Unidirectional sodium and potassium flux in myogenic L6 cells: mechanisms and volume-dependent regulation

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Sen, Chandan K., Osmo Hänninen, and Sergei N. Orlov. Unidirectional sodium and potassium flux in myogenic L6 cells: mechanisms and volume-dependent regulation. *J. Appl. Physiol.* 78(1): 272–281, 1995.—To clarify the relative participation of particular ion transport systems in net univalent cation fluxes under basal conditions and altered volume of skeletal muscle-derived cells, the effect of inhibitors of the Na^+-K^+ pump (ouabain), univalent ion cotransporters [bumetanide, furosemide, and (dihydroindenyl)oxy alkanoic acid], and Na^+/H^+ exchanger (ethylisopropylamiloride) on ^{86}Rb and ^{22}Na fluxes has been studied in L6 myoblasts incubated in isosmotic (320 mosmol/kg) and anisosmotic media. Under the isosmotic condition, the relative contribution of ouabain-inhibited and ouabain-insensitive bumetanide-inhibited component of ^{86}Rb influx was ~15–20 and 60%, respectively. ^{22}Na influx was inhibited by bumetanide and ethylisopropylamiloride by 25 and 15%, respectively. Under isosmotic conditions, an increase of L6 cell volume was observed after addition of extracellular acetylcholine, extracellular K^+-induced depolarization, or lowering of the pH of the incubation medium. High extracellular glutathione (150 μM) did not affect the cell volume of the muscle-derived cells bathed in isosmotic medium. Results of this study suggest that the bumetanide-inhibited component of K^+ influx plays a key role in the adjustment of transmembrane K^+ gradient in L6 myoblasts. The Na^+/H^+ exchanger appears to be important in regulatory volume increase.

muscle; ion transport; sodium/hydrogen exchanger; sodium-potassium pump; univalent ion cotransporters; glutathione; inhibitors

ELECTROLYTE REGULATION in skeletal muscle is fundamental to electromechanical coupling and to the development of fatigue (4, 22). Intense muscular contraction remarkably modifies intracellular electrolyte concentrations. The sum total of such ionic changes is manifested as a marked increase in intracellular H^+ concentration (22). This intracellular acidosis has been suggested to impair regulatory and contractile protein function and calcium regulation and thus contributes to the development of skeletal muscle fatigue. Na^+-K^+ pump and carriers play a key role in the adjustment of electrochemical univalent ion gradients and cell volume. The introduction of the skeletal muscle-derived clonal L6 cell line as a model for experimental research (33) was soon followed by a series of studies related to nerve-muscle interaction and development of action potentials (12, 15). All such investigations employed electrophysiological techniques to study membrane potentials and permeability of ion channels. Although the properties of several ionophores have been studied in L6 cells (9, 27), data on the relative activity of the Na^+-K^+ pump and univalent ion carriers in L6 cells or any other skeletal muscle-derived cultured cells are lacking. We were therefore interested to study the participation of different ion transport systems in inward and outward potassium (^{86}Rb) and sodium (^{22}Na) fluxes in L6 cells.

It is known that feedback regulation of volume is a fundamental property of most cell types (13). In human and animal skeletal muscles, marked shifts of water and electrolytes after electrical stimulation or intense exercise have been documented. Intense muscular contraction was described to be accompanied by cell swelling, a 6–20% decrease in intracellular K^+ concentration, a twofold increase in intracellular Na^+ concentration ($[Na^+]_i$), and variable increases in intracellular Cl^- concentration ($[Cl^-]_i$). Potassium efflux from contracting muscle cells dramatically decreases the intracellular-to-extracellular potassium ratio, leading to depolarization of sarcolemmal t-tubular membranes (22). To our knowledge, there is no report on the volume-dependent mechanisms of ion flux regulation, regulatory volume increase (RVI), or regulatory volume decrease (RVD) in skeletal muscle-derived cells. We carried out experiments on the volume-dependent regulation of sodium and potassium fluxes in L6 cells. This study provides the first direct evidence regarding the nature in which K^+ and Na^+ transport systems in the skeletal muscle-derived cells are affected by changes in cell volume, which are known to be associated with intense exercise. We also studied the effects of high extracellular K^+-induced depolarization, lowered extracellular pH, and high extracellular glutathione on cell volume. All the above-cited conditions may be manifested as consequences of exhaustive exercise. Reduced glutathione (GSH), a sulfhydryl tripeptide anionic in physiological pH, is known to serve antioxidant and detoxicant functions in the body and has been shown to be synthesized by L6 myoblasts as well (29). Thiols are known to modulate K^+-Cl^- cotransport in erythrocytes. The sulfhydryl reagent iodoacetamide and *N*-methylmaleimide sharply reduced the capability of Cl^--dependent K^+ transport to regulate its activity in response to cell volume changes (19). Because exhaustive exercise is associated with a remarkable rise in plasma GSH (20), we were also interested to know about the possible effect of high concentrations of extracellular GSH on muscle cell volume. We also studied the effect of acetylcholine-induced hyperpolarization on the cell volume of L6 myoblasts.

MATERIALS AND METHODS

Materials. L6 cells (33) from American Type Culture Collection (Rockville, MD) were provided by Dr. P. Rahkila of

the University of Jyväskylä. Dulbecco's modified Eagle's medium, fetal calf serum, and other reagents for cell culture were purchased from GIBCO (Grand Island, NY). The radioisotopes ^{86}RbCl, ^{22}NaCl, and 3-*O*-methyl-D-[^{14}C]glucose were obtained from Amersham (Buckinghamshire, UK). Ethylisopropylamiloride (EIPA) was obtained from Ciba Geigy (Basel, Switzerland). (Dihydroindenyl)oxy alkanoic acid (DIOA) was synthesized (8) and provided as a gift by Dr. Edward J. Cragoe, Jr. Ouabain was obtained from Serva Feinbiochemica (Heidelberg, Germany). *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), choline chloride, 3-*O*-methyl-D-glucose, bumetanide, and furosemide were purchased from Sigma Chemical (St. Louis, MO). All other chemicals were of the highest purity available and were obtained from Merck AG (Darmstadt, Germany) or BDH (Poole, UK). Cell culture dishes were purchased from A/S Nunc (Roskilde, Denmark).

Cell culture. Monolayer cultures were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 5 mM glutamine, 0.3% D-glucose, 50 U/ml of penicillin, and 50 μg/ml of streptomycin in humidified air containing 10% CO_2 at 37°C. Cell viability was >95% as estimated by trypan blue exclusion. The cells were split once every 2 days. All experiments were carried out with myoblast of the fourth to the tenth passage. About 12–14 h (lag phase of cell multiplication) before ion-flux studies, cells were seeded in either 24-well ($4–5 \times 10^5$ cells/well; for measurements of ^{86}Rb uptake and loss) or 6-well ($1.5–2 \times 10^6$ cells/well; for measurement of ^{22}Na uptake and determination of volume of intracellular water) culture dishes as required.

Determination of ^{86}Rb influx. ^{86}Rb influx was studied as described previously (25), with minor modifications reported below. L6 cells (seeded in 24-well plates; $4–5 \times 10^5$ cells/well) were washed with 2 × 1-ml aliquots of *medium A* (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ in 10 mM Tris-HEPES buffer, pH 7.4) and preincubated in 0.5 ml of *medium B* (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, and 10 mM D-glucose in 20 mM Tris-HEPES buffer, pH 7.4) for 10 min at 37°C. In some experiments, this preincubation medium contained ion transport inhibitors or other compounds at twice the concentration indicated in the legends of Figs. 2, 6, and 8–10. After the preincubation as indicated above, 0.5 ml of *medium B* containing 1–2 μCi/ml of ^{86}RbCl was added to each well and the incubation was continued for another 2, 5, 10, or 20 min to study the uptake kinetics (see Fig. 1*A*). For other experiments (see Figs. 2, 6, and 8–10), the incubation with the radioisotope salt was carried out for 10 min. When required (see Figs. 6 and 8–10), changes in the total osmolality of the incubation medium (in the range 200–620 mosmol/kg) were made by either decreasing the concentration of NaCl (up to 20 mM) or increasing the concentration of sucrose (up to 600 mM) in the incubation medium (*medium B* with ^{86}Rb). Osmolalities of the incubation media were measured by a milliosmometer (Knauer, Hamburg, Germany). In some experiments (see Figs. 8 and 9), NaCl of the incubation medium was substituted by an equimolar amount of choline chloride. The uptake of ^{86}Rb was terminated after appropriate time intervals by the addition of 1.5 ml of ice-cold *medium C* (100 mM $MgCl_2$ in 5 mM Tris-HEPES, pH 7.4). Dishes were immediately placed on ice, and the cells were washed with 5 × 1.5 ml of ice-cold *medium C*. After the aspiration of *medium C*, 1 ml of 5% sodium dodecyl sulfate solution containing 2 mM Na_2EDTA was added to each well to lyse the cells. The cell lysate (or incubation medium, 1 ml) followed by 4 ml of OptiPhase HiSafe3 (LKB Wallac, Turku, Finland) was then added to scintillation vials. Radioactivities in the incubation medium and in the cell lysate were determined by a 1214 RACKBETA liquid scintillation counter (LKB Wallac). ^{86}Rb influx (nmol · 10^6 cells^{-1} · min^{-1}) was calculated as $A(ant)^{-1}$, where A is the radioactivity (counts/min) in the cell lysate, a is the specific radioactivity (counts · min^{-1} · nmol^{-1}) of the incubation medium, n is the number of 10^6 cells per well, and t is the time interval (in minutes) of incubation of the cells with the isotope.

Determination of ^{86}Rb efflux. L6 cells (seeded in 24-well plates; $4–5 \times 10^5$ cells/well) were prelabeled with ^{86}Rb by incubating the cells with 0.5 ml/well of *medium B* containing 3–4 μCi/ml of ^{86}RbCl. The incubation was carried out at 37°C and lasted for 4 h. After the incubation, the 24-well culture plates were placed on ice and the radioactive incubation medium was aspirated. The cells were then washed with 5 × 1.5-ml aliquots of *medium A*. To initiate ^{86}Rb efflux, 1 ml of prewarmed (37°C) *medium B* containing 1 mM ouabain was added to each well. Cells were incubated at 37°C for 2, 5, 10, 15, 20, or 25 min for the study of ion loss kinetics (see Fig. 1*B*). For other experiments, the incubation for ^{86}Rb efflux was carried out for 5 min. After the appropriate time interval of incubation, 1.5 ml of an ice-cold solution of 150 mM NaCl in 10 mM Tris-HEPES (pH 7.4 at 4°C) were added and the medium (overlaying buffer) from each well was transferred to scintillation vials. To determine the amount of ^{86}Rb still remaining within the cells, cell lysates were obtained as described above and transferred to scintillation vials. Values of ^{86}Rb efflux were expressed as the percentage of initial ^{86}Rb content (as a result of prelabeling) in the cells and was calculated as $(100A_1)(A_1 + A_2)^{-1}$, where A_1 is the radioactivity in the withdrawn medium (overlaying buffer) and A_2 is the residual radioactivity in the cell lysate.

Determination of ^{22}Na influx. ^{22}Na influx was studied as described previously (26, 32), with minor modifications as described below. L6 cells (seeded in 6-well plates; $1.5–2 \times 10^6$ cells/well) were washed with 2 × 3-ml aliquots of *medium A* at room temperature. The cells were then preincubated in 0.5 ml of *medium B* containing 1 mM ouabain for 10 min at 37°C. In some experiments (see Figs. 3 and 7), the preincubation medium contained either bumetanide or EIPA in twice the concentration indicated in the legends of Figs. 3 and 7. After the 10 min of preincubation, 1 ml of *medium B* containing 3–4 μCi ^{22}NaCl was added to each well and the incubation was continued for another 1, 2, 3, 5, 7, or 10 min to study the uptake kinetics (see Fig. 1*C*). For other experiments (see Figs. 3 and 7), the incubation with the radioisotope was carried out for 3 min. When required (see Fig. 7), changes in the total osmolality of the incubation medium were made as described above. After the appropriate time interval of incubation, ^{22}Na uptake was terminated by the addition of 3 ml of ice-cold *medium C* (100 mM $MgCl_2$ in 5 mM Tris-HEPES, pH 7.4) to each well. The multiwell plates were placed on ice, and the cells were washed with 5 × 3 ml of *medium C* per well. After the final aspiration of *medium C*, cell lysates were prepared and radioactivity was determined as described earlier. ^{22}Na influx (nmol · 10^6 cells^{-1} · min^{-1}) was calculated as $A(ant)^{-1}$.

Determination of intracellular water space. The principle of equilibrium distribution of the nonmetabolizable hexose, 3-*O*-methyl-D-[^{14}C]glucose, as suggested by Kleitzen et al. (16) and described previously (26), was used. L6 cells (seeded in 6-well plates; 2×10^6 cells/well) were washed with 2 × 3-ml aliquots of *medium A* at room temperature. The cells were then incubated in 1 ml (per well) of a solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM 3-*O*-methyl-D-glucose in 20 mM Tris-HEPES buffer, pH 7.4 at 37°C) containing 0.8 μCi/ml of 3-*O*-methyl-D-[^{14}C]glucose. In some experiments, either the composition of the incubation medium was altered or other compounds were added as mentioned in the legend of Fig. 5. Osmolality of the incubation medium

was adjusted as described earlier. After 30 min of incubation, the radioactivity-containing buffer was collected from each well and the cells were washed with 5 × 3-ml aliquots of ice-cold *medium A*. Cell lysates were prepared as described earlier, and the radioactivities in the cell lysate fraction and the incubation buffer samples were quantitated by liquid scintillation counting as described earlier. Intracellular water space (V) was calculated as $V = (A_l V_m) A_m^{-1}$, where A_l is the radioactivity of the lysate, A_m is the radioactivity of the medium, and V_m is the volume of the medium used for the determination of A_m.

Statistical analysis. Results are expressed as means ± SD of either three or four different experiments as indicated in the respective figure legends. Data of each experiment were obtained as means of quadruplicates. As required, the difference between means was tested by Student's *t*-test for unpaired data or one-way analysis of variance.

RESULTS

Kinetics of potassium (^{86}Rb) and sodium (^{22}Na) uptake and potassium (^{86}Rb) loss. In the L6 myoblasts, the kinetics of potassium uptake were studied for up to 20 min of incubation with the radioisotope. The kinetics were observed to be linear over the studied period (Fig. 1*A*). Figure 1*B* shows that the curve representing the kinetics of potassium loss was linear at least up to the first 10–15 min. Figure 1*C* represents the kinetics of sodium uptake into the L6 myoblasts incubated in a buffer containing ^{22}Na. Linear uptake kinetics were observed up to 5 min, after which the curve tended to plateau. On the basis of the above-mentioned observations, 10, 5, and 3 min were chosen to be the time intervals of incubation to estimate ^{86}Rb uptake, ^{86}Rb loss, and ^{22}Na uptake, respectively, in subsequent experiments.

Effect of ion transport inhibitors on ^{86}Rb and ^{22}Na flux under isosmotic conditions. Under isosmotic conditions, ^{86}Rb influx in L6 myoblasts was ~3 nmol · 10^6 cells^{-1} · min^{-1} (Fig. 2). Addition of 1 mM ouabain to the incubation medium led to a decrease in ^{86}Rb influx by 10–20%. In the presence of 1 mM ouabain in combination with 10 μM bumetanide, ^{86}Rb influx was 70–80% lower compared with the control. No effect on ^{86}Rb influx was observed on addition of 30 μM DIOA to the combination of ouabain + bumetanide inhibitors. Figure 2 (*inset*) illustrates the relative contribution of ouabain-inhibited, ouabain-insensitive bumetanide-inhibited, ouabain + bumetanide-insensitive DIOA-inhibited, and ouabain + bumetanide + DIOA-insensitive components of ^{86}Rb influx. Data are expressed as percentage of total ^{86}Rb influx. The relative contribution of the Na^+-K^+ pump (ouabain-inhibited component), Na^+-K^+-$2Cl^-$ cotransport (ouabain-insensitive bumetanide-inhibited component), and passive permeability ("leakage") for potassium (ouabain + bumetanide + DIOA-insensitive component) to the total ^{86}Rb influx in L6 myoblasts are also represented in Fig. 2 (*inset*).

Direct evidence of inhibition by bumetanide of electroneutral sodium, potassium, and chloride symport (Na^+-K^+-$2Cl^-$ cotransport) had been obtained for erythrocytes of several avian and mammalian species, as well as for the Ehrlich ascites tumour cells (10). Recently it was shown that DIOA is a specific inhibitor of K^+-Cl^- cotransport in human erythrocytes (8). We did not observe any effect of DIOA on ^{86}Rb influx in the L6 myoblasts (Fig. 2, *inset*).

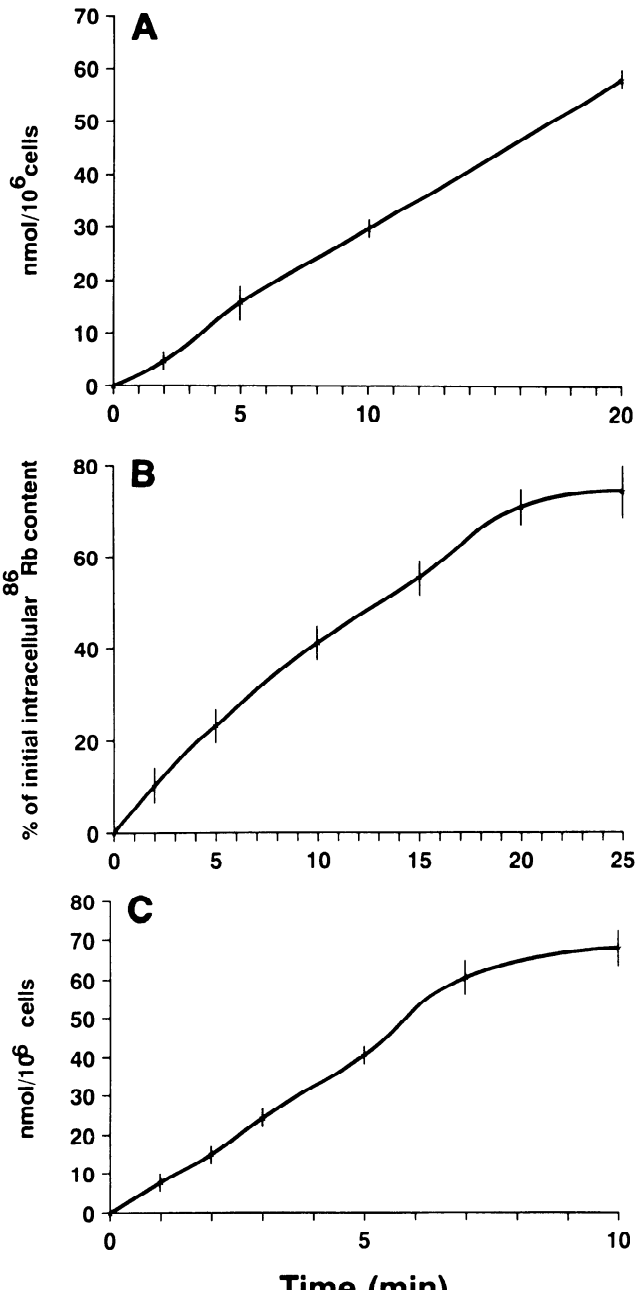

FIG. 1. Kinetics of ^{86}Rb uptake (*A*), ^{86}Rb loss (*B*), and ^{22}Na uptake (*C*) in L6 myoblasts at 37°C. For these experiments, isosmotic transport medium contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 10 mM D-glucose in 20 mM HEPES-Tris buffer, pH 7.4 (*medium B*). For ^{86}Rb uptake study (*A*), no ion transport inhibitor was added to *medium B*. For experiments concerning ^{86}Rb loss (*B*) and ^{22}Na uptake (*C*), 1 mM ouabain was added to *medium B*. Data are means ± SD; $n = 4$ experiments.

In experiments related to the study of ^{86}Rb efflux from prelabeled L6 myoblasts, 1 mM ouabain was added to the incubation medium to block Na^+-K^+ pump-mediated efflux of K^+ by way of K^+/K^+ exchange (6). No significant effect of either 10 μM bumetanide or 30 μM DIOA on ^{86}Rb efflux was observed (results not shown).

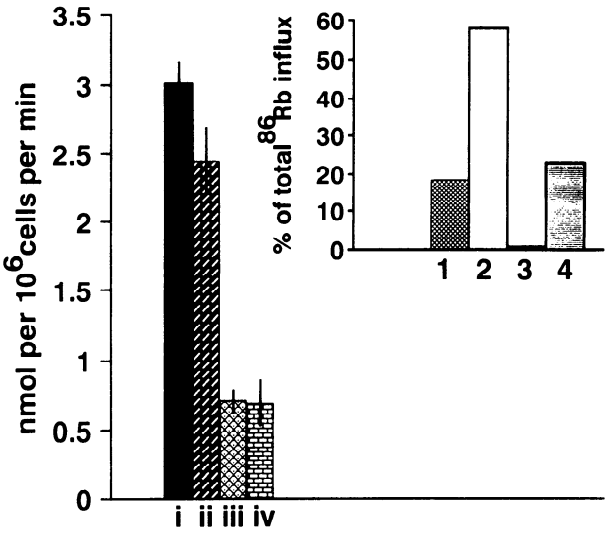

FIG. 2. Effect of ion transport inhibitors on ^{86}Rb influx in L6 myoblasts. Influx experiment was carried out in isosmotic *medium B* at 37°C. i, Control (*medium B* containing no ion transport inhibitor); ii, *medium B* containing 1 mM ouabain; iii, *medium B* containing 1 mM ouabain + 10 μM bumetanide; iv, *medium B* containing 1 mM ouabain + 10 μM bumetanide + 30 μM (dihydroindenyl)oxy alkanoic acid (DIOA). Data are means ± SD; n = 4 experiments. *Inset*: relative contribution of different ion transport system studied to total ^{86}Rb influx capacity (i of main graph) of L6 cells. 1, Ouabain-sensitive component; 2, ouabain-insensitive bumetanide-inhibited component; 3, ouabain + bumetanide-insensitive DIOA inhibited component; 4, ouabain + bumetanide + DIOA-insensitive component. Incubation of cells with ^{86}RbCl was carried out for 10 min.

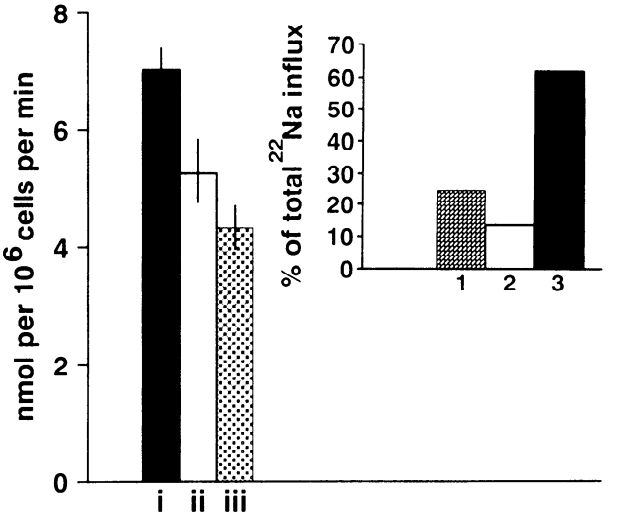

FIG. 3. Effect of ion transport inhibitors on ^{22}Na influx in L6 myoblasts. Influx experiment was carried out in isosmotic *medium B* at 37°C. i, Control (*medium B* containing 1 mM ouabain); ii, *medium B* containing 1 mM ouabain + 10 μM bumetanide; iii, *medium B* containing 1 mM ouabain + 10 μM bumetanide + 10 μM ethylisopropylamiloride (EIPA). Data are means ± SD; n = 4 experiments. *Inset*: relative contribution of different ion transport systems studied to total ^{22}Na influx capacity of L6 cells. 1, Ouabain-insensitive bumetanide inhibited component; 2, ouabain + bumetanide-insensitive EIPA-inhibited component; 3, ouabain + bumetanide + EIPA-insensitive component. Incubation of cells with ^{22}NaCl was carried out for 3 min.

Figure 3 illustrates that in the presence of ouabain ^{22}Na influx in L6 myoblasts is ~7 nmol · 10^6 cells^{-1} · min^{-1}. After addition of 10 μM bumetanide and combined addition of bumetanide and 10 μM EIPA, ^{22}Na influx decreased to ~5 and 4 nmol · 10^6 cells^{-1} · min^{-1}, respectively. Therefore, we see that the relative contribution of Na^+-K^+-$2Cl^-$ cotransport (ouabain-insensitive bumetanide-inhibited component) and Na^+/H^+ exchanger (ouabain + bumetanide-insensitive EIPA-inhibited component) to unidirectional ^{22}Na influx under basal conditions was ~25 and 15%, respectively. The ion transport system(s) involved in ouabain + bumetanide + EIPA-insensitive ^{22}Na influx in L6 myoblasts is yet unknown.

Effect of osmolality, high extracellular K^+, pH, extracellular glutathione, acetylcholine, and inhibitors of ion transport systems on cell volume. Boyle-van't Hoff's law states that the volume of an ideal osmometer is inversely proportional to the osmolality of the incubation medium. Figure 4 demonstrates that in the L6 cells the line representing the dependence of the volume of intracellular water on the inverse osmolality of the incubation medium is rather linear at the physiological domain (280–420 mosmol/kg); however, at the two (i.e., hypo- and hyper-) extremes of osmolality bending of the line was observed. This anomalous behaviour of the cells at the two extremes of osmolality may be due to increased solute influx at very high osmolality (bending of the line toward the x-axis due to RVI) and the absence of bending in the same direction due to the absence of RVD. An upward slope change suggests additional cell swelling not compensated by RVI as borne

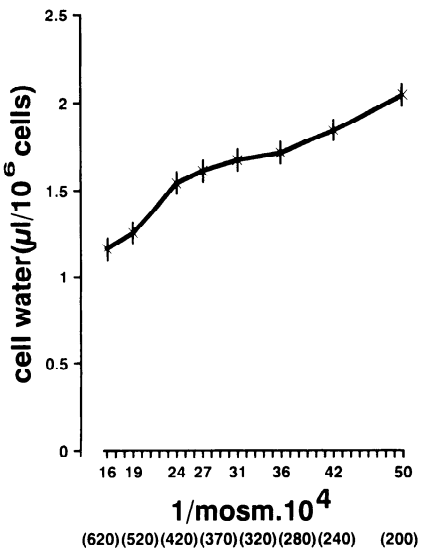

FIG. 4. Dependence of volume of intracellular water in L6 myoblasts on inverted osmolality (Π^{-1}) of incubation medium. Values of (mosmol/kg)$^{-1}$ × 10^4 values are plotted on y-axis with corresponding values of osmolality enclosed in parentheses. Data are means ± SD; n = 4 experiments.

out by the data on ^{86}Rb and ^{22}Na leaks not sensitive to inhibitors. Results presented in Fig. 4 were used for the presentation of data on volume-dependent regulation of univalent ion flux (see Figs. 6–9).

Under isosmotic conditions (320 mosmol/kg), extracellular GSH (150 μM), ouabain (1 mM), ouabain + bumetanide (10 μM), or ouabain + bumetanide + DIOA (30 μM) did not affect intracellular water content (Fig. 5). Extracellular presence of 1 mM acetylcholine or low-

ered pH (7.0) caused a significant swelling of the cells. About 12–16% increase of cell volume was observed when cells were exposed to a medium with high concentration of K^+.

Dependence of univalent cation fluxes on intracellular water content. Compared with cells under isosmotic conditions (320 mosmol/kg), swelling of the cells in a hyposmotic medium increased Na^+-K^+ pump activity. At 200 mosmol/kg (2.04 μl cell water/10^6 cells), the activities of the Na^+-K^+ pump and the ouabain-insensitive bumetanide-inhibited component of ^{86}Rb influx (Fig. 6) were about the same. The activity of the pump decreased in hyperosmotic conditions until its activity could be hardly detected at 420 mosmol/kg. A further shrinkage of the cell remarkably increased the contribution of the pump to inward potassium (^{86}Rb) flux. At 620 mosmol/kg (1.16 μl cell water/10^6 cells), the activity of the Na^+-K^+ pump was ~60% of the bumetanide-inhibited component of ^{86}Rb influx.

Shrinkage of the L6 myoblasts under hyperosmotic conditions resulted in a remarkable increase in the ouabain-insensitive bumetanide-inhibited component of ^{86}Rb influx. Compared with cells under isosmotic conditions, an 8% decrease in intracellular water content caused a 100% increase in the activity of the above-mentioned component (Fig. 6). However, a further increase in the activity of the ouabain-insensitive bumetanide-inhibited component of ^{86}Rb influx was not seen in cells exposed to a more hyperosmotic environment. The ouabain- and bumetanide-insensitive component of ^{86}Rb influx, usually identified as a passive diffusion or leakage (2), was not dependent on intracellular water content of the L6 myoblasts (Fig. 6). Swelling of cells in hyposmotic medium also caused a 25–35% increase in the activity of the transport system(s) involved in ^{86}Rb efflux (results not shown). Unlike ^{86}Rb influx, the activity of the pathways of ^{86}Rb efflux was not affected by even 25% of cell shrinkage. However, further shrinkage of the cell appeared to activate ^{86}Rb efflux by ~15%.

Unlike that during ^{86}Rb influx (Fig. 6), alterations in cell volume did not have any effect on the ouabain-insensitive bumetanide-inhibited component of ^{22}Na influx in the L6 myoblasts (Fig. 7). The activity of the Na^+/H^+ exchanger (ouabain + bumetanide-insensitive EIPA-inhibited component of ^{22}Na influx) was considerably higher in shrunken cells placed in hyperosmotic medium. Compared with cells in isosmotic medium, a 25% decrease in intracellular water content resulted in an eightfold increase in the activity of the exchanger. The activity of the ouabain + bumetanide + EIPA-insensitive component of ^{22}Na influx in the L6 myoblasts increased by 25% with cell swelling. No comprehensive effect was seen even at 25% of cell shrinkage. A further decrease in cell volume sharply increased (20–30%) the activity of the above-described component of ^{22}Na influx.

Effect of extracellular sodium (Na_o^+) on volume-dependent regulation of ^{86}Rb influx. Figure 8 illustrates that under isosmotic conditions an equimolar substitution of extracellular sodium with choline resulted in an 80% decrease in the activity of the Na^+-K^+ pump (ouabain-inhibited ^{86}Rb influx). Such an effect is proba-

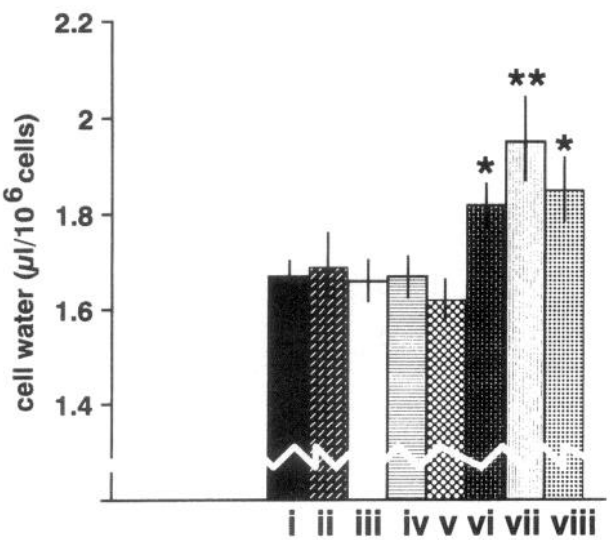

FIG. 5. Intracellular water content of L6 myoblasts under various conditions of incubation medium (isosmotic *medium B* at 37°C). i, Control (incubation medium comprised of *medium B* only); ii, *medium B* containing 150 μM of reduced glutathione; iii, *medium B* containing 1 mM ouabain; iv, *medium B* containing 1 mM ouabain + 10 μM bumetanide; v, *medium B* containing 1 mM ouabain + 10 μM bumetanide + 30 μM DIOA; vi, *medium B* containing 1 mM acetylcholine; vii, *medium B* containing higher concentration of K^+ (90 mM NaCl and 55 mM KCl instead of 140 mM NaCl and 5 mM KCl; other composition of *medium B* remained unchanged); viii, medium (140 mM NaCl and 5 mM KCl in 20 mM Tris-HEPES) with lowered pH (pH 7.0 at 37°C). Data are means ± SD; $n = 4$ experiments. Means were compared with control using Student's t-test for unpaired data: $^*P < 0.05$; $^{**}P < 0.01$. Under isosmotic conditions (320 mosmol/kg), extracellular reduced glutathione (150 μM), ouabain (1 mM), ouabain + bumetanide (10 μM), or ouabain + bumetanide + DIOA (30 μM) did not affect intracellular water content. Extracellular presence of 1 mM acetylcholine or lowered pH caused a significant swelling of the cells (vi and viii, respectively, compared with control). About 12–16% increase of cell volume was observed when cells were exposed to a medium with high concentration of K^+ (vii).

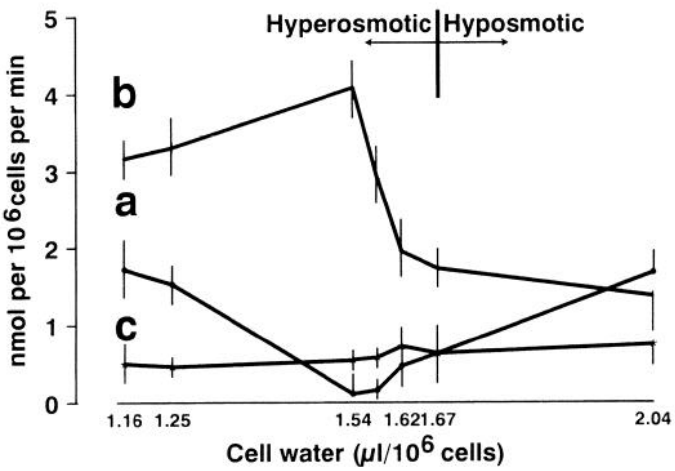

FIG. 6. Dependence of ^{86}Rb influx on intracellular water content of L6 myoblasts adjusted in incubation medium with different osmolalities as shown in Fig. 5. *Line a*: ouabain-inhibited component of ^{86}Rb influx. *Line b*: ouabain-insensitive bumetanide-inhibited component of ^{86}Rb influx. *Line c*: ouabain + bumetanide-insensitive component of ^{86}Rb influx. Incubation of cells with $^{86}RbCl$ was carried out for 10 min. Data are means ± SD; $n = 4$ experiments.

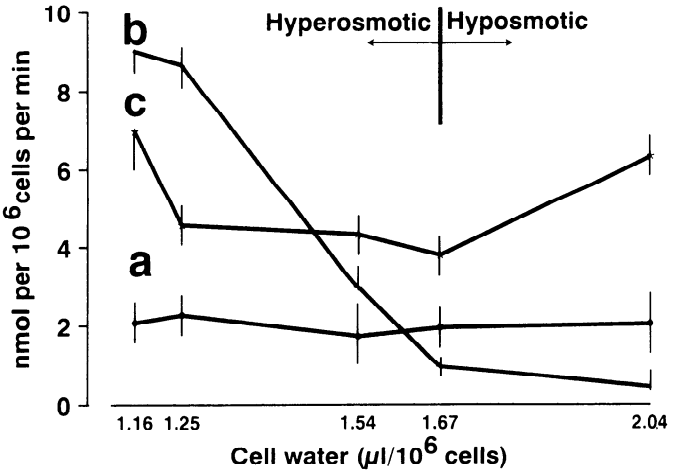

FIG. 7. Dependence of ^{22}Na influx on intracellular water content of L6 myoblasts adjusted in incubation medium with different osmolalities as shown in Fig. 5. Incubation medium used for influx experiment contained 1 mM ouabain to inhibit Na^+-K^+-adenosinetriphosphatase activity. *Line a*: bumetanide-inhibited component of ^{22}Na influx. *Line b*: bumetanide-insensitive 10 μM EIPA-inhibited component of ^{22}Na influx. *Line c*: bumetanide + EIPA-insensitive component of ^{22}Na influx. Incubation of cells with $^{22}NaCl$ was carried out for 3 min. Data are means ± SD; n = 4 experiments.

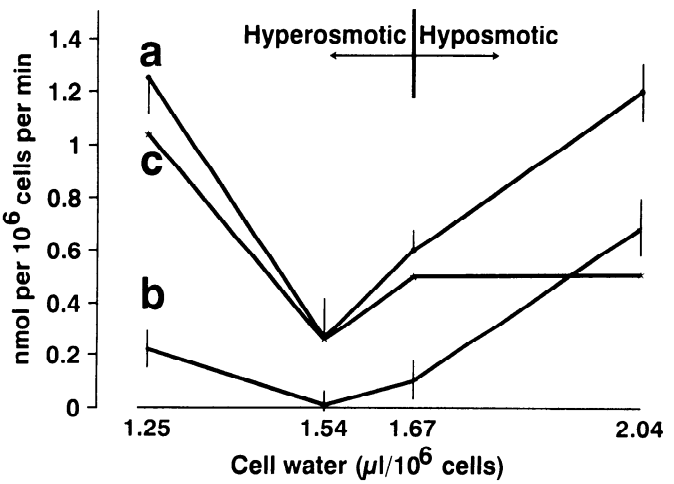

FIG. 8. Dependence of ouabain-inhibited component of ^{86}Rb influx on volume of intracellular water in L6 myoblasts. Effect of extracellular Na^+ on above-said dependence is represented. *Line a*: incubation medium (*medium B*) contains Na^+. *Line b*: influx experiment was carried out in Na^+-free medium (NaCl in *medium B* was substituted by equimolar amounts of choline chloride). *Line c*: Na^+-dependent (i.e., *line a* − *line b*) component of ouabain-inhibited ^{86}Rb influx. Incubation of cells with $^{86}RbCl$ was carried out for 10 min. Data are means ± SD; n = 4 experiments.

bly caused by a drastic decrease in $[Na^+]_i$ of L6 myoblasts in the sodium-replaced medium. Figure 8 also shows the sodium-dependent component of ouabain-inhibited ^{86}Rb influx. No effect of extracellular sodium (Na_o^+) on the increment of the activity of the Na^+-K^+ pump under hyposmotic condition was observed. However, the remarkable activation of the Na^+-K^+ pump that accompanied cell shrinkage (from 1.54 to 1.25 μl intracellular water space/10^6 cells) was almost entirely dependent on the presence of sodium in the incubation medium.

Under isosmotic condition, the equimolar substitution of sodium in the incubation medium with choline decreased the ouabain-insensitive bumetanide-inhibited component of ^{86}Rb influx by ~15% only (Fig. 9). In hyposmotic medium the above-mentioned component of ^{86}Rb influx was absolutely (Na_o^+) insensitive. The cell shrinkage-dependent increase (~90%) in the ouabain-insensitive bumetanide-inhibited component of ^{86}Rb influx was almost entirely dependent on extracellular sodium (Na_o^+). Figure 9 also shows the sodium-dependent component of the ouabain-insensitive bumetanide-inhibited component of ^{86}Rb influx.

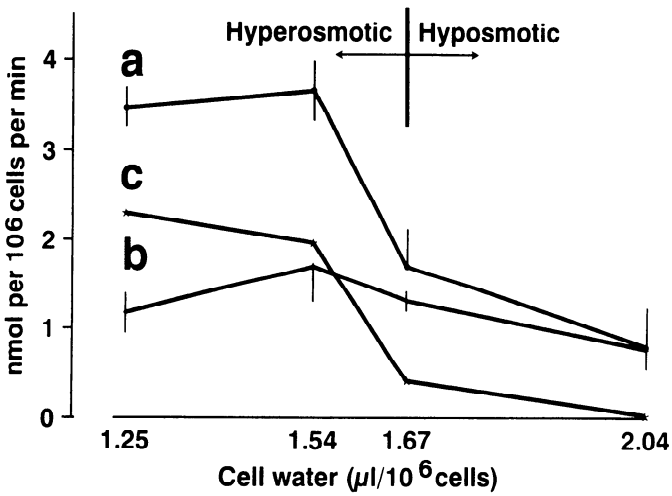

FIG. 9. Dependence of ouabain-insensitive bumetanide-inhibited component of ^{86}Rb influx on volume of intracellular water in L6 myoblasts. Effect of extracellular Na^+ on above-said dependence is represented. *Line a*: incubation medium (*medium B*) contains Na^+. *Line b*: influx experiment was carried out in Na^+-free medium [NaCl of *medium B* (used as incubation medium) was substituted by equimolar amounts of choline chloride]. *Line c*: Na^+-dependent (i.e., *line a* − *line b*) component of ouabain-insensitive bumetanide-inhibited ^{86}Rb influx. Incubation of cells with $^{86}RbCl$ was carried out for 10 min. Data are means ± SD; n = 4 experiments.

Effect of bumetanide, furosemide, and DIOA on ^{86}Rb influx in swollen and shrunken myoblasts. With human erythrocytes it was shown that bumetanide and DIOA are selective inhibitors of Na^+-K^+-$2Cl^-$ and K^+-Cl^- cotransport, respectively, whereas furosemide at concentrations of 0.5–1.0 mM inhibits both of the above-mentioned ion transport pathways (8). On the basis of the above-mentioned properties of bumetanide, furosemide, and DIOA and our findings regarding the different effect of (Na_o^+) on ouabain-insensitive ^{86}Rb influx in L6 myoblast in hyper- and hyposmotic medium (Fig. 9), we compared the effect of the above-mentioned compounds on ouabain-insensitive ^{86}Rb influx in swollen (2.04 μl intracellular water space/10^6 cells in 200 mosmol/kg medium) and shrunken (1.54 μl intracellular water space/10^6 cells in 420 mosmol/kg medium) myoblasts. Figure 10 shows that the values of bumetanide- and furosemide-inhibited components are about the same with relation to each other in both shrunken and swollen cells. However, in the shrunken cells the value of each of the above-mentioned components was almost twice of that observed in the swollen cells. The DIOA-sensitive component of ouabain-insensitive ^{86}Rb influx is also shown. In the swollen myoblasts, the

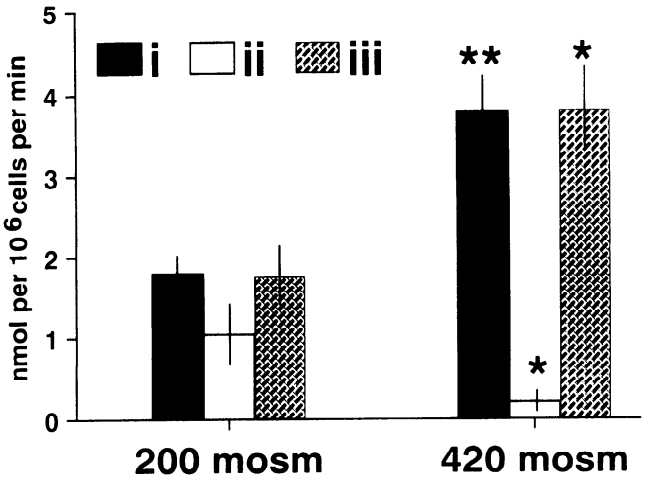

FIG. 10. Effect of 10 μM bumetanide (i), 30 μM DIOA (ii), and 0.5 mM furosemide (iii) on ^{86}Rb influx in swollen myoblasts suspended in 200 mosmol/kg medium (comprised of 80 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose, 1 mM Na_2HPO_4, and 1 mM ouabain in 20 mM HEPES-Tris buffer, pH 7.4 at 37°C) and shrunken (myoblasts suspended in a 400 mosmol/kg medium comprised of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose, 100 mM sucrose, 1 mM Na_2HPO_4, and 1 mM ouabain in 20 mM HEPES-Tris buffer, pH 7.4 at 37°C) L6 myoblasts. Incubation of cells with ^{86}RbCl was carried out for 10 min. Mean (±SD) values of bumetanide-, DIOA-, and furosemide-inhibited components of ^{86}Rb influx are represented. Means of values at 420 mosmol/kg were compared with those at 200 mosmol/kg using Student's *t*-test for unpaired data: * $P < 0.005$; ** $P < 0.001$.

DIOA-sensitive component accounted for ~50% of the individual contribution of the bumetanide- and furosemide-inhibited components. However, in the shrunken myoblasts, the activity of the DIOA-sensitive component of ouabain-insensitive ^{86}Rb influx was negligible compared with the individual contribution of the bumetanide- and furosemide-inhibited components.

DISCUSSION

Ouabain-inhibited ^{86}Rb influx. Comparing data on potassium efflux during the propagation of the action potential, ouabain-inhibited ^{86}Rb influx, Na^+-K^+-adenosinetriphosphatase activity, and [^{3}H]ouabain binding, it was proposed that during the contractile activity of skeletal muscles the activity of the Na^+-K^+ pump may be a rate-limiting step in clearing potassium from the interfiber space to the sarcoplasm (4). Our results show that, at least in the myoblasts used in this study, under basal conditions the ouabain-inhibited component of ^{86}Rb influx accounts for ~20% of the total capacity for ^{86}Rb influx. ^{86}Rb influx was primarily mediated by a ouabain-insensitive bumetanide-inhibited transport system (Fig. 2). It is known that in the resting skeletal muscle $[Na^+]_i$ is only 8–16% of the level required to produce maximal activation of Na^+-K^+-adenosinetriphosphatase. Factors affecting $[Na^+]_i$ play a key role in the regulation of the activity of the ion transport pathway (see Ref. 4). It may be assumed that low $[Na^+]_i$ is the main cause of the relatively low Na^+-K^+ pump activity in the resting L6 myoblasts. This assumption is in agreement with the approximately fourfold activation of the ouabain-inhibited component of ^{86}Rb influx in shrunken cells (Figs. 6 and 8) where $[Na^+]_i$ may have sharply increased due to a loss (20–25%) of intracellular water (Fig. 4) and a powerful activation of the Na^+/H^+ exchanger (Fig. 7). Figure 8 shows that the shrinkage-induced activation of the Na^+-K^+ pump was absent when cells were incubated in Na^+-free medium. This observation indicates that shrinkage-induced sodium influx may play a key role in the activation of Na^+-K^+ pump under hyperosmotic conditions. Unlike shrinkage, swelling of L6 myoblasts did not modify the total capacity for ^{22}Na influx (Fig. 7, compare at 320 and 200 mosmol/kg). Therefore, it appears that the activity of the Na^+-K^+ pump was not sensitive to a further lowering of $[Na^+]_i$ as caused by a 20% increase in intracellular water content. However, it should be noted that the activity of the ouabain-inhibited component of ^{86}Rb influx was increased in swollen cells (in hyposmotic medium) and that such an increment was not dependent on the presence or absence of (Na_o^+) (Fig. 8). The mechanism of (Na_o^+)-independent activation of the Na^+-K^+ pump in swollen L6 myoblasts is yet unknown. However, recently we have observed (Na_o^+)-independent swelling-induced activation of the Na^+-K^+ pump in excitable rat brain synaptosomes (1).

Ouabain-insensitive bumetanide-inhibited ^{86}Rb and ^{22}Na influxes. The main pathway for inward potassium flux in L6 myoblasts was ouabain insensitive and bumetanide inhibited (Fig. 2). In erythrocytes and Ehrlich ascites cells, this pathway was identified as an electroneutral transport system for Na^+-K^+-$2Cl^-$ cotransport (10). The ouabain-insensitive bumetanide-inhibited pathway for ^{86}Rb influx in L6 myoblasts was barely dependent on the presence of (Na_o^+) when the cells were placed in hyposmotic medium. However, such a dependence was observed in cells bathed in iso- and hyperosmotic media, indicating the possibility that the ion transport system responsible for the potassium influx may have been Na^+-K^+-$2Cl^-$ cotransport. Our observation supports the contention that the stoichiometry of ^{86}Rb and ^{22}Na influx through the bumetanide-sensitive pathway was close to unity (Figs. 2 and 3). Eveloff and Calamina (7) observed Na^+-K^+-$2Cl^-$ cotransport in rabbit medullary thick ascending limb cells only under hypertonic conditions. The apparently (Na_o^+)-independent mechanism of inward K^+ transport in the cells bathed in hyposmotic medium is yet unclear.

K^+-Cl^- cotransport in mammalian erythrocytes was inhibited by 0.5–1 mM furosemide and 30 μM DIOA, was insensitive to 10 μM bumetanide, and can be activated by one to two orders of magnitude by cell swelling (3, 8, 11). However, in L6 myoblasts the activities of the 10 μM bumetanide- and 0.5 mM furosemide-inhibited components of ^{86}Rb influx were equal in both shrunken and swollen cells (Fig. 10). The activity of the DIOA-inhibited component of ^{86}Rb influx was less by ~40% and one order of magnitude compared with the activities of the 10 μM bumetanide- or 0.5 mM furosemide-inhibited components in the swollen (200 mosmol/kg) and shrunken (420 mosmol/kg) cells, respectively (Fig. 10). Both 10 μM bumetanide- and 0.5 mM furosemide-inhibited components of ^{86}Rb influx were increased in shrunken cells, whereas the activity of the DIOA-inhib-

ited component was decreased under the same conditions (Fig. 10). As is evident from Fig. 2, no effect of DIOA on ^{86}Rb influx in L6 myoblasts was observed in the presence of bumetanide in the incubation medium (Fig. 2, *inset*). In erythrocytes, K^+-Cl^- cotransport operates in both directions (3, 23). In L6 myoblasts, neither bumetanide nor DIOA modified ^{86}Rb efflux both under isosmotic and anisosmotic conditions (data not shown). Unlike our observation concerning ^{86}Rb influx, no significant effect of cell shrinkage on ^{86}Rb efflux was observed (results not shown).

Bumetanide-insensitive EIPA-inhibited ^{22}Na influx. In resting L6 myoblasts, the activity of the Na^+/H^+ exchanger (bumetanide-insensitive EIPA-inhibited component of ^{22}Na influx) accounted for ~10–15% of the total ^{22}Na influx (Fig. 3, *inset*). As was shown previously in a wide variety of cell types, with the exception of human erythrocytes (24), the activity of the Na^+/H^+ exchanger in L6 myoblasts was increased by hyperosmotic shrinkage (Fig. 7).

Ion transport inhibitor-insensitive ^{86}Rb and ^{22}Na fluxes. ^{86}Rb influx in the presence of ouabain, bumetanide, and DIOA is said to represent the passive diffusion (leakage) of ions through the membrane lipid bilayer or lipid-protein contact area (2). In L6 myoblasts bathed in an isosmotic medium, the above-mentioned pathway mediated ~20% of the total inward flux of ^{86}Rb (Fig. 2). The inward passive diffusion of ^{86}Rb was not dependent on the osmolality of the incubation medium (Fig. 6). Under isosmotic conditions, ~60% of the total ^{22}Na influx in L6 myoblasts was mediated by bumetanide + EIPA-insensitive pathway(s) (Fig. 3, *inset*). The ion transport system involved in such pathway(s) of inward flux of ^{22}Na or in the pathway(s) mediating the ion transport inhibitor-insensitive ^{86}Rb efflux is yet unknown.

Relative contribution of ion transport system(s) in transmembrane sodium and potassium fluxes and volume adjustment in L6 myoblasts. Figure 11 displays a hypothetical (developed on the basis of the findings of the present study) schematic representation of the relative activity of Na^+ and K^+ transport systems of L6 myoblasts and their involvement in cell volume regulation. Under isosmotic condition, most of the ^{86}Rb influx is mediated by a bumetanide-inhibited pathway. It is likely that transmembrane Cl^- gradient is the driving force for the bumetanide-inhibited pathway. It is known that unlike the majority of other cells, $[Cl^-]_i$ in the resting skeletal muscle is rather low, varying between 2 and 20 mM (21). The presence of a low $[Cl^-]_i$ and/or low affinity of the cotransporter to intracellular chloride may be viewed as a main cause for the absence of any effect of bumetanide on outward ^{86}Rb flux in the L6 myoblasts.

No effect of ouabain, bumetanide, DIOA (Fig. 5), or EIPA (data not shown) on the volume of L6 myoblasts incubated in an isosmotic medium could be observed. Previously we have reported that vascular smooth muscle cell volume was slightly decreased after inhibition of the Na^+-K^+ pump but was unaltered by the addition of bumetanide and EIPA (26). Under conditions of moderate shrinkage (Fig. 11) of the myoblast, a twofold activation of the bumetanide-sensitive ^{86}Rb influx was observed (Fig. 6). Previously, we reported a similar shrinkage-associated activation of the bumetanide-inhibited component of univalent cation flux in erythrocytes and vascular smooth muscle cells (23, 24, 26). However, in rat erythrocytes (23, 24) and vascular smooth muscle cells (26), shrinkage of cells increased both inward and outward bumetanide-sensitive fluxes, thereby decreasing the efficiency of the pathway to participate in RVI. In L6 myoblasts, the volume-dependent bumetanide-sensitive K^+ transport pathway was unidirectional and extremely sensitive to volume alterations. The pathway was markedly (100%) activated in response to a slight (5%) decrease in intracellular water space (Fig. 6). Previously we have shown that in vascular smooth muscle cells a similar activation of the bumetanide-sensitive K^+ transport pathway was caused by 10% cell volume decrease (26). Thus, in L6 myoblasts the bumetanide-sensitive potassium pathway may be viewed as a highly effective mechanism that operates to restore cell volume after moderate shrinkage. Under such conditions of cell shrinkage, the activity of the Na^+/H^+ exchanger was also increased. A higher degree of cell shrinkage was accompanied by a manyfold activation of the Na^+/H^+ exchanger (Fig. 7). Similar results were obtained by Klip et al. (17) using an intracellular fluorescent pH indicator. Thus it appears plausible that Na^+/H^+ exchange plays a key role in RVI of L6 myoblasts after its powerful shrinkage.

No significant response of the ^{86}Rb efflux pathway(s) to hyperosmotic shrinkage was observed. The increase in ^{86}Rb efflux after hyposmotic swelling (20% increase in intracellular water space) was accompanied by almost a similar increase in the activity of the bumetanide + EIPA-insensitive component of ^{22}Na influx (Fig. 7). Perhaps the above-described swelling-induced accelerated univalent cation leakage was mediated by low-selective stretch-activated channels recently revealed in vascular smooth muscle cells by the patch-clamp technique (5). Activity of the channel in L6 myoblasts appeared to be quite low, and therefore the key role in RVD is expected to be played by the volume-dependent electrogenic (3 intracellular Na^+/2 extracellular K^+; see Fig. 11) Na^+-K^+ pump (Fig. 11). However, to substantiate the relative participation of the Na^+-K^+ pump and univalent ion carriers in cell volume regulation, additional experiments on the effect of the inhibitors of these ion transport pathways on L6 cell volume under anisotonic conditions should be carried out.

Figure 5 illustrates that prolonged depolarization in a medium containing high K^+ concentration or hyperpolarization caused by acetylcholine caused a marked increase in intracellular water content. This effect on cell volume may be mediated via sodium and chloride influxes through potential-operated ion channels. Such an assumption is supported by the reported data on the twofold increase of intracellular Na^+ (14), four- to fivefold increase of intracellular Cl^- content (21), and 7–9% increase of intracellular water content (31) in skeletal muscle after intense exercise. Intense exercise is also associated with GSH oxidation, mobilization of the hepatic GSH pool, and a remarkable rise in plasma

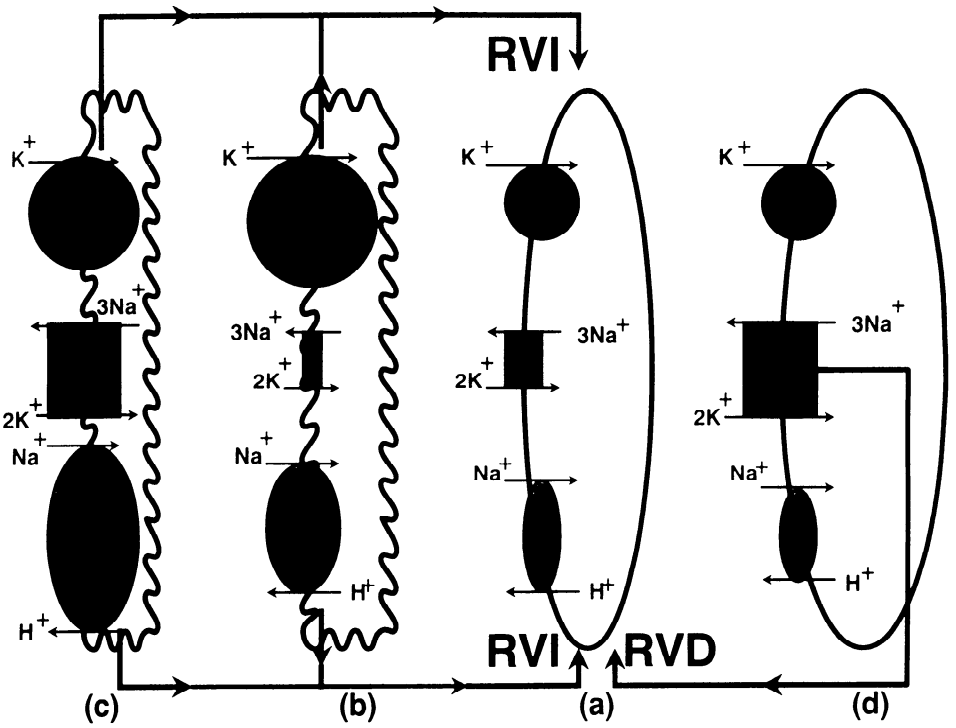

FIG. 11. Hypothetical model describing involvement of Na^+-K^+ pump (solid rectangle), bumetanide-inhibited K^+-flux (solid circle), and Na^+/H^+ exchange (solid ellipse) in regulatory volume increase (RVI) and decrease (RVD) in L6 myoblasts as observed in this study. Depending on their intracellular water content, 4 states of cell are illustrated: *a*, control cells in isosmotic medium; *b*, cells with moderate hyperosmotic-shrinkage (5–7% decreased cell volume); *c*, cells with powerful hyperosmotic-shrinkage (~20% decreased cell volume); and *d*, cells with hyposmotic-swelling (~20% increased cell volume). Size of each ion transport system is approximately proportional to its respective relative contribution (activity) to net transmembrane ion flux in control, shrunken, and swollen cells. Bold line with arrows indicates bumetanide-inhibited K^+-flux and Na^+/H^+ exchange may play key role in RVI and that Na^+-K^+ pump may be important in RVD.

glutathione content (20, 28, 30). A high concentration (150 μM) of extracellular GSH did not have any effect in modifying the cell volume of L6 myoblasts (Fig. 5). It is known that intense exercise may be accompanied by a rapid acidification of extracellular fluids (see Ref. 22). Data obtained in this study showed that acidification of the incubation medium from pH 7.4 to 7.0 increased cell volume by ~10%. Such an acidification may thus be expected to cause an insufficiency in the activity of RVD provided by the Na^+-K^+ pump. Insufficiency in the activity of the pump will also tend to dissipate the transsarcolemmal K^+ gradient and may play a key role in the pathogenesis of fatigue.

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REFERENCES

1. **Aksentsev, S. L., A. A. Mongin, S. N. Orlov, A. A. Rakovich, G. V. Kaler, and S. V. Konev.** Osmotic regulation of sodium pump in rat brain synaptosomes: the role of cytoplasmic sodium. *Brain Res.* 644: 1–6, 1994.
2. **Bernhardt, I., A. C. Hall, and J. C. Ellory.** Transport pathways for monovalent cations through erythrocyte membrane. *Stud. Biophys.* 126: 5–21, 1988.
3. **Brugnara, C., and D. C. Tosteson.** Cell volume, K transport, and cell density in human erythrocytes. *Am. J. Physiol.* 252 (*Cell Physiol.* 21): C269–C276, 1987.
4. **Clausen, T.** Regulation of active Na^+-K^+ transport in skeletal muscle. *Physiol. Rev.* 66: 542–578, 1986.
5. **Davis, M. J., J. A. Donovitz, and J. D. Hood.** Stretch-activated single channel and whole cell currents in vascular smooth muscle cells. *Am. J. Physiol.* 262 (*Cell Physiol.* 31): C1083–C1088, 1992.
6. **Duhm, J.** Na^+ and K^+ transport in human and rat erythrocytes: features complicating the interpretation of data. In: *Salt and Hypertension: Dietary Minerals, Volume Homeostasis, and Cardiovascular Regulation,* edited by R. Rettig, D. Ganten, and F. C. Luft. Heidelberg: Springer-Verlag, 1989, p. 35–51.
7. **Eveloff, J. L., and J. Calamina.** Effect of osmolality on cation fluxes in medullary thick ascending limb cells. *Am. J. Physiol.* 250 (*Renal Fluid Electrolyte Physiol.* 19): F176–F180, 1986.
8. **Garay, R. P., C. Nazaret, P. A. Hannaert, and E. J. Cragoe, Jr.** Demonstration of a [K^+,Cl^-]-cotransport system in human red cells by its sensitivity to [(dihydroindenyl)oxy] alkanoic acids: regulation of cell swelling and distinction from the bumetanide-sensitive [Na^+,K^+,Cl^-]-cotransport. *Mol. Pharmacol.* 33: 696–701, 1988.
9. **Gartner, T. K., B. Land, and T. R. Podleski.** Genetic and physiological evidence concerning the development of chemically sensitive voltage dependent ionophores in L6 cells. *J. Neurobiol.* 7: 537–549, 1976.
10. **Geck, P., and E. Heinz.** The Na^+,K^+-cotransport system. *J. Membr. Biol.* 91: 97–105, 1986.
11. **Hall, A. C., and J. C. Ellory.** Evidence for the presence of volume sensitive KCl transport in 'young' human red cells. *Biochim. Biophys. Acta* 858: 317–320, 1986.
12. **Harris, A. J., S. Heinemann, D. Schubert, and H. Tarakis.** Trophic interaction between cloned tissue culture lines of nerve and muscle. *Nature Lond.* 231: 296–301, 1971.
13. **Hoffman, E. K., and L. O. Simonsen.** Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.* 69: 315–382, 1989.
14. **Juel, C.** Potassium and sodium shifts during in vitro isometric muscle contraction, and the time course of the ion-gradient recovery. *Pfluegers Arch.* 406: 458–463, 1986.
15. **Kidokoro, Y.** Development of action potential in a clonal rat skeletal muscle cell line. *Nature New Biol.* 241: 158–159, 1973.
16. **Kleitzen, R. F., M. W. Pariza, J. E. Becker, and V. R. Potter.** A method using 3-*O*-methyl-D-glucose and phloretin for the determination of intracellular water space of cells in monolayer culture. *Anal. Biochem.* 68: 537–544, 1975.
17. **Klip, A., M. Hill., and T. Rambal.** Halothane increases cytosolic Ca^{2+} and inhibits Na^+/H^+ exchange in L6 muscle cells. *J. Pharmacol. Exp. Ther.* 254: 552–559, 1990.
18. **Kregenow, F. M.** Osmoregulatory salt transporting mechanisms: control of cell volume in anisotonic media. *Annu. Rev. Physiol.* 43: 493–505, 1981.
19. **Lauf, P. K.** Thiol-dependent passive K/Cl transport in sheep red

cells. VII. Volume-independent freezing by iodoacetamide, and sulfhydryl group heterogeneity. *J. Membr. Biol.* 98: 237–246, 1987.

20. **Lew, H., S. Pyke, and A. Quintanilha.** Changes in glutathione status of plasma, liver and muscle following exhaustive exercise in rats. *FEBS Lett.* 185: 262–266, 1985.
21. **Lindinger, M. I., and C. J. F. Heigenhauser.** Intracellular ion content of skeletal muscle measured by instrumental neutron activation analysis. *J. Appl. Physiol.* 63: 426–433, 1987.
22. **McKenna, M. J.** The role of ionic processes in muscular fatigue during intense exercise. *Sports Med.* 13: 134–145, 1992.
23. **Orlov, S. N., N. I. Pokudin, T. G. Gurlo, I. M. Okun, S. L. Aksentsev, and S. V. Konev.** On the mechanism of shrinkage-induced potassium influx in human and rat erythrocytes. *J. Gen. Physiol. Biophys.* 10: 359–372, 1991.
24. **Orlov, S. N., N. I. Pokudin, Y. V. Kotelevstev, and P. V. Gulak.** Volume-dependent regulation of ion transport and membrane phosphorylation in human and rat erythrocytes. *J. Membr. Biol.* 107: 105–117, 1989.
25. **Orlov, S. N., T. J. Resink, J. Bernhardt, and F. R. Buhler.** Na^+-K^+-pump and Na^+-K^+ cotransport in cultured vascular smooth muscle cells from spontaneously hypertensive and normotensive rats: baseline activity and regulation. *J. Hypertens.* 10: 733–740, 1992.
26. **Orlov, S. N., T. J. Resink, J. Bernhardt, and F. R. Buhler.** Volume-dependent regulation of sodium and potassium fluxes in cultured vascular smooth muscle cells: dependence on medium osmolality and regulation by signalling systems. *J. Membr. Biol.* 129: 199–210, 1992.
27. **Sastre, A., and T. R. Podleski.** Pharmacologic characterization of the Na^+ ionophore in L6 myotubes. *Proc. Natl. Acad. Sci. USA* 73: 1355–1359, 1976.
28. **Sen, C. K., E. Marin, M. Kretzschmar, and O. Hänninen.** Skeletal muscle and liver glutathione homeostasis in response to training, exercise, and immobilization. *J. Appl. Physiol.* 73: 1265–1272, 1992.
29. **Sen, C. K., P. Rahkila, and O. Hänninen.** Glutathione metabolism in skeletal muscle derived cells of the L6 line. *Acta Physiol. Scand.* 148: 21–26, 1993.
30. **Sen, C. K., T. Rankinen, S. Väisänen, and R. Rauramaa.** Oxidative stress after human exercise: effect of *N*-acetylcysteine supplementation. *J. Appl. Physiol.* 76: 2570–2577, 1994.
31. **Sjogård, G., and B. Saltin.** Extra- and intra-cellular water spaces in muscles of man at rest and dynamic exercise. *Am. J. Physiol.* 243 (*Regulatory Integrative Comp. Physiol.* 12): R271–R280, 1982.
32. **Vallega, G. A., M. L. Canessa, B. C. Berk, T. A. Brook, and R. W. Alexander.** Vascular smooth muscle Na^+/H^+ exchanger. Kinetics and its activation by angiotensin II. *Am. J. Physiol.* 254 (*Cell. Physiol.* 23): C751–C758, 1988.
33. **Yaffe, D.** Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Natl. Acad. Sci. USA* 61: 477–489, 1968.