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Serum Stimulation of Sodium Transport in Human Fibroblasts Containing Low and High Levels of Intracellular Sodium

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Summary. The relationships between intracellular sodium content, sodium transport and serum effects were investigated in human fibroblasts. In the cells with low intracellular sodium (Na^+_{iL}; 0.04 μmol sodium/mg protein), serum stimulated the sodium-potassium pump as measured by ouabain-sensitive sodium efflux and rubidium influx and also exerted a transstimulation of ouabain-insensitive sodium transport resulting in net influx. In cells with high intracellular sodium (Na^+_{iH}; 0.42 μmol sodium/mg protein) all aspects of sodium transport were increased compared to Na^+_{iL} cells. In these cells serum caused no change in sodium-potassium pump activity but significantly increased the ouabain-insensitive sodium fluxes resulting in net efflux. In Na^+_{iL} cells, serum promoted net sodium influx through an amiloride-sensitive pathway that was undetectable in the basal state. In Na^+_{iH} cells the serum-stimulated net efflux was amiloride sensitive but this pathway also contributed to a major portion of sodium transport in the basal state. This study demonstrated that sodium-potassium pump activity is directed by the supply of internal sodium and that serum can increase this supply by promoting net influx, and that serum-induced sodium transport can be modified by intracellular sodium content.

Key Words sodium transport · serum stimulation · fibroblasts

Introduction

Cultured cells can be reversibly arrested in the G_1 phase of the cell cycle when deprived of growth factors or essential nutrients [6, 27]. Introduction of serum stimulates resting cells to reinitiate DNA synthesis and cell division [28]. Recent evidence suggests that the earliest detectable biochemical events initiated by serum occur at the plasma membrane level and include changes in transport rates for P_i, nucleosides, glucose [2, 4, 7, 19] and in the levels of cyclic nucleotides [16, 21]. However, the most striking of these early changes is an increase in sodium influx and sodium-potassium pump activity [18, 29–31, 33]. Various investigators [12, 23] have suggested that the serum-stimulated sodium-potassium pump activity is secondary to an enhanced sodium influx. To support this contention, Smith and Rozengurt [23] used the ionophore monensin to increase sodium entry into 3T3 cells, and they demonstrated a significant increase in sodium-potassium pump activity measured as ouabain-sensitive $^{86}Rb^+$ influx. Similar findings have been demonstrated in other cell types [12]. However, these studies have not ruled out the possibility that the serum may have an allosteric effect at the sodium-potassium pump site since it produced a stimulatory effect in addition to that caused by monensin [23]. Further, monensin at high concentrations (>1.5 μg/ml) causes a net exit of potassium which can interfere with the sodium-potassium pump measurement and thereby with the interpretation of the results.

This study was designed to explore the effects of serum on sodium transport and to evaluate the contribution of intracellular sodium concentration on these effects in cultured human fibroblasts. To avoid the use of ionophores or any other exogenous factors, the intracellular sodium concentration of the cells was increased by incubating the fibroblasts in a medium without potassium (removal of potassium ions causes a reversible inhibition of sodium-potassium pump activity allowing an accumulation of intracellular sodium against its concentration gradient). The possible allosteric effect of serum at the sodium-potassium pump site was investigated by measuring sodium-potassium-ATPase activity enzymatically coupled to NADH oxidation [20].

Materials and Methods

Growth media and plastic ware for tissue culture were obtained from Gibco Europe Ltd. (Paisley, Scotland). $^{22}Na^+$ and $^{86}Rb^+$ were purchased from Amersham International Ltd. (Amersham, Buckinghamshire). Ouabain, enzymes and substrates for the NADH-linked sodium-potassium-ATPase assay were obtained from Sigma Chemical Co., Ltd. (Poole, Dorset). Amiloride was a

generous gift from Merck Sharpe and Dohme Ltd. (Hoddesdon, Herts), and all other chemicals used were Analar Grade obtained from BDH Chemicals, Ltd. (Poole, Dorset).

Cell Culture

Small sections of human foreskin were obtained following routine circumcisions carried out at this hospital. Samples (<0.5 mm) were fixed under glass coverslips in petri dishes containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. After 4 weeks at 37°C in a 95%/5% air/CO_2 atmosphere, during which time the medium was changed twice weekly, the outgrowth of fibroblasts was trypsinized (0.25% wt/vol trypsin) and seeded into plastic culture flasks. For sodium and rubidium transport studies, the cells were reseeded in plastic petri dishes (50 mm diameter) at a density of 5×10^4 cells/ml (5 ml total volume). After 7 days with one change of medium, the fibroblast cultures were confluent and used for transport experiments.

Transport Studies

All sodium and rubidium transport studies were carried out in medium based on Hank's balanced salt solution (HBSS: NaCl 137 mM, $CaCl_2$ 1.26 mM, $MgSO_4 \cdot 7H_2O$ 0.4 mM, $MgCl_2 \cdot 6H_2O$ 0.49 mM, $Na_2HPO_4 \cdot 2H_2O$ 0.17 mM, KH_2PO_4 0.44 mM, glucose 5.5 mM, $NaHCO_3$ 4.17 mM, KCl 5.4 mM). The various modifications to this physiological medium are described below.

$^{22}Na^+$ Efflux

The culture medium was removed and the cells were washed 3 times with 5 ml of either HBSS-potassium (KCl and KH_2PO_4 had been omitted from this solution and replaced with equimolar quantities of NaCl and NaH_2PO_4) or HBSS + ChCl (91 mM choline chloride had been substituted for 91 mM NaCl). The dishes of cells were then incubated in 2 ml of HBSS-potassium + 1 μCi $^{22}Na^+$ or 2 ml of HBSS + ChCl + 1 μCi $^{22}Na^+$. The purpose of incubating in HBSS-potassium was to prepare fibroblasts with high internal sodium concentration (Na^+_{iH}). Preliminary experiments have shown that fibroblasts incubated for 1 hr in HBSS-potassium accumulate 10 times more internal sodium (0.42 μmol sodium/mg protein) than cells incubated in HBSS (0.04 μmol sodium/mg protein). By substituting ChCl for NaCl (HBSS-ChCl), the specific activity of $^{22}Na^+$ relative to sodium was increased, thus facilitating greater $^{22}Na^+$ uptake without causing a rise in the intracellular sodium content. These cells were designated Na^+_{iL}, i.e. fibroblasts with low internal sodium content.

After 1 hr incubation at 37°C, the radioactive medium was removed and the cells were washed and incubated in 5 ml of efflux medium (HBSS ± 1 mM ouabain ± 10% FCS ± 1 mM amiloride) for various time intervals up to 15 min. The dishes of cells were then rapidly washed with 3 × 5 ml of ice-cold choline chloride solution (ChCl 151 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2.2 mM, pH 7.4) and then the cells were solubilized in 2 ml of 0.4 M NaOH, counted for radioactivity and subsequently estimated for protein content [11]. For each time point, and for each set of efflux conditions, quadruplicate sets of petri dish cultures were used.

Estimation of Sodium Efflux Rate Constant

The first-order rate constant (the fraction of intracellular isotope lost from the cells per unit of time) was derived from log cpm/mg protein versus efflux time in minutes. The sodium efflux rate has been shown to remain linear during the 15-min period of study [25]. The slope was calculated by the method of least-squares and the half-time ($t_{0.5}$) was derived from this. Sodium efflux rate constant ($^\circ K_{Na^+}$) was calculated from the equation:

$$^\circ K_{Na^+} = \frac{0.693}{t_{0.5}} \text{ per min.}$$

The 'total' sodium efflux rate constant $^\circ K^T_{Na^+}$ (a) was obtained when efflux was measured in the absence of ouabain. The 'passive' or ouabain-insensitive rate constant $^\circ K^{OI}_{Na^+}$ (b) was calculated from the efflux measured in the presence of 1 mM ouabain and the 'active' or ouabain-sensitive sodium efflux rate constant $^\circ K^{OS}_{Na^+}$ (c) was calculated by subtracting (b) from (a). To express sodium efflux as μmol sodium/g protein/min, the following equation [32] was used;

$$\text{Efflux} = {}^\circ K_{Na^+} \cdot (Na^+ i)$$

where $(Na^+_i)$is the internal sodium content obtained from

$$(Na^+_i) = \frac{\text{cpm/g protein}}{SA_{ex}} \text{ at } t = 0$$

and SA_{ex} is the specific activity of the extracellular medium (cpm/μmol sodium). A value for Na^+_i was obtained during the course of each individual experiment.

$^{86}Rb^+$ Uptake

Potassium influx was measured with $^{86}Rb^+$ as a tracer which is a congever of potassium and has a longer half-life than $^{42}K^+$ [8]. The culture medium was removed and the dishes of fibroblasts were washed three times and incubated for 1 hr at 37°C with 5 ml of either HBSS or HBSS-potassium to obtain (Na^+_{iL}) and (Na^+_{iH}) fibroblasts as explained for $^{22}Na^+$ efflux. After the incubation period the medium was removed and replaced with 2 ml of HBSS + 4 μCi $^{86}Rb^+$ ± 10% FCS ± 1 mM ouabain. After 3 or 6 min, the radioactive medium was removed and the cells were washed, counted for radioactivity and estimated for protein content as described for $^{22}Na^+$ efflux estimation. The $^{86}Rb^+$ uptake was expressed as μmol Rb^+/g protein/min calculated by the following equation [31]

$$\text{influx} = \frac{dRc/dt}{SA_{ex}}$$

where dRc/dt represents the slope of the linear phase of the uptake curve (cpm of $^{86}Rb^+$ taken up per gram of protein per min) and SA_{ex} is the specific activity of the extracellular medium (cpm/μmol potassium).

Table 1. Sodium efflux measured in the presence and absence of 10% FCS in fibroblasts with low (Na^+_{iL}) and high (Na^+_{iH}) internal sodium[a]

Internal sodium	+/− 10% FCS	Sodium efflux (μmol Na/g protein/min)		
		Total	Ouabain-insensitive	Ouabain-sensitive
Na^+_{iL}	−	8.9 ± 0.58	2.91 ± 0.37	5.95 ± 0.57
Na^+_{iL}	+	30.48 ± 4.00	11.08 ± 0.66	19.41 ± 3.69
Serum-stimulated sodium efflux		21.61 ± 3.53	8.17 ± 1.02	13.46 ± 3.13
		$P < 0.001$	$P < 0.001$	$P < 0.005$
Na^+_{iH}	−	108 ± 11*	35 ± 4.0*	72.5 ± 14*
Na^+_{iH}	+	151 ± 16*	77 ± 11*	74 ± 19*
Serum-stimulated sodium efflux		44 ± 7.0	43 ± 12	1.25 ± 7.5
		$P < 0.005$	$P < 0.001$	NS

[a] Results are mean ± 1 SD for 4 separate estimations. * $P < 0.001$ compared with corresponding values in (Na^+_{iL}) cells. NS = not significant.

$^{22}Na^+$ Influx

For sodium influx measurements, fibroblasts were preincubated as described for sodium efflux to obtain (Na^+_{iL}) and (Na^+_{iH}) fibroblasts. 2 ml of HBSS + 1 mM ouabain (to prevent sodium efflux via the sodium-potassium pump) + 1 μCi $^{22}Na^+$ ± 10% FCS ± 1 mM amiloride were then added and the cells left for periods up to 4 min. After washing with choline chloride solution, radioactivity and protein content were determined and the sodium influx was calculated and expressed as μmol sodium/g protein/min using the equation described for $^{86}Rb^+$ uptake.

Measurement of Sodium-Potassium-ATPase Activity

Canine kidney sodium-potassium-ATPase activity was measured with a coupled enzyme assay in which the hydrolysis of ATP (10 mM) to ADP by sodium-potassium-ATPase (0.05 units) was coupled to NADH (0.25 mM) oxidation via the enzymes pyruvate kinase (10 units) and lactic dehydrogenase (10 units), with the intermediate substrate phosphoenolypyruvate (1.2 mM) in excess. NADH oxidation was recorded spectrophotometrically as the loss in absorption at 340 nm [20].

Statistical Methods

All data are presented as means ± one standard deviation. Student's *t*-test was used to determine significance between the effects of high and low intracellular sodium content and the addition of 10% FCS and 1 mM amiloride on $^{22}Na^+$ and $^{86}Rb^+$ transport measurements.

Results

Effect of Serum on $^{22}Na^+$ Efflux

Sodium efflux was measured in fibroblasts loaded to contain either low (Na^+_{iL}) or high (Na^+_{iH}) intracellular sodium concentrations as described in the Materials and Methods. The results of these experiments are summarized in Table 1. In the absence of serum, sodium efflux from Na^+_{iH} cells was 12-fold higher than the rate measured in Na^+_{iL} cells ($P < 0.001$). The increase affected both the ouabain-insensitive as well as ouabain-sensitive components. The addition of 10% FCS caused significant rise in efflux rates in both Na^+_{iH} and Na^+_{iL} cells but there were striking differences between the magnitude of these effects on the ouabain-insensitive and ouabain-sensitive components. While the total sodium efflux increased by 244% from 8.9 ± 0.6 to 30.5 ± 4.0 μmol sodium/g protein/min in Na^+_{iL} fibroblasts, it only increased by 41% from 108 ± 11 to 151 ± 16 μmol sodium/g protein/min in Na^+_{iH} cells. Unlike the Na^+_{iL} cells, which showed 226% ($P < 0.005$) increase in ouabain-sensitive (sodium-potassium pump-mediated) sodium efflux, in the Na^+_{iH} cells the serum-induced increase in sodium efflux was confined to the ouabain-insensitive component which increased by 123% from 35.0 ± 4.0 to 77 ± 11 μmol sodium/g protein/min ($P < 0.001$) and the sodium-potassium pump-mediated sodium efflux remained unchanged.

Effect of Serum on Rubidium Influx

To confirm the results for sodium efflux studies, we performed a parallel set of experiments measuring rubidium influx using the ouabain-sensitive component as an index of sodium-potassium pump activity. The results are presented in Table 2. The total, ouabain-insensitive and ouabain-sensitive rubidium influx were significantly higher in Na^+_{iH} cells compared with Na^+_{iL} cells. In keeping with the results

Table 2. Rubidium influx measured in the presence and absence of 10% FCS in fibroblasts with low (Na^+_{iL}) and high (Na^+_{iH}) internal sodium[a]

Internal sodium	+/− 10% FCS	Rubidium influx (μmol Rb/g protein/min)		
		Total	Ouabain-insensitive	Ouabain-sensitive
Na^+_{iL}	−	11.9 ± 1.2	4.6 ± 0.5	7.3 ± 0.8
Na^+_{iL}	+	17.0 ± 1.1	5.9 ± 0.7	11.1 ± 0.5
Serum-stimulated rubidium influx		5.1 ± 0.8 $P < 0.005$	1.3 ± 0.6 NS	3.8 ± 0.6 $P < 0.005$
Na^+_{iH}	−	30.4 ± 1.8*	10.6 ± 0.7*	19.8 ± 1.1*
Na^+_{iH}	+	29.4 ± 1.6*	10.0 ± 0.5*	19.4 ± 1.5*
Serum-stimulated rubidium efflux		−1.0 ± 1.2 NS	−0.6 ± 0.3 NS	−0.4 ± 1.4 NS

[a] Results are mean ± 1 SD for 3 separate estimations. * $P < 0.001$ as compared with corresponding values in (Na^+_{iL}) cells. NS = not significant.

Table 3. Effect of serum on ouabain-insensitive sodium influx and efflux measured in fibroblasts containing low (Na^+_{iL}) and high (Na^+_{iH}) internal sodium[a]

Internal sodium	+/− 10% FCS	Sodium movement (μmol Na^+/g protein/min)		Significance of difference between influx and efflux (P)
		Sodium influx	Sodium efflux	
Na^+_{iL}	−	21.24 ± 2.89	2.91 ± 0.37	<0.001
	+	36.80 ± 1.44	12.43 ± 1.72	<0.001
Serum-stimulated sodium transport		16.25 ± 3.90 $P < 0.001$	9.52 ± 1.35 $P < 0.001$	$P < 0.025$
Na^+_{iH}	−	46.29 ± 8.70*	37.80 ± 6.46*	NS
	+	66.64 ± 6.80	82.08 ± 11.90	<0.02
Serum-stimulated sodium transport		19.47 ± 4.37 $P < 0.001$	44.28 ± 10.02 $P < 0.001$	<0.001

[a] Results are mean ± 1 SD of four separate estimations. * $P < 0.001$ as compared with corresponding values in (Na^+_{iL}) cells. NS = not significant.

presented in Table 1, serum caused a stimulation of sodium-potassium pump-mediated rubidium influx in Na^+_{iL} cells but had no effect on this component in Na^+_{iH} cells. Serum had no effect on the ouabain-insensitive rubidium influx in either Na^+_{iL} or Na^+_{iH} fibroblasts.

Direct Effect of Serum on Sodium-Potassium-ATPase Activity

Sodium-potassium-ATPase activity was measured by enzymatically linking ATP hydrolysis to NADH oxidation [20]. The inclusion of 10% FCS in the assay cuvette did not alter the activity of the enzyme. The enzyme activity was measured over a range of sodium concentrations (4 to 20 mM) and a K_m of 11.11 mM sodium was obtained. This value did not change in the presence of serum (*data not shown*). Since the result presented in Table 1 had shown a serum-induced increase in ouabain-insensitive sodium efflux, we considered the possibility that the serum could have reduced the inhibitory effect of ouabain by binding with it and making it inaccessible to sodium-potassium-ATPase. To investigate this we measured the ouabain-inhibition of sodium-potassium-ATPase activity in the presence and absence of 10% FCS and found that there were no differences (*data not shown*). These results would suggest that the stimulation of sodium transport activity seen with serum is unlikely to be due to alterations in the kinetic properties of the enzyme sodium-potassium-ATPase.

Effect of Serum on Ouabain-Insensitive Sodium Transport

The effect of serum on ouabain-insensitive sodium movements was investigated by measuring both sodium influx and efflux rates in the presence of 1 mM ouabain in fibroblasts that had low and high internal

sodium concentrations. As shown in Table 3, Na^+_{iL} fibroblasts had 7 times more influx than efflux in the absence of serum. The presence of serum increased both influx and efflux; the serum-stimulated influx (16.3 ± 3.90 μmol sodium/g protein/min) was higher than serum-stimulated efflux (9.5 ± 1.4 μmol sodium/g protein/min) ($P < 0.025$). Similar measurements were made on cells preincubated in HBSS-potassium to achieve Na^+_{iH} status. In the absence of serum sodium influx exceeded efflux but to a lesser degree (only 1.2 times) than in Na^+_{iL} cells and this difference was not statistically significant. When serum was present, the rates of both sodium influx and efflux were significantly increased, but in contrast with the Na^+_{iL} cells, there was greater serum-stimulated efflux (44.3 ± 10.0 μmol sodium/g protein/min) than serum-stimulated influx (19.5 ± 4.4 μmol sodium/g protein/min) ($P < 0.001$). Thus depending on the level of intracellular Na^+ concentration, serum stimulates ouabain-insensitive sodium transport to produce either net influx or efflux.

Effect of Serum on Intracellular Sodium Content

Fibroblasts were incubated with 1 μCi $^{22}Na^+$ in HBSS for 1 hr in the presence and absence of 10% FCS and the internal sodium content (Na_i^+) was estimated at the end of this period. Similar measurements were made in the presence of 1 mM ouabain and the results are shown in the Figure. There were no differences in Na_i^+ between the cells incubated with and without serum. However, when the sodium-potassium pump was inhibited with ouabain, serum caused a significant rise in Na_i^+ ($P < 0.001$). This finding is in agreement with the results presented in Table 3 where serum-stimulated sodium influx exceeded efflux in ouabain-treated Na^+_{iL} cells.

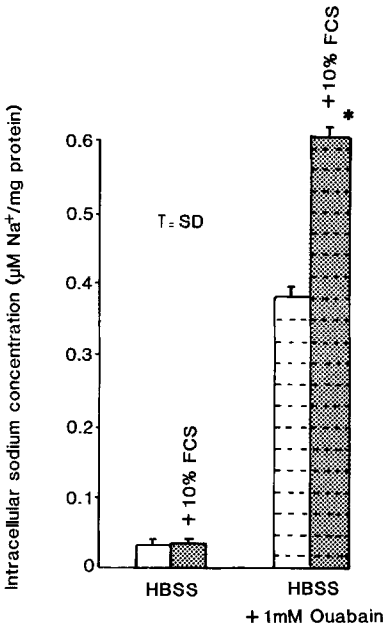

Figure. Effect of serum on intracellular sodium content of fibroblasts. Intracellular sodium was measured in fibroblasts after 1-hr incubation in HBSS (*see* Materials and Methods) in the absence (nonhatched bars) and presence (hatched bars) of 10% FCS. The dotted bars represent the presence of 1 mM ouabain in the incubation medium. * $P < 0.001$ compared to the corresponding value in the absence of 10% FCS

Effect of Amiloride

Several reports have shown that serum and growth factors act by stimulating an electroneutral amiloride-sensitive Na^+/H^+ exchange system [14, 15, 17]. To investigate if any of the above increases in sodium transport, either as a result of high intracellular sodium content or the addition of serum, could be attributable to this pathway, we measured the effect of 1 mM amiloride on ouabain-insensitive sodium influx and efflux in both Na^+_{iL} and Na^+_{iH} fibroblasts, in the presence and absence of 10% FCS. The results are shown in Tables 4 and 5. In Na^+_{iL} fibroblasts, the basal rate of sodium influx was amiloride-insensitive (Table 4). When serum was added, the increase in sodium influx (16.4 μmol sodium/g protein/min) was substantially inhibitable (61%) by 1 mM amiloride. On the other hand, in the Na^+_{iH} cells a significantly greater proportion of the influx was inhibitable by amiloride (19.4 μmol sodium/g protein/min) ($P < 0.01$). The addition of serum increased the sodium influx rate but the amiloride-sensitive component remained unchanged (18.5 μmol sodium/g protein/min).

Ouabain-insensitive sodium efflux measurements are shown in Table 5. In Na^+_{iL} cells the basal rate of sodium efflux was unchanged in the presence of amiloride and the serum-stimulated increase was not significantly inhibited by amiloride. In Na^+_{iH} cells a significant component of efflux was amiloride-sensitive (25 μmol sodium/g protein/min) and when serum was present this component was elevated (35.4 μmol sodium/g protein/min) but this only accounted for 31% of the serum-stimulated sodium efflux.

Collectively, these results indicate that the ouabain-insensitive serum-stimulated sodium fluxes are not completely amiloride-sensitive; other passive processes for sodium transport (e.g., sodium/sodium exchange) must also be involved.

Discussion

This study confirms other investigators' findings that serum increases sodium influx and sodium-po-

Table 4. Effect of 1 mM amiloride on ouabain-insensitive sodium influx[a]

Internal sodium status	+/− 10% FCS	+/− 1 mM amiloride	Sodium influx	Amiloride-sensitive sodium influx	Serum-stimulated sodium influx	% of serum-stimulated sodium influx inhibited by amiloride
			(μmol Na^+/g protein/min)			
Na^+_{iL}	−	−	22.4 ± 2.3			
	−	+	20.2 ± 3.0	2.2 NS		
	+	−	38.8 ± 4.6		16.4 $P < 0.01$	
	+	+	26.6 ± 1.9	10.2 $P < 0.02$	6.4 $P < 0.05$	61%
Na^+_{iH}	−	−	52.6 ± 5.8			
	−	+	33.2 ± 3.4	19.4 $P < 0.01$		
	+	−	69.4 ± 7.0		16.8 $P < 0.05$	
	+	+	50.9 ± 6.6	18.5 $P < 0.05$	17.7 $P < 0.02$	0%

[a] Results are mean ± 1 SD of three separate estimations. NS = not significant.

Table 5. Effect of amiloride on ouabain-insensitive sodium efflux[a]

Internal sodium status	+/− 10% FCS	+/− 1 mM amiloride	Sodium efflux	Amiloride-sensitive sodium efflux	Serum-stimulated sodium efflux	% of serum-stimulated sodium efflux inhibited by amiloride
			(μmol Na^+/g protein/min)			
Na^+_{iL}	−	−	2.4 ± 0.4			
	−	+	2.4 ± 0.4	0		
	+	−	10.8 ± 1.7		8.4 $P < 0.005$	
	+	+	9.4 ± 1.5	1.4 NS	7.0 $P < 0.005$	17%
Na^+_{iH}	−	−	36.6 ± 3.4			
	−	+	11.6 ± 1.5	25.0 $P < 0.001$		
	+	−	70.1 ± 6.8		33.5 $P < 0.005$	
	+	+	34.7 ± 4.2	35.4 $P < 0.001$	23.1 $P < 0.001$	31%

[a] Results are mean ± 1 SD of three separate estimations. NS = not significant.

tassium pump activity [18, 29–31,33]. However, our chief objective was to focus attention on sodium influx which other workers have concluded to be the activator of the sodium pump under the influence of serum [12, 23]. The results presented here show that intracellular sodium concentration rather than sodium influx is the chief activator of the sodium pump; indeed the rate of sodium influx itself is governed by the intracellular sodium status of the cell. Using fibroblasts with high and low intracellular concentrations of sodium (Na^+_{iH} and Na^+_{iL}), we have shown that serum increases both active and ouabain-insensitive components of sodium transport in Na^+_{iL} cells with a net influx. In contrast, serum does not cause any significant change in sodium-potassium pump activity in Na^+_{iH} cells as measured by active sodium efflux and rubidium influx; although it increased both sodium influx as well as ouabain-insensitive sodium efflux, it favored a net sodium efflux in Na^+_{iH} cells. These findings

emphasize the principal rule of cellular sodium transport, namely that cells regulate sodium fluxes through various processes to maintain their intracellular sodium concentration within normal limits. Activation of the sodium-potassium pump activity by elevated Na_i^+ has been reported in various cells including erythrocytes, nerve and muscle cells [5, 22, 26]. In this study, the intracellular sodium status determined the magnitude of changes in sodium fluxes caused by serum and the direction of the net ouabain-insensitive flux. Thus serum did not cause any increase in the active sodium efflux in Na_{iH}^+ cells which was probably at its maximum capacity under the influence of the high intracellular sodium concentration. Current methods of measuring cellular sodium are not sophisticated enough to determine the sodium content at the pump site before and during incubation of fibroblasts with serum or to detect any transient small changes. There is some evidence to suggest that sodium distribution inside the cell is heterogeneous [9, 24]. The increase of sodium influx caused by serum must make extra sodium available at the pump site and thereby activate the sodium pump and prevent any accumulation of intracellular sodium. This conclusion is borne out by the experiments summarized in the Figure; serum only caused an increase in cellular sodium content when the sodium pump activity was inhibited by ouabain. Further, serum does not directly stimulate sodium-potassium-ATPase activity (*unpublished observations*). These results together with those obtained from the Na_{iL}^+ and Na_{iH}^+ cells support the contention that serum stimulates the sodium-potassium pump by promoting the availability of sodium at the pump site. The results of this study suggest that the serum-induced effects are unlikely to be due to a simple link between an increase in sodium influx and activation of the sodium-potassium pump since serum affects both the active and ouabain-insensitive efflux channels. It could be argued that such a generalized effect of serum may be a result of increased permeability of the cell membrane. However, a comparison of serum-induced changes between Na_{iL}^+ and Na_{iH}^+ cells suggests that the serum effects are modified subject to intracellular sodium concentrations, a part of the overall strategy of cellular ion transport. The precise mechanism of these changes remains obscure.

There is accumulating evidence to show that serum or growth factors act by stimulating an electroneutral Na^+/H^+ exchange mechanism which is inhibitable by a diuretic drug amiloride [14, 15, 17]. We tested this drug on ouabain-insensitive sodium transport to assess the contribution of the Na^+/H^+ pathway to the stimulation of sodium fluxes resulting from the addition of serum and/or the elevation of Na_i^+ concentration. The results using fibroblasts in the Na_{iL}^+ state were easy to interpret. There were no basal levels of amiloride-sensitive sodium transport which is in agreement with other reports [14, 15, 33] and, like other workers, we found that a significant proportion (61%) of serum-stimulated influx was amiloride sensitive. Although sodium efflux rates were stimulated by serum these were not sensitive to amiloride and presumably did not use the Na^+/H^+ exchange pathway. When amiloride was tested on Na_{iH}^+ cells, there was a significant inhibition of the basal rates of sodium influx and efflux. Previous studies have shown that there are two ways to activate amiloride-sensitive sodium influx. First, the addition of serum or growth factors, and secondly an increase in intracellular Ca^{2+} concentration using Ca^{2+} specific ionophores can result in a dramatic increase in amiloride-sensitive sodium influx in fibroblasts [31–33]. There is also evidence to show that high Na_i^+ can trigger the release of Ca^{2+} from intracellular bound pools [1, 3, 10] which would explain the high levels of amiloride-sensitive sodium influx in Na_{iH}^+ cells. Addition of serum to these cells caused an increase in sodium influx but the amiloride-sensitive component remained unchanged.

In Na_{iH}^+ cells, there was a high basal level of amiloride-sensitive efflux and this level could be further stimulated by serum. It has been demonstrated in mouse neuroblastoma cells loaded with sodium and then exposed to sodium-free medium, that there was a net uptake of protons that was amiloride sensitive [13] which implied that the direction and magnitude of the transmembrane sodium gradient can affect the direction of Na^+/H^+ exchange. In Na_{iH}^+ cells, there would obviously be a reduction in the transmembrane sodium gradient which would explain how Na^+/H^+ exchange was occurring under these conditions in the Na^+-out, H^+-in mode.

In summary, we have shown that sodium-potassium pump activity is directed by the supply of Na_i^+ and that serum can increase this supply by promoting net influx. Under physiological conditions of internal and external sodium, this influx occurs via a unidirectional amiloride-sensitive pathway. In the absence of serum, this pathway can be activated by high levels of Na_i^+ but under these conditions this amiloride-sensitive pathway is bi-directional with a balance of sodium being effluxed. In addition serum shows a transstimulation of sodium fluxes in both cell states which we interpret as a serum-induced permeability change to sodium at the membrane level. Whether any, or all of these membrane-linked events are important in the mitogenic action of serum remains to be elucidated.

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