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EFFECTS OF OUABAIN AND OSMOLARITY ON BUMETANIDE-SENSITIVE POTASSIUM TRANSPORT IN SIMIAN VIRUS-TRANSFORMED 3T3 CELLS

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Unidirectional potassium influx in simian virus-transformed 3T3 cells was dissected into a ouabain-inhibitable 'pump' component, a bumetanide-sensitive and chloride-dependent 'cotransport' component, and a residual 'leak' flux. The bumetanide-sensitive component was stimulated 2–3-fold by a 60-min preincubation with ouabain. Subsequent washing of the cells and incubation in ouabain-free saline reversed both the inhibition of the Na^+ pump and the stimulation of bumetanide-sensitive flux. Bumetanide-sensitive potassium influx was also stimulated by hypertonic cell shrinkage (induced by 0.1 M or 0.2 M sorbitol). This latter observation suggests that the bumetanide-sensitive system may play a role in cellular volume regulation.

Viral transformation profoundly affects the properties of the cell membrane [1]. Several reports have appeared in which K^+ transport in normal and transformed cells was compared (Ref. 1, Chapter 4). Some years ago, Brown and Lamb [2] found that continuous exposure to ouabain induced a time-dependent increase in unidirectional K^+ influx and efflux in virally-transformed, but not in normal 3T3 cells. One of us [3] also observed a similar ouabain-induced K^+ flux in a certain strain of ascites cells, which, in this cell type, was both sensitive towards 'high-ceiling' diuretics and Cl^--dependent. Recently, evidence has been accumulating linking those characteristics to a transport system separate from the Na^+ pump [4,5]. Cation transport through this system is generally Cl^--dependent [6–9], and at least under some conditions an electroneutral cotransport of ($1\,K^+ + 1\,Na^+ + 2\,Cl^-$) has been demonstrated [6,7]. Diuretic-sensitive cotransport has been postulated to play a role in cellular volume regulation [6,7].

In view of these developments, we have now reinvestigated the ouabain-induced K^+ flux in SV-3T3 cells. We show here that the ouabain-induced flux is sensitive to the diuretic bumetanide [10] and towards anion replacement. Moreover, also increases in osmolarity stimulated bumetanide-sensitive K^+ influx. This latter observation suggests that the bumetanide-sensitive transport system may be involved in cellular volume regulation.

For the following experiments, $^{86}Rb^+$ was used as a tracer for K^+ [2,11]. In Cl^--saline, bumetanide inhibited $^{86}Rb^+$ influx approximately 50%. By contrast, a 60-min preincubation with ouabain stimulated influx by about the same amount (Table I, Expt. 1A). This confirms the finding reported in Ref. 2. When Cl^- as the main anion was replaced by NO_3^-, bumetanide did not have any effect, either in the presence or absence of ouabain (Table I, Expt. 1B). These results are similar to those obtained for the ascites cells in [3], except that in the latter cells the Cl^--dependent

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TABLE I

EFFECT OF PREINCUBATION IN OUABAIN ON THE COMPONENTS OF $^{86}Rb^+$ INFLUX IN SV-3T3 CELLS

Experiments were performed essentially as in Ref. 2. Briefly, cells were grown on plastic culture dishes (5 cm diameter). Dishes were rinsed with a saline containing (mM): NaCl, 137; KCl, 5.4; $CaCl_2$, 2.8; $MgSO_4$, 1.2; NaH_2PO_4, 0.3; KH_2PO_4, 0.4; HCl, 12; Tris base, 14; glucose, 10, and 1% newborn-calf serum (pH 7.4, 37°C). For Expt. 1B, all Cl^--salts were replaced by NO_3^--salts. Cells were preincubated for 1 h, or as indicated, at 37°C, in the absence or presence of 1.2 mM ouabain. Subsequently, the preincubation medium was aspirated and 2 ml saline was added containing 0.4 μCi/ml $^{86}Rb^+$ (Amersham) as a tracer for K^+ [2,11], and 1.2 mM ouabain and/or 0.1 mM bumetanide as indicated. After 10 min at 37°C the labeled solution was aspirated and the dishes were rapidly rinsed with ice-cold saline. The cells were trypsinized and part of the cell suspension from each plate was used to determine cell number and mean cell volume by a Coulter counter. $^{86}Rb^+$ was measured in the remaining part by its Cerenkov radiation. $^{86}Rb^+$ influx is linear over at least 10 min [2]. Influx was calculated from cellular $^{86}Rb^+$ content and the activity of the supernatant, and differentiated into components as indicated in the text. All incubations were performed in triplicate. Values are given plus or minus one standard error.

	Additions during		Rate of influx (nmol K^+/min per 10^6 cells)	Flux components (nmol K^+/min per 10^6 cells)		
	Preincubation	$^{86}Rb^+$-flux		Bumetanide-sensitive	Ouabain-sensitive	Residual
Expt. 1						
A: Cl^--saline						
60-min	–	–	7.08 ± 0.33	3.3	3.0	0.7 [a]
	–	bumetanide	3.76 ± 0.46			
	ouabain	ouabain	10.8 ± 0.2	10.1	0	0.7
	ouabain	ouabain plus bumetanide	0.72 ± 0.12			
B: NO_3^--saline						
60-min	–	–	4.53 ± 0.21	0	3.6	0.9 [a]
	–	bumetanide	4.54 ± 0.17			
	ouabain	ouabain	0.79 ± 0.11	(−0.2)	0	0.9
	ouabain	ouabain plus bumetanide	1.01 ± 0.06			
Expt. 2						
Preincubation						
60-min	–	–	6.41 ± 0.40			
	ouabain	ouabain	12.8 ± 1.0	12.1	0	0.7
	ouabain	ouabain plus bumetanide	0.74 ± 0.02			
120-min	–	–	8.22 ± 1.01			
60-min plus 60-min	ouabain	–	10.1 ± 0.51	3.9	5.5	0.7
	–	bumetanide	6.16 ± 0.41			
		ouabain plus bumetanide	0.69 ± 0.04			

[a] The residual flux in the control cells was assumed to be equal (within the experimental error) to that determined for the cells preincubated in ouabain. This assumption was validated in two other experiments (not shown). Cell density and mean volume of the control cells were $3 \cdot 10^4$ cells/cm^2 and 2.87 ± 0.13 pl/cell for Expt. 1, and $6 \cdot 10^4$ cells/cm^2 and 2.47 ± 0.02 pl/cell for Expt. 2, respectively.

stimulation of K^+ influx by ouabain was virtually immediate. The data show that (i) the SV-3T3 cells mediated a diuretic-sensitive, Cl^--dependent K^+ influx, and (ii) whereas bumetanide did not affect the ouabain-sensitive K^+ influx (Expt. 1B), preincubation in ouabain stimulated the bumetanide-sensitive flux. We therefore took, as in Ref. 3, the ouabain-sensitive component as the difference in influx in the presence of bumetanide plus or minus ouabain. The bumetanide-sensitive component was

simply calculated as the difference in influx in the presence and absence of bumetanide, and the residual component as the influx in the presence of both inhibitors. Defined this way, those three components presumably are associated with the Na^+ pump, the diuretic-sensitive cotransport system, and the diffusional leak pathway, respectively [3,11]. From Table I it can be seen that under control conditions the diuretic-sensitive flux contributed 47%, and the ouabain-sensitive flux 42% of the total K^+ influx. For SV-3T3 cells of comparable density Spaggiare et al. [11] have reported values of 20% and 64%, respectively. After preincubation in ouabain the bumetanide-sensitive flux was 3-fold enhanced. The stimulation of bumetanide-sensitive flux quantitatively accounted for the ouabain-induced increase in total influx (Table I). Furosemide at 0.1 mM had the same effect as bumetanide (result not shown).

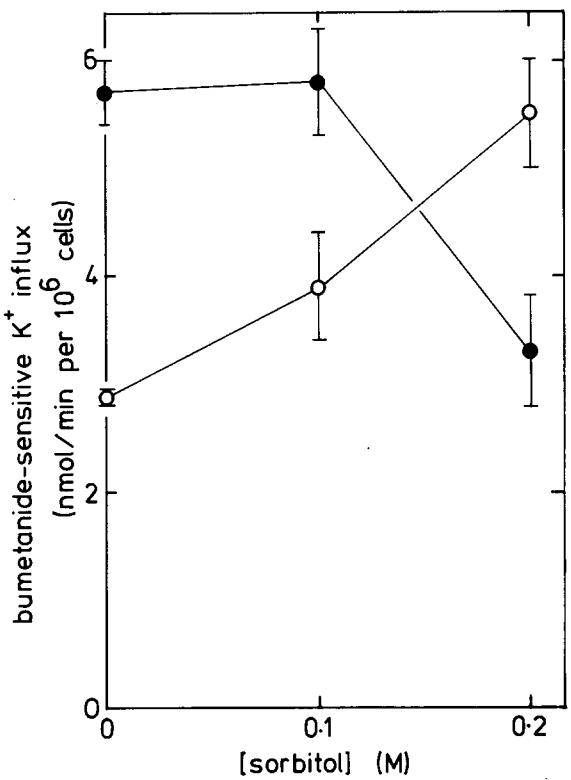

Fig. 1. Effect of increased osmolarity on bumetanide-sensitive K^+ influx. Cells were preincubated for 1 h with sorbitol added at the indicated concentrations, plus or minus 1.2 mM ouabain. $^{86}Rb^+$ influx was allowed to proceed over 14 min and the bumetanide-sensitive flux fraction was determined as in the experiments of Table I. O——O, control; ●——●, plus 1.2 mM ouabain. All points were taken in triplicate. Distance between error bars is 2 S.E. Cell density was $5 \cdot 10^4$ cells/cm^2. Mean volume (pl/cell) in control saline: 1.55±0.10; in saline plus 0.2 M sorbitol: 1.30±0.02.

The data in Ref. 2 had raised some doubt as to the reversibility of the ouabain-induced K^+ flux. However, we now found (Table I, Expt. 2) that the effects of ouabain both on the Na^+ pump and on bumetanide-sensitive K^+ flux were reversible: after removal of ouabain and a 1-h recovery period in ouabain-free saline, ouabain-sensitive flux had returned to 60% of total influx. Concomitantly, bumetanide-sensitive flux had decreased again by a factor 3.

Whatever the nature of the ouabain effect, it probably is indirect, based for instance on a change in intracellular ion concentrations. In line with this supposition we found that ouabain did not stimulate bumetanide-sensitive flux when the cells were preincubated with the glycoside at 18°C instead of 37°C (Table II, Expt. A; see also Ref. 2).

Since the $(Na^+ + K^+ + 2\,Cl^-)$-cotransport system has been implicated in volume control [6,7], we tested the effect of a change in medium osmolarity on the diuretic-sensitive K^+ influx in our cells. Fig. 1 shows that addition of the inert sugar sorbitol stimulated bumetanide-sensitive K^+ influx (open circles). This suggests that in these cells, as in ascites cells [7], cellular shrinkage activates

TABLE II

EFFECT OF PREINCUBATION TEMPERATURE ON THE STIMULATION OF BUMETANIDE-SENSITIVE K^+ INFLUX BY OUABAIN OR SORBITOL

Cells were preincubated for 1 h at either 18°C or 37°C, with 1.2 mM ouabain (Expt. A) or 0.2 M sorbitol (Expt. B). Bumetanide-sensitive K^+ influx was subsequently determined at 37°C, as described in the legend to Table I. For Expt. B, sorbitol was also present during the flux measurement. Cell density was $2.2 \cdot 10^5$ cells/cm^2.

Preincubation conditions	Bumetanide-sensitive K^+ influx (nmol/min per 10^6 cells)
Control	1.28±0.02
A: +ouabain, 37°C	3.67±0.29
+ouabain, 18°C	1.29±0.04
B: +sorbitol, 37°C	2.97±0.18
+sorbitol, 18°C	3.50±0.25

the cotransport system. In agreement with the notion that the effect of sorbitol was purely osmotic, the temperature during preincubation (and, by inference, preincubation itself) was not crucial (Table II, Expt. B). The effects of sorbitol on the one hand and of preincubation with ouabain on the other hand were clearly not additive: at 0.2 M sorbitol they even appeared to cancel each other (Fig. 1, black circles).

Whereas in our experiments on SV-3T3 cells ouabain consistently stimulated bumetanide-sensitive K^+ influx 2–3-fold, results with normal 3T3 cells have so far been variable. In one batch (Flow laboratories, passage number 129; cell density $2 \cdot 10^4$ cells/cm^2), a 75-min preincubation in ouabain did not significantly affect bumetanide-sensitive flux. This corroborates the original observation by Brown and Lamb [2]. By contrast, in another, unspecified batch (cell density $8 \cdot 10^4$ cells/cm^2) ouabain stimulated bumetanide-sensitive flux 2.2-fold. Tupper [12] has noted also that other K^+ transport properties in 3T3 cells vary as a result of subcultivation.

More generally it appears that ouabain stimulates bumetanide-sensitive flux in some cells [2,3,14] but not in others (Ref. 2; other strains of ascites cells [6]; human red blood cells [8]). This stimulating effect is probably not specific for ouabain: other inhibitors of the Na^+ pump may behave analogously [3]. We have no clue yet as to the underlying mechanism. Meanwhile the available evidence suggests that the division between the cells that are susceptible to the effect and those that are not is gradual rather than absolute, and that it may reflect subtle differences in the regulation of bumetanide-sensitive volume control. A final question concerns the physiological significance of the ouabain effect itself. Brown and Lamb [2] observed that the ouabain-induced component of unidirectional K^+ efflux (and that component exclusively) was abolished by the removal of K^+ from the medium. This suggests that the ouabain-induced component represents K^+-K^+ exchange, a process which, in its strictly-coupled form, is biologically futile. Clearly, net fluxes will have to be measured to settle this point.

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