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CELL AND MOLECULAR PHYSIOLOGY

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Cell-volume-dependent vascular smooth muscle contraction: role of Na^+, K^+, $2Cl^-$ cotransport, intracellular Cl^- and L-type Ca^{2+} channels

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Abstract This study elucidates the role of cell volume in contractions of endothelium-denuded vascular smooth muscle rings (VSMR) from the rat aorta. We observed that hyposmotic swelling as well as hyper- and isosmotic shrinkage led to VSMR contractions. Swelling-induced contractions were accompanied by activation of Ca^{2+} influx and were abolished by nifedipine and verapamil. In contrast, contractions of shrunken cells were insensitive to the presence of L-type channel inhibitors and occurred in the absence of Ca^{2+}_o. Thirty minutes preincubation with bumetanide, a potent Na^+,K^+,Cl^- cotransport (NKCC) inhibitor, decreased Cl^-_i content, nifedipine-sensitive ^{45}Ca uptake and contractions triggered by modest depolarization ($[K^+]_o$=36 mM). Elevation of $[K^+]_o$ to 66 mM completely abolished the effect of bumetanide on these parameters. Bumetanide almost completely abrogated phenylephrine-induced contraction, partially suppressed contractions triggered by hyperosmotic shrinkage, but potentiated contractions of isosmotically shrunken VSMR. Our results suggest that bumetanide suppresses contraction of modestly depolarized cells via NKCC inhibition and Cl^-_i-mediated membrane hyperpolarization, whereas augmented contraction of isosmotically shrunken VSMR by bumetanide is a consequence of suppression of NKCC-mediated regulatory volume increase. The mechanism of bumetanide inhibition of contraction of phenylephrine-treated and hyperosmotically shrunken VSMR should be examined further.

Keywords Ca^{2+} channels · Cell volume · Contraction · Intracellular Cl^- · Na^+,K^+,$2Cl^-$ cotransport · Smooth muscle

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Introduction

Since the first observations on increased monovalent ion exchange in vascular smooth muscle rings (VSMR) [25] and red blood cells [51] from spontaneously hypertensive rats, several research teams have reported that, at least partially, these abnormalities are caused by enhanced electroneutral Na^+,K^+,$2Cl^-$cotransport (NKCC) (for review see [41, 47]). Two isoforms of this carrier have been cloned from vertebrate cDNA libraries. A ubiquitous NKCC1 isoform has been detected in all types of cells studied so far, whereas expression of the renal-specific NKCC2 isoform is limited to the apical membrane of epithelial cells from the thick ascending limb and macula densa [37, 53].

The hypothesis on NKCC1 involvement in the pathogenesis of hypertension is based on three major observations. First, in erythrocytes from F_2 hybrids of spontaneously hypertensive and normotensive rats, NKCC activity correlated positively with blood pressure [10, 28]. Second, $NKCC1^{-/-}$ knockout mice exhibited decreased blood pressure as well as attenuated vascular tone and constriction of VSMR treated with the α-adrenoceptor agonist phenylephrine (PE) [16, 35]. Third, NKCC inhibition with bumetanide reduced contractions of rat VSMR and the guinea pig ureter triggered by K^+_o-induced depolarization, PE and electrical stimulation [4, 29]. Brown et al. were the first to suggest that NKCC contributes to the maintenance of vascular tone via

adjustment of the transmembrane Cl^- gradient [11]. Indeed, bumetanide decreased $[Cl^-]_i$, hyperpolarized vascular smooth muscle cells (VSMC) [15] and abolished differences in these parameters between normotensive and DOCA-salt-hypertensive rats [11].

In experiments on bone marrow transplantation from progenitors to X-ray-irradiated F_1 hybrids of spontaneously hypertensive and normotensive rats, Bianchi et al. showed that enhanced NKCC detected in this experimental model of primary hypertension is genetically determined rather than a consequence of the hypertensive milieu [10]. On the other hand, other researchers detected augmented NKCC in VSMR from rodents with secondary hypertension [15, 24] and enhanced content of endogenous NKCC inhibitor in the plasma of salt-sensitive rats [5], suggesting that the carrier is subjected to cell-type-specific regulation by diverse stimuli. Indeed, no systematic involvement of Ca^{2+}, cAMP and cGMP has been detected in studies of NKCC in human and rat erythrocytes [17], whereas in VSMR and cultured VSMC, this carrier was activated by $Ca^{2+}{}_i$-raising vasoconstrictors, including angiotensin II, and was inhibited by cAMP- and cGMP-mediated vasodilators [3, 4, 42, 49, 55, 57]. Opposing regulation by vasodilators was documented in a study of Na^+-independent K^+,Cl^- cotransport (KCC) [2], i.e. another member of the Cl^--coupled cotransporter superfamily contributing to outwardly directed Cl^- transport [1].

Similarly to "ying-yang" regulation by vasodilators, NKCC and KCC are also affected reciprocally by cell volume modulation. Indeed, shrinkage-induced activation of inwardly directed NKCC, Na^+/H^+ exchange, and Na^+-coupled transport of organic osmolytes contributes to regulatory volume increase (RVI), whereas swelling-induced and outwardly directed KCC, K^+ and anion channels provide regulatory volume decrease (RVD) [20, 31, 36]. It is important to underline that cell shrinkage activates NKCC1 in all types of nucleated cells studied so far [39]. Keeping this in mind, we employed hyperosmotic and isosmotic shrinkage to further explore the role of NKCC in VSMC contraction. The results of our study were reported in part at the 13th European Meeting on Hypertension [7].

Materials and methods

Preparation of VSMR

Endothelium-denuded VSMR were obtained from the thoracic aorta of 11- to 13-week-old Wistar rats euthanized under deep intraperitoneal anesthesia with sodium pentobarbital (Nembutal, 70 mg/kg) in accordance with institutional animal care guidelines. The isolated aorta was placed in physiologically balanced salt solution (PSS). Connective tissue and fat were taken out with scissors, whereas the endothelium was removed by careful rotation of a wooden manipulator inside the VSMR lumen just before the experiments. The 2- to 3-mm VSMR were either used immediately or stored at 4°C for up to 24 h. In preliminary experiments, we documented that 24 h storage did not affect VSMR contractile responses.

Isometric VSMR contraction

VSMR were mounted in 1-ml baths with stainless steel hooks inserted into the vascular ring orifice. One hook was fastened to a mechanical force transducer MX2B (Tomsk, Russia) with silk thread; the other served as an anchor. The tissues were bathed in PSS, buffered with TRIS at pH 7.4 (37°C) and bubbled with 95%O_2/5% CO_2 at a volumetric speed of ~1 ml/min. To control the contractile response, the VSMR were equilibrated for 1 h at tension of 0.5–1 g and exposed to $K^+{}_o$-induced depolarization caused by isosmotic substitution of 30 mM NaCl with KCl. The VSMR were then exposed to modified PSS containing either altered ions concentration or sucrose in the presence or absence of the test compounds. Isometric changes in VSMR tension were recorded with an XY recorder (Carl Zeiss, Jena, Germany).

Cultured VSMC

The precise measurement of cell volume and inward ion fluxes in VSMR are complicated by a relatively large extracellular space, the presence of fibroblasts, and VSMC heterogeneity [18]. On the other hand, cultured VSMC rapidly downregulate the expression of several specific genes that define their contractile phenotype in vivo. Keeping this in mind, we used a low-density seeding strategy for VSMC derived from the aortae of Wistar-Kyoto (WKY) rats to select a cell line possessing the highest expression of smooth muscle-specific α-actin, SM22 protein and myosin light chain kinase. This cell line, WKY-7, also possessed the highest sensitivity to angiotensin II and endothelin-1, measured by mitogen-activated protein kinase ERK1/2 phosphorylation [14]. In the present study, WKY-7 cells were grown for 48–72 h in DMEM supplemented with fetal and newborn calf serum (10% of each), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). To establish quiescence, VSMC were incubated before experiments during 48 h in the presence of 0.2% calf serum.

NKCC activity

NKCC activity was measured as a bumetanide-sensitive component of the ^{86}Rb influx rate. WKY-7 cells seeded in 24-well plates were washed twice with 2-ml aliquots of PSS. The medium was then aspirated, and 0.25 ml of PSS with 1 μCi/ml ^{86}Rb and 1 mM ouabain with or without bumetanide was added. After 5-min incubation at 37°C, isotope uptake was terminated by the addition of 2 ml of ice-cold medium W containing 100 mM $MgCl_2$ and 10 mM HEPES-TRIS buffer (pH 7.4). Radioactivity of the

incubation medium and cell lysate was measured with a liquid scintillation analyzer, and the rate of ^{86}Rb influx (V, nmol per mg of protein per 5 min) was calculated as $V=A/am$, where A was the radioactivity of the samples (cpm), a was the specific radioactivity of K^+ (^{86}Rb) in the medium (cpm/nmol), and m was protein content measured with the modified Lowry method. For more details see [46].

Intracellular water volume in WKY-7 cells seeded in 12-well plates was measured as [^{14}C]-urea available space according to a previously described protocol [46] and calculated as $V=A_c/A_m m$, where A_c was the radioactivity of the cells after 30 min incubation with 2 μCi/ml [^{14}C]-urea (dpm), A_m was the radioactivity of the incubation medium (dmp/μl), and m was protein content in the cell lysate (mg).

Intracellular Cl^- content in WKY-7 cells was measured as the steady-state distribution of ^{36}Cl as described previously in detail [44]. To calculate $[Cl^-]_i$, the volume of intracellular water was quantified in parallel experiments with the protocol presented above. To measure Cl^-_i content in VSMR, the rings were subjected to 5 h preincubation in PSS containing 2 μCi/ml ^{36}Cl; in some experiments, bumetanide and sucrose were added in the last 30 min of incubation. The VSMR were then rinsed in 3×50-ml aliquots of ice-cold PSS and solubilized in scintillation cocktail containing Triton X-100:toluene 1:2 (v/v), 4 g/l 2,5-diphenyl-1,3,4-oxadiazole (PPO) and 0.1 g/l 1,4-*bis*[5-phenyl-2-oxazolyl]-benzene; 2–2′-*p*-phenylene-*bis*[5-phenyloxazole] (POPOP). Intracellular Cl^- content (nmol/mg) was calculated as $[Cl^-]_i=A/am$, where A was the radioactivity of the samples (cpm), a was the specific radioactivity of Cl^- in the medium (cpm/nmol), and m was the VSMR wet weight (mg) measured before incubation.

Voltage-gated L-type Ca^{2+} channel activity was estimated as verapamil- or nifedipine-sensitive component of the ^{45}Ca influx rate. WKY-7 cells seeded in 24-well plates were preincubated for 30 min with PSS. Then, this medium was aspirated, and 0.25 ml of PSS containing 0.1 mM $CaCl_2$ with or without nifedipine and verapamil was added. Isotope uptake was initiated by the addition of 0.25 ml of the same PSS with 3 μCi/ml ^{45}Ca, and terminated in 5 min as indicated above. To induce cell depolarization, KCl in isotope-containing medium was increased up to 60 mM by equimolar substitution of NaCl. For more details see [45].

Solutions and chemicals

PSS contained 120.4 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.5 mM glucose, and 15 mM TRIS. Ca^{2+}-free PSS contained 0.5 mM EGTA and 3.6 mM $MgCl_2$. To avoid the impact of Cl^-/HCO_3 exchange on intracellular Cl^- handling, bicarbonate-free PSS was employed in all experiments, and pH was adjusted to 7.4 by the addition of 0.1 N HCl. PSS osmolality was increased by the addition of 50–300 mM sucrose as a cell-impermeable osmolyte. In Cl^--free PSS, KCl, NaCl and $MgCl_2$ were substituted with Na-gluconate, K-gluconate and $MgSO_4$, respectively, whereas $CaCl_2$ was omitted. Previously, it was shown that the

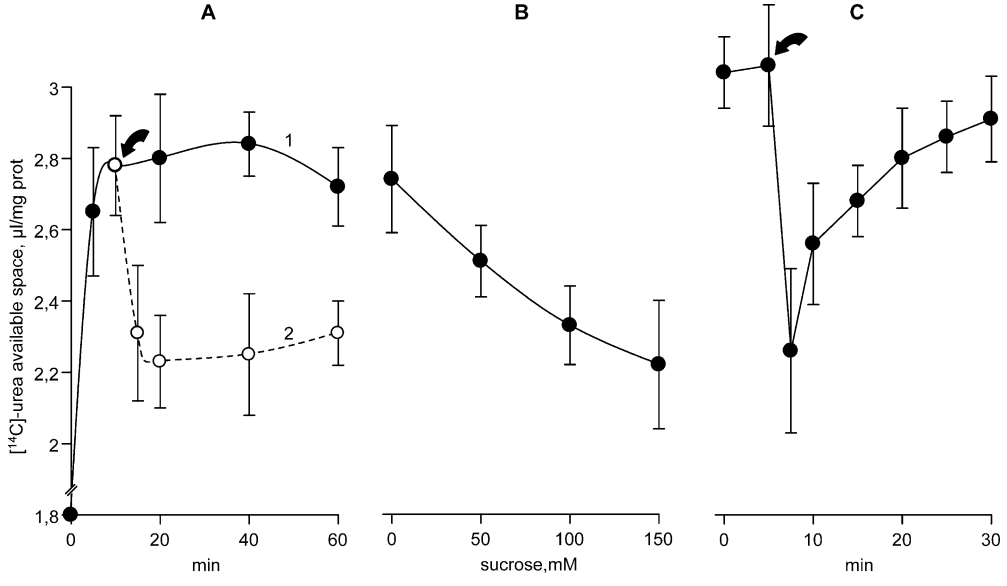

Fig. 1 Cell volume modulation in hyperosmotically (**a**, **b**) and isosmotically (**c**) shrunken vascular smooth muscle rings (VSMC). **a** Kinetics of cell volume modulation in hyperosmotic medium. WKY-7 cells were incubated for time intervals indicated on the *x*-axis in 0.5 ml physiologically balanced salt solution (PSS) containing 2 μCi/ml [^{14}C]-urea. At the time point indicated by the *arrow*, 0.5 ml of PSS (*1*) or PSS containing 300 mM sucrose (*2*) was added. In both cases, the media contained 2 μCi/ml [^{14}C]-urea. **b** Dependence of cell volume on medium osmolality. WKY-7 cells were incubated for 30 min in PSS containing 2 μCi/ml [^{14}C]-urea and sucrose at concentrations indicated on the *x*-axis. **c** Kinetics of cell volume modulation under isosmotic shrinkage. The cells were incubated for 45 min in hyposmotic PSS containing 40 mM NaCl and 2 μCi/ml [^{14}C]-urea. At the time point indicated by the *arrow*, this medium was aspirated, and isosmotic PSS (120.4 mM NaCl) with 2 μCi/ml [^{14}C]-urea was added. Mean±SE from experiments performed in triplicate are shown

absence of Ca^{2+}_o does not affect volume-dependent regulation of ouabain-resistant ^{86}Rb fluxes in VSMC [43]. Chemicals were obtained from Sigma (St. Louis, Mo., USA) with the exception of EGTA, POPOP, PPO (Serva, Heidelberg, Germany), verapamil (Orion, Helsinki, Finland) and cell culture medium (Gibco BRL, Gaithersburg, Mo., USA). Radiochemicals were from New England Nuclear (Boston, Mass.) and Amersham (Mississauga, Ont.). Stock solutions of bumetanide were prepared in DMSO, whereas nifedipine was dissolved in 70% ethanol. Neither DMSO nor ethanol at a final concentration of 0.1% (v/v), respectively, affected the parameters measured.

Statistics

The data, presented as mean±SE, were analyzed by Student's *t*-test or the *t*-test for dependent samples, as appropriate. Significance was defined as $P<0.05$.

Results

Cell volume modulation in hypo- and isosmotic media

Ten minutes incubation of WKY-7 cells was sufficient to establish the steady-state distribution of [^{14}C]-urea between the extra- and intracellular compartment (Fig. 1A, curve 1). In the presence of 150 mM sucrose, VSMC volume was decreased by ~20% in the first 5 min, and did not change significantly during the next 40 min of incubation (Fig. 1a, curve 2). Figure 1b shows the dependence of cell volume on sucrose added to isosmotic PSS at concentrations from 50 to 150 mM.

In additional experiments, we triggered the loss of intracellular osmolytes by preincubation of WKY-7 cells in hyposmotic medium, and then transferred them to isosmotic PSS. This approach is commonly taken to verify the role of isosmotic shrinkage in the regulation of cellular functions [40]. In contrast to hyperosmotic shrinkage, transfer from hypo- to isosmotic medium transiently decreased WKY-7 cell volume that was normalized in 25 min (Fig. 1c). This observation is consistent with previous studies revealed RVI in isosmotically-shrunken VSMC only [46, 48].

NKCC activity

In the presence of the Na^+,K^+-ATPase inhibitor ouabain, the addition of 10 μM bumetanide decreased the K^+ (^{86}Rb) uptake rate by threefold. Further elevation of this compound up to 100 μM did not significantly affect the rate of K^+ influx (data not shown) that is consistent with ID_{50} values of ~1 μM reported in studies on the effect of bumetanide on NKCC1 activity in other cell types [21, 22, 53].

The elevation of medium osmolality by the addition of up to 150 mM of sucrose led to ca. threefold activation of NKCC, measured as a component of ouabain-resistant ^{86}Rb influx inhibited by 10 μM bumetanide (Fig. 2a, curve 1). Suppression of NKCC detected at a sucrose concentration of 300 mM is probably caused by feedback inhibition of this carrier with Cl^-_i [37, 53]. We also noted that elevation of medium osmolality decreased the (ouabain+bumetanide)-resistant K^+ influx rate from ~7 to 2 nmol mg protein^{-1} 5 min^{-1} (Fig. 2a, curve 2). This effect was completely abolished in Cl^--free medium (Fig. 2a, curve 3). These results suggest that suppression of bumetanide-resistant K^+ influx in hyperosmotic medium is caused by the inhibition of KCC reciprocally regulated by cell volume and detected in our previous studies [6, 43].

The increment of NKCC detected after 5 min of 150 mM sucrose addition was preserved with up to 30 min of incubation in hyperosmotic medium (Fig. 2b, curve 2). In contrast, isosmotic shrinkage led to transient activation of this carrier that was completely abolished in 30 min of cell transfer from hypo- to isosmotic medium (Fig. 2b, curve 3).

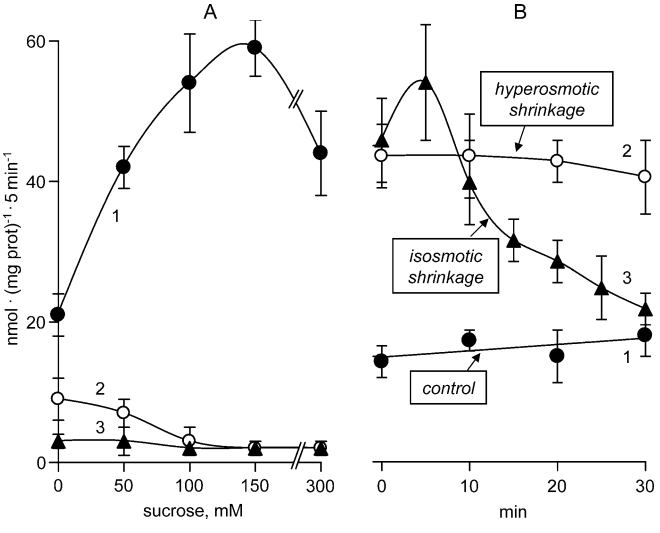

Fig. 2a, b Volume-dependent modulation of Na^+,K^+,Cl^- cotransport (NKCC) in VSMC. **a** Dependence of NKCC activity (*1*) and the bumetanide-resistant component of ^{86}Rb influx (*2* and *3*) on medium osmolality. *Curves 1*, *2*: cells were incubated for 5 min in PSS containing 1 mM ouabain, 1 μCi/ml ^{86}Rb±10 μM bumetanide. *Curve 3*: cells were incubated for 5 min in Cl^--free PSS containing 1 mM ouabain, 1 μCi/ml ^{86}Rb and 10 μM bumetanide. The concentration of sucrose added to control (*1* and *2*) or Cl^--free (*3*) PSS is indicated on the *x*-axis. **b** Kinetics of NKCC modulation in hyperosmotically (*2*) and isosmotically (*3*) shrunken cells. Cells were incubated for 45 min in isosmotic (*1*, *2*) or hyposmotic (40 mM NaCl) (*3*) PSS. These media were then aspirated, and 0.25 ml of isosmotic PSS (*1* and *3*) or PSS containing 150 mM sucrose (*2*) was added. At the indicated time points, isotope uptake was initiated by the addition of 0.25-ml aliquots of the same media containing 1 mM ouabain, 1 μCi/ml ^{86}Rb±10 μM bumetanide and terminated in the next 5 min. Mean±SE from experiments performed in quadruplicate are shown

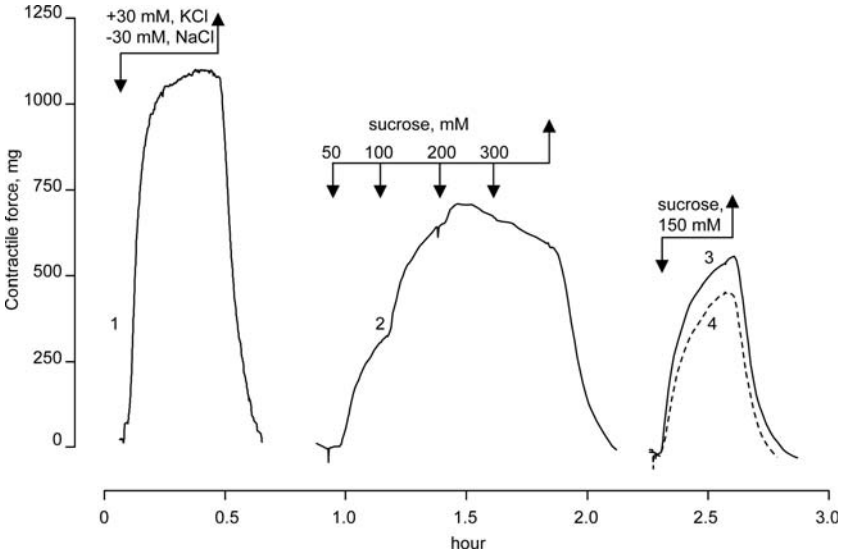

Fig. 3 Isometric contractile force recording from vascular smooth muscle rings (VSMR) under hyperosmotic shrinkage. VSMR were subjected to K^+_o-induced depolarization (*curve 1*), subsequent elevation of sucrose concentration up to 300 mM (*curve 2*), and by the addition of 150 mM sucrose in the absence (*curve 3*) and presence (*curve 4*) of bumetanide. Bumetanide was added at a concentration of 10 μM 5 min before sucrose

Contractions of hyperosmotically and isosmotically shrunken VSMR: role of NKCC

Elevation of sucrose concentration from 50 to 200 mM led to dose-dependent VSMR contractions (Fig. 3, curve 2). The contractile response triggered by 150 mM sucrose (curve 3) remained stable for at least three subsequent additions made with 30-min washout intervals, and its amplitude with 20 min of sucrose addition was 51.8±9.0% (n=78) of the maximal contraction detected in the presence of 36 mM KCl (curve 1).

Figure 4 shows representative tracing of the contractile response of depolarized and PE-treated VSMR in control and hyperosmotic medium. In these experiments, we observed that contractions triggered by equimolar substitution of 30 mM NaCl with KCl and by 1 μM PE were suppressed after 20 min of sucrose addition by 89.6±8.8% and 82.8±10.8% (n=6, P<0.00001), compared to contractions, triggered by these stimuli in isosmotic PSS.

To study the effect of isosmotic shrinkage on VSMR contraction, we applied a protocol developed with cultured cells (Fig. 1c). Application of the hyposmotic solution resulted in VSMR contractions that were completely abolished in 30–45 min (Fig. 5, curve 2). Subsequent transfer of VSMR to isosmotic medium led to transient contractions (curve 3) with a maximal amplitude of 21.6 ±8.7% (n=7) compared to K^+_o-induced contractions. Like hyperosmotic shrinkage, presumed isosmotic shrinkage of VSMR sharply attenuated contraction triggered by K^+_o depolarization and PE (Fig. 6).

Five minutes of pretreatment with 10 μM bumetanide led to 12% inhibition of sucrose-induced contractions (Fig. 3, curve 4). It should be underlined that in contrast to rapid inhibition of ^{86}Rb influx detected with 10 μM bumetanide in WKY-7 cells (Fig. 2a), its action on

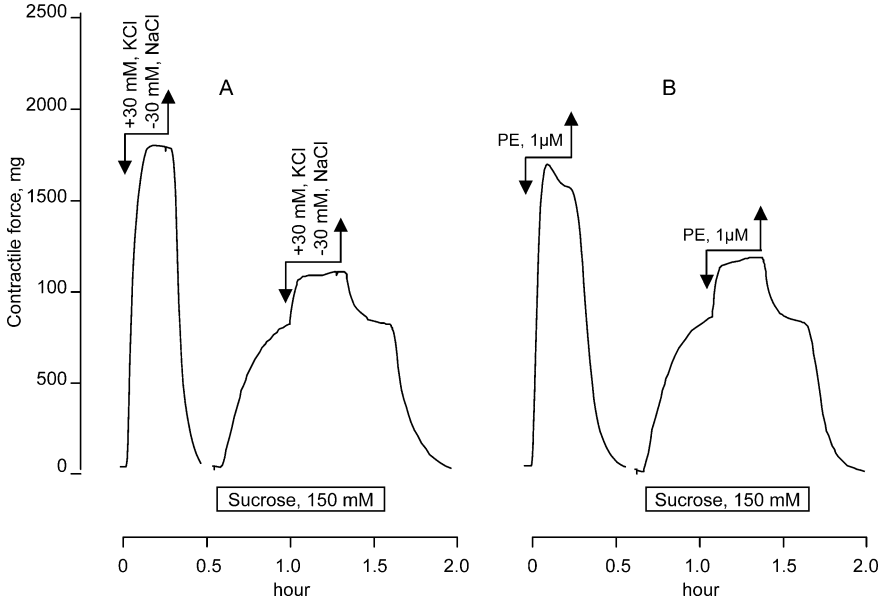

Fig. 4 Effect of hyperosmotic shrinkage on VSMR contractions triggered by K^+_o-depolarization (**a**) and phenylephrine (*PE*) (**b**)

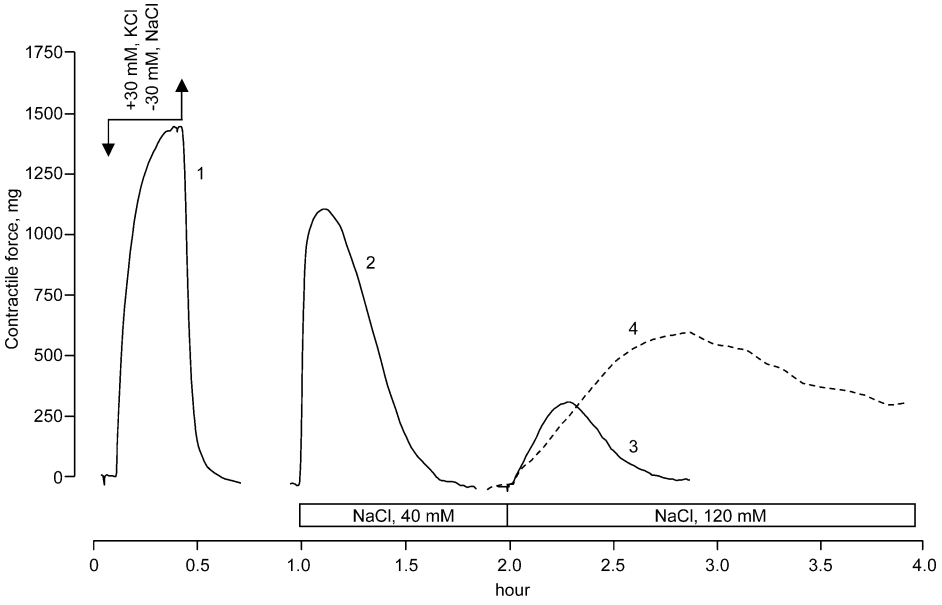

Fig. 5 Isometric contractile force recording from VSMR under isosmotic shrinkage. VSMR were subjected to K^+_o-induced depolarization (*curve 1*), reduced osmolality (PSS containing 40 mM NaCl, *curve 2*), and transfer from hypo- to isosmotic medium (PSS) in the absence (*curve 3*) or presence (*curve 4*) of 10 μM bumetanide . For more details see text

sucrose-induced VSMR contractions increased with time (Fig. 7a) and reached 33% of inhibition after 30-min preincubation (n=4, P<0.005). In contrast to hyperosmotic shrinkage, both maximal amplitude and duration of contractions detected in isosmotically shrunken VSMR were potentiated rather than attenuated by bumetanide (Fig. 5, curve 4 versus 3, and Table 1).

Keeping in mind the distinct effect of bumetanide in hyperosmotically and isosmotically shrunken VSMR, we examined its action on contractions triggered by 1 μM PE and by depolarization in modest- (36 mM) and high- (66 mM) K^+ medium. Similarly to hyperosmotic shrinkage, contractions triggered by $[K^+]_o$ elevation up to 36 mM were slightly inhibited by 5 min incubation with 10 μM bumetanide, reaching 35±12% and 53±10% of inhibition after 30 min preincubation with 10 or 100 μM bumetanide, respectively (Fig. 7b). A more striking action of bumetanide was detected in the study of PE-induced contractions. In this case, 30 min preincubation with 10 or 100 μM bumetanide suppressed contractions by 42% (n=4, P<0.02) and 89% (n=9, P>0.0000001) (Fig. 7c). In

Table 1 Effect of bumetanide on vascular smooth muscle ring (VSMR) contraction triggered by isosmotic shrinkage. Isosmotic shrinkage was triggered as indicated in the legend to Fig. 5. Bumetanide was added in the last 5 min of incubation in hyposmotic medium. The maximal amplitude of contractions triggered by K^+_o-induced depolarization was taken as 100%. Mean±SE obtained in n experiments is shown

Additions	Maximal amplitude of contractions (%)	Duration of contractions (min)
None (control)	21.6±8.7 (n=7)	38.8±1.6 (n=8)
Bumetanide (10 μM)	39.5±14.7 (n=6)	143.8±8.5* (n=4)

*P<0.005

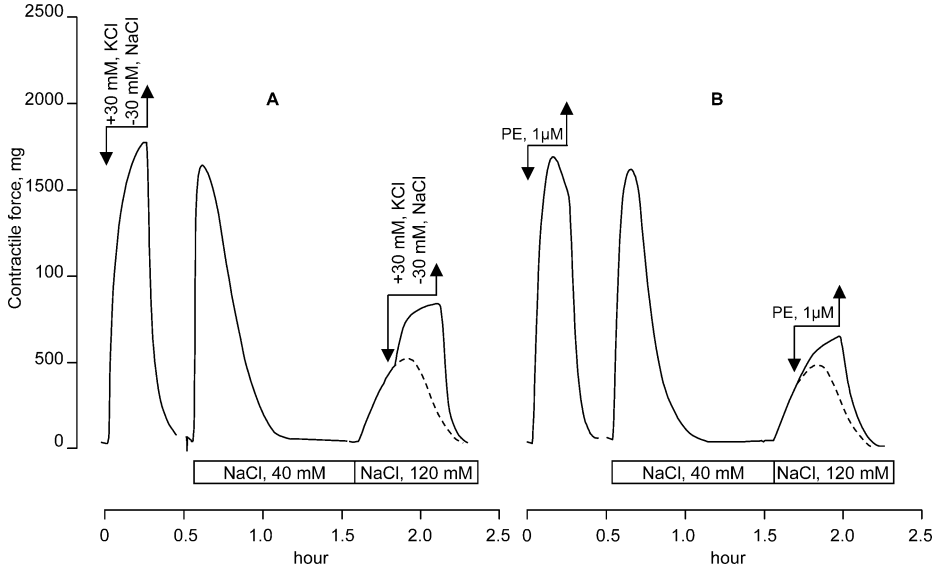

Fig. 6 Effect of isosmotic shrinkage on VSMR contractions triggered by K^+_o-depolarization (**a**) and PE (**b**). *Broken lines* show contractions in the absence of stimuli mentioned above

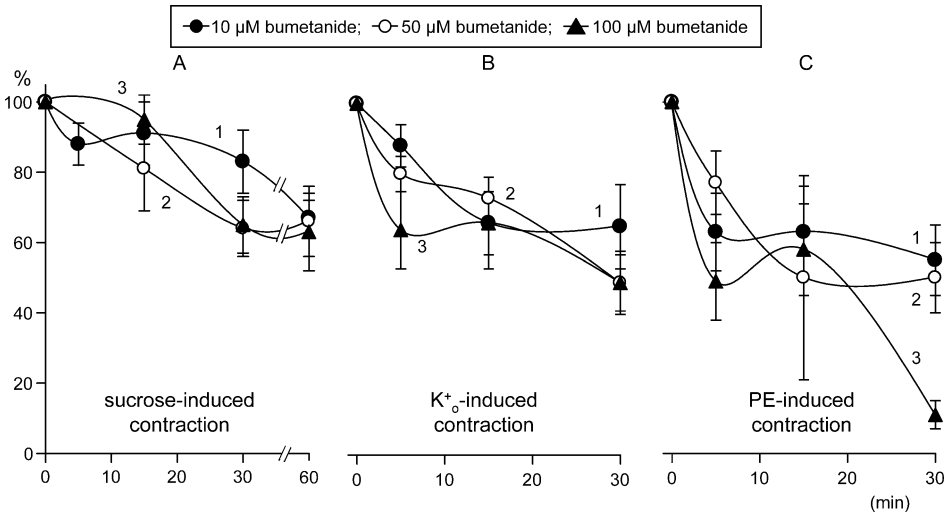

Fig. 7 Kinetics of inhibition of sucrose- (**a**), K^+_o- (**b**) and PE- (**c**) induced contractions by bumetanide. VSMR were preincubated with 10 (*1*), 50 (*2*) or 100 μM (*3*) bumetanide, and then contractions were triggered by the addition of 150 mM sucrose, 1 μM PE or equimolar substitution of NaCl with 30 mM KCl. The amplitude of contractions in the absence of bumetanide was taken as 100%. Mean±SE from 4–7 experiments are shown

contrast to PE (Fig. 8a) and modest elevation of $[K^+]_o$ (Fig. 8B), contractions in high-K^+ medium were completely insensitive to bumetanide (Fig. 8c), and after 30 min preincubation with this compound at 100 μM concentration the maximal amplitude was 96±6% of control values (n=6).

Role of intracellular Cl^-

Bumetanide did not affect cell volume in hyperosmotically shrunken VSMC, but suppressed RVI detected in VSMC subjected to isosmotic shrinkage (Table 2). Comparison of the sucrose effect on cell volume and Cl^-_i content shows that $[Cl^-]_i$ elevation by ~40% was mainly caused by attenuation of cell volume rather than by NKCC activation. Indeed, NKCC inhibition with bumetanide decreased the sucrose-induced increment of $[Cl^-]_i$ by only10–15%. After 15 min of isosmotic shrinkage, $[Cl^-]_i$ was increased in the absence and presence of bumetanide by 75% and 30%, respectively, which was also consistent with the magnitude of volume modulation observed in control and bumetanide-treated, isosmotically shrunken VSMC (Table 2).

Long-term maintenance in culture affects diverse cellular functions, including the activity of ion transporters [9, 52]. Thus, in VSMC subjected to more than ten passages and in the absence of any stimuli, such as cell shrinkage, NKCC operates as a (Na^+,Cl^-)-dependent K^+/K^+ exchanger [44]. These data are consistent with the modest effect of bumetanide on $[Cl^-]_i$ in cultured WKY-7 cell line (Table 2) and contrast with the 30–40% decrease of $[Cl^-]_i$ in freshly isolated rat femoral arteries [12]. Keeping this in mind, we studied the effect of bumetanide on $[Cl^-]_i$ in control and shrunken aortic rings. Table 3 shows that 30 min preincubation with 100 μM bumetanide decreased Cl^-_i content by ~40%, and completely abolished the rise of this parameter caused by 20 min hyperosmotic shrinkage in the presence of 150 mM sucrose.

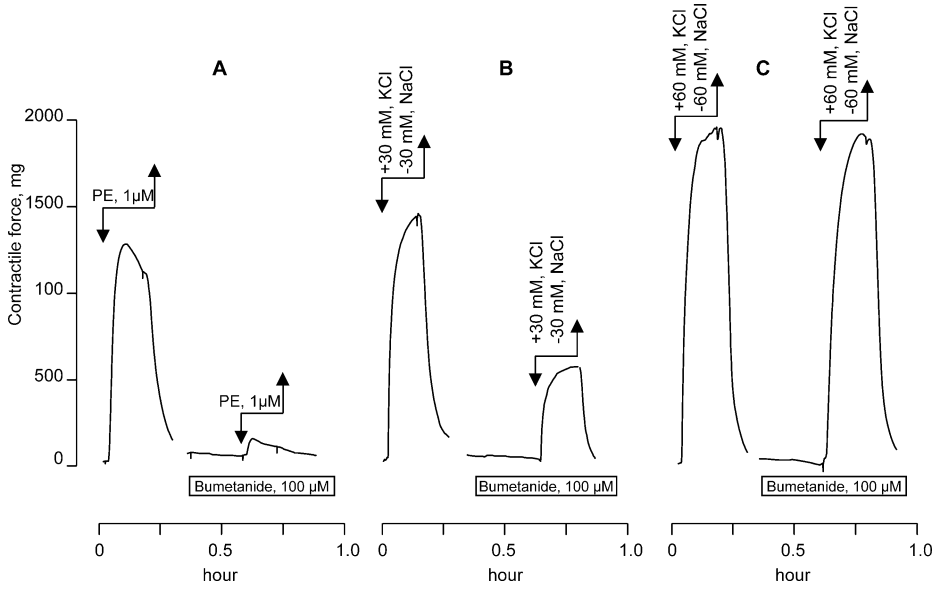

Fig. 8 Effect of bumetanide on contractile force generation by VSMR induced by PE (**a**) or by depolarization in moderate- (**b**) or high-K^+ (**c**) medium

Table 2 Effect of hyper- and isosmotic shrinkage on intracellular water content and Cl^-_i concentration in WKY-7 cells. To establish the steady-state distribution of isotopes, cells were preincubated with [^{14}C]-urea and ^{36}Cl for 2 h. Hyperosmotic shrinkage was then induced by the addition of 150 mM sucrose, and isosmotic shrinkage was triggered as indicated in the legend to Fig. 1. Bumetanide was added 10 min before cell shrinkage. Mean±SE obtained in experiments performed in quadruplicate is shown

Type of cell shrinkage and its duration	Intracellular water (μl per mg protein)		$[Cl^-]_i$ (mmol/l cell water)	
	Control	Bumetanide	Control	Bumetanide
Control	2.71±0.14	2.63±0.07	46±4	43±4
Hyperosmotic (5 min)	2.33±0.19	2.30±0.12	59±6	53±3
Hyperosmotic (15 min)	2.24±0.11*	2.27±0.13*	63±3*	56±4*
Control	2.87±0.18	2.78±0.09	28±4[a]	-
Isosmotic (5 min)	2.30±0.10*	2.21±0.14*	39±4*	31±3
Isosmotic (15 min)	2.71±0.13	2.38±0.09*	49±7*	36±5

*P<0.05 compared to control values
[a]Note that the lower $[Cl^-]_i$ values obtained in these conditions compared to control values presented in lane 1 were due to Cl^- depletion caused by 45 min preincubation in hyposmotic medium

Table 3 Effect of bumetanide and sucrose on intracellular Cl^- content in VSMR. Bumetanide was applied 5 or 30 min before sucrose addition. Mean±SE obtained in experiments performed in quadruplicate is shown. For more details of methodology see Materials and methods

Addition of bumetanide (concentration/ preincubation time)	Addition of sucrose (concentration/ incubation time)	Intracellular Cl^- (nmol/mg wet weight)
None	None	22.8±2.7
10 μM/5 min	None	20.0±4.0
10 μM/30 min	None	18.7±1.9
100 μM/30 min	None	14.1±1.3*
None	150 mM/20 min	31.4±2.2*
100 μM/30 min	150 mM/20 min	19.1±3.7

*P<0.05 compared to values obtained in the absence of bumetanide and sucrose

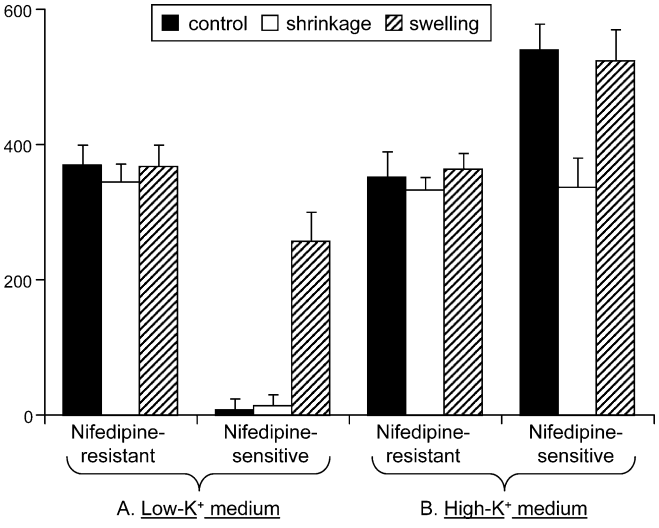

Fig. 9 Effect of hyperosmotic shrinkage and hyposmotic swelling on baseline (**a**) and depolarization-induced (**b**) ^{45}Ca uptake by WKY-7 cells. Cells were incubated for 5 min with ^{45}Ca (2–3 μCi/ml) in control (~6 mM KCl, 120 mM NaCl) or high-K^+ PSS (~43 mM KCl, 83 mM NaCl) containing 0.1 mM $CaCl_2$ with or without 1 μM nifedipine. To induced shrinkage, 150 mM sucrose was added. To induce swelling, NaCl concentration was decreased by 60 mM. Mean±SE from three experiments performed in triplicate are shown

Role of L-type Ca^{2+} channels and extracellular Ca^{2+}

It is generally accepted that electrical excitation leads to VSMC contraction caused by Ca^{2+} influx via voltage-gated long-lasting (L-type) Ca^{2+} channels (for references see [30]). K^+_o-induced depolarization of WKY-7 cells

Table 4 Effect of bumetanide on ^{45}Ca uptake by WKY-7 cells. Cells were preincubated for 30 min in 0.25 ml physiologically balanced salt solution (PSS) containing 0.2 mM $CaCl_2$ with or without 20 μM bumetanide. Aliquots of Ca^{2+}-free PSS containing 3–4 μCi/ml ^{45}Ca, with or without 2 μM nifedipine and with NaCl and KCl at final concentrations indicated in the left column, were then added. Mean±SE from experiments performed in quadruplicate is shown

Concentration of monovalent cations in PSS (mM)	Addition of bumetanide	^{45}Ca uptake (pmol mg protein^{-1}/5 min)		
		In the absence of nifedipine	In the presence of nifedipine	Nifedipine sensitive ($\Delta_{1,2}$)
Na 120; K 6	None	387±33	392±51	-
	10 μM	391±44	388±22	3
Na 96; K 30	None	972±70	368±40	604
	10 μM	689±51*	411±44	278
Na 66; K 60	None	1,188±88	354±29	834
	10 μM	1,117±71	361±47	756

*P<0.02 compared to values obtained in the absence of bumetanide

caused two- to threefold elevation of the ^{45}Ca influx rate. Neither 1 μM nifedipine nor 30 μM verapamil, potent inhibitors of L-type Ca^{2+} channels, significantly affected baseline Ca^{2+} uptake, but completely abolished the depolarization-induced increment of the Ca^{2+} influx with a ID_{50} of ~0.01 and 1 μM, respectively (data not shown). These values are consistent with data obtained for L-type Ca^{2+} channels by the patch-clamp technique [34]. Neither hyperosmotic shrinkage nor hyposmotic swelling affected nifedipine-resistant Ca^{2+} uptake in control and depolarized VSMC (Fig. 9). Hyposmotic swelling sharply increased nifedipine-sensitive Ca^{2+} influx under baseline conditions, whereas hyperosmotic shrinkage inhibited by ~40% nifedipine-sensitive Ca^{2+} influx triggered by K^+_o-induced depolarization (Fig. 9).

Keeping in mind the K^+_o-dependent effect of bumetanide on VSMR contractions (Fig. 8), we studied ^{45}Ca uptake at three different K^+_o concentrations. Thirty minutes of preincubation with 10 μM bumetanide did not alter ^{45}Ca uptake at $[K^+]_o$=6 and 60 mM, whereas at modest depolarization ($[K^+]_o$=30 mM) the nifedipine-sensitive component of ^{45}Ca uptake was decreased in bumetanide-treated VSMC by twofold (Table 4).

Consistent with previous data [19], both verapamil and nifedipine almost completely inhibited depolarization-induced VSMR contractions and partially suppressed the contractions triggered by PE (Table 5). We also observed sharp inhibition by L-type channel blockers of VSMR contractions in hypotonic medium. In contrast, neither verapamil nor nifedipine affected hyperosmotically and isosmotically shrunken VSMR contractions (Table 5).

To further examine the role of Ca^{2+}, we subjected VSMR to 1 h preincubation in Ca^{2+}-free solution containing the Ca^{2+} chelator EGTA. In contrast to almost complete inhibition of K^+_o- and PE-induced contractions by Ca^{2+} depletion documented in previous studies [19] and confirmed in our experiments, this procedure attenuated the amplitude of contractions of hyperosmotically and isosmotically shrunken VSMR by ~50 and 35%, respectively (Table 6).

To determine whether or not the lack of complete suppression of shrinkage-induced contractions in Ca^{2+}-free medium is caused by increased sensitivity of the contractile machinery to Ca^{2+}, we compared the effect of extracellular Ca^{2+} on depolarization-induced contractions of control and shrunken VSMR. The addition of 1 mM $CaCl_2$ led to the development of full-scale contraction of VSMR subjected to depolarization in Ca^{2+}-free medium (Fig. 10). In contrast, $[Ca^{2+}]_o$ elevation up to 10 mM did not trigger contractions of hyperosmotically shrunken VSMR subjected to subsequent depolarization (Fig. 10). Negative results were also obtained in isosmotically shrunken VSMR (Fig. 11).

Discussion

The data presented here drew us to a number of conclusions: (1) both cell shrinkage and swelling lead to VSMR contractions; (2) contractions triggered by shrinkage are not mediated by activation of L-type Ca^{2+} channels and are at least partially Ca^{2+}_o-independent. In contrast, contractions of swollen VSMR are accompanied by elevated Ca^{2+} uptake and abolished by L-type Ca^{2+} channel inhibitors; (3) both hyperosmotic and isosmotic shrinkage inhibits contractions triggered by K^+_o-induced depolarization and PE; (4) NKCC inhibition with bumetanide decreases $[Cl^-]_i$ and contractions triggered by modest depolarization as well as contractions triggered by PE and hyperosmotic shrinkage, but potentiated contractions of isosmotically shrunken VSMR; (5) suppression by bumetanide of contractions evoked by modest $[K^+]_o$ elevation is caused by partial inactivation of L-type Ca^{2+} channels, probably because of Cl^-_i-mediated membrane hyperpolarization; and (6) in isosmotically shrunken cells, NKCC inhibition enhances contractions via RVI

Table 5 Effect of L-type channel blockers on the amplitude of VSMR contraction. VSMR were treated with Ca^{2+} channel blockers 15 min before the application of contractile stimuli. The maximal amplitude of K^+_o-induced contractions was taken as 100%. *ND* These parameters were not determined. Mean±SE from *n* experiments is shown

Additions (μM)	Depolariza-tion-induced contractions[a] (%)	PE-induced contractions[b] (%)	Contractions induced by hyperosmotic medium[c] (%)	Contractions induced by hyposmotic medium[d] (%)	Contractions induced by isosmotic shrinkage[e] (%)
None (control)	100	92.1±10.6 (*n*=52)	51.9±8.8 (*n*=78)	75.7±8.9 (*n*=18)	21.6±8.7 (*n*=7)
Nifedipine (3)	5.6±2.2** (*n*=4)	ND	46.1±3.4* (*n*=5)	4.5±2.3** (*n*=4)	18.4±6.1 (*n*=4)
Verapamil (10)	4.6±1.6** (*n*=5)	25.8±7.3* (*n*=5)	48.3±2.7 (*n*=4)	3.8±1.1** (*n*=4)	ND

*,**$P<0.05$ and $P<0.00005$ compared to controls, respectively
[a]Depolarization-induced contractions were triggered by medium containing 36 mM KCl and 90 mM NaCl
[b]PE was added at a final concentration of 1 μM
[c]Hyperosmotic medium contained 150 mM sucrose added to PSS (Fig. 3, curve 3)
[d]Hyposmotic PSS contained 40 mM NaCl (Fig. 4, curve 2)
[e]Isosmotic shrinkage was triggered as indicated in Fig. 5

Table 6 Effect of extracellular Ca^{2+} on the maximal amplitude of VSMR contractions. The maximal amplitude of K^+_o-induced contractions was taken as 100%. Mean±SE from *n* experiments is shown

Incubation medium	Depolarization-induced contractions (%)	PE-induced contractions (%)	Contractions induced by hyperosmotic shrinkage (%)	Contractions induced by isosmotic shrinkage (%)
Control (PSS)	100	92.1±10.6 (*n*=52)	51.9±8.8 (*n*=78)	21.6±8.7 (*n*=7)
Ca^{2+}-free PSS	1.7±2.1** (*n*=9)	6.6±2.0*[a] (*n*=4)	25.6±7.8** (*n*=12)	14.0±3.7* (*n*=8)

*,**$P<0.05$ and $P<0.00005$, respectively, compared to controls
[a]VSMR were treated in control or Ca^{2+}-free PSS containing 0.5 mM EGTA for 60 or 15 min before contractile stimuli were applied

suppression. Data supporting these conclusions are considered below.

Cell volume modulation is sufficient to evoke VSMR contractions

Several lines of evidence indicate that VSMR contractions detected in anisosmotic media are caused by cell volume modulation rather than by altered medium osmolality per se. First, the magnitude of VSMR contractions triggered by sucrose at concentrations from 50 to 150 mM (Fig. 3) was in proportion to cell volume reduction measured as the volume of [^{14}C]-available space (Fig. 1b). Second, the volume of hyposmotically swollen VSMC was rapidly normalized by RVD occurring via activation of K^+ and Cl^- channels [6]. These data are consistent with our results showing slight cell volume elevation after 45 min incubation of VSMC in hyposmotic medium (3.03±0.16 versus 2.85±0.18 µl/mg protein in control; *n*=3) and transient VSMR contractions (Fig. 5, curve 2). Third, sustained versus transient contractions detected in sucrose-treated (Fig. 3) and isosmotically shrunken VSMR (Fig. 5) are consistent with the dynamics of cell volume modulation observed under these conditions (Fig. 1a, c). Fourth, the transient kinetics of volume decrease in isosmotically shrunken VSMC are caused by NKCC-mediated RVI [46]. Both cell volume decrease (Table 2) and force generation in isosmotically shrunken VSMR were prolonged under NKCC inhibition with bumetanide (Table 2 and Fig. 5).

Contractions of shrunken and swollen VSMR: distinct impact of Ca^{2+}-mediated signaling

Despite the monotonous impact on force generation, the cellular mechanisms underlying the contractile responses of shrunken and swollen VSMR are essentially different. Indeed, similarly to K^+_o-induced depolarization, hyposmotic swelling of VSMC augmented ^{45}Ca influx inhibited by nifedipine (Fig. 9). Moreover, both nifedipine and verapamil abolished contractions of swollen VSMR (Table 5). These results strongly suggest that swelling-induced VSMR contractions were mediated by L-type Ca^{2+} channel activation (Fig. 12). In contrast, contractions triggered by hyper- and isosmotic shrinkage were resistant to L-type Ca^{2+} channels blockers (Table 5). Contractions of shrunken VSMR were also preserved in the absence of Ca^{2+}_o and depletion of intracellular Ca^{2+} stores by incubation in Ca^{2+}-free, EGTA-containing medium, i.e. conditions abolishing K^+_o- and PE-induced contractions (Table 6).

In additional experiments, we observed that hyperosmotic shrinkage sharply attenuates rather than enhances

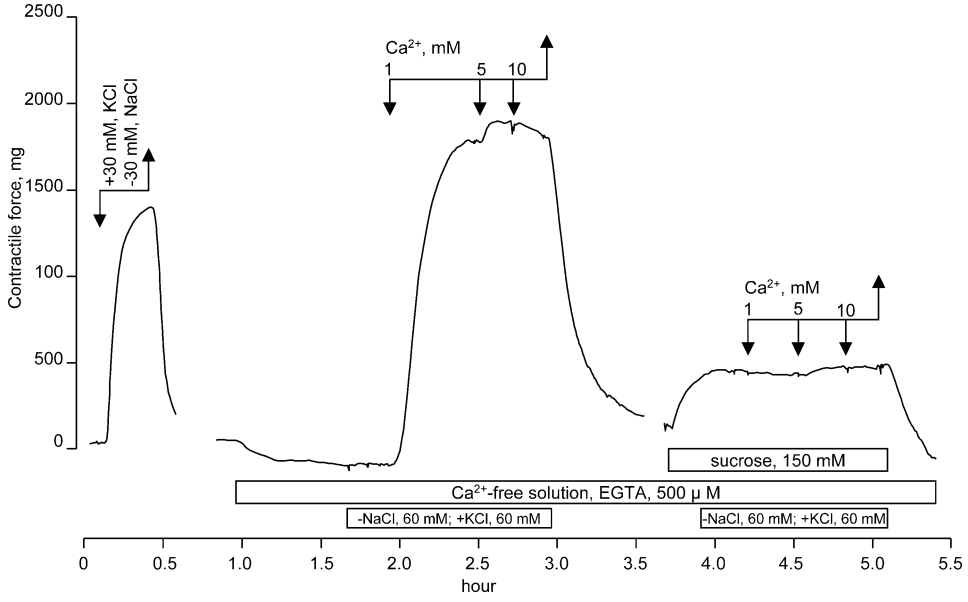

Fig. 10 Effect of extracellular Ca^{2+} on depolarization-induced contractions of control and hyperosmotically shrunken VSMR

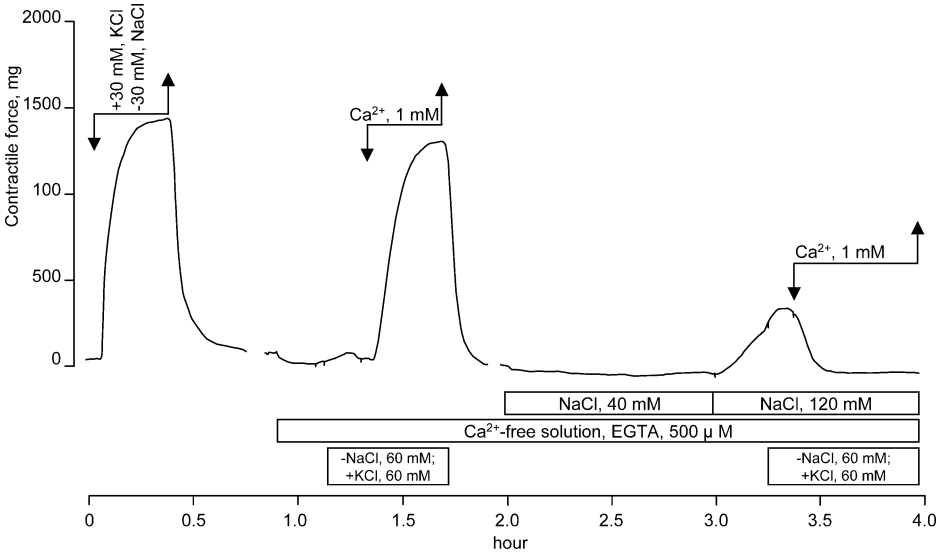

Fig. 11 Effect of extracellular Ca^{2+} on depolarization-induced contractions of control and isosmotically shrunken VSMR

contractions triggered by K^+_o-depolarization and PE (Fig. 4). This finding is consistent with data obtained on VSMR shrunken in the presence of 100 mM urea [58]. Inhibition of K^+_o- and PE-induced contractions was also seen in isosmotically shrunken VSMR (Fig. 6). Importantly, 30 min preincubation in hyposmotic medium preceding isosmotic shrinkage increased the magnitude of K^+_o-induced contractions (148.2±28.7 versus 100% in control; n=9, P<0.05) and did not influence contractions triggered by PE (100±14.4%, n=6). Viewed collectively, these results demonstrate the distinct mechanism of contractions triggered by cell shrinkage and physiological stimuli.

Several attractive hypotheses have been generated to explain the Ca^{2+}-independent mechanism of excitation-contraction coupling detected in shrunken VSMR. Thus, Kravtsov and co-workers reported that K^+_o-induced contractions in Mg^{2+}-free medium are not mediated by Ca^{2+}-influx and occur in the presence of EDTA [30]. It should be underlined, however, that in contrast to shrunken VSMR, K^+_o-induced contractions in Mg^{2+}-free medium were suppressed by L-type channel blockers [30]. It was shown that Ca^{2+}_o-independent VSMC contractions triggered by activators of protein kinase C, tyrosine and Rho-associated kinases were mediated by calcium sensitization of the contractile machinery [8, 23, 38]. Both myosin light chain phosphorylation and Rho kinase-dependent myosin II translocation have been detected in hyperosmotically shrunken cells [26, 27, 50, 54, 56]. However, in contrast to K^+_o- and PE-induced contractions, contractions of Ca^{2+}-

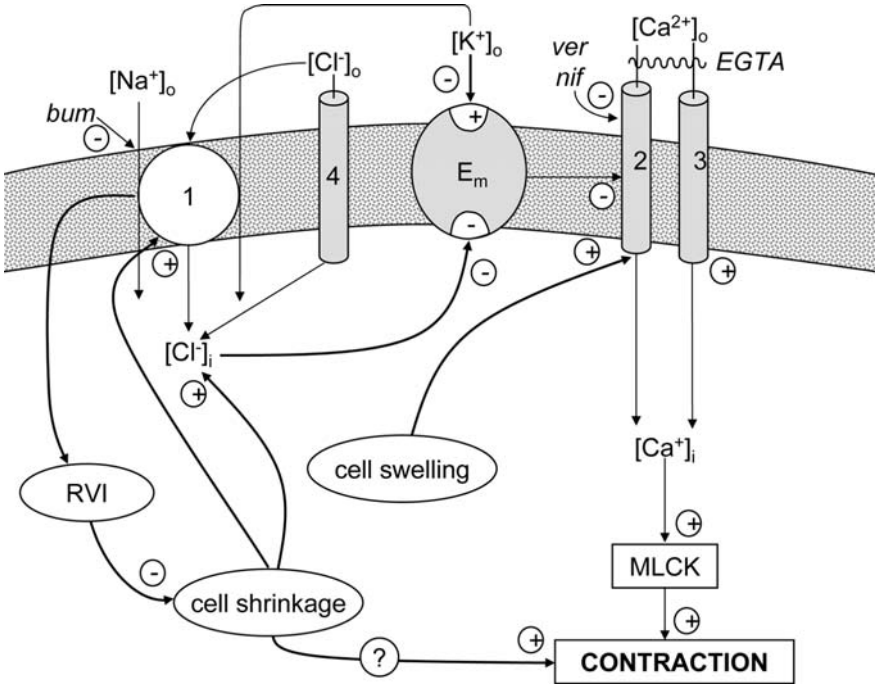

Fig. 12 Cross-talk of cell volume, Na^+,K^+,Cl^--cotransport, intracellular Cl^-_i and Ca^{2+} in the regulation of vascular smooth muscle contraction. *1* NKCC; *2* L-type Ca^{2+} channels; *3* calcium release-activated Ca^{2+} channels; *4* Ca^{2+}-activated anion channels; E_m electrical membrane potential; *MLCK* myosin light chain kinase; *RVI* regulatory volume increase; *bum*, *ver*, *nif* bumetanide, verapamil and nifedipine, respectively. +, − activatory and inhibitory stimuli; *?* unknown intermediates of signal transduction. For more details see text

depleted, shrunken VSMR were not evoked by the addition of Ca^{2+} (Figs. 10, 11). Keeping in mind that the total content of macromolecules in shrunken cells is increased and that modest elevation of this parameter affects diverse intracellular proteins, including cytoskeleton organization [13, 33], the role of macromolecular crowding in contractions of shrunken VSMR deserves further investigation.

Role of NKCC and intracellular Cl^-

Unlike the dominant contribution of K^+ permeability to resting membrane potential in skeletal and cardiac muscles, the P_K/P_{Cl} ratio in VSMC varies from 0.8 to 0.5 [12]. These data led Brown et al. to suggest that NKCC contributes to vascular tone via the maintenance of $[Cl^-]_i$ [11]. Later on, it was shown that NKCC inhibition with bumetanide decreases $[Cl^-]_i$, and hyperpolarizes the sarcolemma in freshly isolated VSMR [15]. These data suggest that NKCC inhibition will decrease VSMC contractions triggered by modest depolarization, but will not affect contractions in sharply depolarized cells (Fig. 12). Indeed, 30 min pretreatment with bumetanide reduced the increment of nifedipine-sensitive Ca^{2+} uptake and VSMR contractions evoked by 30 mM KCl but did not affect these parameters at $[K^+]_o$=60 mM (Table 4, Fig. 8). It may be assumed that the lack of effect of bumetanide in high-K^+ medium is caused by partial inactivation of NKCC due to reduction of $[Na^+]_o$ from 120 to 66 mM. However, the high affinity of VSMC NKCC for Na^+_o, documented in our previous study ($K_{0.5}$~25 mM) [44] did not support this assumption.

We observed that at 100 μM concentration bumetanide almost completely blocked the contractions triggered by PE (Fig. 8). Akar et al. reported that at 10 μM and 20 min preincubation, bumetanide inhibited VSMR contractions triggered by 1 μM PE by only 5–10% [4]. Several explanations for this controversy might be proposed. First, complete and rapid inhibition of NKCC, detected in cultured VSMC in the presence of 10 μM bumetanide (Fig. 2), does not mean that this concentration will lead to rapid hyperpolarization. Indeed, the effect of bumetanide on K^+_o-induced contractions was increased with concentration and time (Fig. 7b). We also detected that 30 min of incubation with 10 or 100 μM bumetanide decreased $[Cl^-]_i$ by 18% and 38%, respectively (Table 3). Second, the above-mentioned discrepancy is caused by differences between rat strains. Indeed, the PE EC_{50} of $10^{-6.9}$ M, detected in VSMR from rats in our study (data not shown), was an order of magnitude higher than values reported by Akar et al. [4]. Third, at high concentrations, bumetanide affects VSMR contractions independently of NKCC inhibition. This assumption makes sense in analyzing the mechanisms of almost complete inhibition of PE-induced contractions after 30 min preincubation with 100 μM bumetanide (Fig. 8a). However, keeping in mind the lack of effect of bumetanide on contractions triggered by high $[K^+]_o$ (Fig. 8c), upstream PE-specific intermediates of signaling, such as α-adrenoceptors, phospholipase C, calcium release-activated Ca^{2+} channels, rather than downstream elements of the contractile machinery, should be examined as potential targets for high-ceiling diuretics. Fourth, at high concentrations loop diuretics partially suppress KCC [32]. Recent data suggest that KCC activation contributes to vasodilatory action of nitric oxide derivatives [1]. The role of this carrier in modulation of VSMR contraction caused by 100 μM bumetanide should be examined further.

Rapid elevation of $[Cl^-]_i$ in sucrose-treated VSMC cells is a direct consequence of cell shrinkage. In contrast, NKCC activation is a major determinant of $[Cl^-]_i$ elevation in isosmotically shrunken cells subjected to preliminary Cl^- depletion in hyposmotic medium. A key role of NKCC in $[Cl^-]_i$ adjustment is consistent with a rapid decline of this parameter in isosmotically shrunken VSMC treated with bumetanide (Table 2). Thus, it can be suggested that shrinkage-induced contraction is caused by $[Cl^-]_i$ elevation and membrane depolarization. It should be underlined, however, that the lack of involvement of L-type Ca^{2+} channels in contractions of isosmotically and hyperosmotically shrunken VSMR (Table 6) contradicts this assumption. Moreover, we observed that inhibition of NKCC providing inwardly directed Cl^- transport potentiates rather than inhibits the contraction of isosmotically shrunken VSMR (Fig. 5). Prolongation of isosmotically shrunken VSMR contractions by bumetanide is probably caused by suppression of NKCC-mediated RVI (Figs. 1c, 2c, Table 2). Two hypotheses can be offered to explain the partial inhibition with bumetanide of nifedipine-insensitive contraction of hyperosmotically shrunken VSMR (Fig. 7A). First, hyperpolarization developing under NKCC inhibition suppressed the activity of inwardly directed Ca^{2+} transporters distinct from L-type channels. It should be mentioned, however, that contractions of shrunken VSMR were only partially suppressed in Ca^{2+}-free medium (Table 6). Second, $[Cl^-]_i$ modulation affects intracellular proteins involved in assembly of the contractile machinery independently of the regulation of membrane potential.

In conclusion, our results show that both swelling and shrinkage lead to VSMR contractions. Swelling-induced contractions are caused by activation of L-type Ca^{2+} channels. In contrast, contractions of shrunken VSMR are insensitive to the presence of L-type channel inhibitors and occur in the absence of Ca^{2+}_o. Augmented contractions detected in isosmotically shrunken VSMR in the presence of bumetanide are probably caused by suppression of NKCC-mediated RVI. The mechanism of bumetanide-sensitive, Ca^{2+}-independent contractions in hyperosmotically shrunken VSMR deserves further investigations.

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References

1. Adragna N, White RE, Orlov SN, Lauf PK (2000) K-Cl cotransport in vascular smooth muscle and erythrocytes: possible implication in vasodilation. Am J Physiol 278:C381–C390
2. Adragna N, Di Fulvio M, Lauf PK (2004) Regulation of K-Cl cotransport: from function to genes. J Membr Biol (in press)
3. Akar F, Skinner E, Klein JD, Jena M, Paul RJ, O'Neill WC (1999) Vasoconstrictors and nitrovasodilators reciprocally regulate the Na^+-K^+-$2Cl^-$ cotransporter in rat aorta. Am J Physiol 276:C1383–C1390
4. Akar F, Jiang G, Paul RJ, O'Neill WC (2001) Contractile regulation of the Na^+-K^+-$2Cl^-$ cotransporter in vascular smooth muscle. Am J Physiol 281:C579–C584
5. Alvarez-Guerra M, Nazaret C, Garay RP (1998) The erythrocyte Na,K,Cl cotransporter and its circulating inhibitor in Dahl salt-sensitive rats. J Hypertens 16:1499–1504
6. Anfinogenova YJ, Rodriguez X, Grygorczyk R, Adragna N, Lauf PK, Hamet P, Orlov SN (2001) Swelling-induced K^+ fluxes in vascular smooth muscle cells are mediated by charybdotoxin-sensitive K^+ channels. Cell Physiol Biochem 11:295–310
7. Anfinogenova YJ, Kilin AA, Kovalev IV, Baskakov MB, Orlov SN (2003) Vascular smooth muscle contraction in hyperosmotic medium: role of Ca^{2+}, anion channels and cell volume-sensitive Na^+,K^+,Cl^- cotransport. J Hypertens 21:S101
8. Barandier C, Ming X-F, Yang Z (2003) Small G proteins as novel therapeutic targets in cardiovascular medicine. News Physiol Sci 18:18–22
9. Berk BC, Vallega G, Muslin AJ, Gordon HM, Canessa M, Alexander RW (1989) Spontaneously hypertensive rat vascular smooth muscle cells in culture exhibit increased growth and Na$^+$/H^+ exchange. J Clin Invest 83:822–829
10. Bianchi G, Ferrari P, Trizio P, Ferrandi M, Torielli L, Barber BR, Polli E (1985) Red blood cell abnormalities and spontaneous hypertension in rats. A genetically determined link. Hypertension 7:319–325
11. Brown RA, Chipperfield AR, Davis JPL, Harper AA (1999) Increased ($Na^+K^+Cl^-$) cotransport in rat arterial smooth muscle in deoxycorticosterone (DOCA)/salt-induced hypertension. J Vasc Res 36:492–501
12. Chipperfield AR, Harper AA (2001) Chloride in smooth muscle. Prog Biophys Mol Biol 74:175–221
13. Cuneo P, Margi E, Verzola A, Grazi E (1992) "Macromolecular crowding" is a primary factor in the organization of the cytoskeleton. Biochem J 281:507–512
14. Davis A, Hogarth K, Fernandes D, Solway J, Niu J, Kolenko V, Browning D, Miano JM, Orlov SN, Dulin NO (2003) Functional significance of protein kinase A (PKA) activation by endothelin-1 and ATP: negative regulation of SRF-dependent gene expression by PKA. Cell Signal 15:597–604
15. Davis JPL, Chipperfield AR, Harper AA (1993) Accumulation of intracellular chloride by (Na-K-Cl) cotransport in rat arterial smooth muscle is enhanced in deoxycorticosterone acetate (DOCA)/salt hypertension. J Mol Cell Cardiol 25:233–237
16. Flagella M, Clarke LL, Miller ML, Erway LC, Giannella RA, Andriga A, Gawenis LR, Kramer J, Duffy JJ, Doetschman T, Lorenz JN, Yamoah EN, Cardell EL, Shull GE (1999) Mice lacking the basolateral Na-K-2Cl cotransporter have impaired epithelial chloride secretion and are profoundly deaf. J Biol Chem 274:26946–26955
17. Garay RP (1982) Inhibition of the Na^+/K^+ cotransport system by cyclic AMP and intracellular calcium in human red cells. Biochim Biophys Acta 688:786–792
18. Glukhova MA, Frid MG, Koteliansky VE (1994) Phenotypic changes of human aortic smooth muscle cells during development and in adult. J Atheroscler Thromb 1 [Suppl 1]:S47–S49
19. Godfraind T (1994) Calcium antagonists and vasodilation. Pharmacol Ther 64:37–75
20. Hoffmann EK, Simonsen LO (1989) Membrane mechanisms in volume and pH regulation in vertebrate cells. Physiol Rev 69:315–382
21. Isenring P, Forbush III B (1997) Ion and bumetanide binding by the Na-K-Cl cotransporter. Importance of transmembrane domains. J Biol Chem 272:24556–24562
22. Isenring P, Jacoby SC, Payne JA, Forbush BI (1998) Comparison of Na-K-Cl cotransporters: NKCC1, NKCC2 and HEK cell Na-K-Cl cotransporter. J Biol Chem 273:11295–11301
23. Janssen LJ, Lu-Chao H, Netherton S (2001) Excitation-contraction coupling in pulmonary vascular smooth muscle involves tyrosine kinase and Rho-kinase. Am J Physiol 280: L666–L674
24. Jiang G, Cobbs S, Klein JD, O'Neill WC (2003) Aldosterone regulates the Na-K-Cl cotransporter in vascular smooth muscle. Hypertension 41:1131–1135
25. Jones AW (1973) Altered ion transport in vascular smooth muscle from spontaneously hypertensive rats. Influence of aldosterone, norepinephrine and angiotensin. Circ Res 33:563–572
26. Klein JD, O'Neill WC (1993) Myosin light chain phosphorylation in endothelial cells is regulated by cell volume and correlates with volume-regulatory Na-K-2Cl cotransport (abstract). J Gen Physiol 102:18a
27. Klein JD, O'Neill WC (1995) Volume-sensitive myosin phosphorylation in vascular endothelial cells: correlation with Na-K-2Cl cotransport. Am J Physiol 269:C1524–C1531
28. Kotelevtsev YuV, Orlov SN, Pokudin NI, Agnaev VM, Postnov YuV (1987) Genetic analysis of inheritance of Na^+,K^+ cotransport, calcium level in erythrocytes and blood pressure in F_2 hybrids of spontaneously hypertensive and normotensive rats. Bull Exp Biol Med 103:456–458
29. Kovalev IV, Baskakov MB, Anfinogenova YJ, Borodin YL, Kilin AA, Minochenko IL, Popov AG, Kapilevich LV, Medvedev MA, Orlov SN (2003) Effect of Na^+,K^+,$2Cl^-$ cotransport inhibitor bumetanide on electrical and contractile activity of smooth muscle cells in guinea pig ureter. Bull Exp Biol Med 136:145–149
30. Kravtsov GM, Bruce IC, Wong TK, Kwan CY (2003) A new view of K^+-induced contraction in rat aorta: the role of Ca^{2+} binding. Pflugers Arch 446:529–540
31. Lang F, Busch G, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D (1998) Functional significance of cell volume regulatory mechanisms. Physiol Rev 78:247–306
32. Lauf PK, Adragna NC (2000) K-Cl cotransport: properties and molecular mechanism. Cell Physiol Biochem 10:341–354
33. Madden TL, Herzfeld J (1993) Crowding-induced organization of cytoskeletal elements. I. Spontaneous demixing of cytoplasmic proteins and model filaments to form filament bundles. Biophys J 65:1147–1154
34. McDonald TF, Pelzer S, Trautwein W, Pelzer DJ (1994) Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. Physiol Rev 74:365–512
35. Meyer JW, Flagella M, Sutliff RL, Lorenz JN, Nieman ML, Weber GS, Paul RJ, Shull GE (2002) Decreased blood pressure and vascular smooth muscle tone in mice lacking basolateral Na^+-K^+-$2Cl^-$ cotransporter. Am J Physiol 283:H1846–H1855
36. Mongin AA, Orlov SN (2001) Mechanisms of cell volume regulation and possible nature of the cell volume sensor. Pathophysiology 8:77–88
37. Mount DB, Delpire E, Gamba G, Hall AE, Poch E, Hoover RS, Hebert SC (1999) The electroneutral cation-chloride cotransporters. J Exp Biol 201:2091–2102
38. Nakao F, Kobayashi S, Mogami K, Mizukami Y, Shirao S, Miwa S, Todoroki-Ikeda N, Ito M, Matsuzaki M (2002) Involvement of Src family protein tyrosine kinases in Ca^{2+} sensitization of coronary artery contraction mediated by a sphindosylphosphorylcholine-Rho-kinase pathway. Circ Res 91:953–960
39. O'Neill WC (1999) Physiological significance of volume-regulated transporters. Am J Physiol 276:C995–C1011

40. O'Neill WC, Klein JD (1994) Regulation of vascular endothelial cell volume by Na-K-2Cl cotransport. Am J Physiol 262: C436–C444
41. Orlov SN (2003) Hypertension. In: Bernhardt I, Ellory JC (eds) Red cell membrane transport in health and disease. Springer, Berlin Heidelberg New York, pp 587–602
42. Orlov SN, Resink TJ, Bernhardt J, Buhler FR (1992) Na^+-K^+ pump and Na^+-K^+ co-transport in cultured vascular smooth muscle cells from spontaneously hypertensive rats: baseline activity and regulation. J Hypertens 10:733–740
43. Orlov SN, Resink TJ, Bernhardt J, Buhler FR (1992) Volume-dependent regulation of sodium and potassium fluxes in cultured vascular smooth muscle cells: dependence on medium osmolality and regulation by signalling systems. J Membr Biol 126:199–210
44. Orlov SN, Tremblay J, Hamet P (1996) Bumetanide-sensitive ion fluxes in vascular smooth muscle cells: lack of functional Na^+,K^+,$2Cl^-$ cotransport. J Membr Biol 153:125–135
45. Orlov SN, Tremblay J, Hamet P (1996) cAMP signaling inhibits dihydropyridine-sensitive Ca^{2+} influx in vascular smooth muscle cells. Hypertension 27:774–780
46. Orlov SN, Tremblay J, Hamet P (1996) Cell volume in vascular smooth muscle is regulated by bumetanide-sensitive ion transport Am J Physiol 270:C1388–C1397
47. Orlov SN, Adragna N, Adarichev VA, Hamet P (1999) Genetic and biochemical determinants of abnormal monovalent ion transport in primary hypertension. Am J Physiol 276:C511–C536
48. Orlov SN, Pchejetski D, Taurin S, Thorin-Trescases N, Maximov GV, Pshezhetsky AV, Rubin AB, Hamet P (2004) Apoptosis in serum-deprived vascular smooth muscle cells: evidence for cell volume-independent mechanism. Apoptosis 9:55–66
49. Owen NE, Ridge KM (1989) Mechanism of angiotensin II stimulation of Na-K-Cl cotransport of vascular smooth muscle cells. Am J Physiol 257:C629–C636
50. Pedersen SF, Beisner KH, Hougaard C, Willumsen BM, Lambert IH, Hoffmann EK (2002) Rho family GTP binding proteins are involved in the regulatory volume decrease in NIH3T3 mouse fibroblasts. J Physiol (Lond) 541:779–796
51. Postnov YuV, Orlov SN, Gulak PV, Shevchenko AS (1976) Altered permeability of the erythrocyte membrane for sodium and potassium in spontaneously hypertensive rats. Pflugers Arch 365:257–263
52. Raat NJ, Hartog A, van Os CH, Bindels RJ (1994) Regulation of Na^+-K^+-$2Cl^-$ cotransport in rabbit proximal tubule primary culture. Am J Physiol 267:F63–F69
53. Russell JM (2000) Sodium-potassium-chloride cotransport. Physiol Rev 80:212–276
54. Shrode LD, Klein JD, O'Neill WC, Putnam RW (1995) Shrinkage-induced activation of Na^+/H^+ exchange in primary rat astrocytes: role of myosin light-chain kinase. Am J Physiol 269:C257–C266
55. Smith JB, 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 Membr Biol 99:51–63
56. Takeda M, Homma T, Breyer MD, Horiba N, Hoover RL, Kawamoto S, Ichikawa I, Kon V (1993) Volume and agonist-induced regulation of myosin light-chain phosphorylation in glomerular mesangial cells. Am J Physiol 264:F421–F426
57. Tseng H, Berk BC (1992) The Na/K/2Cl cotransporter is increased in hypertrophied vascular smooth muscle cells. J Biol Chem 267:8161–8167
58. Wagner CA, Huber SM, Warntges S, Zempel G, Kaba NK, Fux R, Orth N, Busch GL, Waldegger S, Lambert I, Nilius B, Heinle H, Lang F (2000) Effect of urea and osmotic cell shrinkage on Ca^{2+} entry and contraction of vascular smooth muscle cells. Pflugers Arch 440:295–301