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Perturbation of Na^+ and K^+ gradients in human fibroblasts incubated in unsupplemented saline solutions

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Changes in the intracellular concentrations of Na^+ and K^+ of fetal human fibroblasts have been followed after replacement of serum-containing growth media with unsupplemented and serum-supplemented saline solution (Earle's balanced salt solution). Incubation in unsupplemented salt solution was followed by a progressive increase of the internal Na^+ counterbalanced by a decrease of internal K^+, without major alterations of the internal osmolarity. After 3 h incubation the intracellular Na^+ and K^+ concentrations were 120 mM and 50 mM, respectively. These intracellular ion derangements were not associated with a failure of the ($Na^+ + K^+$)-ATPase pump, whose activity actually increased with enhanced intracellular Na^+ concentration. Ion changes did not take place when serum (in excess of 0.5%, final concentration) was present in the saline solution and a complete restoration to normal of the Na^+ and K^+ gradients occurred upon addition of serum to cells previously incubated in plain saline solution. The effects of serum were mimicked by furosemide, thus suggesting that channels sensitive to this diuretic are involved in the movement of Na^+ and K^+ following fibroblast incubation in unsupplemented saline solution.

Introduction

Most mammalian cells maintain a high steady-state K^+ concentration and a low steady-state Na^+ concentration relative to their surrounding physiologic fluids. When cultured human fibroblasts are grown in a complete medium containing 145 mM Na^+ and 5.4 mM K^+, intracellular K^+ typically ranges between 150 and 160 mM, whereas intracellular Na^+ is lower than 20 mM. These transmembrane gradients depend on the activity of ($Na^+ + K^+$)-ATPase, the enzyme responsible for pumping Na^+ out of, and K^+ into the cell. In human fibroblasts, Na^+ and K^+ fluxes and redistribution across the cell membrane take place also through other pathways as an amiloride-sensitive Na^+/H^+ antiport (sodium inward [1,2]) and a furosemide-sensitive channel (Na^+-K^+-$2Cl^-$ symport [3]). Other routes less well characterized in fibroblast-like cells include a Na^+/Ca^{2+} exchanger [4], neurotoxin-sensitive Na^+ channels [5–7], Ca^{2+}-insensitive and Ca^{2+}-activated K^+ channels [8].

During studies on the transport of amino acids in cultured human fibroblasts [9–11], we became aware that the presence of serum was an obligatory requirement to maintain optimal metabolic conditions of the cells when they were transferred to a saline solution (derepressive phase of adaptive regulation, [11]). Recently we found that, in the absence of serum, cells incubated in saline solu-

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tions undergo a slight, though definite contraction of their volume and exhibit a dramatic change in their K^+ and Na^+ content [12]. Authors who used to incubate tissue slices or pieces in buffered salt solutions made similar observations [13,14]. The present study was undertaken to investigate the kinetics of the intracellular Na^+ and K^+ changes following serum deprivation and readdition to cells incubated in a saline solution. Moreover, attempts were made to identify the channels involved in the underlying transmembrane movements of these cations.

Methods and Materials

Cell culture. Fetal human fibroblasts, obtained from a 9-week gestational age, karyotipically normal male abortus, were routinely grown in 10-cm diameter dishes in Medium 199 containing 10% fetal calf serum and antibiotics (penicillin 100 I.U./ml, streptomycin 100 μg/ml). The conditions of culturing were: pH 7.4, atmosphere 5% CO_2 in air, temperature 37°C. For the experiments, cells were seeded onto 24-well plates (COSTAR) and used 4–5 days following subculture while still subconfluent (30 ± 10 μg of protein/cm^2 corresponding to $5–10 \cdot 10^4$ cells/cm^2). The culture medium was always renewed 48 h before the experiment. Fetal human fibroblasts were used between the 4th and 15th passage in vitro.

Incubations. In all experiments described in this paper cell monolayers were incubated at 37°C (atmosphere 5% CO_2 in air) in Earle's balanced salt solution (116 mM NaCl/26 mM $NaHCO_3$/5.4 mM KCl/1.8 mM $CaCl_2$/1 mM NaH_2PO_4/0.8 mM $MgSO_4$/5.5 mM glucose) in the absence or in the presence of dialyzed fetal calf serum or any other designated compound.

Measurement of Na^+ and K^+ content. Cell monolayers were rapidly washed four times with ice-cold 0.1 M $MgCl_2$ and drained for a few minutes. Ethanol (0.1 ml) was added to each well and allowed to dry; then 2 ml of cold 10 mM CsCl were added to each well. Intracellular sodium and potassium contents were determined with a Varian AA-275 atomic absorption spectrophotometer using NaCl and KCl in 10 mM CsCl as standards. After absorption spectroscopy determinations, cell monolayers were dissolved with 0.5% sodium deoxycholate in 1 M NaOH and assayed for proteins directly in the wells using a modified Lowry procedure [15] as described previously [16].

Measurement of K^+ influx. $^{86}Rb^+$ was used as a tracer for K^+ movements. This isotope has been shown to mimic K^+ movements in cultured human fibroblasts [3]. The uptake was assessed using the method for rapid measurement of solute fluxes in adherent cultured cells described by Gazzola et al. [16]. Human fibroblast monolayers were washed and incubated for 1 min at 37°C in 0.2 ml Earle's balanced salt solution containing 2 μCi/ml of ^{86}Rb (as a tracer of the 5.4 mM K^+ content of the solution). When present, ouabain (1 mM) was added 2 min before the uptake assay. The incubations were terminated by rinsing the cells four times with 4 ml of ice-cold 0.1 M $MgCl_2$ and drained for a few minutes. Ethanol (0.1 ml) was added to each well and allowed to dry; then 0.5 ml of 10 mM CsCl was added to each well. Extracts were added to 2.5 ml of scintillation fluid and counted for radioactivity with a Packard 460C liquid scintillation spectrometer. Protein content was determined as described above.

Measurement of cell water and data expression. Intracellular ion concentrations were expressed as

TABLE I

CELL WATER CONTENT IN FETAL HUMAN FIBROBLASTS INCUBATED IN EARLE'S BALANCED SALT SOLUTION IN THE PRESENCE OR IN THE ABSENCE OF SERUM

Cells grown in 10% fetal calf serum-containing Medium 199 were washed and incubated for 6 h in Earle's balanced salt solution (EBSS) or EBSS supplemented with 10% dialyzed fetal calf serum (FCS). At the times indicated, intracellular water was measured as indicated in Methods and Materials. The values are shown with the standard error for three determinations within four to six complete experiments.

Incubation time (h)	μl cell water/mg protein	
	EBSS	EBSS + 10% FCS
0.5	7.51 ± 0.41	7.65 ± 0.43
1	7.05 ± 0.46	7.55 ± 0.46
1.5	7.08 ± 0.50	7.50 ± 0.35
3	6.03 ± 0.70	6.74 ± 0.98
6	5.97 ± 0.58	6.66 ± 0.67

μmol per ml cell water. The intracellular fluid volume was estimated either from the steady-state distribution of 3-*O*-methyl-D-glucose [17] or from the difference between total water ([^{14}C]urea space) and extracellular fluid volume ([^{3}H]inulin space) [18]. Measurements were performed under each experimental condition at successive intervals during the experiment. Volume changes for fibroblasts incubated in the absence and in the presence of serum are shown in Table I. The addition of the various inhibitors for the proper time intervals did not substantially alter the values obtained in their absence.

Materials. Fetal calf serum, growth media and antibiotics were purchased from GIBCO. 3-*O*-Methyl-D-[U-^{14}C]glucose (329 Ci/mol), [^{14}C]urea (40 Ci/mol), [*methoxy*-^{3}H]inulin (152.5 mCi/g) and ^{86}Rb (20 Ci/g) were obtained from New England Nuclear. Furosemide was from Hoechst AG and amiloride was a generous gift of Merck, Sharp & Dohme. Sigma was the source of ouabain and all other chemicals.

Results

Changes in Na^+ and K^+ content upon serum deprivation and readdition

When the incubation medium of human fibroblasts was changed from Medium 199 containing 10% fetal calf serum to serum-free Earle's balanced salt solution, the intracellular concentration of Na^+ rose from 15 mM to 130 mM within 6 h of incubation. The rate of change was rather high during the first 3 h and low thereafter (Fig. 1A). Serum readdition to cells incubated in serum-free Earle's balanced salt solution for 3 h caused a rapid decrease of the accumulated internal cation. Within 2 h the concentration of Na^+ fell to values comparable to those observed in cells maintained in saline solution supplemented with 10% fetal calf serum. The intracellular K^+ concentration varied in an opposite manner (Fig. 1B), decreasing from 150 mM to 40 mM within 6 h incubation upon serum deprivation and being restored to normal within 2 h by serum readdition. Comparable results were obtained when fetal calf serum-containing minimal essential medium or Dulbecco's modified Eagle's medium replaced medium 199 for fibroblast culturing (not shown).

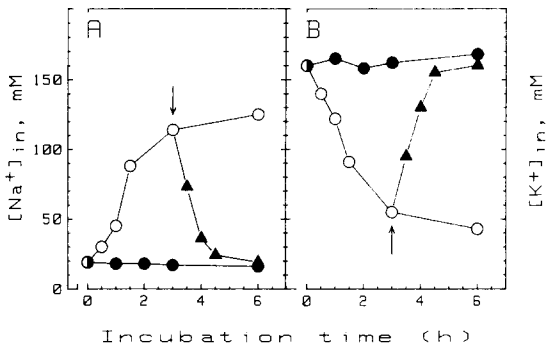

Fig. 1. Changes of Na^+ (panel A) and K^+ (panel B) intracellular concentration in fetal human fibroblasts incubated in Earle's balanced salt solution. Effects of serum deprivation and readdition. Cells grown in 10% fetal calf serum-containing Medium 199 (◑) were incubated for 6 h in Earle's balanced salt solution in the presence (●) or in the absence (○) of 10% dialyzed fetal calf serum. Dialyzed fetal calf serum (10%, final concn.) was added (▲) to cells incubated for 3 h in serum-free Earle's balanced salt solution (arrow). At the times indicated, cell monolayers were washed and the intracellular Na^+ and K^+ concentrations were determined as described in Methods and Materials. The points are means of triplicate determinations in a representative experiment. The experiment repeated five times yielded similar results.

TABLE II

CHANGES OF Na^+ AND K^+ INTRACELLULAR CONTENT IN FETAL HUMAN FIBROBLASTS INCUBATED IN EARLE'S BALANCED SALT SOLUTION SUPPLEMENTED WITH DECREASING CONCENTRATIONS OF SERUM

Cells grown in 10% fetal calf serum-containing Medium 199 were incubated for 3 h in Earle's balanced salt solution (EBSS) containing dialyzed fetal calf serum at the indicated concentrations. At the end of the incubation period, Na^+ and K^+ intracellular concentrations were assayed as described in Fig. 1. The values are means of triplicate determinations in a representative experiment. The experiment repeated twice yielded similar results.

Serum concentration (%) in EBSS	Intracellular concentration (mM)	
	Na^+	K^+
10	18	155
5	19	153
1	20	158
0.5	31	149
0.25	44	135
0.1	86	82
0.05	92	86
0.025	112	60
0	115	55

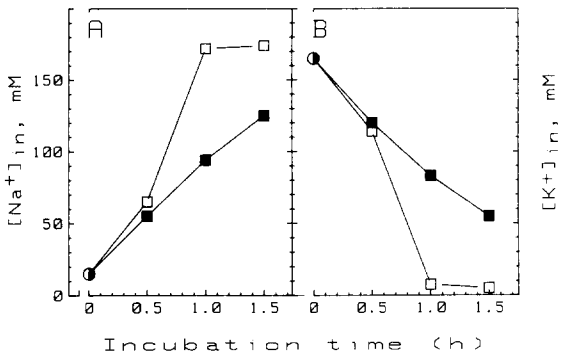

Fig. 2. Changes of Na^+ (panel A) and K^+ (panel B) intracellular concentration in fetal human fibroblasts incubated in Earle's balanced salt solution in the presence of ouabain. Effect of serum. Cells grown in 10% fetal calf serum-containing Medium 199 (◑) were incubated for 90 min in Earle's balanced salt solution containing 1 mM ouabain in the absence (□) or in the presence (■) of 10% dialyzed fetal calf serum. Na^+ and K^+ intracellular concentrations were assayed as described in Fig. 1. The points are means of triplicate determinations in a representative experiment. The experiment repeated three times yielded similar results.

Table II shows changes of Na^+ and K^+ content in fibroblasts whose fetal calf serum-containing Medium 199 was replaced with Earle's balanced salt solution supplemented with decreasing serum concentrations (from 10 to 0.025%). Concentrations of serum equal to or higher than 1% offered a complete protection against ionic derangements within a 3 h incubation period. The protective effect of serum was barely detectable at a 0.05% concentration.

Effect of ouabain

Upon replacement of Medium 199 with Earle's balanced salt solution containing 1 mM ouabain in the absence and in the presence of 10% fetal calf serum (Fig. 2) the fibroblast intracellular concentration of Na^+ increased sharply and the intracellular concentration of K^+ fell to very low values within 90 min incubation. The rate of change was higher in fibroblasts incubated in the absence of serum than in its presence.

Following a 3-h incubation in a serum-free Earle's balanced salt solution, fibroblasts exhibited the expected increase in Na^+ content and the subsequent serum-mediated restoration of Na^+ concentration to physiologic values (Fig. 3A). The effect of serum was fully prevented by ouabain

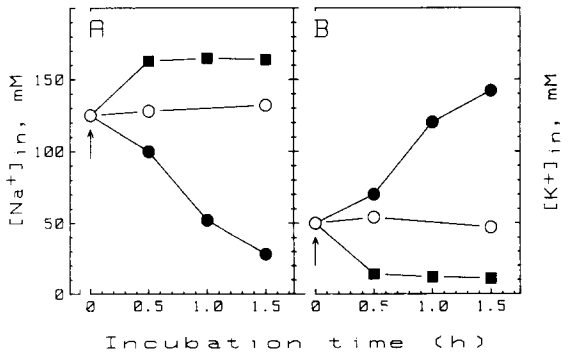

Fig. 3. Changes of Na^+ (panel A) and K^+ (panel B) intracellular concentration in fetal human fibroblasts incubated in Earle's balanced salt solution. Effect of ouabain on the restoration by serum of ionic gradients. Cells grown in 10% fetal calf serum-containing Medium 199 were incubated for 3 h in serum-free Earle's balanced salt solution. After this time (arrow), cells were incubated for additional 90 min in Earle's balanced salt solution (○) and in the same solution supplemented with 10% dialyzed fetal calf serum in the absence (●) or in the presence (■) of 0.1 mM ouabain. Na^+ and K^+ intracellular concentrations were assayed as described in Fig. 1. The points are means of triplicate determinations in a representative experiment. The experiment repeated three times yielded similar results.

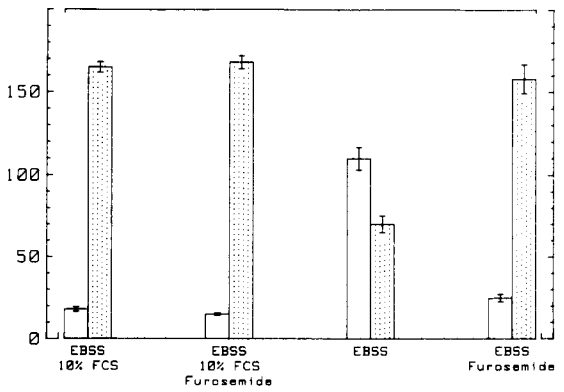

Fig. 4. Intracellular concentration of Na^+ and K^+ of fetal human fibroblasts incubated in Earle's balanced salt solution. Effect of furosemide. Cells grown in 10% fetal calf serum-containing Medium 199 were incubated in Earle's balanced salt solution (EBSS), EBSS plus 10% dialyzed fetal calf serum (FCS), EBSS plus 1 mM furosemide or EBSS containing 10% dialyzed fetal calf serum and 1 mM furosemide as indicated in the figure. Incubations in these media lasted 3 h. At the end, Na^+ (open bars) and K^+ (dotted bars) intracellular concentrations (mM) were assayed as described in Fig. 1. The values are the means of three independent determinations ± S.D.

(0.1 mM, final concentration), whose addition actually caused a further increase of the intracellular Na^+ concentration (up to values comparable to those of the external medium). In these experiments, changes in the internal K^+ concentration were opposite and, in the presence of the (Na^+ + K^+)-ATPase inhibitor, the intracellular to extracellular K^+ ratio approached unity (Fig. 3B).

Effect of furosemide

The addition of furosemide (1 mM, final concentration) to fibroblasts incubated in Earle's balanced salt solution in the absence of serum completely prevented the increase of intracellular Na^+ and the decrease of intracellular K^+ caused by serum deprivation (Fig. 4).

Furosemide was also effective in reestablishing normal intracellular Na^+ and K^+ concentrations when added to fibroblasts whose ionic content was previously altered by a 3-h incubation in serum-free Earle's balanced salt solution (Fig. 5). This restoration was faster and more complete in the presence of serum than in its absence.

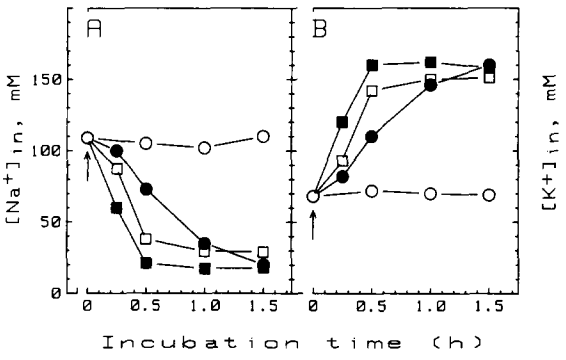

Fig. 5. Changes of Na^+ (panel A) and K^+ (panel B) intracellular concentration in fetal human fibroblasts incubated in Earle's balanced salt solution. Effect of serum and/or furosemide on the restoration of ionic gradients. Cells grown in 10% fetal calf serum-containing Medium 199 were incubated for 3 h in plain Earle's balanced salt solution. After this time (arrow), cells were incubated for additional 90 min in Earle's balanced salt solution (EBSS) (○), in EBSS containing 10% dialyzed fetal calf serum (●), in EBSS containing 1 mM furosemide (□) and in EBSS containing 10% dialyzed fetal calf serum plus 1 mM furosemide (■). Na^+ and K^+ intracellular concentrations were assayed as described in Fig. 1. The points are means of triplicate determinations in a representative experiment. The experiment repeated twice yielded similar results.

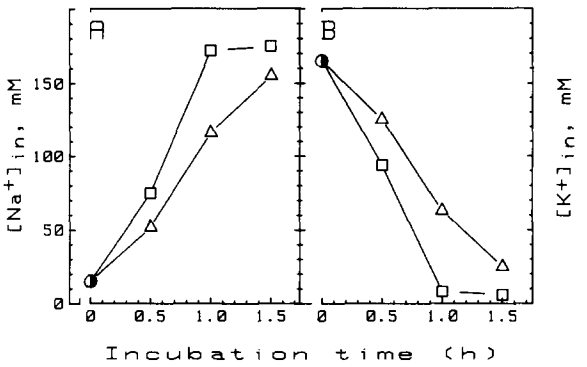

Fig. 6. Chagnes of Na^+ (panel A) and K^+ (panel B) intracellular concentration in fetal human fibroblasts incubated in Earle's balanced salt solution in the presence of ouabain. Effect of furosemide. Cells grown in 10% fetal calf serum-containing Medium 199 (◑) were incubated in Earle's balanced salt solution containing 1 mM ouabain without (□) and with 1 mM furosemide (△). Incubation in these media lasted 90 min. Na^+ and K^+ intracellular concentrations were assayed as described in Fig. 1. The points are means of triplicate determinations in a representative experiment. The experiment repeated twice yielded similar results.

Moreover, furosemide lowered the rate of change of the intracellular concentrations of Na^+ and K^+ following ouabain addition to cultured fibroblasts (Fig. 6).

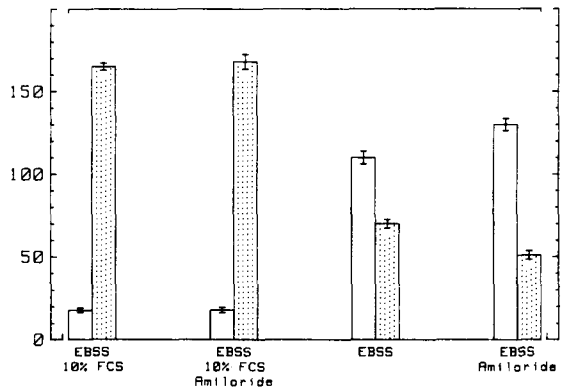

Fig. 7. Intracellular concentration of Na^+ and K^+ of fetal human fibroblasts incubated in Earle's balanced salt solution. Effect of amiloride. Cells grown in 10% fetal calf serum-containing Medium 199 were incubated in Earle's balanced salt solution (EBSS), EBSS plus 10% dialyzed fetal calf serum (FCS), EBSS plus 1 mM amiloride or EBSS containing 10% dialyzed FCS and 1 mM amiloride as indicated in the figure. Incubations in these media lasted 3 h. At the end, Na^+ (open bars) and K^+ (dotted bars) intracellular concentrations (mM) were assayed as described in Fig. 1. The values are the means of three independent determinations ± S.D.

Effect of amiloride

Amiloride (1 mM, final concentration) added to fibroblasts incubated in Earle's balanced salt solution was unable to alter the basal internal concentration of Na^+ and K^+ when serum was present in the incubation medium, and failed to prevent the ionic changes caused by serum deprivation; in fact it amplified them (Fig. 7).

Changes in $(Na^+ + K^+)$-ATPase activity upon serum deprivation and readdition

As measured by ouabain-sensitive ^{86}Rb uptake (Fig. 8) the activity of the fibroblast $(Na^+ + K^+)$-ATPase did not change for at least 6 h upon replacement of the culture medium (Medium 199) with Earle's balanced salt solution containing 10% fetal calf serum. Under these conditions, the ouabain-sensitive component amounted to approximately half of ^{86}Rb total uptake. When the culture medium was replaced with serum-free Earle's balanced salt solution, the activity of the pump increased dramatically within 60 min and decreased slowly thereafter. 6 h later it was still twice as high as in the presence of serum. Serum readdition to cells incubated in serum-free Earle's balanced salt solution for 3 h caused a transient increase of the pump activity, followed by a prolonged decrease of it down to values comparable to those observed in cells maintained in serum-supplemented Earle's balanced salt solution.

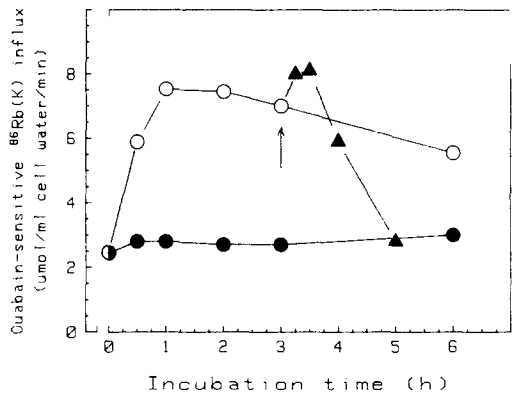

Fig. 8. Changes of ouabain-sensitive K^+ influx in fetal human fibroblasts incubated in Earle's balanced salt solution. Effects of serum deprivation and readdition. Cells grown in 10% fetal calf serum-containing Medium 199 (◑) were incubated for 6 h in Earle's balanced salt solution in the presence (●) or in the absence (○) of 10% dialyzed fetal calf serum. Dialyzed fetal calf serum (10% final concn.) was added (▲) to cells incubated for 3 h in serum-free Earle's balanced salt solution (arrow). At the times indicated, cell monolayers were washed and incubated in Earle's balanced salt solution containing ^{86}Rb (0.4 μCi/μmol of K^+) under conditions approaching initial entry rates (1 min) in the presence and in the absence of 1 mM ouabain. Ouabain-sensitive ^{86}Rb uptake was calculated as the difference between ^{86}Rb uptake in the absence and presence of the inhibitor. The points are means of triplicate determinations in a representative experiment. The experiment repeated twice yielded similar results.

Discussion

A marked increase of the internal Na^+ and a comparable marked decrease of internal K^+ takes place in cultured human fibroblasts when the culture medium containing serum is replaced by a serum-free saline solution. For these pronounced ionic changes to occur, the saline solution (for example Earle's balanced salt solution) must be devoid of serum (or contain serum at a concentration not exceeding 0.1%, see Table II) and of the usual nutrient supplements (except glucose). Indeed, no substantial ionic changes were recorded upon incubation of human fibroblasts in such nutrient-supplemented culture media as minimal essential medium, Dulbecco's modified Eagle's medium and Medium 199, either in the presence or in the absence of serum (unpublished results). Several authors focused their studies on the effects of serum on Na^+ and K^+ fluxes across the plasma membrane of fibroblasts and fibroblast-like cells [1,2,7,19–29]. In these investigations, complete (nutrient-supplemented) serum-free media or low serum-containing media have been used to arrest the cell cycle in the G_1/G_0 phase. Under these conditions, intracellular ionic changes are unlikely to occur or may not have been noticed. Only small decreases of K^+ intracellular concentration have been reported in some of these studies, though K^+ and Na^+ fluxes across the plasma membrane were markedly affected by serum removal and readdition.

In our experiments, Na^+ entering the cells largely counterbalanced K^+ loss, contributing to maintain the internal osmolarity at rather high values even when the internal K^+ concentration dropped to 40 mM. This fact explains why changes of intracellular water volume were quite small

even under extreme ionic derangements. The internal ionic changes caused by serum deprivation were reversible and a complete ionic restoration by serum readdition occurred even after 12 h of cell incubation in serum-free saline solution (not shown).

A furosemide-sensitive channel (Na^+-K^+-$2Cl^-$ symport) has been characterized in human fibroblasts [3]. In our experiments, furosemide completely prevented the alteration of the intracellular Na^+ and K^+ content caused by cell incubation in serum-free Earle's balanced salt solution (see Fig. 4) and allowed a restoration to normal of altered ionic gradients in cells incubated under conditions of serum deprivation (see Fig. 5). Moreover, furosemide partially counteracted ion redistribution associated with the inhibition of ($Na^+ + K^+$)-ATPase by ouabain (Fig. 6). Its restraining effect on the ouabain-mediated ionic changes was comparable to that of serum (see Fig. 2). These results confirm that a furosemide-sensitive channel is operative in human fibroblasts and suggest that this channel must be involved in the movement of Na^+ and K^+ (or at least of one of these cations) when serum deprivation alters the intracellular ionic concentrations.

Although our results suggest that the ionic flow through a furosemide-sensitive channel would be adequate to sustain in full the internal ionic alterations caused by serum deprivation, by no means do they rule out the contribution of other pathways transferring Na^+ and K^+ across the plasma membrane. Failure to observe an inhibitory effect of amiloride on the enhancement of the internal Na^+ content that follows the incubation of human fibroblasts in serum-free Earle's balanced salt solution (Fig. 7) renders the involvement of the amiloride-sensitive Na^+/H^+ antiport unlikely.

Conditions that allow ionic changes to occur (incubation of human fibroblasts in serum-free saline solutions) did not impair the function of ($Na^+ + K^+$)-ATPase and actually enhanced its activity several fold (Fig. 8). It has been reported that the activity of ($Na^+ + K^+$)-ATPase is modulated by internal Na^+, when this cation is the rate-limiting substrate [30]. Under our conditions, the internal Na^+ concentration is much higher than that required for half-maximal Na^+ extrusion [30–32]. Hence, the substrate-mediated modulation of the activity of the pump is likely to be superseded by its operation under saturation conditions. It has also been proposed that one of the two molecular forms of ($Na^+ + K^+$)-ATPase described in mesenchymal cells is hormonally sensitive [31,32]. Serum might stimulate the activity of this form. Indeed, the addition of serum to cells already filled up with saturating concentrations of Na^+ caused a transient enhancement of the pump activity (Fig. 8), and the restoration of altered ionic gradients by a combination of serum and furosemide occurred at a faster rate than restoration caused by furosemide alone (Fig. 5).

In conclusion, we propose that incubation of human fibroblasts in unsupplemented saline solutions renders these cells unable to maintain their steady-state Na^+ and K^+ gradients because of changes in ion permeability that the enhanced activity of the Na^+/K^+ pump is unable to compensate. We suggest that altered fluxes of Na^+ and/or K^+ take place through a deregulated furosemide-sensitive channel. Incubations of the cells in serum-containing media appear to prevent and counteract the effects of this change in cell permeability.

Acknowledgments

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