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Regulation by Cell Volume of Na^+-K^+-$2Cl^-$ Cotransport in Vascular Endothelial Cells: Role of Protein Phosphorylation

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Summary. Na^+-K^+-$2Cl^-$ cotransport in aortic endothelial cells is activated by cell shrinkage, inhibited by cell swelling, and is responsible for recovery of cell volume. The role of protein phosphorylation in the regulation of cotransport was examined with two inhibitors of protein phosphatases, okadaic acid and calyculin, and a protein kinase inhibitor, K252a. Both phosphatase inhibitors stimulated cotransport in isotonic medium, with calyculin, a more potent inhibitor of protein phosphatase I, being 50-fold more potent. Neither agent stimulated cotransport in hypertonic medium. Stimulation by calyculin was immediate and was complete by 5 min, with no change in cell Na + K content, indicating that the stimulation of cotransport was not secondary to cell shrinkage. The time required for calyculin to activate cotransport was longer in swollen cells than in normal cells, indicating that the phosphorylation step is affected by cell volume. Activation of cotransport when cells in isotonic medium were placed in hypertonic medium was more rapid than the inactivation of cotransport when cells in hypertonic medium were placed in isotonic medium, which is consistent with a shrinkage-activated kinase rather than a shrinkage-inhibited phosphatase. K252a, a nonspecific protein kinase inhibitor, reduced cotransport in both isotonic and hypertonic media. The rate of inactivation was the same in either medium, indicating that dephosphorylation is not regulated by cell volume. These results demonstrate that Na^+-K^+-$2Cl^-$ cotransport is activated by protein phosphorylation and is inactivated by a Type I protein phosphatase. The regulation of cotransport by cell volume is due to changes in the rate of phosphorylation rather than dephosphorylation, suggesting the existence of a volume-sensitive protein kinase. Both the kinase and the phosphatase are constitutively active, perhaps to allow for rapid changes in cotransport activity.

Key Words phosphorylation · cell volume · okadaic acid · calyculin · phosphatase · endothelial cells

Introduction

Endothelial cells respond to mechanical stresses in a number of ways, including changes in morphology [25, 42] that may serve to maintain endothelial barrier function, and changes in the secretion of vasoactive substances that alter underlying smooth muscle tone [7, 9, 23, 38]. The mechanism by which vascular endothelial cells respond to mechanical stimuli is unknown but may involve activation of ion transporters. Direct stretching of the plasma membrane [24] and the application of shear stress [31] activate K^+ currents in aortic endothelial cells. Such mechanosensitive ion transporters may be closely related to volume-regulatory ion transporters that are activated by changes in cell volume and serve to restore cell volume. In addition to serving as a model of mechanosensitive ion transport, volume-regulatory transporters may govern the size and shape of endothelial cells. Endothelial barrier function is governed in part by intercellular gaps [20], the size of which could vary with cell volume. This was demonstrated in cerebrovascular endothelial monolayers in culture, in which hypertonic shrinkage increased albumin permeability [41]. Rapid adjustments in cell size may also be required to maintain barrier function in the face of mechanical perturbations related to blood flow and pressure.

We have recently shown that both shrinkage and swelling of aortic endothelial cells activate specific ion transporters that restore cell volume [26, 33]. Shrunken cells exhibit rapid volume recovery through activation of Na^+-K^+-$2Cl^-$ cotransport [33]. This transporter is abundant in aortic endothelial cells and is regulated by vasoactive substances [6, 22, 29]. Although there is evidence that Na^+-K^+-$2Cl^-$ cotransport in endothelial cells is activated by intracellular calcium [6, 29, 30] and is inhibited by protein kinases [29, 30], the mechanism by which cell shrinkage activates cotransport in these or other cells [14] is unknown. The elucidation of this mechanism may provide insight into the regulation of ion transport by mechanical forces.

Evidence from several cell types suggests that Na^+-K^+-$2Cl^-$ cotransport is regulated by protein

phosphorylation. The activation of cotransport in duck red cells by agents that increase cyclic AMP levels [40] provided the first evidence. Subsequent studies showed that cotransport is inhibited by metabolic depletion of ATP in human erythrocytes [1, 8] and by removal of ATP from internally dialyzed squid axons [3]. The inhibition in squid axons is delayed by adding vanadate or fluoride to inhibit phosphatases [2]. Interpretation of these findings, however, is difficult due to the nonspecific nature of the compounds or techniques used. Okadaic acid and calyculin have recently been found to be potent and selective inhibitors of protein phosphatases I and IIa [4, 16]. Na^+-K^+-$2Cl^-$ cotransport in red cells [37], salivary acinar cells [35] and cells cultured from the medullary thick ascending limb of Henle [19] is stimulated by okadaic acid, providing further evidence that protein phosphorylation regulates cotransport. The putative Na-K-2Cl cotransporter recently identified in shark rectal gland [27] and avian salt gland [45] is phosphorylated in response to agonists or cell shrinkage. We examined the effect of two protein phosphatase inhibitors and a protein kinase inhibitor on Na^+-K^+-$2Cl^-$ cotransport in aortic endothelial cells, seeking to determine whether protein phosphorylation activates cotransport and whether activation by cell shrinkage involves protein phosphorylation. Preliminary accounts of this work have appeared in abstract form [32, 36].

Materials and Methods

Reagents

^{86}Rb was obtained from DuPont-NEN (Wilmington, DE). Bumetanide was generously donated by Hoffmann-LaRoche, NJ. Okadaic acid was obtained from Biomol (Plymouth Meeting, PA) and UBI (Lake Placid, NY), calyculin was obtained from LC Services (Woburn, MA), and K252a was obtained from Calbiochem (San Diego, CA); stock solutions (0.5 mM) of each were made in dimethylsulfoxide and stored at 4 °C. All other chemicals were purchased from Sigma (St. Louis, MO).

Endothelial Cells

Endothelial cells were obtained from lumina of collagenase-digested calf aortas and grown in Minimal Essential Medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA), 2 mM L-glutamine, penicillin, and streptomycin (GIBCO, Grand Island, NY) at 37 °C in 5% CO_2. Fresh medium was applied every three days, and 12–24 hr prior to assays. Cells were passaged 1 : 5 with trypsin-EDTA (GIBCO) into 12-well cluster dishes (3.8 cm^2 surface per well), and studied between passages 5 and 15. Assays were performed in confluent cells 3–5 day after passage. The average protein content per well is 120 μg.

K^+ Fluxes

K^+ flux was determined using ^{86}Rb as a tracer for K^+ as previously described [22, 33]. The standard isotonic medium was Earle's salts buffered with NaHEPES instead of $NaHCO_3$ (in mM: 116 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 1 PO_4, 26 NaHEPES, pH 7.4). Hypertonic medium consisted of the standard medium with 150 mM sucrose added. Isosmotic cell swelling was produced as previously described [33] by incubating cells in a high K^+ medium (the standard medium with K^+ replacing Na^+). All assays were performed at 37 °C on intact monolayers of cells. Bumetanide-sensitive flux was determined by subtracting the flux in the presence of 50 μM bumetanide from the flux in the absence of bumetanide. For influx, cells were incubated with tracer quantities of ^{86}Rb (0.2 μCi/well) for 10 min, during which time influx is linear. After three washes with ice-cold 110 mM $MgCl_2$ the radioactivity was extracted into 5% trichloroacetic acid and counted by Cerenkov radiation. Rapid changes in cotransport activity were determined through measurements of ^{86}Rb efflux. Cells were preincubated with ^{86}Rb (1 μCi/ml) in standard medium containing 10% fetal bovine serum for 2 hr at 37 °C. The cells were then washed free of preincubation medium and the appropriate efflux medium was added. Medium was removed every minute and replaced with fresh medium. At the end of the experiment the cells were lysed to measure the remaining ^{86}Rb. Rate constants for efflux were obtained by dividing the amount of ^{86}Rb in each one minute supernatant by the amount of ^{86}Rb remaining in the cells at the end of the previous minute.

Na^+ and K^+ Content

Cells were washed rapidly four times with ice-cold 110 mM $MgCl_2$ and then lysed in 1 ml water for 15 min. The Na^+ and K^+ contents of the lysates were determined by atomic absorption spectrophotometry.

Data Analysis

Values for K_m and K_i were obtained from Eadie-Hofstee transformations by linear regression of the least-squares method. Kinetic analysis was based on a simple two-state model of cotransport shown below, where k_1 and k_2 are the rate constants for activation and inactivation respectively.

$$\text{Inactive Cotransport} \underset{K_2}{\overset{K_1}{\rightleftharpoons}} \text{Active Cotransport}$$

The cotransport flux rate during transition to a new steady state is given by:

$$J = J_B + [J_A - J_B] \exp -(k_1 + k_2)t$$

where J_A is the flux rate at the previous steady state and J_B is the flux rate at the new steady state, k_1 and k_2 are the rate constants for activation and inactivation at the new steady state and $(k_1 + k_2)^{-1}$ is the time constant [18]. The data were fitted to this equation using an iterative, nonlinear regression program contained within Sigmaplot Version 4.1 (Jandel, Corte Madera, CA). All data are presented as means ± SE. Unless otherwise stated, significance was determined by analysis of variance.

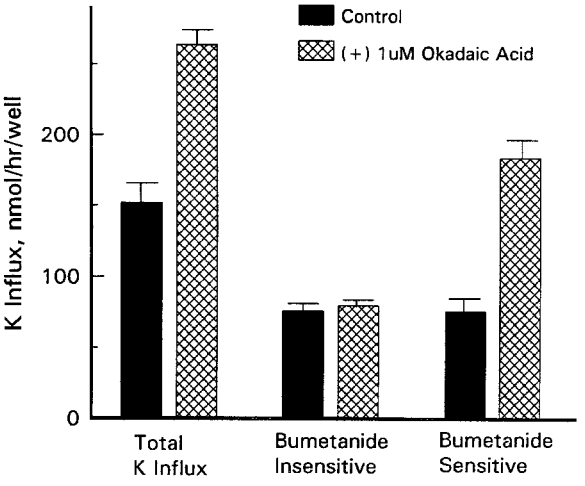


Fig. 1. Effect of okadaic acid on K influx in bovine aortic endothelial cells. Cells were incubated in standard isotonic medium with and without 50 μM bumetanide in the presence and absence of 1 μM okadaic acid for 5 min. ^{86}Rb was added and influx was measured over 10 min as described in Materials and Methods. The results shown are the means of six separate experiments, each performed in triplicate wells. Error bars, SE.

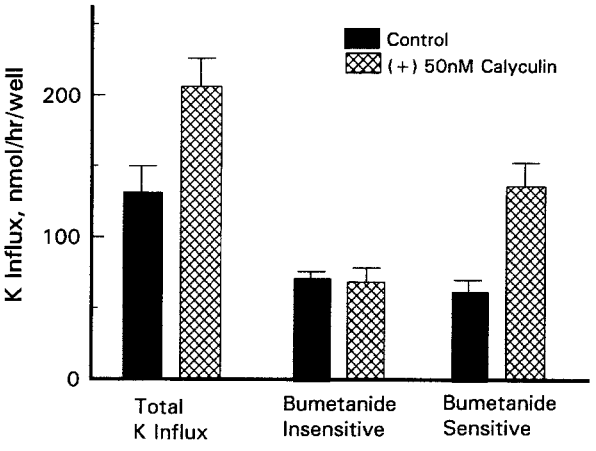


Fig. 2. Effect of calyculin on K influx in bovine aortic endothelial cells. Procedure was as described for Fig. 1, using 50 nM calyculin instead of okadaic acid. Results are the means of five separate experiments, each performed in triplicate wells. Error bars, SE.

Results

Both okadaic acid (Fig. 1) and calyculin (Fig. 2) increased K^+ influx into bovine aortic endothelial cells in isotonic medium (normal volume). The increased influx was inhibited by 50 μM bumetanide, and there was no stimulation of bumetanide-insensitive influx. The results of ion substitutions in the external medium are shown in Fig. 3. Bumetanide-sensitive influx into control cells was blocked by removing external Cl^- (substituted with NO_3^-) or Na (substituted with N-methyl-D-glucamine), as previously reported [33]. Similar results were obtained in the presence of okadaic acid, indicating that phosphatase inhibition specifically stimulated Na^+-K^+-$2Cl^-$ contransport. As shown in Fig. 4A, the half-maximal stimulatory concentration of okadaic acid was 401 ± 72 nM. This is substantially higher than the half-maximal concentration (IC_{50}) required to inhibit type IIa protein phosphatases (0.5–1 nM) [4, 16], but is in the range of concentrations reported to inhibit type I phosphatases (60–500 nM) [4, 16]. Calyculin (Fig. 4*B*) was at least 50-fold more potent than okadaic acid in stimulating cotransport, with a half-maximal concentration of 7.4 ± 1.4 nM. This concentration is similar to the IC_{50} for inhibition of type I and IIa protein phosphatases by calyculin: 2 nM for cell-free systems, [16]; 30 nM intact muscle, [17]. The effect of calyculin was dependent on the volume of medium (amount of calyculin) as well as the concentration of calyculin, probably reflecting the lipophilic nature of this compound. Doubling the volume increased the stimulation of cotransport by 68% at 10 nM, 24% at 20 nM, and had no effect at 40 nM calyculin.

Since cell volume affects cotransport activity in endothelial cells [33], it was necessary to ensure that phosphatase inhibition was not stimulating cotransport indirectly through cell shrinkage. Okadaic acid had no effect on the microscopic appearance of the endothelial cells. Calyculin, however, caused the cells to retract and round up, similar to the effect of cytochalasins on these cells (J.D. Klein and W.C. O'Neill, *unpublished data*). Cell Na^+ and K^+ content was measured as an assessment of cell volume. We have found that intracellular [Na] + [K] ranges from 180–200 mM and that cell Na^+ + K^+ content varies proportionately with cell volume under isosmotic conditions [33], indicating that these cations and their associated anions account for almost all osmotically active intracellular molecules. Since activation of cotransport could counteract and mask cell shrinkage, measurements were made in the presence and absence of bumetanide. In control wells the Na content was 12.5 ± 1.8 nmol/well and the K content was 141 ± 7 nmol/well. In the absence of phosphatase inhibitors, cell Na^+ + K^+ content was not altered when cotransport was inhibited by bumetanide, consistent with our previous results showing that cotransport does not mediate net influx at normal cell volume [33]. Neither okadaic acid nor calyculin significantly altered Na^+ + K^+ content in the absence or in the presence of bumetanide (Table). Okadaic acid also had no effect on the individual ion contents (*data not shown*); calyculin produced a small, bumetanide-sensitive increase in Na

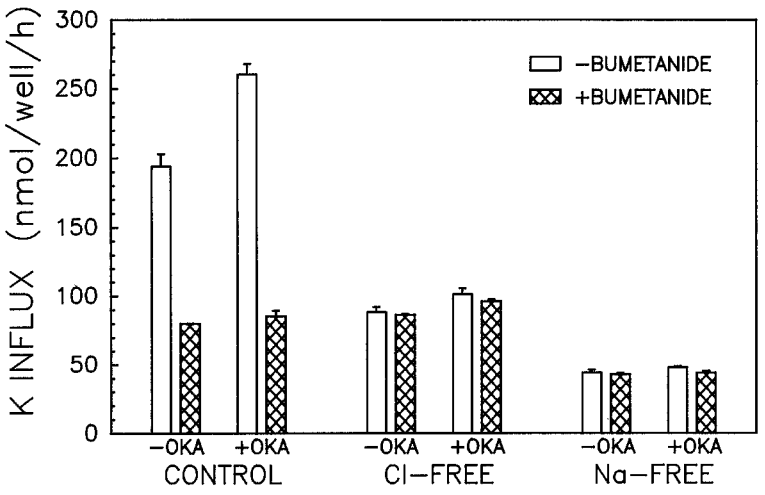
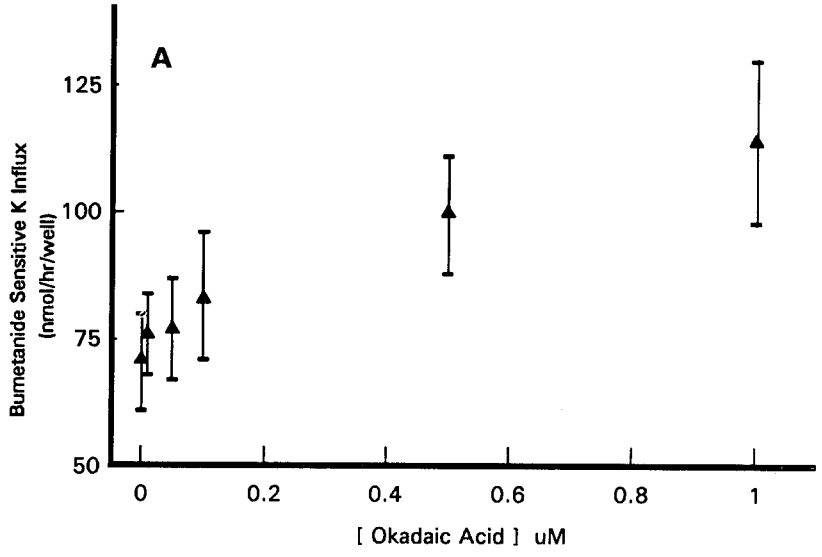


Fig. 3. Dependence of bumetanide-sensitive influx in bovine aortic endothelial cells on external Na^+ and Cl^-. Cells were preincubated for 5 min in standard isotonic medium or isotonic medium with NO_3^- replacing Cl^- (Cl-free) or N-methyl-D-glucamine replacing Na^+ (Na-free), with or without 1 μM okadaic acid. ^{86}Rb was added and influx measured over 10 min as described in Materials and Methods. Results are the means of triplicate wells from one experiment. Error bars, SE.



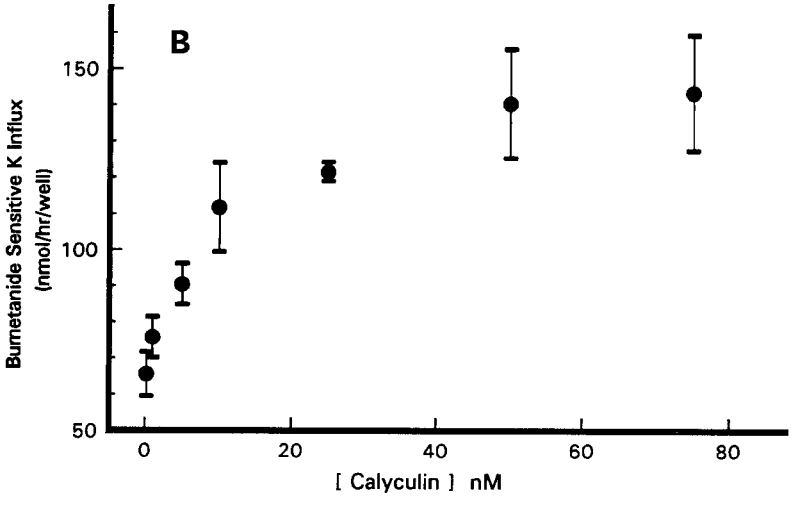


Fig. 4. Stimulation of Na-K-2Cl cotransport in bovine aortic endothelial cells by different concentrations of okadaic acid and calyculin. Procedure was as described in Fig. 1. (*A*) 0 to 1.0 μM okadaic acid. Results are means of four experiments, each performed in triplicate wells. (*B*) 0 to 75 nM calyculin. Results are the means of three experiments, each performed in triplicate wells. Error bars, SE.

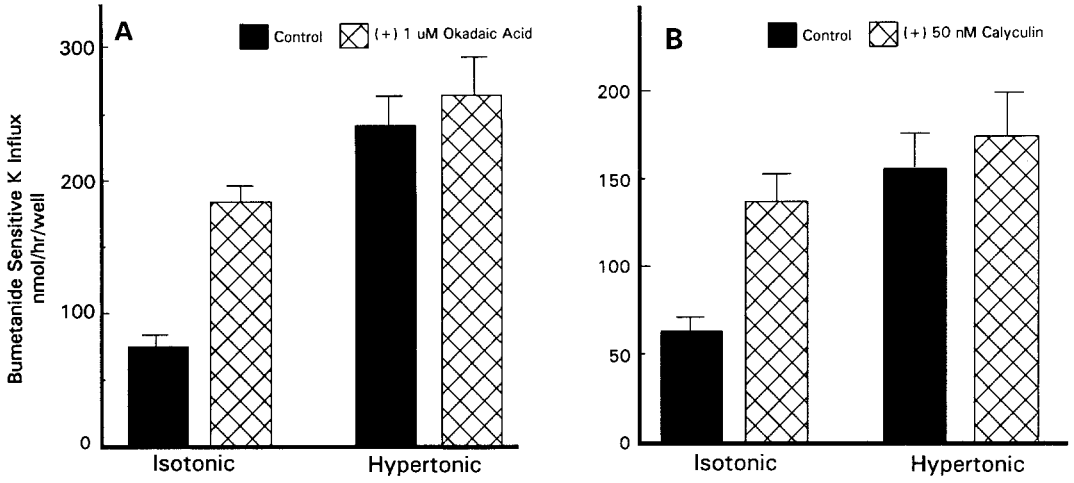


Fig. 5. Effect of cell shrinkage and phosphatase inhibition on Na^+-K^+-$2Cl^-$ cotransport in bovine aortic endothelial cells. Influxes were performed over 10 min in standard isotonic medium or hypertonic medium (isotonic medium containing 150 mM sucrose) with or without preincubation with phosphatase inhibitors. (*A*) Okadaic acid, 1 μM. (*B*) Calyculin, 50 nM. Results are the means of five (okadaic acid) and four (calyculin) separate experiments, each performed in triplicate wells. Error bars, SE.

Table. Effect of okadaic acid and calyculin on ion content of endothelial cells

Addition	Cell Na + K content (% of control)	
	−Bumetanide	+Bumetanide
None (n = 9)	100 ± 5.9	103.4 ± 3.3
Okadaic acid (1 μM) (n = 6)	105.8 ± 5.9	103.9 ± 5.0
Calyculin (50 nM) (n = 9)	108.2 ± 4.6	103.4 ± 2.3

Results are expressed as a % of ion content of cells incubated in standard isotonic medium without bumetanide. Cells were incubated for 10 min 37 °C. Values are the means ± SE.

content (24.3 ± 3.6%). Thus, phosphatase inhibition was not inducing a net efflux of ions leading to secondary stimulation of cotransport.

The combined effects of phosphatase inhibition and cell shrinkage on cotransport are shown in Fig. 5. Hypertonic (440 mosmol/kg) shrinkage increased bumetanide-sensitive K influx, as previously reported [33]. Despite marked stimulation of cotransport in normal cells, okadaic acid and calyculin did not increase cotransport further in hypertonically shrunken cells. Since endothelial cells do not restore their volume after hypertonic shrinkage [33], the cells remained shrunken throughout the influx assay. The relationship between protein phosphorylation and the regulation of cotransport by cell volume was also examined kinetically through measurements of ^{86}Rb efflux. The rate of activation of cotransport by calyculin should depend solely on the rate of phosphorylation since dephosphorylation has been blocked. If the rate of phosphorylation is governed by cell volume, then the rate of activation of cotransport by calyculin should vary with cell volume. Cell swelling, which inhibits cotransport [33], should delay activation while cell shrinkage, which stimulates cotransport [33], should accelerate activation. A high concentration of calyculin (100 nM) was used to ensure maximal inhibition of phosphatases. Studies were performed only with calyculin due to the prohibitive cost of okadaic acid. In isotonic medium (Fig. 6), an increase in bumetanide-sensitive ^{86}Rb efflux was apparent within the first minute after addition of calyculin and the new steady state was reached in 4 to 5 min (time constant for activation: 2.63 ± 0.22 min). Consistent with the influx data, calyculin had no effect on bumetanide-insensitive efflux. In order to examine the effect of cell swelling (Fig. 7), cells were swollen prior to efflux measurements by an incubation in isotonic KCl medium. Isosmotic swelling was required because aortic endothelial cells rapidly restore their volume (<5 min) after hypotonic swelling [26]. Since isosmotic swelling is not instantaneous, preincubation was required. The 20-min preincubation employed results in approximately 25% swelling, based on measurement of Na and K content [34]. Efflux was measured in isotonic KCl medium to maintain cell swelling. The baseline rate constant for bumetanide-sensitive ^{86}Rb efflux was reduced, consistent with inhibition of cotransport in swollen cells. The stimulation was delayed, beginning after 3.6 ± 0.4 min and reaching a plateau at 8.0 ± 0.3 min. This was not due to

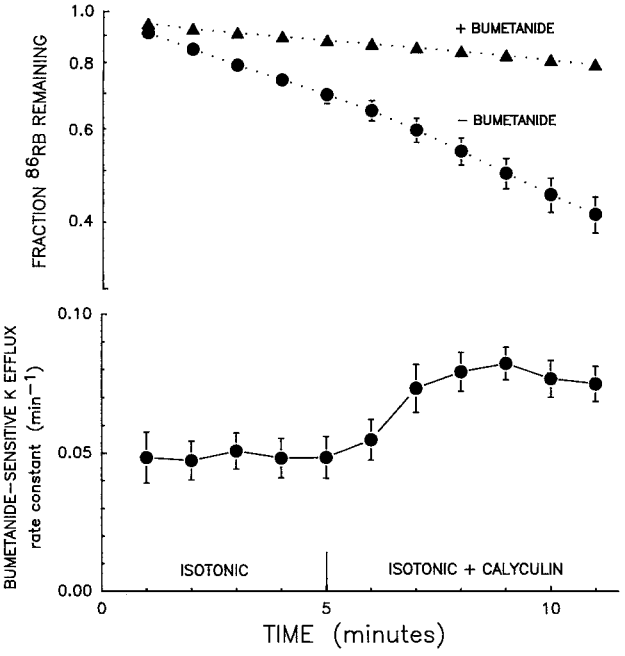


Fig. 6. Time course of stimulation of Na^+-K^+-$2Cl^-$ cotransport by calyculin in bovine aortic endothelial cells. Efflux of ^{86}Rb was measured over one-minute intervals in standard isotonic medium with or without 50 μM bumetanide as described in Materials and Methods. After 5 min, 100 μM calyculin was added to the efflux medium. Top panel: the fraction of ^{86}Rb remaining in the cells after each one-minute efflux. Bottom panel: rate constant of bumetanide-sensitive efflux for each one-minute efflux. Results are the means of five separate experiments, each performed in a single well. Error bars, SE.

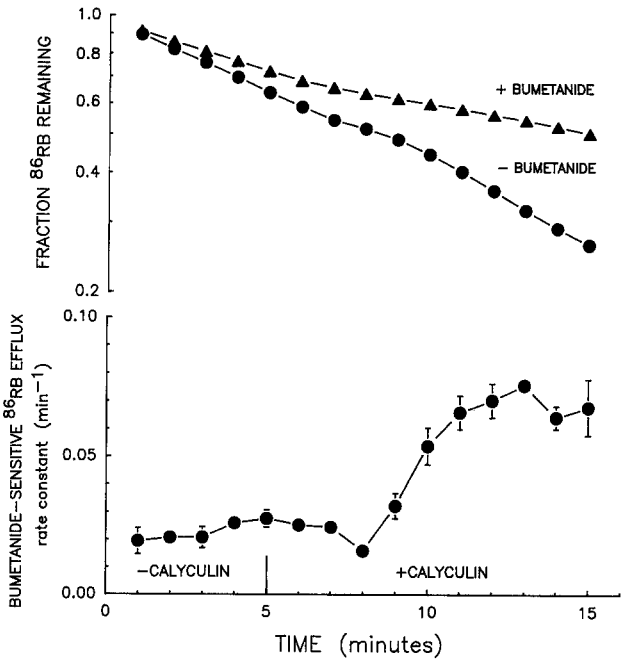


Fig. 7. Effect of cell swelling on the activation of Na^+-K^+-$2Cl^-$ cotransport by calyculin in cultured bovine aortic endothelial cells. Cells were swollen for 20 min in an isotonic KCl medium (standard medium with K^+ replacing Na^+) and efflux was measured in the same medium. Calyculin, 100 nM, was added to the efflux medium after 5 min. Top panel: the fraction of ^{86}Rb remaining in the cells after each one-minute efflux. Bottom panel: rate constant of bumetanide-sensitive efflux for each one-minute efflux. Results are the means of five separate experiments, each performed in a single well. Error bars, SE.

decreased penetration of calyculin into the cells since the morphologic change was not delayed. When cells were exposed to calyculin in KCl medium without prior incubation in KCl medium, there was no delay in the activation of cotransport (*data not shown*). This indicates that it is the cell swelling rather than the high K concentration that is delaying the activation of cotransport. Once cotransport began to increase, however, the time constant (3.30 ± 0.68 min) was similar to that in normal cells. Hypertonic shrinkage did not increase the time constant for activation of cotransport by calyculin (3.41 ± 0.47 min, *data not shown*).

Further information about the regulation of cotransport by cell volume was obtained by comparing the kinetics of activation and inactivation of cotransport (relaxation kinetics) by changes in cell volume in the absence of phosphatase inhibition. This technique has recently been applied to volume-sensitive KCl cotransport in rabbit red cells to show that cell swelling activates KCl cotransport by inhibiting a protein kinase [18]. The rate constant for relaxation to a new steady state is the sum of the rate constants for activation and inactivation, $k_1 + k_2$ [18]. If cell shrinkage stimulates phosphorylation, k_1 increases during activation and decreases during inactivation, and k_2 is unchanged. $k_1 + k_2$ is then greater, and the rate of relaxation faster, during activation than during inactivation. The opposite occurs if cell shrinkage inhibits dephosphorylation. Figure 8 shows the time course for activation, and Fig. 9, the time course for inactivation of cotransport by changes in cell volume. Cells were loaded with ^{86}Rb and then incubated in either isotonic or hypertonic medium. Efflux was measured in this medium for 5 min, and then in the opposite medium for an additional 10 min. Isotonic to hypertonic activation of cotransport occurred more quickly than hypertonic to isotonic inactivation, consistent with k_1 (phosphorylation) being the volume-sensitive step. However, direct comparison of time constants was not possible because the inactivation data could not be adequately fitted to a single exponential decay. The regulation of cotransport may therefore be more complex than the simple two-state model proposed. The time constant for activation was 3.76 ± 0.31 min. In order to quantify the difference between activation and inactivation times, we determined the

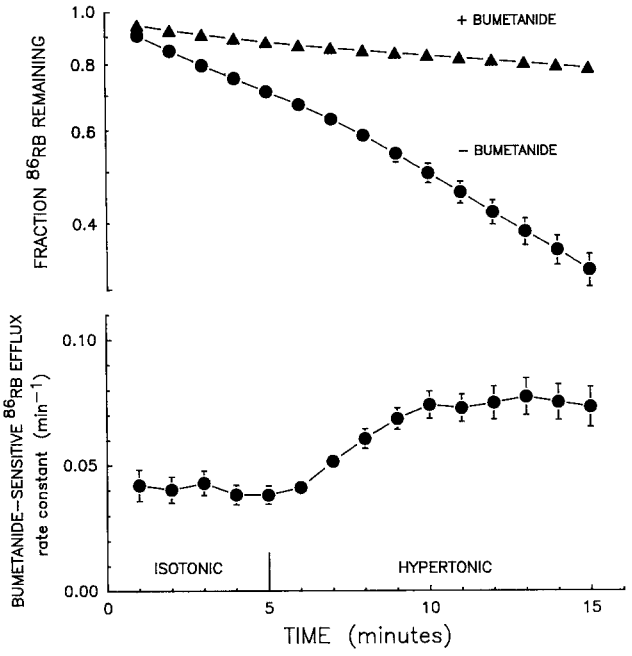


Fig. 8. Time course of activation of Na^+-K^+-$2Cl^-$ cotransport by cell shrinkage in cultured bovine aortic endothelial cells. Efflux of ^{86}Rb was measured over one-minute intervals in standard isotonic medium and after 5 min the medium was changed to hypertonic medium. Top panel: the fraction of ^{86}Rb remaining in the cells after each one-minute efflux. Bottom panel: rate constant of bumetanide-sensitive efflux for each one-minute efflux. Results are the means of 13 separate experiments, each performed in a single well. Error bars, SE.

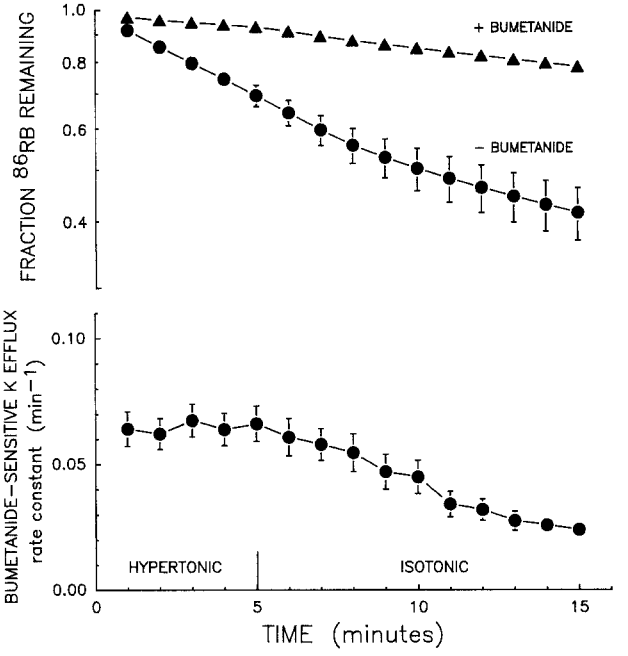


Fig. 9. Time course of inactivation of Na^+-K^+-$2Cl^-$ cotransport upon restoring cell volume in shrunken bovine aortic endothelial cells. Efflux of ^{86}Rb was measured over one-minute intervals in standard hypertonic medium and after 5 min the medium was changed to isotonic medium. Top panel: the fraction of ^{86}Rb remaining in the cells after each one-minute efflux. Bottom panel: rate constant of bumetanide-sensitive efflux for each one-minute efflux. Results are the means of 13 separate experiments, each performed in a single well. Error bars, SE.

time required to reach new steady state in each experiment. For this purpose the time to new steady state was defined as the time at which the flux was greater than or equal to (for activation) or less than or equal to (for inactivation) the flux at the subsequent two time points. The time required to reach new steady state was shorter for activation than for inactivation (6.4 ± 0.5 min *vs.* 8.3 ± 0.5 min, $n = 11$, $P < 0.02$). The value for inactivation is an underestimate since steady state was not reached by 10 min (the last time point) in several experiments but was taken to be 10 min.

K252a is a nonspecific inhibitor of protein kinases [28, 39] that inhibits activation of Na-K-2Cl cotransport in duck red cells [37]. In aortic endothelial cells this agent inhibited basal and shrinkage-activated cotransport in a dose-dependent manner (Fig. 10). The K_i was 1.6 ± 0.3 μM in hypertonic medium; it appeared to be in the same range in isotonic medium but could not be accurately determined. This K_i is identical to that for inhibition of cAMP-stimulated Na-K-2Cl cotransport in duck red cells but is more than 10-fold less than that for inhibition of shrinkage-activated cotransport in duck red cells [37]. The time constants for inactivation of cotransport by K252a (Fig. 11) were similar in isotonic and hypertonic media (2.65 ± 0.40 min *vs.* 2.14 ± 0.21 min) indicating that the dephosphorylation step is not altered by cell volume.

Discussion

Okadaic acid and calyculin, two compounds that inhibit protein phosphatases, stimulated Na^+-K^+-$2Cl^-$ cotransport in bovine aortic endothelial cells. Since cell shrinkage also activates cotransport in aortic endothelial cells [33], it is important to determine whether or not activation of cotransport by other agents is secondary to a change in cell volume. There was no significant change in cell volume with either phosphatase inhibitor despite the morphologic effect of calyculin. Whereas it is possible that shape change without a change in volume could also activate cotransport, morphologic changes were not associated with the activation of cotransport by okadaic acid. It appears then that the activation of cotransport by phosphatase inhibitors is a direct effect, independent of changes in cell volume. The

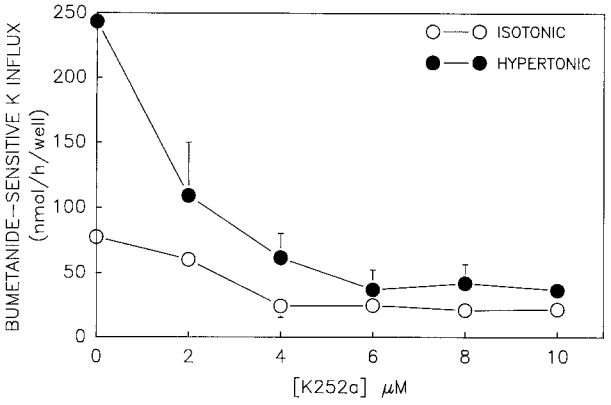


Fig. 10. Inhibition of Na^+-K^+-$2Cl^-$ cotransport in bovine aortic endothelial cells by K252a. Influx was measured over 10 min in standard isotonic or hypertonic medium containing varying concentrations of K252a as denoted. Results are the means of duplicate wells from one experiment. Error bars, SE.

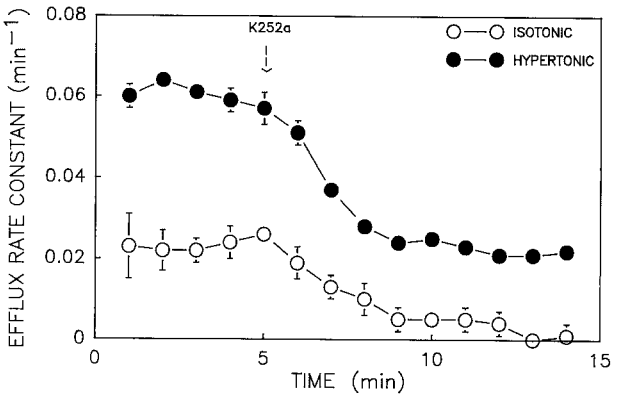


Fig. 11. Time course of inactivation of Na^+-K^+-$2Cl^-$ cotransport in bovine aortic endothelial cells by K252a. Efflux was measured in standard isotonic or hypertonic medium as described in Materials and Methods. K252a (100 μM) was added immediately after the 5-min time point. Results are the means of 5 experiments, each performed in a single well. Error bars, SE.

results in endothelial cells are consistent with the effect of phosphatase inhibition on Na^+-K^+-$2Cl^-$ cotransport in duck red cells [37], squid axons [2], and rat medullary thick ascending limb cells [19], and indicate that Na^+-K^+-$2Cl^-$ cotransport is activated by protein phosphorylation. The evidence for phosphorylation is based on the effects of protein kinase and protein phosphatase inhibitors. Specificity of inhibitors is always a concern, but okadaic acid and calyculin have not been found to interfere with other signaling processes [15, 43, 44]. The fact that these structurally dissimilar compounds both stimulate cotransport makes it unlikely that their effect is nonspecific. Okadaic acid is a potent inhibitor of protein phosphatase IIa (IC_{50} of about 1 nM) with weaker activity (IC_{50} = 60–500 nM) against protein phosphatase I [4, 16]. Calyculin is equipotent to okadaic acid in inhibiting type IIa protein phosphatases but is a more potent inhibitor (IC_{50} = 0.3–2.0 nM) of type I protein phosphatases [16]. Activation of cotransport required high concentrations of okadaic acid and much lower concentrations of calyculin, suggesting the involvement of a type I phosphatase. Some caution must be exercised in interpreting the K_i values since the true intracellular concentrations that were obtained are unknown. It is unlikely that intracellular levels are limited by cell permeability given the lipophilic nature of these compounds and the rapidity with which calyculin inhibited cotransport. The free concentration could be reduced by binding to cellular components. The increased effect of calyculin with increased amounts of medium at a constant calyculin concentration is consistent with such an effect.

The results with phosphatase inhibitors indicated that Na-K-2Cl cotransport in endothelial cells is regulated by protein phosphorylation. The inhibition of cotransport by K252a, a protein kinase inhibitor, provided further evidence for this. We next sought to determine whether the regulation of cotransport by cell volume also occurs through protein phosphorylation. The stimulation of cotransport by cell shrinkage was not enhanced by phosphatase inhibition, indicating that the effects of shrinkage and phosphatase inhibition were not additive. This would be expected if shrinkage inhibited a phosphatase. If shrinkage activated a kinase, some small degree of additivity might be expected. However, there may not be a direct correlation between phosphorylation and cotransport activity, so that further phosphorylation may not result in further activation. Although not significant, there was a small increase in cotransport activity when okadaic acid or calyculin were added to shrunken cells (Fig. 5).

More definitive information was obtained from kinetic analyses. The activation of cotransport by calyculin was delayed by cell swelling, indicating that the phosphorylation step is affected by cell volume. This effect is difficult to interpret since the rate of activation, once it began, was equivalent to that in unswollen cells. There may be a second step in the activation cascade subsequent to and independent of phosphorylation. This latter step could be rate-limiting, explaining why activation of cotransport was not accelerated by cell shrinkage. Such a model could also explain the lack of additivity in the effects of phosphatase inhibitors and cell shrinkage on cotransport. In the absence of phosphatase inhibitors, activation of cotransport upon transfer from isotonic to hypertonic medium was faster than inactivation upon transfer from hypertonic to isotonic medium,

consistent again with phosphorylation (activation) being the volume-sensitive step. Finally, the rate of inactivation of cotransport by K252a did not differ in isotonic and hypertonic media, indicating that the dephosphorylation step is not affected by cell volume. Although there are some assumptions in the interpretation of these data, and the models may be oversimplified, the simplest explanation for the results is that cell volume regulates cotransport by altering the activity of a protein kinase.

The results of this study are in agreement with results recently obtained in duck red cells and in shark rectal gland. In duck red cells, stimulation of Na^+-K^+-$2Cl^-$ cotransport by cAMP but not by shrinkage is blocked by inhibitors of cAMP-dependent protein kinase [37]. One of these inhibitors, K252a, did block stimulation of cotransport by shrinkage but at 10-fold higher concentrations, suggesting that a separate kinase was responsible for activation by hypertonic shrinkage. Activation of cotransport in these cells by okadaic acid was also inhibited by the higher concentrations of K252a, suggesting that phosphatase inhibition and cell shrinkage were acting through a common pathway. However, the interaction of cell volume and phosphatase inhibition was not directly investigated. These investigators also provided evidence that the 150 kD bumetanide-binding protein in these cells is a phosphoprotein. The Na-K-2Cl cotransporter, recently identified and purified from shark rectal gland, is phosphorylated in response to cAMP-dependent agonists or hypertonic shrinkage [27]. An immunologically identical protein in the avian salt gland is phosphorylated by agonists [45]. We have not observed enhanced phosphorylation of any proteins in the range of 150–180 kD in shrunken aortic endothelial cells (J.D. Klein, S. Bakir, and W.C. O'Neill, *manuscript in preparation*). Although it is likely that the Na^+-K^+-$2Cl^-$ cotransporter is being phosphorylated in endothelial cells, we cannot rule out phosphorylation of a separate, regulatory protein rather than the cotransporter protein itself. This will await identification of the Na-K-2Cl cotransporter in endothelial cells.

This study provides evidence for a volume-sensitive protein kinase in aortic endothelial cells. Preliminary work has identified two proteins in these cells whose phosphorylation is increased by cell shrinkage [21], lending further support to this conclusion. A similar kinase may be present in lymphocytes, in which the Na^+/H^+ antiporter is activated by hypertonic shrinkage [10]. This activation is blocked by prior depletion of ATP [11] and is not additive with the stimulation of Na^+/H^+ by okadaic acid [5], suggesting that activation by shrinkage involves protein phosphorylation. Although shrinkage induces phosphorylation of specific proteins in lymphocytes [12], the putative antiporter protein is not phosphorylated [13].

The rapidity with which calyculin stimulated cotransport was surprising, and was on the same time scale as activation by cell shrinkage. This indicates that the phosphorylation step and, by necessity, the dephosphorylation step are constitutively active in cultured aortic endothelial cells. Such a constitutively active phosphorylation-dephosphorylation system is not unique to endothelial cells since a similarly rapid activation of Na^+-K^+-$2Cl^-$ cotransport by okadaic acid occurs in duck red cells [37]. While this constant phosphorylation and dephosphorylation would appear to be inefficient, it may prime the cells for rapid changes in cotransport activity, implying that acute changes in cotransport activity and, possibly, cell volume occur in these cells and are important in endothelial function.

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