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Volume-Dependent Regulation of Sodium and Potassium Fluxes in Cultured Vascular Smooth Muscle Cells: Dependence on Medium Osmolality and Regulation by Signalling Systems

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Summary. To identify ion transport systems involved in the maintenance of vascular smooth muscle cell volume the effects of incubation medium osmolality and ion transport inhibitors on the volume and ^{86}Rb and ^{22}Na transport in cultured smooth muscle cells from rat aorta (VSMC) have been studied. A decrease of medium osmolality from 605 to 180 mosm increased intracellular water volume from 0.6 to 1.3 μl per 10^6 cells. Under isosmotic conditions, cell volume was decreased by ouabain (by 10%, $P < 0.005$) but was not influenced by bumetanide, furosemide, EIPA and quinidine. These latter compounds were also ineffective in cell volume regulation under hypotonic buffer conditions. Under hyperosmotic conditions, cell volume was decreased by bumetanide (by ≈7%, $P < 0.05$) and by ethylisopropyl amiloride (by ≈13%, $P < 0.005$). Ouabain-sensitive ^{86}Rb influx was decreased by 30–40% under hypoosmotic conditions. An increase in medium osmolality from 275 to 410 mosm resulted in an ≈eightfold increase in bumetanide-inhibited ^{86}Rb influx and ^{86}Rb efflux. The (ouabain and bumetanide)-insensitive component of ^{86}Rb influx was not dependent on the osmolality of the incubation medium. However (ouabain and bumetanide)-insensitive ^{86}Rb efflux was increased by ≈1.5–2 fold in VSMC incubated in hypotonic medium. Ethylisopropyl amiloride-inhibited ^{22}Na influx was increased by ≈sixfold following osmotic-shrinkage of VSMC. The data show that both Na^+/H^+ exchange and $Na^+/K^+/2Cl^-$ cotransport may play a major role in the regulatory volume increase in VSMC. Basal and shrinkage-induced activities of $Na^+/K^+/2Cl^-$ cotransport in VSMC were similarly sensitive to inhibition by either staurosporin, forskolin, R24571 or 2-nitro-4-carboxyphenyl N,N-diphenylcarbomate (NCDC). In contrast basal and shrinkage-induced $Na^+/K^+/2Cl^-$ cotransport were differentially inhibited by NaF (by 30 and 65%, respectively), suggesting an involvement of guanine nucleotide binding proteins in the volume-sensitive activity of this carrier. Neither staurosporin, forskolin, R24571 nor NCDC influenced shrinkage-induced Na^+/H^+ exchange activity. NaF increased Na^+/H^+ exchanger activity under both isosmotic and hyperosmotic conditions. These data demonstrate that different intracellular signalling mechanisms are involved in the volume-dependent activation of the $Na^+/K^+/2Cl^-$ cotransporter and the Na^+/H^+ exchanger.

Key Words vascular smooth muscle cells · $Na^+/K^+/2Cl^-$ cotransport · Na^+/H^+ exchange · regulatory volume increase · regulatory volume decrease · intracellular signalling

Introduction

Cell volume regulation via activation or inhibition of several types of ion transport systems involved in the fluxes of sodium, potassium and chloride across plasma membrane is a fundamental property of the majority of cells. Regulatory volume increase (RVI) has been associated with the activation of Na^+/H^+ exchange in rat lymphocytes (Grinstein et al., 1984) and rat and rabbit erythrocytes (Jennings, Douglas & Andrew, 1986; Orlov et al., 1989). RVI has also been associated with activation of $Na^+/K^+/2Cl^-$ cotransport in duck and rat erythrocytes (Schmidt & McManus, 1977; Duhm & Göbel, 1984; Orlov et al., 1989), endothelial cells from bovine thoracic aorta (O'Donnell & Owen, 1991) and rabbit ventricular myocytes (Drewnowska & Baumgarten, 1991). Regulatory volume decrease (RVD) has been demonstrated to be mediated via activation of K^+/Cl^- cotransport in erythrocytes from man (Canessa, Spalrins & Nagel, 1986; Brugnara & Tosteson, 1987) and several other animal species (Lauf, 1982, 1984; Parker, 1983; MacManus et al., 1985; Al-Rohil & Jennings, 1989). RVD has also been associated with the activation of K^+/H^+ exchange in *Amphiuma* erythrocytes (Cala, 1983) or with the opening of potassium and chloride channels in rat hepatocytes (Bakker-Grunwald, 1983), lymphocytes (Grinstein et al., 1984) and Ehrlich ascites tumor cells (Hoffman, Simonsen & Lambert, 1984).

In epithelial cells, which normally experience large changes in osmolality, volume-dependent ion transport systems play a key role in transcellular solute transport (Larson & Spring, 1987). In nonepithelial cells these volume-dependent ion-transport systems are probably involved in the cell volume regulation that occurs both during growth and differ-

entiation, and also following the action of external stimuli which generate net fluxes of electrolytes and water (Hoffmann & Simonsen, 1989; Gukovskaya & Zinchenko, 1990).

Vascular smooth muscle cells are under the permanent control of spontaneous electrical activity, neurotransmitters and peptide hormones, all of which elicit activation of transmembrane electrolyte transport via modulation of the activity of ion channels (Van Breemen & Saida, 1989), Na^+/H^+ exchange (Vallega et al., 1988), $Na^+/K^+/2Cl^-$ cotransport (Smith & Smith, 1987; O'Donnell & Owen, 1991) and the Na^+/H^+ pump (Smith & Brock, 1983). However, to date there is no data pertaining to either volume-dependent regulation of ion transport across the sarcolemma of vascular smooth muscle cells or to the involvement of ion-transport systems in the maintenance of vascular smooth muscle cell volume. This study has investigated the volume-dependent regulation of ^{86}Rb and ^{22}Na fluxes in cultured rat vascular smooth muscle cells (VSMC). We demonstrate that Na^+/H^+ exchange and $Na^+/K^+/2Cl^-$ cotransport are involved in RVI. An unidentified K^+-transport system is responsible for K^+ efflux in swollen cells. The effects of several pharmacological compounds on the basal and volume-induced activities of these ion transport systems have also been investigated.

Materials and Methods

Materials

All materials and media for tissue culture were obtained from Gibco AG (Basel, Switzerland), except for fetal calf serum which was purchased from Fakola AG (Basel, Switzerland). Phorbol 12-myristate, 13-acetate (PMA), angiotensin II, ouabain, furosemide, quinidine, quercetin, meclofenamic acid, 2-nitro-4-carboxyphenyl N,N-diphenylcarbomate (NCDC), 8-bromo-cyclic GMP (8-Br-cGMP) were purchased from Sigma (St. Louis, MO). Staurosporine and forskolin were from Calbiochem AG (Lucerne, Switzerland), and R24571 was from Boehringer Mannheim (Schweiz) AG (Rotkreuz, Switzerland). Ethylisopropyl amiloride (EIPA) was provided by Ciba Geigy (Basel, Switzerland) and bumetanide was a kind gift of Prof. J. Duhm (Institute of Physiology, Munich University, Germany). Dowex-AG 1-x8 was from Bio Rad Laboratories AG (Glattbrugg, Switzerland). The isotopes $^{86}RbCl$, $^{22}NaCl$, 3-O-[methyl-^{14}C]-D-glucose and myo-[2-^{3}H]-inositol were obtained from Amersham (Bucks, UK). All other chemicals were purchased from Merck AG (Darmstadt, Germany) or Fluka AG (Buchs, Switzerland) and were of the highest purity available.

Isolation and Culture of Smooth Muscle Cells

Aortic smooth muscle cells (VSMC) from 20-week old, male Wistar Kyoto rats were isolated, phenotypically characterized, and propagated as described in detail previously (Scott-Burden et al., 1989). For the experiments described herein isolates of VSMC at between passage 14 and 17 were used. VSMC were seeded into 6- (for intracellular water determination), 12- (for measurement of ^{22}Na influx) or 24- (for measurements of ^{86}Rb influx and efflux, and phosphoinositide turnover) well multiwell dishes at a seeding density of $\approx 3.5 \times 10^4$ cells/cm^2 and grown to confluence ($7–9 \times 10^4$ cells/cm^2). Prior to all experimentation confluent cultures of VSMC were rendered quiescent by serum deprivation and maintenance in serum-free medium containing 0.1% (wt/vol) bovine serum albumin for 48 hr. Cell numbers were routinely obtained by counting aliquots of cell suspensions in Isoton with a Coulter counter (Coulter Electronics, Haileah, FL) after enzymatic disaggregation of cell layers (Scott-Burden et al., 1989).

Cell Volume Measurement

The equilibrium distribution of 3-O-[methyl-^{14}C]-D-glucose was used to measure cellular water space (Kletzien et al., 1975). VSMC (in 6-well dishes, $\approx 1.5–2.6 \times 10^6$ cells/well) were washed twice with 3-ml aliquots of phosphate buffered saline and then incubated for 30 min at 37°C in buffer (1 ml/well) containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES-Tris-HCl (pH 7.4 at 37°C), 1 mM 3-O-methyl-D-glucose and 0.8–1.0 μCi 3-O-[methyl-^{14}C]-D-glucose/ml. In some experiments 1 mM ouabain (inhibitor of Na^+/K^+ pump) was included in this buffer (*see* Results, tables and figures). In order to change the osmolality range (between 170–615 mosm) either the NaCl concentration was reduced or sucrose was added. Buffer osmolality was controlled in all experiments, including those measuring ion fluxes (*see below*), using a milliosmometer (Knauer, Germany). To assess the participation of different ion transport systems in the regulation of cell volume, bumetanide (10 μM), furosemide (0.5 mM), quinidine (50 μM) or ethylisopropyl amiloride (EIPA, 10 μM) were variously included during the last 10 min of incubation (*see* Results, tables and figures). On completion of the full 30 incubation period, radioactive-containing buffer was withdrawn and cells were washed with 5 × 3 ml aliquots of an ice-cold solution containing 150 mM NaCl/10 mM HEPES-Tris-HCl (pH 7.4 at 4°C), and then lysed with 1 ml of 1% sodium dodecyl sulphate/4 mM EDTA. The radioactivity in cell lysates and incubation buffer samples was quantitated by liquid scintillation counting.

^{86}Rb Influx

^{86}Rb influx was studied according to the methods described by Smith and Smith (1987) and Duhm et al. (1990), with minor modifications. VSMC (in 24-well plates, $\approx 1.5–2.2 \times 10^5$ cells per well) were washed with 3 × 1 ml aliquots of buffer containing 150 mM NaCl/10 mM HEPES-Tris-HCl (pH 7.4 at 37°C), and then preincubated for 30 min at 37°C in 0.5 ml of medium A (140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES-Tris-HCl, pH 7.4). Thereafter the medium was replaced with a fresh 0.25 ml aliquot of medium A either without or with various inclusions of 1 mM ouabain and 20 μM bumetanide, and cells were further preincubated for 10 min. Any other additions made during this 10 min preincubation period have been specified in Results and the legend to Table 2. Thereafter a further 0.25 ml of medium A (at different osmolalities) containing 2–3 μCi $^{86}RbCl$/ml was added to each well and incubation at 37°C continued. Changes in the total osmolality of medium A were

made by varying the concentration of NaCl and by addition of sucrose. The uptake of ^{86}Rb was terminated at appropriate time intervals (*see* Results) by addition of 1.5 ml of ice-cold stop buffer containing 100 mM $MgCl_2$/10 mM HEPES-Tris-HCl (pH 7.4). Dishes were immediately transferred onto ice and cells thereafter washed with 5 × 1.5 ml stop buffer followed by lysis with 1 ml of 1% sodium dodecyl sulphate/4 mM EDTA. Radioactivity in the incubation medium and cell lysates were determined by liquid scintillation spectrometry. The rate of ^{86}Rb influx was calculated as A/ant where A is the radioactivity in cell lysate (cpm), a is the specific radioactivity of incubation medium (cpm/pmol), n is the cell number per well and t is the time of incubation. The different cation transport systems were evaluated as described by Smith and Smith (1987) and Duhm et al. (1990): Ouabain-inhibited ^{86}Rb influx represents Na^+/K^+ pump activity; ouabain-insensitive, bumetanide-inhibited ^{86}Rb influx represents $Na^+/K^+/2Cl^-$-co-transport activity.

^{86}Rb Efflux

Quiescent VSMC (in 24-well plates, ≈1.5–2.2 × 10^5 cells per well) were prelabeled by overnight incubation in serum-free culture medium containing 2 μCi $^{86}RbCl$/ml. Dishes were then transferred onto ice, the radioactive medium was aspirated and cells were washed with 5 × 1.5 ml aliquots of an ice-cold solution containing 150 mM NaCl/10 mM HEPES-Tris-HCl (pH 7.4 at 4°C). To initiate ^{86}Rb efflux, 0.25 ml medium A (at varied osmolalities) containing 1 mM ouabain was added to the wells and dishes transferred into a water bath (37°C, 50 shakes/min). Where indicated (*see* Results and Fig. 5) 0.5 mM furosemide, 0.1 mM quinidine, and 20 μM bumetanide were variously included in the ouabain-containing medium A. ^{86}Rb efflux was terminated at selected time intervals by addition of 1.5 ml of an ice-cold solution containing 150 mM NaCl/10 mM HEPES-Tris-HCl (pH 7.4 at 4°C). Dishes were transferred onto ice and 1 ml of incubation medium/buffer overlay withdrawn from each well. The rate of ^{86}Rb efflux is expressed as the % of initial ^{86}Rb content in cells and was determined as the ratio $A_1/(A_2 \times 100)$, where A_1 is the ^{86}Rb radioactivity in withdrawn overlay and A_2 is the initial ^{86}Rb content in cells. A_2 was determined in cells which had been lysed with 1.5 ml of 1% sodium dodecyl sulphate/4 mM EDTA immediately after the prelabeling, aspiration and washing procedures described above.

^{22}Na Influx

The method described by Vallega et al. (1988) was applied, with minor modifications. VSMC (in 12-well plates; ≈4–7 × 10^5 cells/well, respectively) were washed at 37°C with 2 × 3 ml aliquots of a solution containing 150 mM NaCl/10 mM HEPES-Tris-HCl (pH 7.4). Cells were sequentially preincubated at 37°C with 1 ml medium A (60 min) and 1 ml of fresh medium A containing 1 mM oubain and 10 μM bumetanide (10 min). Any other additions made during the second 10 min preincubation period have been specified in Results and legend to Table 3. ^{22}Na uptake was initiated by addition of 1 ml of incubation medium (at 37°C) containing 60 mM NaCl, 70 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES-Tris (pH 7.4), 1 mM ouabain, 20 μM bumetanide and 2–3 μCi $^{22}NaCl$/ml, and either without or with inclusion of 10 μM EIPA. For these experiments changes in the total osmolality of the incubation medium were made by either decreasing the concentration of choline chloride or by addition of sucrose. ^{22}Na uptake was terminated at selected time intervals by addition of 3 ml ice-cold stop buffer containing 100 mM $MgCl_2$/10 mM HEPES-Tris-HCl (pH 7.4). Dishes were transferred onto ice, the cells washed with 5 × 3 ml of stop buffer and then lysed by addition of 1 ml 4 mM EDTA/1% sodium dodecyl sulphate. Radioactivity in the incubation medium and cell lysates was quantitated by liquid scintillation counting. ^{22}Na-influx was calculated as A/ant where A is the radioactivity in cell lysate (cpm), a is the specific radioactivity of incubation medium (cpm/nmol), n is the cell number per well, and t is the time of incubation with ^{22}Na.

Phosphatidylinositol Catabolism

Confluent VSMC (in 24-well plates; ≈1.5–2.2 × 10^5 cells per well) were rendered quiescent as described above but with inclusion of 2.5 μCi myo[2-^{3}H]-inositol/ml to prelabel inositol phospholipids. Prior to experimentation, radioactive medium was aspirated, and the cells were washed with 3 × 1 ml aliquots of buffer containing 150 mM NaCl/10 mM HEPES-Tris-HCl (pH 7.4 at 37°C) and then preincubated for 30 min at room temperature in 0.5 ml/well Medium B (containing 130 mM NaCl, 15 mM LiCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES-Tris-HCl (pH 7.4 at 37°C)). Thereafter this medium was replaced with a fresh 0.25-ml aliquot of medium B and cells were further incubated for 10 min. Then 0.25 ml of medium B (at different osmolalities) either without or with 10^{-7} M Ang II was added to the cells. Changes in the total osmolality of medium B were made by varying the concentration of NaCl and by addition of sucrose. Incubations were terminated after 5 min by addition of 1 ml 4 mM EDTA/1% sodium dodecyl sulphate. Cell lysates were applied to columns containing 0.5 g Dowex-AG 1-X8 (formate form) and inositol phosphates were resolved as described previously (Resink et al., 1987). Radioactivity of inositol phosphates was quantitated by liquid scintillation spectrometry, and data for [^{3}H]-inositol content are expressed as dpm per 10^5 cells.

Statistical Analysis

All experimental manipulations were performed in quadruplicate within any single experiment. Where appropriate, statistical significance was determined using Student's t-test for unpaired data.

Results and Discussion

The Dependence of VSMC Volume on the Osmolality of the Incubation Medium and on the Presence of Ion Transport Inhibitors

In accordance with the Boyle-van't Hoff law the volume of an ideal osmometer is inversely proportional to the osmolality of the medium. Figure 1 demonstrates that in VSMC the dependence of the volume of intracellular water on the inverse osmolality ($1/\pi$) of the incubation medium is not linear and that the effect of osmolality on cell volume diminishes with the swelling of VSMC. Thus it may be assumed that, during cell volume alterations under anisosmotic medium conditions, the activation of

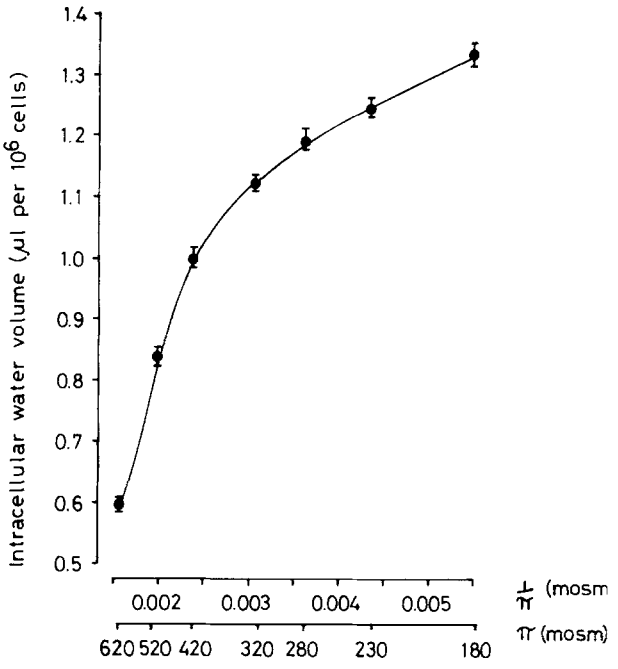

Fig. 1. The dependence of intracellular water volume in VSMC on the osmolality of the incubation medium. Experimental conditions are detailed in Materials and Methods. Values given are means ± SEM and were obtained from eight separate experiments.

RVI and RVD takes place and/or VSMC possess some properties which limit cellular swelling (*see* Macknight, 1987).

Under isosmotic (312 mosm) conditions the loss of intracellular potassium following ouabain-mediated inhibition of Na^+/K^+ pump activity resulted in a 8–12% decrease ($P < 0.005$) of cell volume (Table 1, column 1). Under isosmotic conditions and in the presence of ouabain, neither bumetanide (an inhibitor of $Na^+/K^+/2Cl^-$ cotransport), furosemide (an inhibitor of $Na^+/K^+/2Cl^-$ and K^+/Cl^- cotransport), EIPA (an inhibitor of Na^+/H^+ exchange) nor quinidine (an inhibitor of Ca^{2+}-activated K^+ channels) significantly further modulated cell volume (Table 1, column 1). Neither bumetanide, furosemide nor quinidine had any effect on cell volume when VSMC were incubated under ouabain-containing hypoosmotic (181 mosm) medium conditions (Table 1, column 2). However, under ouabain-containing hyperosmotic (504 mosm) medium conditions the shrinkage of VSMC was increased further with inclusion of either bumetanide (by 5–8%, $P < 0.05$) or EIPA (by 11–15%, $P < 0.005$) (Table 1, column 3). Cell volume under hyperosmotic, ouabain- and bumetanide-containing medium conditions was not further modulated by inclusion of furosemide (Table 1, column 3).

In a variety of cell types, including erythrocytes, lymphocytes and endothelial cells, cell shrinkage induces activation of $Na^+/K^+/2Cl^-$ cotransport and Na^+/H^+ exchange, whereas cell swelling activates K^+/Cl^- cotransport, K^+/H^+ exchange or K^+ and Cl^- channels (reviewed in Introduction, and Hoffmann & Simonsen, 1989). The lack of influence of furosemide and quinidine on cell volume under hypoosmotic buffer conditions (Table 1, column 2) indicates that, in VSMC, neither K^+/Cl^- cotransport nor Ca^{2+}-activated K^+ channels are activated by cell swelling. However, the effects of bumetanide and EIPA on cell volume under hyperosmotic conditions (Table 1, column 3) suggest that shrinkage of VSMC also induces activation of $Na^+/K^+/2Cl^-$ cotransport and Na^+/H^+ exchange.

The Dependence of Univalent Cation Fluxes on Incubation Medium Osmolality

In order to demonstrate that VSMC indeed possess ion transport systems which are sensitive to changes in cell volume, we investigated the influence of cell-shrinkage (hyperosmotic buffer conditions) and cell swelling (hypoosmotic buffer conditions) on univalent cation fluxes, namely K^+ (^{86}Rb) and Na^+ (^{22}Na).

Kinetics of ^{86}Rb and ^{22}Na Fluxes

The kinetics of potassium (^{86}Rb) and sodium (^{22}Na) fluxes in VSMC under isosmotic buffer conditions are shown in Fig. 2. In VSMC, ^{86}Rb influx (Fig. 2*A*) and ^{22}Na influx (Fig. 2*B*) occurred linearly for up to 10 and 3 min, respectively. ^{86}Rb efflux from ^{86}Rb-preloaded VSMC was linear for up to 5 min, and the initial isotope content in VSMC decreased by 50% after 15 min of incubation (Fig. 2*C*). For subsequent determinations of the influence of osmolality on the initial rates of ion fluxes, the incubation times were limited either to 5 min for ^{86}Rb influx and ^{86}Rb efflux, or to 3 min for ^{22}Na influx.

Swelling of VSMC Decreases Na^+/K^+ Pump Activity

The activity of the Na^+/K^+ pump (ouabain-inhibited component of ^{86}Rb influx) was decreased by ≈30–40% under hypotonic buffer conditions (Fig. 3, curve *1*). Since intracellular Na^+ in the rate-limiting substrate for the Na^+/K^+ pump (Brock & Smith, 1982), the inhibitory effect of hypoosmolality on pump activity is probably a consequence of the decreased intracellular sodium concentration in swollen VSMC.

Table 1. Effects of ion transport-system inhibitors on the volume of intracellular water in VSMC

Addition of inhibitors	Intracellular water volume (μl per 10^6 cells)		
	Isosmotic (Column 1)	Hypoosmotic (Column 2)	Hyperosmotic (Column 3)
None (control)	1.18 ± 0.03	1.32 ± 0.04	0.60 ± 0.03
Ouabain	1.05 ± 0.02*(1)	1.28 ± 0.03	0.78 ± 0.02
Ouabain + bumetanide	1.04 ± 0.02*(1)	1.26 ± 0.03	0.73 ± 0.02**(2)
Ouabain + bumetanide + furosemide	1.03 ± 0.02*(1)	1.26 ± 0.03	0.73 ± 0.02**(2)
Ouabain + EIPA	1.06 ± 0.02*(1)	1.27 ± 0.03	0.68 ± 0.02***(2)
Ouabain + quinidine	1.05 ± 0.02*(1)	1.28 ± 0.03	ND

The volume of intracellular water in VSMC was determined as detailed under Materials and Methods. The osmolality and composition of the different incubation media was as follows: Column 1 (312 mosm): 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM 3-O-methyl-D-glucose, 20 mM HEPES-Tris-HCl; Column 2 (181 mosm): the same as in column 1 but with NaCl reduced to 70 mM; Column 3 (504 mosm): the same as in column 1 but with inclusion of 200 mM sucrose. Inhibitors were added at the following concentrations: 10^{-3} M ouabain, 10^{-5} M bumetanide, 5.10^{-4} M furosemide, 10^{-5} M EIPA, 10^{-4} M quinidine. Results are given as means ± SD and were obtained from six separately performed experiments. (1) Cell volumes are compared under isosmotic conditions, and asterisks indicate significance of difference ($*P < 0.005$) from control. (2) Cell volumes are compared under hyperosmotic conditions, and asterisks indicate significance of difference ($**P < 0.05$, $***P < 0.005$) from incubations in the presence of ouabain alone. ND, not determined.

Shrinkage of VSMC Activates the Na^+/H^+-Exchange Exchanger

Cell volume alterations did not affect the ouabain-, bumetanide-, and EIPA-insensitive component of ^{22}Na influx in VSMC (Fig. 4, curve *1*). Na^+/H^+-exchange activity (EIPA-inhibited component of ^{22}Na influx) was negligible under hypotonic buffer conditions (0.041 ± 0.029 nmol/10^5 cells/min at 220 mosm), but increased markedly (up to ≈30–40 fold) with increases in buffer osmolality (Fig. 4, curve *2*).

Na^+/K^+2Cl^--Cotransport-Mediated ^{86}Rb Influx and ^{86}Rb Efflux in VSMC are Highly Sensitive to Changes in Cell Volume

The ouabain- and bumetanide-insensitive component of ^{86}Rb influx (inward K^+ leakage) was not dependent on the osmolality of the incubation medium (Fig. 3, curve *3*). The $Na^+/K^+/2Cl^-$ cotransporter operates bidirectionally and mediates both the influx and efflux of univalent cations (Geck & Heinz, 1986). The inward Na^+/K^+2Cl^--cotransport activity (ouabain-insensitive, bumetanide-inhibited component of ^{86}Rb influx) increased by ≈5–6 fold when the buffer osmolality was increased from 180 to ≈500 mosm (Fig. 3, curve *2*). Further shrinkage of VSMC (osmolality >550 mosm) resulted in an inhibition of shrinkage-induced $Na^+/K^+/2Cl^-$ cotransport, with a return of the rate of ^{86}Rb influx to that observed under isosmotic buffer conditions (Fig. 3, curve *2*). These data demonstrate that in VSMC, cation (^{86}Rb) influx mediated by this carrier is strongly dependent on cell volume. A similar volume dependence was found with respect to $Na^+/K^+/2Cl^-$ cotransport-mediated ^{86}Rb efflux (Fig. 5, curve *2*). The ≈15–17% decrease of cell volume that is caused by increasing medium osmolality from 270 to 420 mosm (Fig. 1), resulted in an increase of the ouabain-insensitive, bumetanide-inhibited component of ^{86}Rb efflux by almost one order of magnitude (Fig. 5, curve *2*).

The data presented in Figs. 3–5 demonstrate that osmotic shrinkage of VSMC is accompanied by activation of both the Na^+/H^+ exchanger and the $Na^+/K^+/2Cl^-$ cotransporter. However, the two carriers apparently differ with respect to their volume-dependent activation. In particular, for the $Na^+/K^+/2Cl^-$ cotransporter, we observed an extremely sharp dependence on the osmolality of the incubation medium (Figs. 3 and 5). When compared with data obtained previously for rat erythrocytes (Orlov et al., 1990) and bovine vascular endothelial cells (O'Donnell & Owen, 1991), the present findings show that the sensitivity of this carrier in VSMC to cell volume alterations is higher. However, for VSMC incubated in hypertonic medium the relative participation of $Na^+/K^+/2Cl^-$ cotransport in RVI is less than that of the Na^+/H^+ exchanger (*compare* data in Figs. 3 and 4). Indeed, as can be seen from Table 1, the shrinkage of VSMC in hypertonic me-

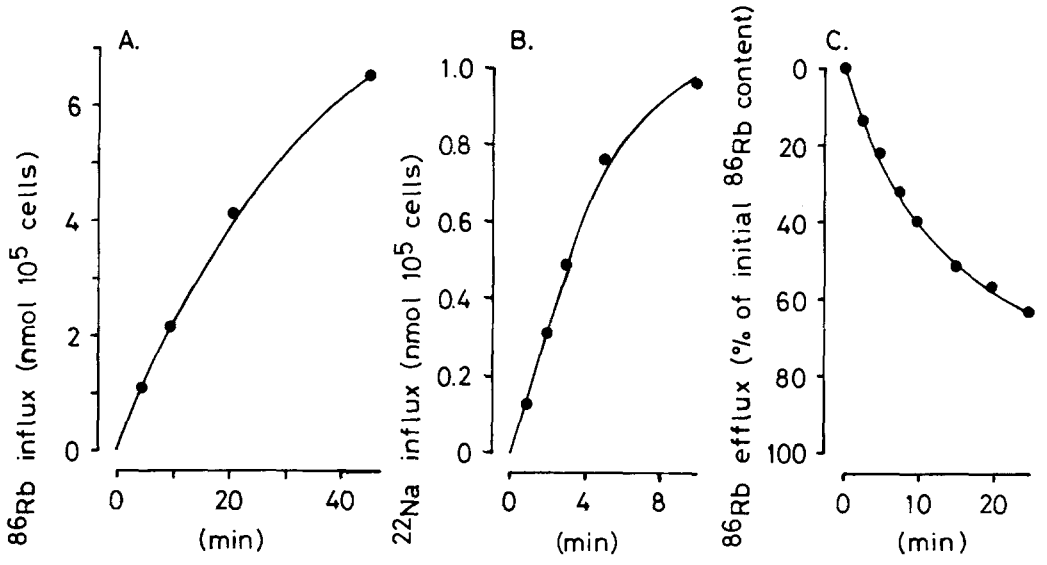

Fig. 2. The kinetics of ^{86}Rb influx (*A*), ^{22}Na influx (*B*) and ^{86}Rb efflux (*C*) in VSMC. Experimental protocols for measurement of univalent cation fluxes are detailed under Materials and Methods. Means from four separate experiments are given.

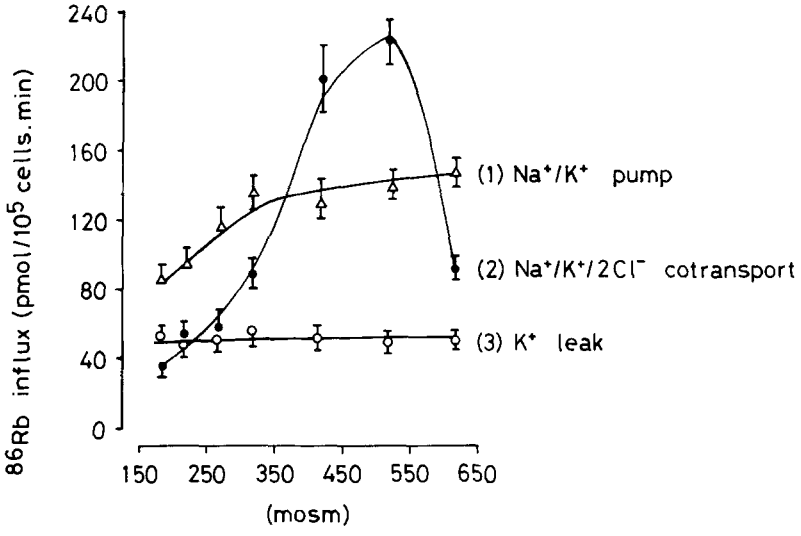

Fig. 3. Dependence of the activity of the Na^+/K^+ pump (ouabain-inhibited ^{86}Rb influx; △—curve *1*), $Na^+/K^+/2Cl^-$ cotransport (ouabain-insensitive, bumetanide inhibited ^{86}Rb influx; ●—curve *2*), and K^+ leak (ouabain- and bumetanide-insensitive ^{86}Rb influx; ○—curve *3*) on the osmolality of the incubation medium. Experimental procedures are described under Materials and Methods. Means ± SEM from nine separate experiments are given.

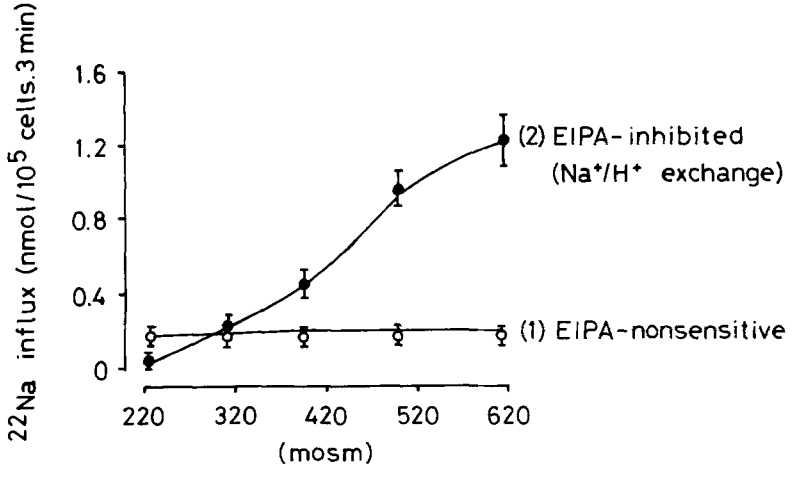

Fig. 4. Dependence of the EIPA-insensitive (○—curve *1*) and EIPA-inhibited (Na^+/H^+ exchange; ●—curve *2*) components of ^{22}Na influx on the osmolality of the incubation medium. Experimental protocols are detailed under Materials and Methods. Means ± SEM from nine separate experiments are given.

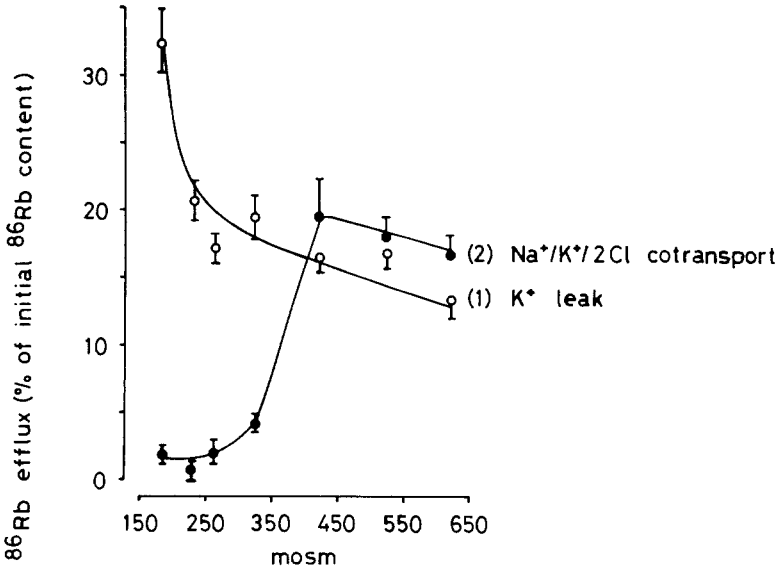

Fig. 5. Dependence of the bumetanide-insensitive ^{86}Rb influx (K^+ leakage; ○—curve *1*) and bumetanide-inhibited ^{86}Rb influx ($Na^+/K^+/2Cl^-$ cotransport; ●—curve *2*) on the osmolality of the incubation medium. Experimental procedures are described under Materials and Methods. Means ± SEM from nine separate experiments are given.

dium is more pronounced in the presence of EIPA as compared with that in the presence of bumetanide. This differential participation may reflect the bidirectional mode of operation of the $Na^+/K^+/2Cl^-$ cotransporter and its mediation of both shrinkage-induced univalent cation influx and efflux.

Swelling of VSMC Activates K^+ Leakage

Under hypotonic medium conditions (osmolality <250 mosm) the ouabain- and bumetanide-insensitive component of ^{86}Rb efflux (outward K^+ "leakage") increased by ≈60–80% (Fig. 5, curve *1*). Neither furosemide nor quinidine influenced this swelling-induced component of ^{86}Rb efflux further (data not presented), thus indicating that neither K^+/Cl^- cotransport nor Ca^{2+}-activated potassium channels are involved in the potassium efflux-mediated RVD of VSMC. We are presently unable to identify the ion-transport system mediating swelling-induced K^+ efflux, and the insensitivity of this flux to bumetanide, furosemide and quinidine does not permit an examination of its relative participation in regulatory volume changes.

Signalling Mechanisms: Compounds Affecting Volume-Induced Ion Fluxes

The mechanisms of intracellular signalling involved in the volume-induced activation of ion flux-mediated RVD and RVI are not well understood. In *Amphiuma* erythrocytes, Ca^{2+} appears to act as a modulator of swelling-induced K^+/H^+ exchange (Cala, 1983). In lymphocytes, Ehrlich ascites tumor cells and epithelial cells there is strong evidence to support the involvement of Ca^{2+} and calmodulin in the swelling-induced activation of K^+ transport pathways (Hoffmann et al., 1984; Sarkadi, Mack & Rothstein, 1984; Foskett & Spring, 1985). It has been demonstrated that during RVD in Ehrlich ascites cells the synthesis of leukotrienes is stimulated while prostaglandin synthesis is reduced. Addition of LTD4 accelerates RVD while addition of inhibitors of leukotriene synthesis block the volume response (Lambert, Hoffmann & Christensen, 1987). In contrast, for rat erythrocytes RVD mediated by K^+/Cl^- cotransport is blocked by inhibitors of cyclooxygenase (Gurlo et al., 1991). Based on the data demonstrating activation of Na^+/H^+ exchange by protein kinase C it has been proposed that this enzyme is also involved in volume-dependent regulation of Na^+/H^+ exchange (Grinstein & Rothstein, 1986). Shrinkage of rat erythrocytes leads to increased incorporation of ^{32}P into di- and tri-phosphoinositides (Orlov et al., 1989), thus indicating that RVI may be mediated via alteration of polyphosphoinositide metabolism.

To identify signalling mechanisms involved in RVI and RVD in VSMC we studied basal and volume-induced ^{86}Rb and ^{22}Na fluxes in the presence of the following compounds: phorbol 12-myristate, 13-acetate (PMA) (a protein kinase C activator); staurosporine (an inhibitor of protein kinase C); forskolin (an activator of adenylate cyclase); 8-Br-cyclic GMP (the permeant cyclic GMP analogue); calmidazolium (R24571) (an inhibitor of calmodulin-dependent reactions); 2-nitro-4-carboxyphenyl N,N-diphenylcarbomate (NCDC) (a putative inhibitor of phospholipase C; Turla & Webb, 1990); sodium fluoride (an

Table 2. Compounds affecting basal and shrinkage-induced $Na^+/K^+/2Cl^-$-cotransport activities in VSMC

Compounds added		$Na^+/K^+/2Cl^-$-cotransport activity (pmol per 10^5 cells per min)	
		Basal	Shrinkage-induced increment
None	(control)	58.7 ± 4.8	223.0 ± 24.1
1.5 mM	EGTA	49.1 ± 3.9	201.7 ± 18.5
10^{-7} M	PMA	54.6 ± 3.9	233.7 ± 17.0
5.10^{-7} M	staurosporin	29.5 ± 3.7[a]	105.7 ± 26.7[a]
10^{-5} M	forskolin	35.5 ± 5.0[a]	130.3 ± 29.6[a]
10^{-4} M	8-Br-cGMP	50.6 ± 4.4	215.6 ± 16.6
10^{-5} M	R 24571	16.8 ± 2.0[a]	73.2 ± 15.0[a]
10^{-4} M	NCDC	38.7 ± 4.6[a]	180.0 ± 24.5[a]
10^{-2} M	NaF	40.0 ± 4.4[a]	76.5 ± 11.8[a]
10^{-5} M	meclofenamic acid	53.4 ± 5.5	240.0 ± 25.3
10^{-6} M	quercetin	60.1 ± 7.8	215.9 ± 17.7

$Na^+/K^+/2Cl^-$-cotransport activity (ouabain-insensitive, bumetanide-inhibited component of ^{86}Rb influx) was determined as detailed in Materials and Methods. VSMC were preincubated with the listed compounds for 10 min prior to flux measurements. Basal activity was determined in a medium with osmolality at 282 mosm. Osmolality was increased to 416 mosm by addition of sucrose. The difference between the rates of transport activity in these media represents the shrinkage-induced increment of $Na^+/K^+/2Cl^-$-cotransport activity. Data are given as mean ± SD and were obtained from six separately performed experiments.

[a] Activity in the presence of a given compound differed significantly ($P < 0.05$) from that in the respective control.

activator of GTP-binding proteins); meclofenamic acid and quercetin (inhibitors of cyclooxygenase and lipoxygenase, respectively; Needleman et al., 1986); and EGTA (removal of extracellular Ca^{2+}).

Swelling-Induced K^+ Efflux is Insensitive to Second Messengers

None of the compounds listed above influenced the ouabain- and bumetanide-insensitive component of ^{86}Rb efflux (K^+ leakage) in VSMC under either isosmotic (312 mosm) or hypoosmotic (181 mosm) buffer conditions (*data not shown*).

Regulation of $Na^+/K^+/2Cl^-$-Cotransport Activity by Cyclic AMP, Calmodulin and the Phosphoinositide Signalling Pathway

Neither the basal nor shrinkage-induced activity of the $Na^+/K^+/2Cl^-$ cotransporter was influenced by EGTA, 8-Br-cGMP, meclofenamic acid or quercetin (Table 2). The lack of effect of these compounds indicates that neither transarcolemmal Ca^{2+} fluxes, cGMP-dependent systems nor arachidonic acid metabolites are involved in the volume-dependent signalling system coupled with this carrier. Forskolin, staurosporin, R24571, NCDC and NaF inhibited both basal (282 mosm) and shrinkage-induced (416 mosm) activation of $Na^+/K^+/2Cl^-$ cotransport (Table 2). The inhibition of basal $Na^+/K^+/2Cl^-$-cotransport activity in VSMC by forskolin and R24571 is in agreement with previous observations on the effects of other compounds which elevate cyclic AMP (e.g., isoproterenol, cholera toxin, isobutylmethylxanthine) or which function as calmodulin antagonists (e.g. trifluoperazine, naphthalenesulfonamides) (Owen, 1984; Smith & Smith, 1987). Our present additional findings of inhibition of VSMC $Na^+/K^+/2Cl^-$ cotransport by staurosporin, NCDC and NaF indicate that guanine nucleotide-binding protein(s) and the phosphoinositide signalling pathway (i.e., protein kinase C and phospholipase C) may also be involved in the modulation of both basal and volume-dependent activity of this carrier.

Dissociation between Regulation of Cell Volume and Modulation of $Na^+/K^+/2Cl^-$-Cotransport Activity

To determine the contribution of these signalling pathways towards an associated regulation of both cell volume and cotransport activity, those com-

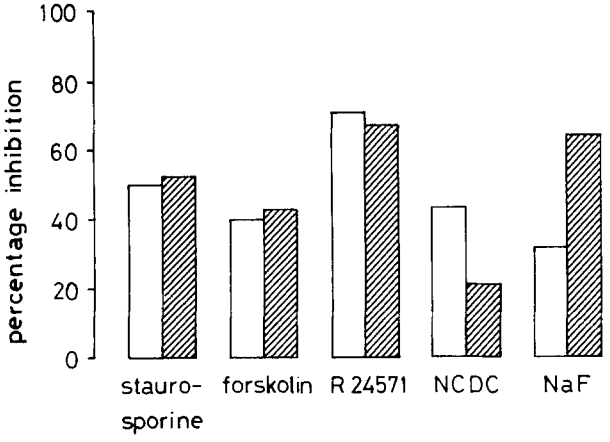

Fig. 6. The percentage of inhibition of the basal (open bars) and shrinkage-induced (hatched bars) $Na^+/K^+/2Cl^-$-cotransport activity by staurosporin, forskolin, R24571, NCDC and NaF. Refer to Table 2 for details of absolute cotransport activities.

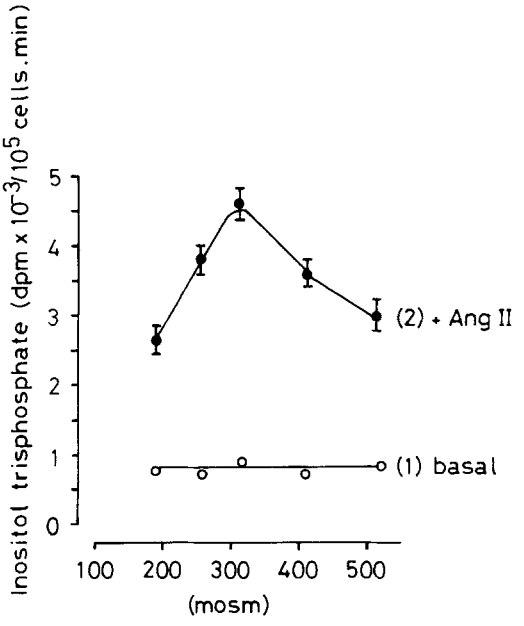

Fig. 7. The dependence of the basal (○—curve *1*) and Ang II-stimulated (10^{-7} M, 5 min, ●—curve *2*) content of [^{3}H]-inositol triphosphate on the osmolality of the incubation medium. Experimental protocols are described under Materials and Methods. Data are given as means ± SEM and were obtained from six separate experiments.

pounds which inhibited $Na^+/K^+/2Cl^-$ cotransport activity were further evaluated (from data presented in Table 2) in terms of their degrees of inhibition of cotransport activity under isosmotic or hyperosmotic conditions. Basal and shrinkage-induced $Na^+/K^+/2Cl^-$-cotransport activities were similarly sensitive (% inhibition) to either forskolin, staurosporin or R24571 (Fig. 6), suggesting that these particular compounds decrease the maximal activity of the carrier but do not affect those mechanisms mediating activation of cotransport under hyperosmotic conditions. These findings agree with other observations on agonist-elicited alterations in cotransport activity in VSMC. Stimulation of $Na^+/K^+/2Cl^-$ cotransport by angiotensin II (Ang II), a vasoactive peptide which mediates many of its cellular effects via phospholipase C-mediated phosphoinositide hydrolysis and subsequent intracellular Ca^{2+} mobilization and activation of protein kinase C (Smith, 1986), has previously been demonstrated for VSMC (Smith & Smith, 1987). Beta-adrenergic stimulation of VSMC, which increases cyclic AMP, inhibits cotransport activity (Smith & Smith, 1987). However, the intracellular volume of water in VSMC was not affected by either Ang II (Smith & Brock, 1983) or isoproterenol (Smith, 1984). Therefore, in VSMC, the modulation of $Na^+/K^+/2Cl^-$ cotransport by cyclic AMP, Ca^{2+}-calmodulin and protein kinase C in VSMC appears to be unrelated to the regulation of cell volume.

In contrast, the putative inhibitor of phospholipase C, NCDC, decreased basal and shrinkage-induced $Na^+/K^+/2Cl^-$-cotransport activities by 44 and 20%, respectively (Fig. 6), suggesting some involvement of phospholipase C in the regulation of cotransport activity under hyperosmotic conditions. To investigate this possibility we studied the influence of osmolality on phospholipase C-mediated phosphoinositide catabolism in the absence (basal) and presence of Ang II. The ability of Ang II to stimulate phospholipase C (≈sixfold increase in [^{3}H]-$InsP_3$ generation under isosmotic conditions) was highly sensitive to changes in buffer osmolality, and Ang II-induced [^{3}H]-$InsP_3$ accumulation was reduced by 40–60% under both hypotonic and hypertonic buffer conditions (Fig. 7, curve *2*). On the other hand, basal (i.e., without agonist) production of [^{3}H]-inositol trisphosphate ([^{3}H]-$InsP_3$) in VSMC was not modified by changes in osmolality (Fig. 7, curve *1*). These data indicate that although receptor-mediated activation of phospholipase C depends on cell volume, phospholipase C activity in resting cells is insensitive to alterations in osmolality. Since the data pertaining to the effects of NCDC on $Na^+/K^+/2Cl^-$ cotransport in VSMC (Fig. 6 and Table 2) were performed under resting (i.e., without agonist stimulation) conditions, it may be concluded that phospholipase C *per se* is not involved in volume-dependent regulation of ion transport.

Table 3. Effects of forskolin, NCDC and NaF on ^{22}Na influx in VSMC under isosmotic and hyperosmotic conditions

Additions	^{22}Na influx (nmol/10^5 cells/3 min)			
	EIPA-insensitive component		EIPA-inhibited component	
	312 mosm	612 mosm	312 mosm	612 mosm
None (control)	0.24 ± 0.04	0.27 ± 0.06	0.22 ± 0.04	1.37 ± 0.28
Forskolin (10^{-5} M)	0.25 ± 0.05	0.23 ± 0.06	0.27 ± 0.06	1.31 ± 0.03
Staurosporin (5.10^{-7} M)	0.21 ± 0.04	0.25 ± 0.05	0.09 ± 0.02[a]	1.25 ± 0.27
NCDC (10^{-4} M)	0.29 ± 0.04	0.25 ± 0.04	0.26 ± 0.07	1.80 ± 0.32
NaF (10^{-2} M)	0.70 ± 0.12[a]	0.39 ± 0.09	1.95 ± 0.28[a]	2.84 ± 0.41[a]

^{22}Na influx was determined under isosmotic (312 mosm) and hyperosmotic (612 mosm) buffer conditions as described in Materials and Methods. VSMC were preincubated for 10 min with the indicated compounds before flux determinations. Na^+/H^+ exchange activity is represented by the EIPA-inhibited component of ^{22}Na influx. Data are given as mean ± SD and were obtained from four separately performed experiments.

[a] Activity in the presence of a given compound differed significantly ($P < 0.05$) from that in the respective control.

Role for Guanine Nucleotide Binding Proteins in Volume-Dependent Regulation of Cotransport Activity

For several Ca^{2+}-mobilizing hormones, including Ang II, the coupling between cell surface receptors and polyphosphoinositide-specific phospholipase C is mediated via guanine nucleotide binding protein (G protein) (Smith, 1986). G-proteins have also been shown to be involved in the transduction coupling of receptors to several other signalling systems such as adenylate cyclase, phosphodiesterase, phospholipase A_2, K^+ channels, Ca^{2+} channels and ion pumps (Taylor, 1990). NaF (more specifically AlF_4^-) is a well known pharmacological probe for establishing the significance of G protein activation in cellular systems (Gilman, 1987). Our data demonstrate that the inhibitory effect of NaF on shrinkage-induced $Na^+/K^+/2Cl^-$ cotransport was ≈twofold greater than that on the basal activity (isosmotic conditions) of this carrier (Table 2 and Fig. 6), suggesting that guanine nucleotide-binding proteins may indeed be involved in the signalling systems which are coupled to and/or mediate the volume-sensitive activity for this carrier.

Na^+/H^+ Exchange and Volume-Independent Regulation by G Protein

Under isosmotic (312 mosm) buffer conditions Na^+/H^+ exchange was insensitive to forskolin and NCDC, inhibited by staurosporin ($P < 0.05$) and increased by NaF ($P < 0.05$) (Table 3). Under hyperosmotic (612 mosm) conditions there was a ≈sixfold increase in the EIPA-inhibited component of ^{22}Na influx, and this shrinkage-induced activation of Na^+/H^+ exchange was insensitive to forskolin, NCDC as well as staurosporin (Table 3). The inhibitory effects of staurosporin on the basal activity of this carrier are in agreement with observations that protein kinase C is a stimulator of the Na^+/H^+ exchanger in many cell types including VSMC (Berk et al., 1987; Vigne et al., 1988). The present data, however, demonstrate that protein kinase C is not involved in the volume-dependent regulation of Na^+/H^+ exchange activity. The stimulation of exchanger activity by NaF was still evident under hyperosmotic conditions, and the NaF-dependent increment in activity (≈1.5 nmol/10^5 cells/3 min) was comparable to that under isosmotic conditions (≈1.7 nmol/10^5 cells/3 min). Additionally, shrinkage-induced increments in Na^+/H^+-exchange activity were comparable in the presence and absence of NaF (≈0.9–1.1 nmol/10^5 cells/3 min) (calculated from data in Table 3). These data invoke the involvement of G protein(s) in regulation of the Na^+/H^+ exchanger in a manner which, independently of the volume of VSMC, increases maximal exchanger activity.

It is noteworthy that NaF stimulates a marked influx of sodium via activation of EIPA-sensitive (Na^+/H^+ exchange) and EIPA-insensitive pathways under isosmotic conditions (Table 3). This influx may promote the increase of intracellular sodium concentrations and water volume, thereby increasing the volume of VSMC. As abovementioned, the

$Na^+/K^+/2Cl^-$ cotransporter in VSMC is extremely sensitive to cell volume alterations (*see* Figs. 1, 3 and 5 for relationships between cell volume and cotransport activity). It is thus conceivable that the inhibitory effects of NaF on cotransport activity may reflect a small (NaF-induced) increase in cell volume rather than a "direct" G-protein-mediated effect on this carrier.

Volume-Dependent Activation of $Na^+/K^+/2Cl^-$-Cotransport and Na^+/H^+ Exchange Involve Different Mechanisms of Intracellular Signalling

The $Na^+/K^+/2Cl^-$ cotransporter and Na^+/H^+ exchanger in VSMC differ with respect to the mechanisms of intracellular signalling involved in volume-dependent activation as follows: (i) the two systems differ with respect to their dependencies on osmolality of the incubation medium (*cf.* Figs. 3 and 5 *vs.* Fig. 4); (ii) staurosporin, forskolin, R24571 and NCDC inhibit shrinkage-induced $Na^+/K^+/2Cl^-$ cotransport, but do not modulate shrinkage-induced activation of Na^+/H^+ exchange; (iii) NaF inhibits the shrinkage-induced activation of $Na^+/K^+/2Cl^-$ cotransport but stimulates shrinkage-induced Na^+/H^+ exchange. The stimulatory effect of NaF on Na^+/H^+ exchange is, however, apparently independent of cell volume.

Conclusions and Physiological Implications

Using changes in buffer osmolality to promote either cell shrinkage (hyperosmotic conditions) or swelling (hypoosmotic conditions), we have demonstrated that VSMC possess ion transport systems which are sensitive to changes in cell volume. Hypoosmotic swelling of VSMC induces the activation of an ion-transport system which mediates K^+ efflux, while hyperosmotic shrinkage of VSMC activates the $Na^+/K^+/2Cl^-$ cotransporter and the Na^+/H^+ exchanger. G-proteins(s), either directly and/or indirectly (via activation of Na^+/H^+ exchange) appear to be involved in the intracellular signalling mechanisms for the volume-sensitive regulation of $Na^+/K^+/2Cl^-$ cotransport. Further studies are required to clarify the role of G-proteins in volume-induced activation of $Na^+/K^+/2Cl^-$ cotransport in VSMC.

The physiological implications of RVD and RVI mediated via the ion-transport systems described in this paper have not been investigated. However, it may be assumed that these systems participate in the maintenance of cell volume following hormone- and neurotransmitter-triggered excitation-contraction coupling as well as during cell growth. In healthy vessels, VSMC are protected from the direct effects of blood-borne stimuli by the endothelial monolayer. However, under conditions where this barrier is damaged, such as in atherosclerotic lesions, the volume-dependent regulation of ion transport and cell volume maintenance in smooth muscle cells is likely to play a critical role in blood vessel wall structure and function.

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