obtained in the present study show that the mode of operation of BS transporter in VSMC is controlled by cell volume. Indeed, both hyperosmotic and isosmotic shrinkage of VSMC activated BS ^{86}Rb and ^{36}Cl uptake and did not alter EIPA-resistant ^{22}Na influx (Fig. 5). In contrast to basal conditions, the increments of BS ^{86}Rb and ^{36}Cl influx in shrunken VSMC were not statistically different (Table 2). Shrinkage-induced BS ^{86}Rb uptake was dependent on the presence of Na^+_o or Cl^-_o, whereas shrinkage-induced BS ^{36}Cl uptake was dependent on the presence of Na^+_o and K^+_o (Table 3). These results indicate that shrinkage-induced BS ion fluxes are mediated by activation of Na^+_o-dependent K^+,Cl^- cotransport. This suggestion is supported by data on cell volume measurement. In contrast to equimolar K^+/K^+ exchange, BS K^+,Cl^- cotransport should be involved in net movement of salt and osmotically obliged water. Indeed, as seen from Fig. 7, shrunken VSMC display a BS regulatory volume increase. Table 4 shows that intracellular Cl^- concentration in isosmotically shrunken cells was 4-fold less as compared with control VSMC. Based on these results, the increment of electrochemical gradient of Cl^- can be viewed as the driving force for inward K^+,Cl^- cotransport in isosmotically shrunken cells.

The affinity of bumetanide for an isoform of ion transporter localized in basolateral membranes of secretory epithelia (NKCC1) varied between 0.1–0.5 μM, and these values were one order of magnitude higher than those reported for the carrier localized in apical membranes of absorptive epithelia (NKCC2) (Haas, 1994). On the basis of data on the half-maximal inhibition of basal ^{86}Rb influx and shrinkage-induced ^{36}Cl influx in VSMC by bumetanide (0.2–0.3 μM), it may be assumed that the NKCC1 isoform is involved in BS K^+/K^+ exchange and K^+,Cl^- cotransport in VSMC. This hypothesis is also confirmed by Northern blot analysis of poly(A)$^+$ RNA. These studies revealed that mRNA encoding rat NKCC2 is selectively expressed in the kidney (Payne et al., 1995; Gamba et al., 1994), whereas mRNA encoding human and mouse NKCC1 is present in epithelial cells as well as in heart, brain and skeletal muscle cells (Delpire et al., 1994; Payne et al., 1995). It has been shown that NKCC1 mediates (Na^+ + Cl^-)-dependent BS ^{86}Rb influx in Cl^--depleted, HEK-293-

transfected cells (Xu et al., 1994; Payne et al., 1995). However, the modes of operation of expressed isoforms of BS transporters have not yet been studied. It is also unknown whether the differences in regulation by intracellular signaling pathways mentioned above are related to different isoforms of SBA-sensitive transporter or whether they are caused by the tissue specific expression of other proteins involved in controlling of the activity of the carrier.

In conclusion, our results do not fit the hypothesis of the involvement of $Na^+,K^+,2Cl^-$ cotransport in SBA-sensitive ion transport in VSMC and suggest that this transport pathway operates as K^+/K^+ exchanger and K^+,Cl^- cotransport. Investigation of the molecular mechanisms of different modes of operation of SBA-sensitive transporters in epithelial and nonepithelial cells should bring new insights into the therapeutic usage of high-ceiling diuretics and should help us to analyze contradictory data on abnormalities of SBA-sensitive ion transport pathways revealed in erythrocytes, kidney tubule epithelial cells and VSMC of rats with spontaneous genetic hypertension (for recent review *see* (Hamet, Orlov & Tremblay, 1995)).

This work was supported by grants from the Medical Research Council of Canada (MT-10802, MT-10803) and the Heart and Stroke Foundation of Canada. Johanne Tremblay is the recipient of a senior scholarship from Fonds de la Recherche en Santé du Québec and of a grant from the Medical Research Council of Canada (MT-11463). Sergei Orlov is the recipient of a visiting professorship from the International Society of Hypertension and the Pfizer Pharmaceutical Company. The technical assistance of Monique Poirier, the secretarial skills of Josée Bédard-Baker and the editorial help of Ovid Da Silva are appreciated.

References

Aull, F. 1972. The effect of external anions on steady-state chloride exchange across ascites tumour cells. *J. Physiol.* **221:**255–771

Burg, M., Stoner, L., Cardinal, J., Green, J. 1973. Furosemide effect on isolated perfused tubules. *Am. J. Physiol.* **225:**119–124

Canessa, M., Brugnara, C., Cusi, D., Tosteson, D. 1986. Modes of operation and variable stoichiometry of furosemide-sensitive Na and K fluxes in human red cells. *J. Gen. Physiol.* **87:**113–142

Davis, J.P.L., Chipperfield, A.R., Harper, A.A. 1993. Accumulation of intracellular chloride in rat arterial muscle is enhanced by deoxycorticosterone acetate (DOCA)/salt hypertension. *J. Mol. Cell. Cardiol.* **25:**233–237

Delpire, E., Rauchman, M.I., Beier, D.R., Hebert, S.C., Gullans, S.R. 1994. Molecular cloning and chromosome localization of a putative basolateral $Na^+,K^+,2Cl^-$ cotransporter from mouse inner medullary collecting duct (mIMCD-3) cells. *J. Biol. Chem.* **269:**25677–25683

Fleming, W.W. 1980. The electrogenic Na^+,K^+ pump in smooth muscle: physiological and pharmacological significance. *Annu. Rev. Pharmacol. Toxicol.* **20:**129–149

Franks, D.J., Plamondon, J., Hamet, P. 1984. An increase in adenylate cyclase activity precedes DNA synthesis in cultured vascular smooth muscle cells. *J. Cell Physiol.* **119:**41–45

Gamba, G., Miyanoshita, A., Lombardi, M., Lytton, J., Lee, W.S., Hediger, M., Hebert, S. 1994. Molecular cloning, primary structure, and characterization of two members of the mammalian electroneutral sodium-(potassium)-chloride cotransporter family expressed in kidney. *J. Biol. Chem.* **269:**17113–17122

Geck, P., Pietrzyk, C., Burckhardt, B.-C., Pfeiffer, B., Heinz, E. 1980. Electrically silent cotransport of Na^+,K^+ and Cl^- in Ehrlich cells. *Biochem. Biophys. Acta* **600:**432–437

Greger, R. 1985. Ion transport in thick ascending limb of Henle's loop of mammalian nephron. *Physiol. Rev.* **65:**760–797

Gunn, R.B., Dalmark, M., Tosteson, D.C., Wieth, J.D. 1973. Characteristics of chloride transport in human red blood cells. *J. Gen. Physiol.* **61:**185–206

Haas, M. 1989. Properties and diversity of (Na-K-2Cl) cotransporters. *Annu. Rev. Physiol.* **51:**443–457

Haas, M. 1994. The Na-K-Cl cotransporters. *Am. J. Physiol.* **267:** C869–C885

Haas, M., Forbush, B. III 1988. Photoaffinity labelling of 150 kDa (Na^+ K^+ Cl^-) cotransport protein from duck red cells with a bumetanide analogue. *Biochem. Biophys. Acta* **939:**131–144

Hadrava, V., Tremblay, J., Hamet, P. 1989. Abnormalities in growth characteristics of aortic smooth muscle cells in spontaneously hypertensive rats. *Hypertension* **13:**589–597

Hall, A.C., Ellory, J.C. 1985. Measurement and stoichiometry of bumetanide-sensitive (2Na:1K:3Cl) cotransport in ferret red cells. *J. Membrane Biol.* **85:**205–213

Hamet, P., Orlov, S.N., Tremblay, J. 1995. Intracellular signalling mechanisms in hypertension. *In:* Hypertension: Pathophysiology, Diagnosis and Management (Vol. 1). J.H. Laragh and B.M. Brenner, editors pp. 575–607. Raven Press, New York

Hartee, E.I. 1972. Determination of protein content: a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* **48:**422–427

Lauf, P.K., McManus, T.J., Haas, M., Forbush, B., III, Duhm, J., Flatman, P.W., Saier, M.H., Russell, J.M. 1987. Physiology and biophysics of chloride and cation transport across cell membranes. *Fed. Proc.* **96:**2377–2394

Leung S., O'Donnell, M.E., Martinez, A., Palfrey, H.C. 1994. Regulation by nerve growth factor and protein phosphorylation of Na/K/2Cl cotransport and cell volume in PC12 cells. *J. Biol. Chem.* **269:**10581–10589

Little, P.J., Cragoe, E.J., Bobik, A. 1986. Na-H exchange is a major pathway for Na influx in rat vascular smooth muscle. *Am. J. Physiol.* **251:**C707–C712

Mayer, S.E., Sanders-Bush, E. 1994. 5-Hydroxytryptamine type 2A and 2C receptors linked to $Na^+/K^+/Cl^-$ cotransport. *Mol. Pharmacol.* **45:**991–996

McRoberts, J.A., Erlinger S., Ringler, M.J. 1982. Furosemide-sensitive salt transport in Madin-Darby canine kidney cell line. *J. Biol. Chem.* **257:**2260–2266

Molony, D.A., Reeves, W.B., Adreoli, T.E. 1989. $Na^+:K^+:2Cl^-$ cotransport and the thick ascending limb. *Kidney Int.* **36:**418–426

Musch, M.W., Field, M. 1989. K^+-independent Na-Cl cotransport in bovine tracheal epithelial cells. *Am. J. Physiol.* **256:**C658–C666

O'Donnell, M.E., Owen, N.E. 1988. Reduced Na-K-Cl cotransport in vascular smooth muscle cells from spontaneously hypertensive rats. *Am. J. Physiol.* **24:**C169–C180

O'Donnell, M.E., Owen, N.E. 1994. Regulation of ion pump and carriers in vascular smooth muscle. *Physiol. Rev.* **74:**683–721

O'Grady, S.M., Musch, M.W., Field, M. 1986. Stoichiometry and ion affinities of the Na-K-Cl cotransport system in the intestine of the winter flounder (*Pseudupleuronectes americanus*). *J. Membrane Biol.* **91:**33–41

Orlov, S.N., Cragoe, E.J., Hanninen, O. 1994. Volume- and catechol-

amine-dependent regulation of Na/H antiporter and unidirection transport of potassium in *Salmo trutta* red blood cells. *J. Comp. Physiol. B.* **164:**135–140

Orlov, S.N., Kuznetsov, S.R., Skryabin, G.A., Chernova, T.L., Pokudin, N.I. 1992*a*. The peculiarities of volume-dependent regulation of sodium and potassium fluxes in rabbit erythrocytes. *Biol. Membr.* (*Moscow*) **9:**716–722

Orlov, S.N., Resink, T.J., Bernhardt, J., Bühler, F. 1992*b*. Volume-dependent regulation of sodium and potassium fluxes in cultured vascular smooth muscle cells: dependence on medium osmolality and regulation by signalling systems. *J. Membrane Biol.* **129:**199–210

Orlov, S.N., Tremblay, J., Hamet, P. 1995. Bumetanide-inhibited ion fluxes in vascular smooth muscle cells (VSMC): evidence against Na,K,Cl cotransport. *XIII Int. Congress of Nephrology, Madrid, July 2–6* 245

Orlov, S.N., Tremblay, J., Hamet, P. 1996. Cell volume in vascular smooth muscle cells is regulated by bumetanide-sensitive ion transport. *Am. J. Physiol.* **270:**C1388–C1397

Owen, N.E. 1984. Regulation of Na/K/Cl cotransport in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **125:**500–508

Payne, J.A., Xu, J.C., Haas, M., Lyttle, C.Y., Ward, D., Forbush, B. III. 1995. Primary structure, functional expression, and chromosomal localization of the bumetanide-sensitive Na-K-Cl cotransporter in human colon. *J. Biol. Chem.* **270:**17977–17985

Russell, J.M. 1983. Cation-coupled chloride influx in squid axon. Role of potassium and stoichiometry of the transport process. *J. Gen. Physiol.* **81:**909–925

Sen, C.K., Hanninen, O., Orlov, S.N. 1995. Unidirectional sodium and potassium fluxes in myogenic L6 cells: mechanism and volume-dependent regulation. *J. Appl. Physiol.* **78:**272–281

Smith, J.B., Smith, L. 1987. $Na^+/K^+/Cl^-$ cotransport in cultured vascular smooth muscle cells: stimulation by angiotensin II and calcium ionophores, inhibition by cyclic AMP and calmodulin antagonists. *J. Membrane Biol.* **99:**51–63

Sun, A., Grossman, E.B., Lombardi, M., Hebert, S.C. 1991. Vasopressin alters the mechanism of apical Cl^- entry from Na^+, Cl^- to Na^+:K^+:2Cl^- cotransport in mouse medullary thick ascending limb. *J. Membrane Biol.* **120:**83–94

Unwin, R.J., Ligueros, M., Shakelton, C., Wilcox, C.S. 1995. Diuretics in the management of hypertension. *In:* Hypertension: Pathophysiology and Treatment. J.H. Laragh and B.M. Brenner. editors pp. 2785–2799. Raven Press, New York

Van Winkle, L.J., Campione, A.L. 1992. Novel bumetanide-sensitive K^+ transport in preimplantation mouse conceptuses. *Am. J. Physiol.* **263:**C773–C779

Vuillemin, T., Teulon, J., Geniteau-Legendre, M. 1992. Regulation of Na^+-K^+-Cl^- cotransport in a rabbit thick ascending limb cell line. *Am. J. Physiol.* **263:**C563–C572

Xu, J.C., Lytle, C., Zhu, T.T., Payne, J.A., Benz, E. Jr., Forbush, B. III. 1994. Molecular cloning and functional expression of the bumetanide-sensitive Na-K-Cl cotransporter. *Proc. Natl. Acad. Sci. USA* **91:**2201–2205