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Ouabain-Resistant Na^+, K^+ Transport System in Mouse NIH 3T3 Cells

Henri Atlan, David Snyder, and Rivka Panet
Department of Medical Biophysics, Hadassah University Hospital, Jerusalem, Israel

Summary. It is shown that the ouabain-resistant (OR) furosemide-sensitive $K^+(Rb^+)$ transport system performs a net efflux of K^+ in growing mouse 3T3 cells. This conclusion is based on the finding that under the same assay conditions the furosemide-sensitive $K^+(Rb^+)$ efflux was found to be two- to threefold higher than the ouabain-resistant furosemide-sensitive $K^+(Rb^+)$ influx. The oubain-resistant furosemide-sensitive influxes of both ^{22}Na and ^{86}Rb appear to be Cl^- dependent, and the data are consistent with coupled unidirectional furosemide-sensitive influxes of Na^+, K^+ and Cl^- with a ratio of 1 : 1 : 2. However, the net efflux of K^+ performed by this transport system cannot be coupled to a ouabain-resistant net efflux of Na^+ since the unidirectional ouabain-resistant efflux of Na^+ was found to be negligible under physiological conditions. This latter conclusion was based on the fact that practically all the Na^+ efflux appears to be ouabain-sensitive and sufficient to balance the Na^+ influx under such steady-state conditions. Therefore, it is suggested that the ouabain-resistant furosemide-sensitive transport system in growing cells performs a facilitated diffusion of K^+ and Na^+, driven by their respective concentration gradients: a net K^+ efflux and a net Na^+ influx.

Key Words ouabain-resistant · diuretics-sensitive · Na^+, Rb^+ fluxes

Introduction

A ouabain-resistant (OR), monovalent cation transport system in the plasma membrane of several cell types has been described. It has been found to be sensitive to "loop" diuretics and also to some inhibitors having K^+ ionophore properties. However, the physiological role of this transport mechanism is not clear since conflicting results have been reported concerning the direction of the fluxes. OR diuretics-sensitive K^+ influx was found in red blood cells of several species [4, 10, 19, 22, 26, 29, 30–32, 36, 37] as well as in a few cell lines in culture [3, 5, 11, 20, 28, 34, 35]. Such influx was shown to be coupled to influxes of Na^+ and Cl^- with a $K^+/Na^+/Cl^-$ ratio of 1 : 1 : 2 [37]. This finding led the authors to suggest a mechanism of a diuretics-sensitive cotransport of Na^+, K^+, Cl^-. On the other hand, OR diuretics-sensitive K^+ efflux was found in human red blood cells [8], rabbit and sheep erythrocytes and reticulocytes [18, 26], and in a few cell lines [3, 16, 34, 35]. Outward cotransport has also been reported after increase of Na^+ intracellular concentration by previous loading [8]. However, indirect evidence was provided that Na^+ and K^+ efflux appear to be uncoupled under certain conditions [10].

In addition, the exact role of this K^+ transporter in cell metabolism is not clear. Some studies have shown that it has a role in the regulation of the cell volume [17, 23, 30]. The activity of this transport system was shown to change during cell differentiation, cell division, or under the effects of different stimuli [23, 26, 27, 28, 36]. It was shown that an inactivation of the OR diuretics-sensitive K^+ transport system occurs during differentiation of the reticulocyte to erythrocyte [18, 26]. Moreover, its activity in rabbit reticulocytes appeared to be coupled to ribosomal peptide chain elongation by means of a modification of the ADP/ATP ratio [13, 24]. We also found that the OR furosemide-sensitive K^+ influx was stimulated 10- to 20-fold immediately after release of synchronized mouse 3T3 cells from the G_0 phase of the cell cycle [27, 28]. Other groups have claimed to have found decreased activity of a similar Na^+, K^+ cotransport system in erythrocytes of patients with salt-sensitive hypertension [6]. In view of all these findings, it looked important to us to elucidate the normal function of the OR diuretics-sensitive K^+ carrier as far as the net ion transport is concerned.

In this study:

1) We investigated the diuretics-sensitive net K^+ flux by measuring OR furosemide-sensitive $K^+(Rb^+)$ influx and efflux simultaneously under physiological condition of intra- and extracellular ionic concentration.

2) We studied the kinetics of the K^+ influx and efflux performed by this OR diuretics-sensitive

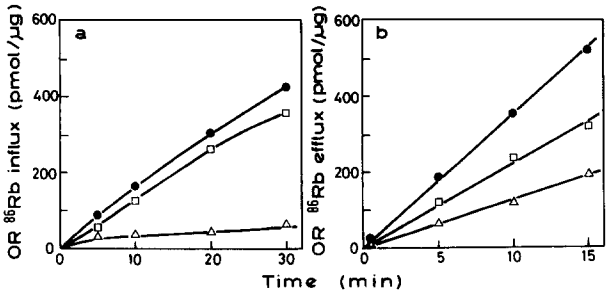

Fig. 1. Kinetics of OR ^{86}Rb influxes and effluxes in NIH 3T3 fibroblasts. Total OR ^{86}Rb influx and efflux was measured on actively dividing cells as described under Materials and Methods. Furosemide (100 μM final concentration) was added directly to the assay mixture and the furosemide-resistant influx or efflux were subtracted from the total OR ^{86}Rb fluxes to yield OR furosemide-sensitive ^{86}Rb influx or efflux. (●—●) Total OR ^{86}Rb flux; (△—△) OR furosemide-resistant ^{86}Rb flux; (□—□) OR furosemide-sensitive ^{86}Rb flux; $n = 3$

transport system with a special interest in its dependency on extracellular Na^+ and Cl^-.

Materials and Methods

^{86}Rb and ^{22}Na were purchased from the Radiochemical Center, Amersham. Furosemide was purchased from Hoechst AG, Frankfurt am Main, FRG. Bumetanide was kindly given by Dr. Bakker-Grunwald, University of Osnabruck, Osnabruck, FRG. NIH 3T3 mouse cells [15] were maintained in RPMI 1640 medium as described before [28].

Since we wanted to measure ionic fluxes under physiological conditions, it was important to do it in such a way that no significant changes in ionic concentrations could occur during the experiments.

For this purpose, neither incubation nor washing of the cells was made under nonphysiological concentration conditions and addition of ouabain and "loop" diuretics, as well as changes in ionic composition of the medium, were produced only in the assay mixture. Therefore if any significant change in ionic concentration was to occur, it was unlikely to be instantaneous and a departure of the unidirectional fluxes from linearity should be observed. Similarly, if a rapid change in cell volume would occur during the assay and affect the fluxes, it would produce a departure from linearity. Since the assays for flux measurements were always limited to the early linear ranges (Fig. 1), we can be fairly confident that the physiological conditions experienced by the cells were not significantly perturbated by the assay itself. Ouabain-sensitive (OS) and ouabain-resistant (OR) ^{86}Rb influxes (by diffusion, and by a "carrier-like" saturable system) were measured as reported before in reticulocytes [26] and in NIH 3T3 mouse fibroblasts [28]. The assay for unidirectional influx measurements was linear up to 20 min (Fig. 1*a*) and was normally conducted for 5 min. ^{86}Rb influx rates presented throughout this work are averages of triplicate cultures. OR K^+ influx was determined from measurements of both the saturable component of OR ^{86}Rb influx [26, 28] and the furosemide-sensitive fraction of this influx. The two methods gave comparable results, which was consistent with the fact that about 100% of the saturable OR $K^+(Rb^+)$ influx was inhibited by "loop" diuretics [26, 28].

^{86}Rb Efflux Measurements

First the cells were loaded with ^{86}Rb (μCi/ml) by overnight incubation in RPMI 1640 medium (2 ml). For ^{86}Rb efflux measurements, the ^{86}Rb-loaded cultures were washed 4–5 times with pre-warmed (37°C) 150 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES-Tris (pH 7.0) and the last wash with the assay mixture (150 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES-Tris (pH 7.0), 2 mM ouabain) preincubated at 37°C. Reactions were started by adding 2 ml assay mixture, and at the indicated intervals samples of 0.2 ml were removed for counting. The assay was linear up to 15 min (Fig. 1*b*). The K^+ specific activity in the ^{86}Rb-loaded cultures was determined by counting total ^{86}Rb and measuring total K^+ content of the cells by Perkin Elmer Atomic absorbance spectrophotometer. These measurements were made at time zero of the assay, i.e., after the last wash of the loaded cultures. The cells were lysed with 1 ml NaOH (0.1 N) and then counting and atomic absorption measurement were done. Thus, possible effect of K^+/Rb^+ exchange during the wash on the internal specific activity were taken care of.

^{22}Na Influx Measurements

Cells (600,000) were plated in 60-mm dishes (Nunc) with RPMI 1640 medium containing 10% calf serum; after two days the medium was replaced by fresh medium for one more day. The medium was aspirated and cells were washed twice with a solution of isotonic choline-chloride containing 10 mM glucose and 10 mM HEPES-Tris at pH 7.0. The reaction was started by adding 2 ml assay mixture (100 mM NaCl, 50 mM choline-chloride, 5 mM RbCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES-Tris at pH 7.0, and 10 μCi ^{22}Na). Incubations were carried out for 2 min at 37°C, and ^{22}Na uptake was terminated by aspiration of the assay mixture. The cells were rapidly washed 3 times with ice cold 125 mM $MgCl_2$ and once with isotonic NaCl. They were lysed with 2 ml NaOH (0.1 N), and radioactivity was counted in a gamma counter. The NaCl wash was added to remove excess of $MgCl_2$ which might precipitate in the NaOH solution.

Results

Unidirectional K^+ Fluxes

In order to determine the net ion flux carried out by the OR furosemide-sensitive K^+ transport system in 3T3 mouse cells, unidirectional OR K^+ influx and efflux were determined under the same assay conditions using ^{86}Rb as an analog for K^+ (Fig. 1, Table 1). Fluxes were measured in subconfluent, actively dividing cells to avoid complications that might occur in arrested cultures (27, 28). ^{86}Rb flux rates presented in Table 1 were determined from the slopes of the linear portion of ^{86}Rb influx or efflux curves (Fig. 1).

Table 1. Components of unidirectional ^{86}Rb influx and efflux in NIH 3T3 cells

Initial cell density	^{86}Rb efflux			^{86}Rb influx					
	Total	Plus furosemide	Furosemide sensitive	Total	Ouabain sensitive	Ouabain resistant	Passive diffusion	OR plus furosemide	OR furosemide-sensitive
	(pmol/min/μg)			(pmol/min/μg)				(pmol/min/μg)	
50,000 cells	21.2 ± 0.13	5.8 ± 0.04	15.4	22.2 ± 0.11	14.6 ± 0.07	5.0 ± 0.23	2.6 ± 0.09	0.5 ± 0.02	4.5
100,000 cells	22.5 ± 0.15	5.3 ± 0.03	17.2	23.2 ± 0.09	12.8 ± 0.09	9.4 ± 0.31	1.0 ± 0.11	1.5 ± 0.01	7.9

Cells (50,000 or 100,000) were seeded and after 48 hr ^{86}Rb fluxes were measured as described in Materials and Methods. Furosemide (100 μM) was added directly to the assay mixture, and the furosemide-sensitive ^{86}Rb influx and efflux were calculated by subtracting the OR furosemide-resistant ^{86}Rb fluxes from the total OR ^{86}Rb influx or efflux.

The results presented in Table 1 confirm the expectation of a steady state, implying that the total K^+ influx rate should be equal to the total efflux rate. This is an indication that the experimental conditions were adequate to maintain a constant intracellular K^+ concentration. Out of the total ^{86}Rb influx 60–80% is ouabain-sensitive (OS), while the ouabain-resistant (OR) furosemide-sensitive ^{86}Rb influx accounts for 20–40% of the total influx. The residual ^{86}Rb influx resistant to both ouabain and furosemide has the characteristics of a passive diffusion [26]. While the total K^+ efflux rate was equal to the total K^+ influx rate, most of it (75–80%) was furosemide sensitive (Fig. 1, Table 1). Thus the furosemide-sensitive ^{86}Rb efflux is two- to threefold higher than the OR furosemide-sensitive ^{86}Rb influx. This result indicates that under physiological conditions the OR furosemide-sensitive K^+ carrier performs net efflux of K^+.

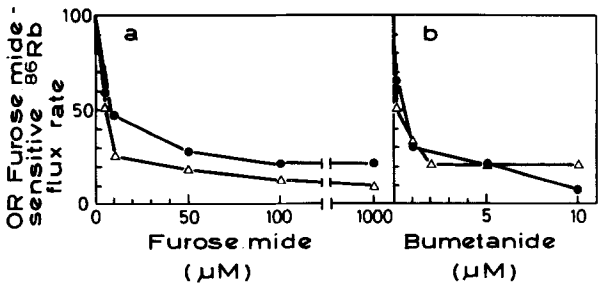

Fig. 2. The effect of furosemide and bumetanide on OR ^{86}Rb influx and efflux. OR ^{86}Rb influx and efflux were measured in actively dividing cells, as described in Materials and Methods. Furosemide (*a*) and bumetanide (*b*) were added directly to the assay mixture to give the final indicated concentrations. The OR ^{86}Rb influx (△—△) and efflux (●—●) in the control cultures (100%) were 8.6 and 32.0 pmol/min/μg, respectively

Characterization of the Ouabain-Resistant ^{86}Rb Fluxes

Sensitivity of OR ^{86}Rb Fluxes to "Loop" Diuretics

The effect of two "loop" diuretics, furosemide and bumetanide, known to inhibit $Na^+/K^+/Cl^-$ cotransport system in avian red cells [22, 23, 30, 31, 32] was tested on OR ^{86}Rb fluxes in mouse 3T3 cells. As seen in Table 1 and Fig. 1 the OR Rb^+ influx can be divided into furosemide-sensitive and furosemide-resistant ^{86}Rb influxes. The dose response curves of furosemide, for OR ^{86}Rb influx and efflux were similar (Fig. 2). The IC-50 for furosemide derived from Fig. 2*a* was 5×10^{-6} M and 8×10^{-6} M for OR ^{86}Rb influx and efflux, respectively. The dose response curves of bumetanide for OR ^{86}Rb influx and efflux in mouse 3T3 cells are similar (IC-50, 0.5×10^{-6} M (Fig. 2*b*)). The OR bumetanide-resistant ^{86}Rb influx (9%) and efflux (20%) are similar to the OR furosemide-resistant fluxes and probably represent passive diffusion. The ouabain-sensitive Rb^+ influx activity was not affected by high concentration of furosemide (1×10^{-3} M) and bumetanide (1×10^{-4} M) (data not shown). Therefore furosemide and bumetanide appear to be more specific than other inhibitors of the OR K^+ carrier previously tested such as valinomycin, dicyclohexyl-18-crown-6 and ethacrynic acid [24, 26, 28].

Effect of Extracellular Na^+ on OR ^{86}Rb Fluxes

The ouabain-resistant Rb^+ influx shown to have a characteristic of a carrier-mediated flux for K^+ (Rb^+) is Na^+ dependent. When Na^+ in the extracel-

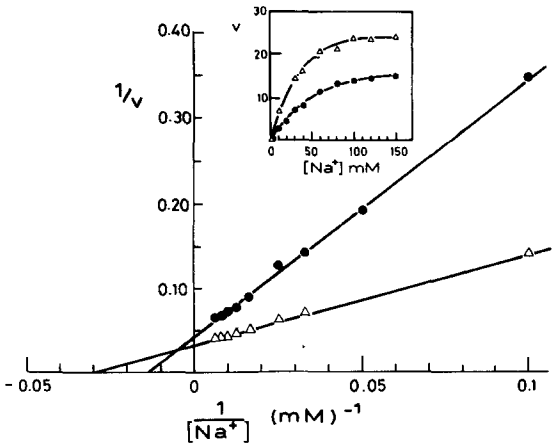

Fig. 3. The effect of Na^+ on OR furosemide-sensitive ^{86}Rb influx and efflux. OR furosemide-sensitive ^{86}Rb influx and efflux were measured as described in Materials and Methods, except that the cells were washed before the assay with isotonic choline and Na^+ concentrations were changed by substitution of Na^+ with choline. The OR furosemide-resistant ^{86}Rb influx rate (3.16 pmol/min/μg) was unaffected by changing NaCl concentrations (data not presented) and was subtracted from the total OR ^{86}Rb infux. The furosemide-resistant ^{86}Rb efflux rate (12.7 pmol/min/μg) was unchanged by varying Na^+ concentrations (not shown here) and was subtracted from the total ^{86}Rb efflux to yield the furosemide-sensitive ^{86}Rb efflux. ^{86}Rb influx and efflux rates (V) were expressed in pmol/min/μg protein. (●—●) OR furosemide-sensitive ^{86}Rb influx; (△—△) OR furosemide-sensitive ^{86}Rb efflux

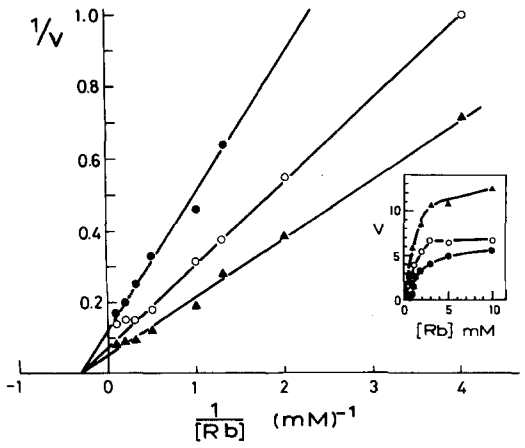

Fig. 4. The effect of extracellular Na^+ on the K_m for Rb^+ of the OR furosemide-sensitive K^+ carrier. OR furosemide-sensitive ^{86}Rb influx was measured in actively dividing cells as described in Materials and Methods, except that the cells were washed with isotonic choline-chloride before the assay and NaCl in the assay mixture was changed to 20 mM (●—●), 50 mM (○—○), and 100 mM (▲—▲) and choline-chloride was added to keep the isotonicity of the assay mixture. The OR furosemide-sensitive ^{86}Rb influx activity (V) was expressed in pmol/min/μg protein

lular medium was replaced with choline, 99% of the OR furosemide-sensitive ^{86}Rb influx was abolished and no effect was found on the residual furosemide-resistant ^{86}Rb influx (Fig. 3). In addition, such substitution of extracellular Na^+ with choline produced a complete inhibition of the furosemide-sensitive ^{86}Rb efflux, while the furosemide-resistant ^{86}Rb efflux, which accounts for 20–30% of the total Rb^+ efflux, was unaffected (Fig. 3). Determination of the external Na^+ requirement for the OR furosemide-sensitive ^{86}Rb fluxes showed hyperbolic response curves, and saturation occurred within the physiological range of Na^+ concentration. The dependence on Na^+ concentration outside was tested not only for Rb^+ influx but also for Rb^+ efflux after it was clear that the OR furosemide-sensitive net flux was directed outward. The rationale was that a possible dependency on Na^+ under these conditions could be related to a necessary occupancy of a site rather than an actual cotransport (*see* Discussion). The K_m for Na^+ calculated from these curves was 68.9 mM for furosemide-sensitive ^{86}Rb influx and 32.3 mM for the furosemide-sensitive efflux (Fig. 3).

By increasing the Na^+ concentration in the extracellular medium the V_m of the OR saturable (furosemide-sensitive) ^{86}Rb influx increased, with no apparent change in the Michaelis constant (K_m) for Rb^+ (Fig. 4). Although the accuracy in K_m determination was not good enough to assess this point with certainty (because of deviation of the plot at high (S) values), this result seems to indicate that the extracellular Na^+ did not affect the binding constant of the Rb^+ to the carrier. However, it suggests that by increasing Na^+ in the extracellular medium more of the carrier molecules are loaded with Rb^+ and can perform $^{86}Rb(K^+)$ influx.

Unidirectional Furosemide-Sensitive ^{22}Na Influx

OR furosemide-sensitive Na^+ influx was measured under the same assay conditions that were used for the $^{86}Rb^+$ influx measurements on growing cells. In order to increase the sensitivity of the ^{22}Na influx measurement we used confluent cells and replaced the medium one day before the assay. This might be the reason why the OR ^{86}Rb influx was found here to be higher than in the previous experiment (Table 1). Table 2 summarizes results comparing OR furosemide-sensitive ^{86}Rb and ^{22}Na influxes. In the standard optimal conditions (NaCl medium, *see* Table 2) the ratio of OR furosemide-sensitive ^{22}Na to ^{86}Rb influx yields a value of 1.05, which is close to the expected value of one, for a Na^+/K^+ cotransport system.

However, in the same experiment the OR furosemide-sensitive ^{86}Rb efflux was found to be 18.21

Table 2. Comparison of OR furosemide-sensitive ^{86}Rb and ^{22}Na influxes at different assay conditions

Assay condition	OR ^{86}Rb influx			OR ^{22}Na influx			
	No addition	Plus furosemide	Furosemide-sensitive influx	No addition	Plus furosemide	Furosemide-sensitive ^{22}Na influx	Influx rate ratio $^{22}Na/^{86}Rb$
	(pmol/min/μg)			(pmol/min/μg)			
NaCl medium	12.49 ± 0.06	1.04 ± 0.01	11.45	22.45 ± 1.18	10.42 ± 1.32	12.03	1.05
Sodium-acetate medium	2.01 ± 0.11	1.89 ± 0.21	0.12	57.05 ± 0.26	56.92 ± 1.15	0.13	—
Choline-chloride medium	4.02 ± 0.15	1.62 ± 0.05	2.40	2.70 ± 0.25	1.51 ± 0.03	1.19	0.49

^{86}Rb and ^{22}Na influxes were assayed as described in Materials and Methods. For measuring fluxes in sodium-acetate medium, the cells were prewashed with isotonic sodium-acetate and sodium-acetate (150 mM) was added to the standard assay mixture instead of NaCl. For measuring fluxes in choline-chloride medium, the cells were prewashed with isotonic choline-chloride solution. Choline-chloride (150 mM) and 5 mM NaCl were added to the standard assay mixture, instead of 150 mM NaCl.

In the same experiment furosemide-sensitive ^{86}Rb efflux was 18.21 ± 0.09 pmol/min/μg, and the OS ^{86}Rb influx was 16.49 ± 0.04 pmol/min/μg. The differences in the values of OR ^{86}Rb influx from 4.5, 7.9 pmol/min/μg (Table 1) to 11.45 (Table 2) result from difference in growth phase, cell density, and possibly cell volume (*see* text).

pmol/min/μg protein (legend to Table 2). When Na^+ concentration in the assay mixture was reduced to 5 mM, which is far below the V_m for OR ^{86}Rb influx (Fig. 4, Table 2), the furosemide-sensitive ^{22}Na influx dropped to 9.9% of its maximal activity. At the same time the OR ^{86}Rb influx was decreased, but to a lower extent, giving a Na^+/Rb^+ influx ratio smaller than 1.0. Note that the ^{22}Na influx as well as the total OR Rb^+ influx were drastically reduced by decreasing the extracellular Na^+ below the normal physiological concentration.

Effect of Extracellular Cl^- on OR Furosemide-Sensitive ^{86}Rb Fluxes and on Furosemide-Sensitive ^{22}Na Influx

We have shown that the OR furosemide-sensitive ^{86}Rb influx in NIH 3T3 cells is Cl^- dependent since inhibition was demonstrated by substitution of Cl^- with acetate, nitrate, and bicarbonate anions [28]. In the present work we have found that the residual OR furosemide-resistant ^{86}Rb influx was not affected by replacing Cl^- with acetate in the extracellular medium, while the OR furosemide-sensitive ^{86}Rb influx was reduced to less than 1% of its maximal activity (Fig. 5, Table 2). When the external Cl^- requirement for the OR furosemide-sensitive ^{86}Rb influx was titrated, a sigmoid rather than hyperbolic relationship was observed for both the influx and the efflux. Both fluxes began to saturate only within the physiological range of Cl^-. Whereas the furosemide-sensitive Rb^+ efflux was inhibited 10-fold after replacing all the Cl^- in extracellular medium with acetate (Fig. 5), the furosemide-resistant Rb^+ efflux was unaffected. Plotting furosemide-sensitive ^{86}Rb influx and efflux rates (V) *versus* Cl^- concentrations squared $(Cl^-)^2$ gave straight lines with slopes of 0.82 and 0.88 for the influx and efflux,

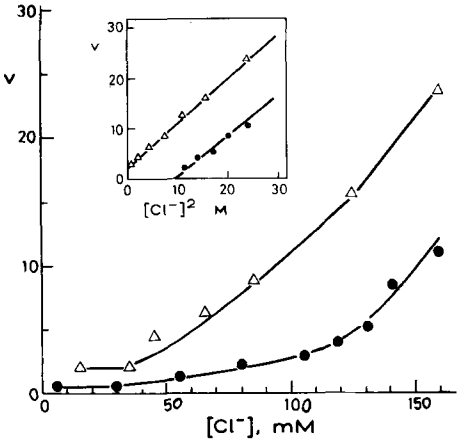

Fig. 5. The effect of Cl^- on OR furosemide-sensitive ^{86}Rb influx and efflux. OR furosemide-sensitive ^{86}Rb influx and efflux were measured as described in Materials and Methods. The cells were washed before the assay with isotonic Na acetate, and Cl^- concentrations were changed by substitution of Cl^- with acetate. The OR furosemide-resistant ^{86}Rb influx (1.9 pmol/min/μg) was not affected by increasing Cl^- concentrations and subtracted from the total OR ^{86}Rb influx to yield OR furosemide-sensitive ^{86}Rb influx. The OR furosemide-resistant ^{86}Rb efflux (9.48 pmol/min/μg) was not affected by Cl^- concentrations (data not shown) and was subtracted from the total ^{86}Rb efflux to yield the OR furosemide-sensitive ^{86}Rb efflux. *Insert:* OR furosemide-sensitive ^{86}Rb influx (●—●) and efflux (△—△) rates, plotted *vs.* Cl^- concentrations squares ($[Cl^-]^2$). ^{86}Rb influx and efflux rates (V) were expressed in pmol/min/μg protein

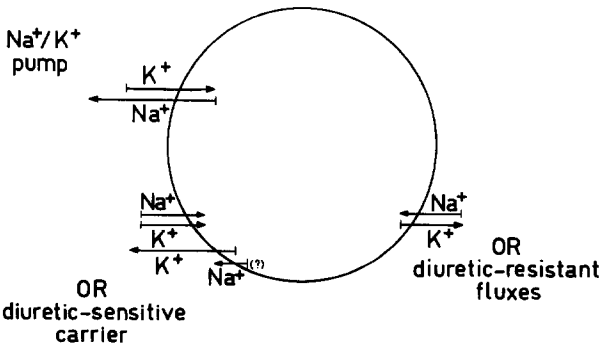

Fig. 6. Suggested balance of unidirectional flows of K^+ and Na^+ transported by different systems under physiological conditions in 3T3 mouse cells. (*See* text)

respectively (Fig. 5). The straight lines of furosemide-sensitive ^{86}Rb fluxes rate (V) *versus* Cl^- concentrations squared ($[Cl^-]^2$), with a slope close to 1.0 indicate stoichiometry of 2 Cl^- per one K^+ as was found in Ehrlich cells [11]. It also indicates two independent but identical binding sites for Cl^- (at least within the range of Cl^- concentrations (squared) which gave straight lines when furosemide-sensitive fluxes were plotted *versus* it). It should be noted that under our experimental conditions, intracellular Cl^- may vary with the external Cl^- concentration. In parallel, replacing Cl^- in the extracellular medium produced an almost complete inhibition of the furosemide-sensitive ^{22}Na influx. However, the total ^{22}Na influx increased significantly, indicating a stimulation of other Na^+ pathways resistant to furosemide.

Discussion

By simultaneous measurements of the OR furosemide-sensitive K^+ influx and efflux in NIH 3T3 mouse fibroblasts, we have found the efflux to be two- to threefold larger than the influx when the cells were kept actively dividing under normal growing conditions.

In previous works on rabbit reticulocytes we have characterized a "carrier-like" activity responsible for a ouabain-resistant net K^+ efflux. As in the present work this was achieved by studying the effects of relatively specific inhibitors (ionophores and diuretics) on a saturable component of OR unidirectional $Rb^+(K^+)$ influx and of the same inhibitors on unidirectional K^+ efflux.

Based on indirect evidence Jayme et al. [16] suggested that the OR diuretic-sensitive K^+ transport in mouse fibroblastic cell line LM(TK−) does perform net K^+ efflux out of the cells. However, their experiments were conducted in low K^+ concentration (0.2 mM) of the growth medium which could have changed the normal activity of this transport system. In fact, the OR furosemide-sensitive K^+ influx and efflux activities are both dependent on the extracellular and intracellular ionic concentrations as shown in previous works [9, 23, 26]. Our results indicate clearly that when the ionic concentrations in the cell and in the growth medium are kept physiological the OR furosemide-sensitive K^+ carrier performs a net K^+ efflux in actively dividing 3T3 cells. The same conclusion was drawn from our previous work, with rabbit reticulocytes and erythrocytes. We found also that this K^+ carrier-like system did not need energy from ATP metabolism, which is consistent with a net efflux of K^+ driven by its concentration gradient across the cell membrane [26].

This conclusion is in disagreement with the observation that in Ascites tumor cells the OR furosemide-sensitive K^+ influx is equal to the furosemide-sensitive K^+ efflux [3, 35]. Differences in the growth conditions, cell volume or in the cell lines could account for the discrepancy. In fact our results seem to indicate that the cell density may affect the OR furosemide-sensitive ^{86}Rb influx but not the efflux (Tables 1 and 2) and thus change the K^+ efflux/influx ratio.

The measurements of OR furosemide-sensitive ^{22}Na influx reported here seem consistent with the existence of a Na^+,K^+ cotransport with a ratio close to one (Table 2), as far as unidirectional fluxes inward are concerned. However, concerning the net fluxes, our results do not support this idea since no net OR efflux of Na^+ can be found to be coupled to the net OR furosemide-sensitive K^+ efflux under physiological conditions of ionic concentrations. This conclusion is based on the following arguments:

1) Although we have not measured OR furosemide-sensitive Na^+ efflux (difficult to assay, under physiological intracellular concentrations) it can certainly be estimated to be much lower than the K^+ efflux. In fact, based on the OS ^{86}Rb influx activity (16.49 pmol/min/μg protein, *see* legend of Table 2) and the known Na^+/K^+ pump 3/2 ratio, the OS Na^+ efflux can be estimated to be 24.73 pmol/min/μg protein. This estimated value is very close to the total ^{22}Na influx actually measured (Table 2), as expected from the fact that we were working under physiological steady-state conditions. Therefore the OR furosemide-sensitive Na^+ efflux in the 3T3 mouse cells under physiological conditions must be negligible.

2) Having in mind the much higher value of the OR diuretic-sensitive K^+ unidirectional (and even net) efflux (Tables 1 and 2), the idea of a OR di-

uretic-sensitive cotransport of Na^+ and K^+ with a similar 1:1 ratio is difficult to accept. Therefore, based on the results presented in this work, we suggest that the OR diuretic-sensitive carrier performs a coupled Na^+, K^+ influx, together with a larger K^+ efflux which is not accompanied by any significant Na^+ efflux. Thus, whereas the characteristics of the unidirectional OR furosemide-sensitive Na^+ and K^+ influxes were consistent with the notion of a cotransport mechanism (as found in other cell lines in different, less physiological experimental conditions), those concerning unidirectional effluxes and net transport do not support this concept.

Rather, this OR furosemide-sensitive transport system, in growing cells under physiological conditions, appears to be working like a facilitated diffusion performing net transport of cations according to the concentration gradient: K^+ outward and Na^+ inward (*see* Fig. 6).

In addition, results presented here have shown a strong dependency on extracellular Na^+ and Cl^-, not only of the OR furosemide-sensitive K^+ influx, but also of the K^+ efflux. This result was obtained in assay conditions such that no time was allowed for significant changes to occur in the intracellular Na^+. Therefore, the coupling between K^+ and Na^+ transport is not simple. It is not a 1:1 K^+/Na^+ exchange since both K^+ unidirectional fluxes are strictly dependent on Na^+ and Cl^- concentration outside the cell membrane. In addition, the coupling mechanisms of the two unidirectional K^+ fluxes with Na^+ influx appears to be different since the kinetics of their dependency on extracellular Na^+ concentrations exhibit different K_m (Fig. 4). One can speculate that, whereas K^+ is inwardly cotransported with Na^+ through this transport system, its outward transport necessitates only the binding of a cooperative site with Na^+ without a concomitant actual transport of sodium ions. In addition, electroneutrality of the carrier seems a necessary condition since the binding of two Cl^- ions is probably necessary together with one K^+ and one Na^+.

Thus, it can be concluded that, under physiological ionic concentration conditions, the Na^+,K^+ ATPase activated pump is working in an opposite direction to this OR "loop" diuretics-sensitive transport system. The need for such a transport system, being regulated by hormones and other ions, was recognized already on the basis of studies of different cellular systems [1, 2, 7, 9, 12, 14, 21, 33]. Further work is necessary to elucidate the mechanism and physiological role of this OR diuretic-sensitive transport system, especially as far as its possible regulatory effects on the ionic composition of the cytoplasm and on the cell metabolism under different growth conditions are concerned.

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