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$Na^+/K^+/Cl^-$ Cotransport in Cultured Vascular Smooth Muscle Cells: Stimulation by Angiotensin II and Calcium Ionophores, Inhibition by Cyclic AMP and Calmodulin Antagonists

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Summary. The specific activity of the $Na^+/K^+/Cl^-$ cotransporter was assayed by measuring the initial rates of furosemide-inhibitable $^{86}Rb^+$ influx and efflux. The presence of all three ions in the external medium was essential for cotransport activity. In cultured smooth muscle cells furosemide and bumetanide inhibited influx by 50% at 5 and 0.2 μM, respectively. The dependence of furosemide-inhibitable $^{86}Rb^+$ influx on external Na^+ and K^+ was hyperbolic with apparent K_m values of 46 and 4 mM, respectively. The dependence on Cl^- was sigmoidal. Assuming a stoichiometry of 1 : 1 : 2 for $Na^+/K^+/Cl^-$, a K_m of 78 mM was obtained for Cl^-. In quiescent smooth muscle cells cotransport activity was approximately equal to Na^+ pump activity with each pathway accounting for 30% of total $^{86}Rb^+$ influx. Growing muscle cells had approximately 3 times higher cotransport activity than quiescent ones. Na^+ pump activity was not significantly different in the growing and quiescent cultures. Angiotensin II (ANG) stimulated cotransport activity as did two calcium-transporting ionophores, A23187 and ionomycin. The removal of external Ca^{2+} prevented A23187, but not ANG, from stimulating the cotransporter. Calmodulin antagonists selectively inhibited $^{86}Rb^+$ influx via the cotransporter. Beta-adrenoreceptor stimulation with isoproterenol, like other treatments which increase cAMP, inhibited cotransport activity. Cultured porcine endothelial cells had 3 times higher cotransport activity than growing muscle cells. Calmodulin antagonists inhibited cotransport activity, but agents which increase cAMP or calcium had no effect on cotransport activity in the endothelial cells.

Key Words endothelial cells · $Na^+/K^+/Cl^-$ cotransport · cyclic AMP · phenothiazines · calcium · angiotensin

Introduction

Smooth muscle cells cultured from rat aorta provide a useful model for investigating the mechanisms by which certain vasoactive hormones regulate ion transport. The cells have functional receptors for both vasoconstrictors and vasodilators (reviewed in Smith, 1986). Angiotensin II (ANG)[1] mobilizes intracellular calcium within a few seconds after hormone addition (Smith et al., 1984). Inositol trisphosphate, which increases rapidly in response to ANG, releases stored calcium apparently by a ligand-binding reaction which opens an intracellular Ca^{2+} channel (Smith, Smith & Higgins, 1985). Isoproterenol stimulates a beta-adrenoreceptor and increases cyclic AMP which inhibits a slower component of $^{45}Ca^{2+}$ release (Smith, 1984). Atriopeptin increases cyclic GMP in the cells (O'Donnell & Owen, 1986; Smith & Lincoln, 1978). ANG and atriopeptin oppose one another in these cells. ANG decreases cyclic GMP production evoked by atriopeptin (Smith & Lincoln, 1987), and atriopeptin decreases the rise in free Ca^{2+} induced by ANG (Hassid, 1986).

Earlier studies of Na^+ and K^+ transport in smooth muscle cells showed that ANG increases the cycling of Na ions (Brock, Lewis & Smith, 1982). ANG stimulates Na^+ entry via an amiloride-inhibitable transporter (Smith & Brock, 1983). Increasing Na^+ entry in turn stimulates Na^+ exodus by supplying the Na^+/K^+ pump with more of its rate-limiting substrate, intracellular Na^+ (Brock & Smith, 1982). The present report concerns another pathway of Na^+ movement: the furosemide-inhibitable cotransporter of Na^+, K^+, and Cl^-. The cotransporter makes a major contribution to the total flux of Na^+, K^+, and Cl^- in a variety of mammalian cell types, and it has been implicated in the regulation of cell volume (Geck & Heinz, 1986). A defect

[1] The abbreviations and trivial names used are: ANG, angiotensin II (human form); Sar[1],leu[8]-ANG, Sarcosyl[1], leucine[8]-angiotensin II; furosemide, 4-chloro-N-(2-furylmethyl)anthanilic acid; bumetanide, 3-butylamino-4-phenoxy-5-sulfamoyl benzoic acid; FBS, fetal bovine serum; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; Me_2SO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Bt_2-cAMP, dibutyryl cyclic AMP; Bt_2-cGMP, dibutyryl cyclic GMP; W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W12, N-(4-aminobutyl)-2-naphthalenesulfonamide; W13, N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide; TMB-8, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate.

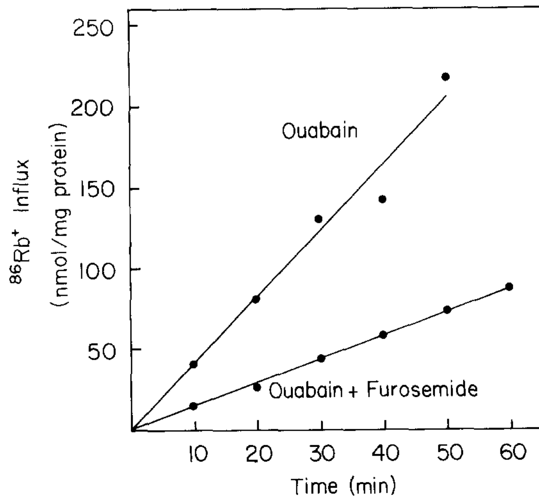

Fig. 1. Time course of $^{86}Rb^+$ uptake by quiescent smooth muscle cells in the presence and absence of furosemide. Ouabain (1 mM) was present during the 10-min incubation before adding $^{86}Rb^+$ as well as during the incubation with $^{86}Rb^+$. Furosemide (0.1 mM) was present only during the incubation with $^{86}Rb^+$. The lines, obtained by linear regression, had slopes of 4.12 and 1.48 nmol/min/mg protein in the absence and presence of furosemide and r^2 values of 0.96 and 0.99, respectively

in this cotransporter appears to be associated with essential hypertension (Garay et al., 1980). Cotransport activity in cultured vascular smooth muscle cells is modulated by cyclic nucleotides. Increasing cellular cyclic AMP inhibited the cotransporter in A7r5 cells (Owen, 1984). A7r5 cells are a putative smooth muscle cell line derived from the thoracic aorta of embryonic DBIX rats (Kimes & Brandt, 1976). Owen (1984) found that 8-bromo cyclic GMP inhibited cotransport activity in A7r5 cells and increased the activity of the cotransporter in early passage smooth muscle cells from rat aorta as did atrial natriuretic factor (O'Donnell & Owen, 1986). Here we show that growing, early passage smooth muscle cells derived from rat aorta have 3 times higher cotransport activity than nongrowing cells, and that ANG stimulates $^{86}Rb^+$ efflux via the cotransporter in both growing and quiescent muscle cells.

Materials and Methods

Materials

Bumetanide was a gift from Dr. Raymond Frizzell. Furosemide was a gift from Hoechst-Roussel Pharmaceuticals Inc. (Somerville, N.J.). Calmidazolium (R 24571) was from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Trifluoperazine HCl, trifluoperazine sulfoxide HCl, and chlorpromazine HCl were gifts of Smith, Kline & French Labs. (Philadelphia, Pa.). W12 and W13 were purchased from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Ionomycin was a gift of E. R. Squibb & Sons (Princeton, N.J.) A23187 was a gift of Lilly Research Laboratories (Indianapolis). Fetal bovine serum was from Hyclone Laboratories (Logan, Utah). Oligomycin, CCCP, W7, rotenone, indomethacin, cholera toxin, and N-methyl-D-glucamine, D-gluconic acid lactone, and the gluconate salts were from Sigma. $^{86}RbCl$ in aqueous solution (37 to 300 mBq/mg Rb) was purchased from Amersham (Arlington Heights, Ill.). All other chemicals were from the sources indicated previously (Smith & Brock, 1983; Smith, 1984).

Cell Culture

Primary cultures of smooth muscle cells were initiated from the tunica media of rat thoracic aorta and subcultured as previously described (Smith & Brock, 1983; Smith, 1984). They were grown in Medium 199 containing 10% FBS and 10 mM HEPES in a humidified atmosphere of 5% CO_2-95% air in the absence of antibiotics. The identity and homogeneity of the smooth muscle cells was indicated by greater than 98% positive staining with a monoclonal antibody (CGA7) to the alpha isoform of actin (Gown et al., 1985). Tissue culture dishes (35 mm Falcon) were seeded in this medium from stock cultures grown in 75 cm^2 culture flasks (Costar, Cambridge, Mass.) which had been passaged between 5 and 15 times as previously described (Smith, 1984). All experiments were done with culture dishes seeded on a given date either before or after the cells became quiescent as indicated by the presence or absence of mitotic cells and daily cell counts per culture dish. Total protein was measured on duplicate cultures at the time of each experiment (Lowry et al., 1951).

Endothelial cells were initiated in culture from intimal scrapings of newborn pig aorta and grown in Medium 199 containing 10% FBS as described above. The cultures had the characteristic morphology of cultured endothelial cells. The absence of spindle-shaped, bipolar cells suggested that the cultures were free of smooth muscle cells. The distinctly different morphology of the smooth muscle cells was confirmed by culturing them from explants of newborn pig aorta as previously described (Brock et al., 1984). The endothelial cells stained positively with Factor VIII antiserum as shown by indirect immunofluorescence which was carried out as described by Del Vecchio and Smith (1981). In addition essentially all of the cells accumulated acetylated low density lipoprotein labeled with the fluorescent chromophore, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate, which is known to be taken up by endothelial, but not smooth muscle cells (Voyta et al., 1984). The cultures were seeded at 2 × 10^5 per mm dish from stock cultures which had been passaged between 5 and 10 times. The cultures formed a confluent monolayer and were used 4 to 6 days after plating.

$^{86}Rb^+$ Influx

Cotransport activity was usually assayed in the presence of ouabain to block $^{86}Rb^+$ entry via the Na^+ pump. The cultures were rinsed 3 times with a buffered salts solution at 37°C containing the following (mM): 120 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, pH 7.4 with Tris. One ml of this solution containing 10 mM glucose and usually 1 mM ouabain was added to each culture, and they were incubated for 10 min. Other compounds were present as indicated during this incubation and/or a second one

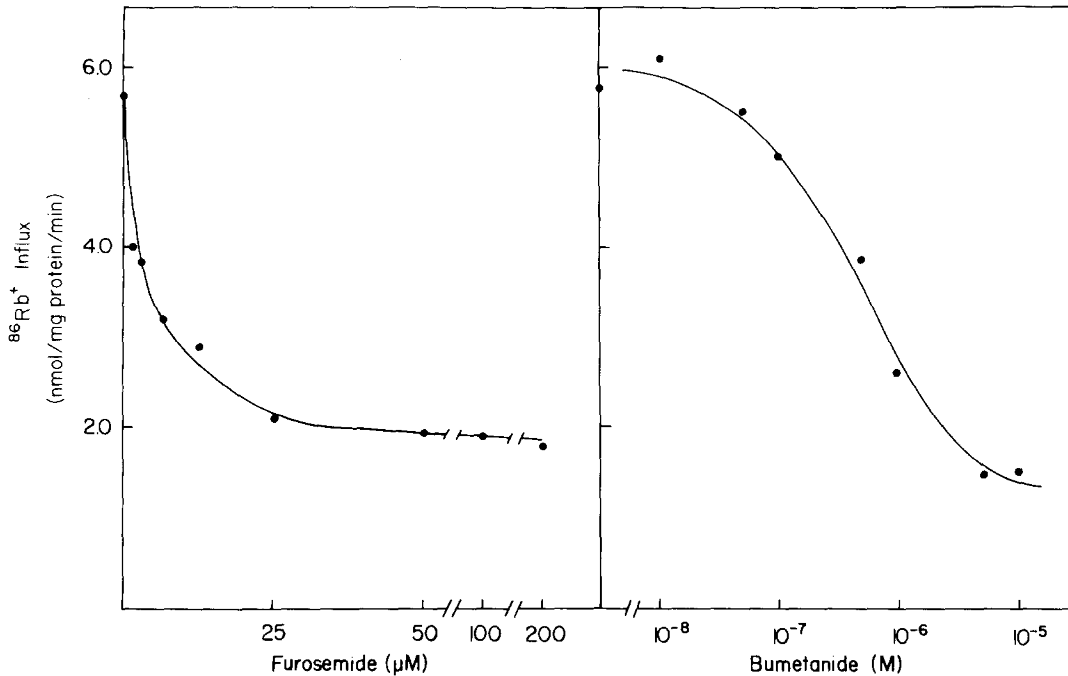

Fig. 2. Dose responses for the inhibition of $^{86}Rb^+$ influx by two loop diuretics in quiescent smooth muscle cells. Ouabain (1 mM) and furosemide or bumetanide were present as indicated during the 10-min incubation before adding $^{86}Rb^+$ and during the 15-min incubation with $^{86}Rb^+$. Values are means of two experiments done in duplicate

(15 min) which was started by adding 20 μl containing approximately 1 μCi $^{86}RbCl$ in water. All incubations were at 37°C, and deviations from this transport medium and/or incubation conditions are indicated in the Figure and Table legends. The specific radioactivity (cpm/nmol K^+) in different experiments was between 50 and 200. To stop the uptake and remove extracellular $^{86}Rb^+$ the cultures were rapidly rinsed 5 times with ice-cold 0.1 M $MgCl_2$-10 mM HEPES adjusted to pH 7.4 with Tris (Smith & Rozengurt, 1978). Intracellular $^{86}Rb^+$ was extracted with 0.1 N HNO_3 and counted by Cerenkov radiation. Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard, and $^{86}Rb^+$ influx was calculated as nmol/min/mg protein.

Furosemide was dissolved directly in the buffered salts solution. Bumetanide (100 mM), A23187 (2 mM), cytochalasin D(1 mM), and calmidazolium (50 mM) were dissolved in Me_2SO. Me_2SO was always less than 0.1% and had no effect on $^{86}Rb^+$ uptake. W7, W12, and W13 (5 mM each), IP (10 mM), and the phenothiazines (10 mM) were dissolved in water and stored in the dark at 4°C. MIX (50 mM), ionomycin (10 mM), CCCP (2 mM), rotenone (2 mM), and oligomycin (2.5 μg/ml) were dissolved in ethanol which at 0.2% or less had no effect on $^{86}Rb^+$ influx. TMB-8 (10 mM) was dissolved in the assay buffer.

$^{86}Rb^+$ Efflux

Forty μl $^{86}RbCl$ (1 μCi) was added directly to the culture medium 4 hr before rapidly rinsing the culture 4 times (less than 15 sec total) with the buffered salts solution described above containing 10 mM glucose at 37°C, and efflux was initiated by the addition of 1 ml of this solution at 37°C with the additions indicated in the legend to Fig. 11. After the indicated time interval the medium was transferred to a scintillation vial and immediately replaced with 1 ml of the same medium. At the end of the efflux period the culture was extracted with 1 ml of nitric acid as indicated above. The total $^{86}Rb^+$ present at the start of the efflux was calculated from the amounts released and remaining in the cells at the end of the experiment.

Results

Time Course of $^{86}Rb^+$ Uptake and Inhibition by "Loop" Diuretics in Cultured Smooth Muscle Cells

$^{86}Rb^+$ uptake increased linearly with time for at least 50 min (Fig. 1). Ouabain was present to inhibit uptake via the Na^+/K^+ pump. Under these conditions furosemide decreased $^{86}Rb^+$ influx from 4.1 to 1.5 nmol/min/mg protein. $^{86}Rb^+$ influx was highly sensitive to inhibition by furosemide and another loop diuretic, bumetanide (Fig. 2). Five μM furosemide and 0.2 μM bumetanide inhibited cotransport activity by 50% (Fig. 2). Furosemide maximally inhibited ouabain-resistant $^{86}Rb^+$ influx by about 65% (Fig. 1, Fig. 2, Table 4).

Dependence of Furosemide-Inhibitable $^{86}Rb^+$ Influx on External Na^+, K^+, Cl^-, and Energy

The deletion of Na^+ and/or Cl^- from the transport medium abolished furosemide-sensitive $^{86}Rb^+$ transport (Table 1). Cotransport activity exhibited a hyperbolic dependence on external Na^+ and K^+ concentrations (Figs. 3 and 4). The apparent K_m values for Na^+ and K^+ were 46 and 4 mM, respectively. The relationship between cotransport activity and Cl^- was sigmoidal (Fig. 5). These data gave a linear Eadie-Hofstee plot when the square of the chloride concentration was used (Fig. 5). The apparent K_m value for Cl^- was 78 mM.

Table 2 shows that three different classes of mitochondrial poisons, (oligomycin, an energy transfer inhibitor; CCCP, an uncoupler; and rotenone, an inhibitor of electron transport), decreased ouabain-insensitive $^{86}Rb^+$ uptake by 84 to 91% (Table 2). The inclusion of glucose in the assay medium greatly reduced the inhibitory effect of the mitochondrial poisons on ouabain-insensitive $^{86}Rb^+$ influx (Table 2). The inhibitory effect of all three compounds was fully reversed by removing the poison and incubating the cultures in the buffered salts solution containing 10 mM glucose (*data not shown*).

Table 1. Both Na^+ and Cl^- are required for furosemide-inhibitable $^{86}Rb^+$ influx in quiescent smooth muscle cells[a]

Additions	$^{86}Rb^+$ Influx (nmol/min/mg)[b]		
	Total	Plus furosemide	Cotransport
Experiment I			
Choline chloride	1.12 ± 0.14 (4)	1.27 ± 0.06 (4)	0
NaCl	4.10 ± 0.06 (4)	0.98 ± 0.12 (4)	3.12
Experiment II			
None	0.45 ± 0.12 (4)	0.69 ± 0.01 (2)	0
Cl^-	0.71 ± 0.12 (3)	0.75 ± 0.05 (2)	0
Na^+	1.60 ± 0.17 (4)	1.14 ± 0.12 (2)	0.4
NaCl	3.66 ± 0.42 (4)	1.15 ± 0.02 (2)	2.51

[a] Experiment I: Equimolar choline chloride replaced NaCl in the transport medium. The cultures were rinsed 3 times with the Na-free medium and incubated for 10 min in transport medium containing NaCl or choline chloride and 1 mM ouabain before adding $^{86}Rb^+$. Experiment II: The assay medium contained 10 mM glucose, 5 mM K gluconate, 3 mM $MgSO_4$, and 20 mM HEPES adjusted to pH 7.4 with Tris and one of the following: 240 mM sucrose, 120 mM choline chloride, 120 mM Na gluconate, or 120 mM NaCl. All cultures were rinsed 3 times with the medium containing no added Na^+ or chloride. Then 1 ml of transport medium containing the additions indicated and 1 mM ouabain was added. The cultures were incubated for 10 min before adding $^{86}Rb^+$.

[b] Values are means ± SEM (n).

Comparison of Cotransport Activity in Growing Versus Quiescent Arterial Muscle Cells

Specific cotransport activity was approximately three times greater in growing than in quiescent muscle cells (Table 3). By comparison, the activity of the Na^+ pump did not differ significantly between growing and quiescent cells (Table 3). In the quiescent cultures cotransport activity approximately equalled Na^+ pump activity, and each pathway constituted about 30% of total $^{86}Rb^+$ influx. In growing cultures cotransport activity comprised about 60% of total $^{86}Rb^+$ influx and was about twofold greater than pump activity (Table 3).

Stimulation of Cotransport Activity in the Muscle Cells by A23187 and Ionomycin and Inhibition by TMB-8

Both ionophores increased furosemide-inhibitable $^{86}Rb^+$ influx by about twofold (Fig. 6). Optimal stim-

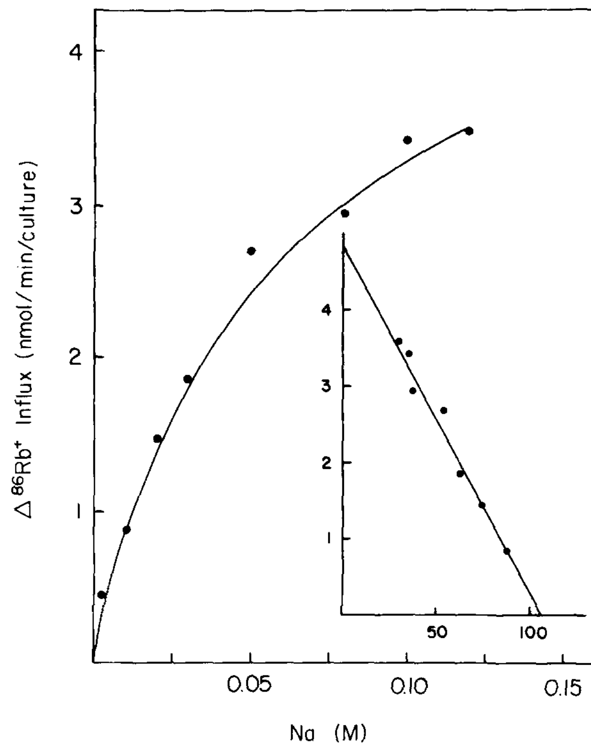

Fig. 3. Effect of Na^+ concentration on $^{86}Rb^+$ influx in the presence of ouabain. The cultures were incubated for 10 min in transport solution containing the indicated concentration of NaCl. Choline chloride plus NaCl always equalled 120 mM. $^{86}Rb^+$ uptake in the absence of Na^+ (0.48 nmol/min/mg protein) was subtracted from total uptake to obtain $^{86}Rb^+$ influx. The inset is an Eadie-Hofstee plot [Δv *vs.* $\Delta v/Na^+$ (M)] of the same data. The line obtained by linear regression has an r^2 value of 0.966 and a slope of −0.046. Values are means of two experiments done in duplicate

ulation occurred at approximately 0.1 μM ionomycin and 0.5 μM A23187 (Fig. 6). Neither ionophore had any significant effect on $^{86}Rb^+$ influx in the presence of furosemide and ouabain (Fig. 6). The removal of external calcium decreased basal cotransport activity by 50% and prevented the stimulatory effect of A23187 (Table 4). Calcium removal inhibited cotransport activity by the same degree in the presence or absence of EGTA (Table 4). Treating the cultures with indomethacin, a fatty acid cyclooxygenase inhibitor, reduced the stimulation of cotransport activity by A23187 from 74 to 62% (*data not shown*). This result suggests that the stimulation of the cotransporter by A23187 is not mediated by prostaglandin synthesis.

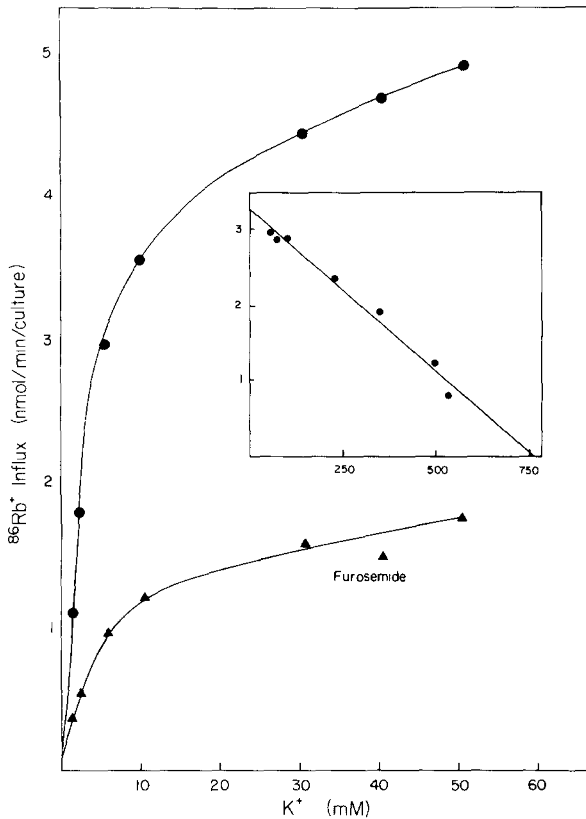

Fig. 4. Dependence of cotransport activity on external K^+. Furosemide (0.1 mM) and ouabain (1 mM) were present as indicated during the 10-min incubation before adding $^{86}Rb^+$ and during the 15-min $^{86}Rb^+$ uptake. The NaCl concentration in the usual transport medium was decreased to 70 mM. KCl plus choline chloride always equalled 55 mM. Values are means of two experiments done in duplicate. The inset is an Eadie-Hofstee plot of the same data. The line obtained by linear regression has an r^2 value of 0.94 and slope of −0.0043 M

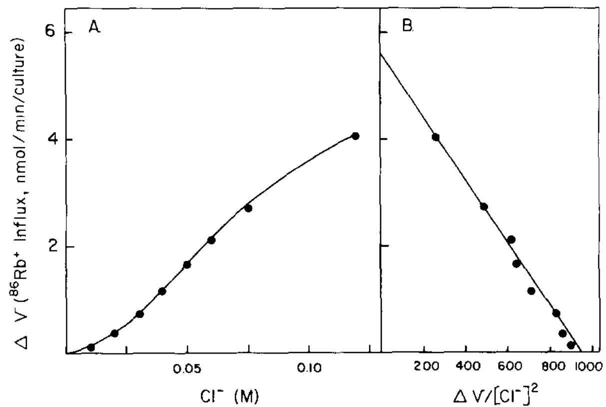

Fig. 5. Dependence of cotransport activity on external Cl^-. (*A*) Quiescent cultures were rinsed 3 times with the assay buffer containing 10 mM Cl^- and incubated for 10 min in the presence of the indicated Cl^- concentration and 1 mM ouabain before adding $^{86}Rb^+$. Influx in the presence of 0.1 mM furosemide and ouabain was not affected by Cl^- concentration, so the average value (1.02 ± 0.03 nmol/min/culture) was subtracted from total influx to obtain furosemide-inhibitable influx (Δv). The assay buffer contained the following (mM): 10 glucose, 20 HEPES-Tris (pH 7.4), 5 K^+ gluconate, 1 Mg gluconate, 2 Ca gluconate, and NaCl plus Na gluconate to equal the indicated Cl^- concentration and 120 Na^+. Values are means of two experiments done in duplicate. (*B*) Eadie-Hofstee plot of the same data [Δv versus $\Delta v/Cl^{-2}$ (M)]. The line, which was obtained by linear regression, has an r^2 value of 0.995 and slope of −0.0061 (M^2). Values are means of two experiments done in duplicate

Table 2. Effects of mitochondrial energy poisons on $^{86}Rb^+$ influx in the smooth muscle cells[a]

Additions	$^{86}Rb^+$ Influx (nmol/min/mg)[b]			
	No glucose	% Inhibition	Plus glucose	% Inhibition
None	9.73 ± 0.05	0	9.72 ± 0.07	0
Oligomycin	0.92 ± 0.17	90.5	8.74 ± 0.12	10.1
CCCP	1.58 ± 0.02	83.8	7.57 ± 0.48	22.1
Rotenone	1.16 ± 0.08	88.1	11.73 ± 0.58	−20.7

[a] Growing cultures were incubated for 20 min with the indicated additions and 1 mM ouabain before adding $^{86}Rb^+$ and assaying influx. The concentrations of glucose, oligomycin, CCCP, and rotenone were 10 mM, 2.5 μg/ml, 2 μM, and 2 μM, respectively. Similar results were obtained for $^{86}Rb^+$ influx via the Na^+/K^+ pump which was assayed in the absence of ouabain and presence of 0.1 mM furosemide.
[b] Values are means ± SEM (4).

The intracellular calcium antagonist, TMB-8 (Malagodi & Chiou, 1974), strongly inhibited ouabain-insensitive $^{86}Rb^+$ influx (Fig. 7). A 50% reduction in influx occurred at approximately 125 μM TMB-8 (Fig. 7). TMB-8 did not inhibit $^{86}Rb^+$ influx in the presence of furosemide which is primarily mediated by the Na^+ pump (*data not shown*). This finding indicates that TMB-8 selectively inhibited $^{86}Rb^+$ influx via the cotransporter.

Inhibition of Cotransport Activity by Calmodulin Inhibitors in Cultured Endothelial and Smooth Muscle Cells

$^{86}Rb^+$ influx by the cultured endothelial cells was linear with time for at least 20 min, and in the presence of ouabain it was 90 to 95% inhibitable by furosemide or bumetanide (*data not shown*). Half-maximal inhibition occurred at about 10 μM furosemide

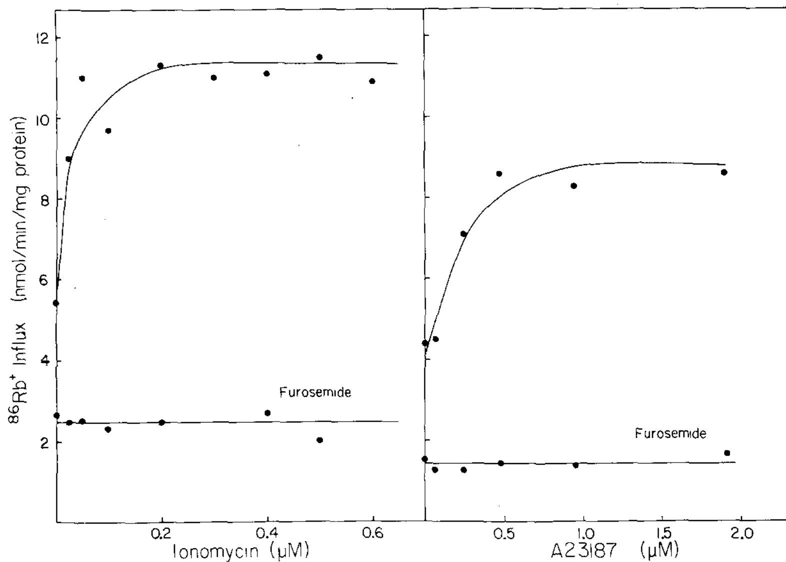

Fig. 6. Stimulation of cotransport activity by ionomycin and A23187 in quiescent smooth muscle cells. For the ionomycin dose-response the cultures were rinsed with the assay buffer (*see* Materials and Methods) and incubated for 15 min with ouabain (1 mM), $^{86}Rb^+$, and furosemide (0.1 mM) as indicated. For the A23187 dose-response the cultures were incubated for 10 min in assay buffer with ouabain (1 mM) and the indicated concentration of A23187 and 0.1 mM furosemide as indicated before adding $^{86}Rb^+$. Values are means of two experiments done in duplicate

Table 3. Comparison of Na^+, K^+, Cl^- cotransport and Na^+ pump activities in growing and quiescent smooth muscle cells[a]

	$^{86}Rb^+$ Influx (nmol/min/mg protein)[b]		
	Quiescent cells	Growing cells	*P*
Total	6.92 ± 0.62	11.48 ± 0.69	
Plus furosemide	4.86 ± 0.69	4.53 ± 0.34	
Cotransport activity	2.06 ± 0.19	6.95 ± 1.00	<0.005
Plus ouabain	4.82 ± 0.65	8.31 ± 0.45	
Na^+ pump activity	2.10 ± 0.44	3.17 ± 0.62	<0.20

[a] The cultures were rinsed with the assay buffer and incubated for 10 min in the assay buffer with 10 mM glucose and the additions indicated before adding $^{86}Rb^+$. The concentrations of ouabain and furosemide were 1.0 and 0.1 mM, respectively. The growing cultures were assayed 3 days after seeding at which time there were many mitotic figures present. The quiescent cultures were assayed 6 or 7 days after seeding at which time there were no cells in mitosis.

[b] Values are means ± SEM for three experiments done in duplicate (n = 6).

Table 4. External Ca is required for the stimulation of Na^+,K^+,Cl^- cotransport in the muscle cells by A23187[a]

Additions	$^{86}Rb^+$ Influx (nmol/min/mg)		
	Total	Plus furosemide	Cotransport
None	3.33 ± 0.56	1.84 ± 0.08	1.49
EGTA	3.51 ± 0.04	2.21 ± 0.18	1.30
A23187	3.69 ± 0.02	1.98 ± 0.03	1.71
A23187 + EGTA	3.25 ± 0.03	1.72 ± 0.09	1.53
Ca	4.37 ± 0.05	1.54 ± 0.06	2.83
Ca + A23187	8.58 ± 0.16	1.44 ± 0.04	7.14

[a] Quiescent cultures were rinsed 4 times with the assay buffer which contained 3 mM $MgCl_2$ (no added Ca) and incubated in this buffer with 1 mM ouabain and the indicated additions for 5 min before adding $^{86}Rb^+$. The concentrations of EGTA, A23187, furosemide, and Ca were: 0.1 mM, 0.25 μg/ml, 0.1 mM, and 2 mM, respectively. When Ca was added, the concentration of $MgCl_2$ was reduced to 1 mM. Values are means for duplicate cultures ± SEM.

and 0.1 to 0.2 μM bumetanide (*data not shown*). The removal of either Na^+ or Cl^- from the transport buffer abolished furosemide-inhibitable $^{86}Rb^+$ influx in the endothelial cells just as in the muscle cells. Specific cotransport activity was 20.8 ± 1.2 (21) nmol/min/mg protein [mean ± SEM (*n*)], which is about threefold higher than in growing muscle cells (Table 3). Brock et al. (1986) obtained a similar value for cotransport activity in porcine aortic endothelial cells.

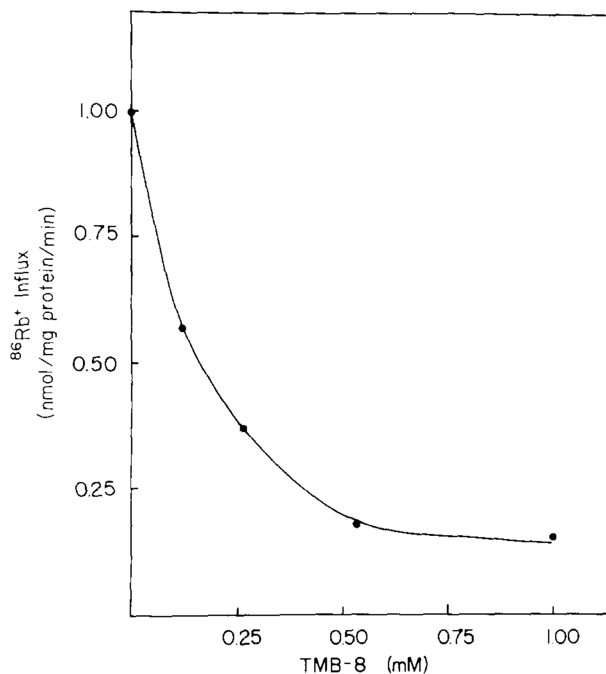

Fig. 7. Inhibition of cotransport activity by the intracellular calcium antagonist, TMB-8. Rapidly growing cultures were incubated for 10 min at 37°C with ouabain (1.0 mM) and the indicated concentration of TMB-8 before adding $^{86}Rb^+$. Under these conditions 0.1 mM furosemide inhibited $^{86}Rb^+$ influx by approximately 75%. Values are means of two experiments done in duplicate

Calmidazolium and trifluoperazine at 10 and 40 μM, respectively, inhibited ouabain-insensitive $^{86}Rb^+$ influx by 50% in the endothelial cells (Fig. 8). The sulfoxide derivative of trifluoperazine had no significant effect on cotransport activity in either smooth muscle or endothelial cells (Fig. 8). Calmidazolium was also more potent than the phenothiazines, trifluoperazine and chlorpromazine, as an inhibitor of ouabain-insensitive $^{86}Rb^+$ influx in the muscle cells (Fig. 8). In the smooth muscle cells calmidazolium, trifluoperazine, and chlorpromazine inhibited ouabain-insensitive $^{86}Rb^+$ influx by 50% at approximately 20, 40, and 100 μM, respectively. The inhibitory effect of trifluoperazine, but not that of calmidazolium, was reversed by rinsing the muscle cells to remove the compound followed by a 20-min incubation in the absence of the compound (*data not shown*). Trifluoperazine prevented A23187 from stimulating cotransport activity in the muscle cells (*data not shown*).

The naphthalenesulfonamides, W12 and W13, inhibited cotransport activity in both cell types (Fig. 9). W13 inhibited cotransport activity by 50% at approximately 125 μM in the endothelial cells and 220 μM in the smooth muscle cells (Fig. 9). W12, which differs from W13 by the absence of a chlorine atom on the naphthalene ring, is about 7 times less potent than W13 as a calmodulin inhibitor (Hidaka, 1982). W12 was considerably less potent than W13 as an inhibitor of cotransport activity in both cell types (Fig. 9). W7, which is similar in potency to W13 as a

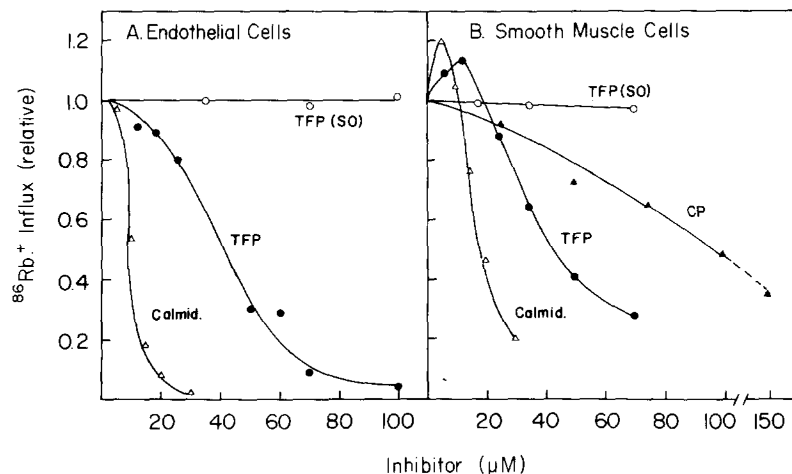

Fig. 8. Effects of calmodulin inhibitors on ouabain-insensitive $^{86}Rb^+$ influx by cultured smooth muscle and endothelial cells. The cultures were incubated for 20 min at 37°C with the indicated concentration of each compound and ouabain (1 mM for the smooth muscle cells and 0.1 mM for the endothelial cells) before adding $^{86}Rb^+$. Values are means of three experiments done on duplicate cultures. The control influx values (nmol/min/mg protein) ranged from 15.4 to 20.2

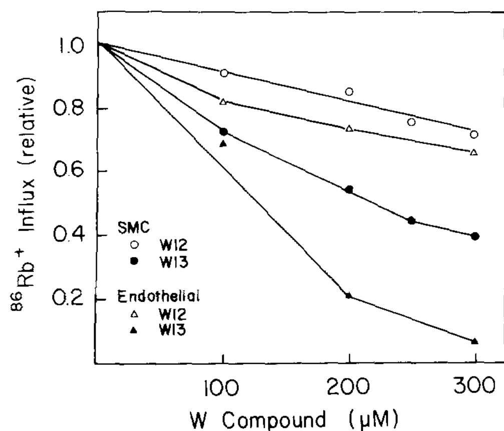

Fig. 9. Effects of W12 and W13 on ouabain-insensitive $^{86}Rb^+$ influx by cultured vascular smooth muscle and endothelial cells. The incubations were the same as for Fig. 7. Values are means of two experiments done in duplicate

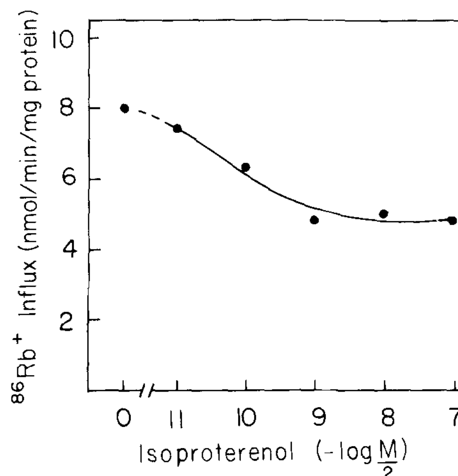

Fig. 10. Inhibition of ouabain-insensitive $^{86}Rb^+$ influx by isoproterenol. Growing muscle cells were incubated for 20 min with the indicated concentration of isoproterenol in the presence of 1 mM ouabain. Then the medium was removed and 1 ml of uptake medium containing 1 mM ouabain and the indicated concentration of isoproterenol was added followed immediately by 20 μl of $^{86}Rb^+$. Values are means of two experiments done in duplicate

calmodulin antagonist, was also similar in potency to W13 as an inhibitor of cotransport activity in the muscle cells (*data not shown*).

It is noteworthy that none of the calmodulin antagonists decreased $^{86}Rb^+$ influx mediated by the Na^+ pump, which was assayed in the presence of furosemide and absence of ouabain. At concentrations that strongly inhibited cotransport activity the calmodulin antagonists either had no effect on Na^+ pump activity (W7, W13) or they stimulated it (calmidazolium, trifluoperazine) (*data not shown*). These data indicate that the calmodulin antagonists selectively inhibited $^{86}Rb^+$ transport mediated by the cotransporter. The fact that the calmodulin inhibitors did not decrease Na^+ pump activity indicates that they probably did not inhibit cotransport activity by decreasing cellular ATP.

Inhibition of Cotransport by Cyclic AMP Production in the Vascular Muscle Cells

Previously we (Smith, 1984) showed that isoproterenol, 3-isobutyl-1-methylxanthine, and cholera toxin markedly increase cAMP in cultured aortic smooth muscle cells. Isoproterenol inhibited ouabain-insensitive $^{86}Rb^+$ influx (Fig. 10). The dose dependence for the inhibition of $^{86}Rb^+$ influx was similar to that reported previously for the production of cAMP by isoproterenol (Smith, 1984). Bt_2 cAMP similarly inhibited cotransport activity as did 3-isobutyl-1-methylxanthine and cholera toxin (Table 5).

Increasing cAMP rounds and arborizes cultured aotic muscle cells (Smith, 1984). Cytochalasin D produces a similar change in the morphology of the cells without increasing cAMP (Smith, 1984). Cytochalasin D at 4 μM rounded and arborized over 90% of the cells, but it had no effect on cotransport activity (Table 5). Therefore, the inhibition of cotransport activity by cAMP is not simply a result of transforming cell morphology.

$^{86}Rb^+$ Efflux in Growing and Quiescent cells

$^{86}Rb^+$ efflux followed first-order kinetics as indicated by the linear relationship between time and the logarithm of the percentage of the isotope remaining in the cells (Fig. 11). The efflux rate constant was 0.0118 ± 0.0008 (6) min^{-1} [mean ± SEM (*n*)] in growing (Fig. 11) as compared to 0.0067 ± 0.0003 (7) in quiescent muscle cells (*data not shown*). Furosemide (Fig. 11) and bumetanide (*not shown*) strongly reduced $^{86}Rb^+$ efflux in growing as well as quiescent cells. In the presence of 0.1 mM furosemide the efflux rate constant was 0.0042 ± 0.0010 (5) in the growing cells and 0.0049 ± 0.0005 (5) in the quiescent ones. These results indicate that a major component of $^{86}Rb^+$ efflux from the growing cells is mediated by the furosemide-inhibitable cotransporter.

The efflux rate constant for furosemide-inhibitable $^{86}Rb^+$ efflux was 4.2 times greater in growing (0.0076 min^{-1}) compared to quiescent cells (0.0018 min^{-1}). The efflux data are in close agreement with the $^{86}Rb^+$ influx data (Table 3) showing that the

Table 5. Effects of Bt_2cAMP, 3-isobutyl-1-methylxanthine, cholera toxin, and cytochalasin D on cotransport activity in cultured smooth muscle cells[a]

Additions	$^{86}Rb^+$ Influx (nmol/min/mg)[b]			
	Total	Plus furosemide	Cotransport	% Inhibition
None	8.89 ± 0.20	2.40 ± 0.06	6.49	0
Bt_2cAMP	6.50 ± 0.20	2.26 ± 0.17	4.24	35
IBMX	5.27 ± 0.20	2.03 ± 0.14	3.24	50
IBMX + IP	5.29 ± 0.33	1.85 ± 0.19	3.44	47
Cholera toxin	6.54 ± 0.63	2.26 ± 0.09	4.28	34
Cytochalasin D	8.86 ± 0.53	2.06 ± 0.09	6.80	0

[a] Growing cultures were incubated for 20 min in the presence of 1 mM ouabain and the indicated additions before adding $^{86}Rb^+$ and assaying influx. The concentrations of Bt_2cAMP, IBMX (by itself), IBMX (with IP), IP, cholera toxin, cytochalasin D, and furosemide were 1 mM, 0.5 mM, 0.1 mM, 0.1 μM, 0.2 μg/ml, 4 μM, and 0.1 mM, respectively. IBMX = 3-isobutyl-1-methylxanthine; IP = isoproterenol.

[b] Values are means ± SEM (4).

growing cells had 3.4 times greater cotransport activity than quiescent ones.

Stimulation of $^{86}Rb^+$ Efflux by Angiotensin and A23187

ANG strikingly increased the efflux rate constant in both growing (Fig. 11) and quiescent (*not shown*) muscle cultures. The magnitude of the increase was greater in the growing cells (68% stimulation by ANG compared to 35% stimulation in the quiescent cells). ANG (0.02 μM) stimulated $^{86}Rb^+$ efflux similarly in the presence (Fig. 11) and absence of ouabain (*not shown*). This finding indicates that the stimulation of the Na^+ pump by ANG (Brock, Lewis & Smith, 1982) is not involved in the stimulation of $^{86}Rb^+$ efflux by ANG. Furosemide (Fig. 11) and bumetanide (*not shown*) strongly reduced, but did not abolish, the stimulation of $^{86}Rb^+$ efflux by ANG. The angiotensin receptor antagonist, Sar[1], leu[8]-ANG, completely blocked the effect of ANG on $^{86}Rb^+$ efflux (Fig. 11) indicating that the effect of ANG is receptor mediated. ANG caused a similar increase in $^{86}Rb^+$ efflux in the presence and absence of external Ca^{2+}. In the medium with 3 mM $MgCl_2$ and no added Ca^{2+} the efflux rate constant was 0.0110 ± 0.0005 (4) and 0.0160 ± 0.0006 (4) min^{-1} in the absence and presence of ANG, respectively. This finding is consistent with measurements of cytoplasmic free Ca^{2+} which indicate that ANG increases free Ca^{2+} in the absence of external Ca^{2+} (*see* refs. cited in Smith, 1986).

A23187 (0.5 μM) increased $^{86}Rb^+$ efflux by approximately the same extent as ANG, as did the

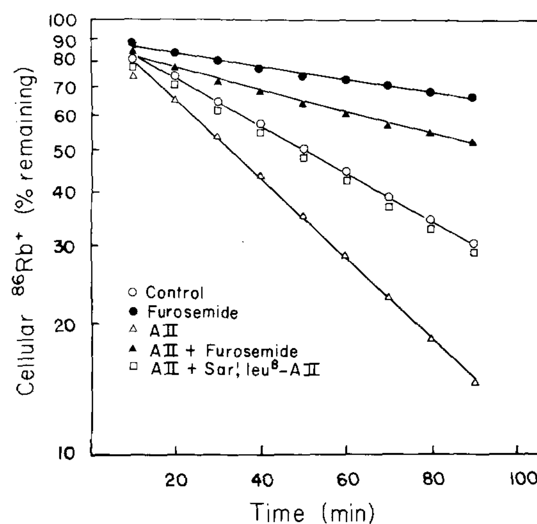

Fig. 11. Effects of angiotensin II, Sar[1],leu[8]-ANG, and furosemide on $^{86}Rb^+$ exodus from growing aortic smooth muscle cells. The cultures were refed 2 days after plating and used on the following day. One mM ouabain was present in all efflux media. The concentrations of ANG, Sar[1],leu[8]-ANG, and furosemide were 0.02, 2, and 100 μM, respectively. The lines, obtained by linear regression, had r^2 values greater than 0.98. The efflux rate constants (min^{-1}) for the control, ANG, Sar[1],leu[8]-ANG + ANG, and furosemide, and furosemide + ANG curves were 0.0125, 0.0210, 0.0131, and 0.0033, and 0.0057, respectively. Sar[1],leu[8]-ANG by itself had no effect on $^{86}Rb^+$ efflux. Values are means of two experiments done in duplicate

addition of 25 mM K^+ to the efflux medium (substituted for 25 mM Na^+) (*data not shown*). Furosemide (0.1 mM) markedly reduced, but did not abolish, the stimulation of efflux by A23187 or 25 mM K^+ (*data not shown*).

Discussion

Similarities between $Na^+/K^+/Cl^-$ Cotransport in Vascular Smooth Muscle and Other Cell Types

The present findings indicate that the "loop" diuretics abolish a major component of $^{86}Rb^+$ influx in cultured vascular smooth muscle and endothelial cells. This component of $^{86}Rb^+$ influx is probably mediated by a $Na^+/K^+/Cl^-$ cotransporter which resembles the cotransporter in Ehrlich ascites, MDCK, and red blood cells in several characteristic features (reviewed in Saier & Boyden, 1984; Geck & Heinz, 1986). The potency of bumetanide in the cultured vascular cells was similar to that reported for isolated kidney tubules (Forbush & Palfrey, 1983), avian red blood cells (Palfrey, Feit & Greengard, 1980), and fibroblast cell lines (Owen & Prastein, 1985; Paris & Pouyssegur, 1986). Furosemide-inhibitable $^{86}Rb^+$ influx in the muscle cells was sigmoidally dependent on external Cl^- (Fig. 5) and hyperbolically dependent on external Na^+ and K^+ (Figs. 3 & 4). A sigmoid relationship between cotransport activity and Cl^- concentration was previously observed in MDCK cells (Saier & Boyden, 1984). These results are consistent with the $Na^+/K^+/Cl^-$ stoichiometry of 1 : 1 : 2 previously proposed for the cotransporter avian red cells (Palfrey & Rao, 1983), Ehrlich ascites (Geck et al., 1980), and MDCK cells (Saier & Boyden, 1984). Since we have not yet shown that Na^+ and Cl^- are cotransported with K^+ in the muscle cells, an alternative interpretation of our data is that Cl^- activates furosemide-sensitive K^+ transport or (Na^+, K^+) cotransport in a cooperative fashion. The apparent K_m values of 46, 4, and 78 mM for Na^+, K^+, and Cl^- in the muscle cells are similar to the values reported for other mammalian cells (*see* Miyamoto et al., 1986 for a review of the K_m values).

The cotransporter in the cultured muscle cells is energy dependent as noted previously for the other cell types (Geck et al., 1980; Rindler, McRoberts & Saier, 1982). The addition of glucose prevented the inhibition of furosemide-sensitive $^{86}Rb^+$ influx by all three types of mitochondrial poisons (Table 2). Therefore, the inhibition of the cotransporter is not a direct effect of the metabolic inhibitors. The finding that glycolysis was sufficient to energize furosemide-inhibitable K^+ transport suggests that the cotransporter is dependent on cellular ATP levels. ATP or a related metabolite apparently activates the cotransporter allosterically rather than by supplying energy for the transport process since furosemide had no effect on ATP turnover in the Ehrlich ascites cells (Geck et al., 1980).

Greater Cotransport Activity in Growing Smooth Muscle Cells

Rapidly growing smooth muscle cells had three- to fourfold greater cotransport activity than quiescent cells as demonstrated by both furosemide-inhibitable influx and efflux of $^{86}Rb^+$ (Table 3, text). Moreover, the increased $^{86}Rb^+$ transport was specific for the cotransport pathway since neither Na^+/K^+ pump activity (Table 3), nor furosemide-insensitive $^{86}Rb^+$ efflux (*see* data in text), was significantly different in growing and quiescent cells. Growing Swiss 3T3 cells also have about 3 times greater cotransport activity than quiescent ones (Amsler et al., 1985).

Tupper, Zorgniotti and Mills (1977) showed that increased furosemide-inhibitable K^+ influx is an "early" response of Balb/c 3T3 cells to mitogenic stimulation. Insulin was the most effective stimulatory mitogen of cotransport in Swiss 3T3 cells (Amsler et al., 1985). Since bumetanide did not interfere with the hormonal stimulation of DNA synthesis in 3T3 cells (Amsler et al., 1985), the activation of the cotransporter appears to be neither necessary nor sufficient for entry into the S phase of the cell cycle. Proliferative stimuli also increase cotransport activity in a mutant strain of Chinese hamster fibroblasts that lack Na^+/H^+ antiport (Paris & Pouyssegur, 1986). Thrombin, which is a potent mitogen for these cells, increased cotransport activity by 4-fold (Paris & Pouyssegur, 1986).

Inhibition of Cotransport Activity by Calmodulin Antagonists in Vascular Smooth Muscle and Endothelial Cells

Calmodulin or a calmodulin-like protein may be required for cotransport activity since three different types of calmodulin antagonists (calmidazolium, phenothiazines, and naphthalenesulfonamides) abolished basal cotransport activity in both the smooth muscle and endothelial cells (Figs. 8 & 9). The calmodulin inhibitors selectively inhibited $^{86}Rb^+$ influx mediated by the cotransporter since they either had no effect on or stimulated furosemide-insensitive $^{86}Rb^+$ influx which is primarily mediated by the Na^+/K^+ pump. Moreover, the relative potency of the various calmodulin antagonists was similar to their relative potencies as inhibitors of calmodulin in vitro and in vivo (Weiss & Levin, 1978; Hidaka, 1982; Nelson, Andrews & Karnovsky, 1983). Nevertheless, the present findings do not exclude the possibility that the calmodulin antagonists inhibit cotransport by binding to a hydrophobic domain of the cotransporter rather than to calmodulin or a calmodulin-like protein.

Regulation of Cotransport Activity by Cyclic Nucleotides and Ca^{2+} in Vascular Smooth Muscle

Beta-adrenergic stimulation (Fig. 10) and other agents known to increase cAMP in the cultured arterial muscle cells (Smith, 1984) inhibited cotransport activity (Table 5) as has been observed in A7r5 cells (Owen, 1984) and HSWP fibroblasts (Owen & Prastein, 1985). Calcium also influences the activity of the cotransporter in the cultured muscle cells. Angiotensin II (Fig. 11) and two ionophores, A23187 and ionomycin (Fig. 6), stimulated cotransport activity in the cultured smooth muscle cells, whereas the intracellular calcium antagonist, TMB-8 (Malagodi & Chiou, 1974), abolished cotransport activity (Fig. 7). Clearly cAMP and calcium have opposing influences on cotransport activity in cultured vascular smooth muscle cells.

ANG rapidly increases polyphosphoinositide metabolism and mobilizes intracellular calcium in cultured vascular smooth muscle cells (Smith, 1986). The potent activator of protein kinase C, phorbol myristate acetate, had no significant effect on $^{86}Rb^+$ efflux (*data not shown*). Therefore, if protein phosphorylation is responsible for the stimulation of cotransport activity by ANG, it is probably mediated by a calcium-activated kinase rather than protein kinase C. Protein phosphorylation may also be necessary for the maintenance of basal cotransport activity since basal cotransport activity was abolished by energy poisons (Table 2) or calmodulin antagonists. There is as yet, however, no convincing evidence that protein phosphorylation modulates cotransport activity.

The stimulation of furosemide-sensitive $^{86}Rb^+$ efflux by ANG is especially interesting in the light of the recent finding that atriopeptin stimulates furosemide-sensitive $^{86}Rb^+$ influx in aortic smooth muscle cells (O'Donnell & Owen, 1986). Atriopeptin increases cyclic GMP in these cells (O'Donnell & Owen, 1986; Smith & Lincoln, 1987), whereas ANG provokes phosphatidylinositol bisphosphate hydrolysis and calcium mobilization (Smith, 1986). Hence, it seems paradoxical that cotransport activity would be stimulated by both vasoconstricting and vasorelaxing hormones. Since cAMP decreased furosemide-inhibitable $^{86}Rb^+$ influx and the calcium ionophores stimulated furosemide-inhibitable $^{86}Rb^+$ influx, we expected that vasoconstricting and relaxing hormones would have opposite effects on cotransport activity. Both cAMP and cGMP mediate vasorelaxation, however, and the former inhibits (Table 5, Fig. 10) and the latter stimulates furosemide-inhibitable $^{86}Rb^+$ influx (O'Donnell & Owen, 1986).

Concerning the stimulation of cotransport activity by ANF and ANG, we wish to call attention to the fact that the present findings show that ANG stimulates $^{86}Rb^+$ *efflux* via the contransporter. On the other hand, ANF has been shown to stimulate furosemide-inhibitable $^{86}Rb^+$ *influx* (O'Donnell & Owen, 1986). It is not yet known if ANF has any effect on furosemide-sensitive $^{86}Rb^+$ efflux or if ANG has any effect on $^{86}Rb^+$ influx via the cotransporter. ANG is known to stimulate $^{86}Rb^+$ influx in these cells, but this occurs primarily via the Na^+/K^+ pump (Brock, Lewis & Smith, 1982; Smith & Brock, 1983).

Physiological Significance

The physiological significance of the greater cotransport activity in the growing cells is unclear, although the cotransporter apparently helps to maintain steady-state cellular levels of Na^+, K^+, Cl^-, and volume. Geck and Heinz (1986) have proposed that the stimulation of cotransport by mitogenic hormones causes a net uptake of osmolarity which is followed by an increase in cell volume. Although mean cell volume and K^+ concentration increase following mitogenic stimulation (Tupper et al., 1977), it is unclear what contribution, if any, the cotransporter makes to the volume increase. The stimulation of cotransport activity by thrombin in hamster fibroblasts appears to be unrelated to cell volume regulation since thrombin had no effect on cell H_2O space measured by [^{14}C]urea equilibration (Paris & Pouyssegur, 1986). Thus, although osmotic shrinkage mimicked the effect of thrombin on cotransport activity (Paris & Pouyssegur, 1986), the stimulation of cotransport by thrombin is probably not a secondary effect of a decrease in cell volume. We previously reported that neither isoproterenol (Smith, 1984), nor ANG (Smith & Brock, 1983), have any effect on cell H_2O space measured by [^{14}C]urea and 3-0-[methyl-^{14}C]-D-glucose distribution. Therefore, the modulation of cotransport activity by cAMP and Ca^{2+} in the smooth muscle cells is also probably not secondary to changes in cell volume.

If it is not related to cell volume regulation, then the modulation of cotransport activity may be needed to compensate for the hormone-evoked changes in other pathways of Na^+, K^+, and/or Cl^- transport. For example, an increased efflux of K^+ via the cotransporter would help to compensate for the increase in K^+ influx via the Na^+/K^+ pump that is produced by ANG (Smith & Brock, 1983). The recent observations of Levinson (1987) suggest that net transport via the cotransporter stops when cell Na^+ and K^+ concentrations attain steady-state lev-

els. Additionally, as pointed out by Gargus and Slayman (1980), hormonal activation of the cotransporter may have a role as an intermediate in the physiological response by conveying information about the altered extracellular environment to another cellular function, rather than the activation of the cotransporter being an end in and of itself. Perhaps the most important missing information concerning these hormones and the regulation of the cotransporter has to do with *net* NaCl or KCl movements. Information on net ion movements via the cotransporter may provide insight to the relationship between cotransport activity, cell growth, and vasoregulation.

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