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Coupling of a loop diuretic-sensitive Na^+ influx with the net loop diuretic-sensitive K^+ efflux in mouse NIH 3T3 cells

Rivka Panet, David Snyder and Henri Atlan

Department of Medical Biophysics, Hadassah University Hospital, Jerusalem (Israel)

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Mouse 3T3 fibroblasts have a loop diuretic sensitive Na^+ transport system, responsible for more than 50% of the total Na^+ influx. This transport system is dependent on the simultaneous presence of all three ions; Na^+, K^+, (Rb^+) and Cl^- in the extracellular medium. The same requirement for these three ions was also found for the loop diuretic-sensitive K^+ efflux. In addition, the sensitivities of Na^+ influx and Rb^+ efflux for the two loop diuretics, furosemide and bumetanide were found to be similar. The similar ionic requirement and sensitivity towards loop diuretics of the two fluxes, support the hypothesis, that this loop diuretic-sensitive Na^+ influx in mouse 3T3 cells, is accompanied by the net loop diuretic-sensitive K^+ efflux.

Introduction

Much attention has been focused recently on the different Na^+ transport systems which carry Na^+ into the cells during proliferation. It has been proposed that early changes in monovalent ion fluxes may act as the transmembrane signal, in the action of mitogens [1]. In particular, a rapid increase in the rate of Na^+ entry, resulting in stimulation of the Na^+/K^+ pump, has been postulated to be a primary event following mitogenic stimulation of G_0/G_1 phase quiescent cells [2–6].

Na^+ ions have been found to be translocated through animal cell membrane by several distinguishable transport systems such as: Na^+/H^+ amiloride-sensitive antiport [2–10], Na^+/Na^+ exchange [11], Na^+/Ca^{2+} antiport [12], tetrodotoxine-sensitive Na^+ influx [13,14] and the loop diuretic-sensitive Na^+ influx, possibly coupled to K^+ and Cl^- fluxes [15–21].

Recently, we demonstrated that in non-synchronized growing NIH 3T3 mouse cells, the loop diuretic-sensitive transport system performs a net flux of K^+ outwards. We have proposed that this transport system is coupled to a net flux of Na^+ inward [19]. Although the K^+ fluxes were found to be dependent on extracellular Na^+, more evidence is needed to substantiate this proposal. To further investigate the coupling between K^+ efflux and Na^+ influx through the loop diuretic-sensitive transporter, two parameters were measured on the two fluxes: (a) sensitivity towards loop diuretics; and (b) kinetic constants for extracellular Na^+, Rb^+ and Cl^-.

Materials and Methods

$^{22}Na^+$ was purchased from the Radiochemical Centre, Amersham, U.K. Furosemide was purchased from Hoechst AG, Frankfurt am Main, F.R.G. Bumetanide was kindly provided by Laborate Leo, B.P.9-28500 Vernouillet, Denmark. Mouse NIH 3T3 fibroblasts were maintained as described before [20].

Abbreviation: Hepes, 4-(2-hydroxethyl)-1-piperazineethanesulfonic acid.

Na^+ influx measurements.

Na^+ influx under physiological conditions was measured as described before [19]. In brief, cells (600 000) were plated in 60-mm dishes (Nunc) with RPMI 1640 medium containing 10% calf serum; after 2 days, the medium was replaced by fresh medium for 1 more day. The medium was aspirated and cells were washed twice with a isotonic choline-chloride solution 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 $MgCl_2$, 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 three times with ice-cold 125 mM $MgCl_2$ and once with isotonic NaCl. They were lysed with 2 ml NaOH (0.1 M), and radioactivity was counted in a gamma counter. Furosemide (1 mM) was added directly to the assay mixture. The furosemide-resistant Na^+ influx was subtracted from the total Na^+ influx, to yield the furosemide-sensitive Na^+ influx.

Since we wanted to measure Na^+ influx under physiological conditions, it was important to do it in such a way that no significant changes in ionic concentrations and in cell volume could occur during the experiments. For this purpose, neither incubation, nor washing of the cells was made under nonphysiological concentration conditions, and the addition of ouabain and loop diuretic, as well as changes in ionic composition of the medium, were introduced only in the assay mixture. Therefore, if any significant change in cell volume was to occur, it was unlikely to be instantaneous. Since the assay for Na^+ influx measurements were always limited to the first 2 min, we can be fairly confident that no significant cell volume change has taken place during the short assay time.

Results

Because of difficulties in the Na^+ influx assay, some of the early studies on Na^+ influx in cell cultures, were conducted in nonphysiological conditions, such as cellular depletion of Na^+ [4,7,9,10], lowering extracellular Na^+ concentration [4,7–10] and changing the internal pH by different means [7,9,10]. We have tested the effect of furosemide and bumetanide on Na^+ influx under the following more physiological conditions: no depletion of cellular Na^+ was conducted before the assay and sodium concentration in the assay was 100 mM. (This Na^+ concentration was found to saturate the furosemide-sensitive Na^+ influx, Fig. 2). In addition, the assay was conducted within a short time (2 min) such that no significant change in the intracellular ionic concentration could occur (see Materials and Methods).

The data presented in Fig. 1 indicate that Na^+ influx is made of two components: loop diuretic-sensitive and loop diuretic-resistant. More than 50% of the total Na^+ influx under these conditions was sensitive to both, furosemide (A) and bumetamide (B) (see also Table II, and Table III in Ref. 19). The furosemide-resistant (Fig. 1A), and the bumetanide-resistant (Fig. 1B) Na^+ influx were found to be of the same magnitude. Other Na^+ channels are probably responsible for it [2–14]. The sensitivity of Na^+ influx to furosemide and bumetanide, was similar to that of both Rb^+ influx and efflux (see Table 1).

Dependence of the loop diuretic-sensitive Na^+ influx on Na^+, Rb^+ and Cl^-

In previous works, both the loop diuretic-sensitive Rb^+ influx and efflux were found to be dependent on the simultaneous presence of all three ions: Na^+, K^+ (Rb^+) and Cl^- in the extracellular medium [5,16–19]. In the present work, we mea-

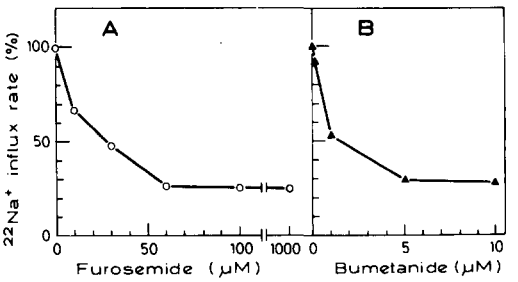

Fig. 1. The effect of furosemide and bumetanide on Na^+ influx. Na^+ influx was measured in log-phase cells as described in Materials and Methods. Furosemide (A) and bumetanide (B) were added directly to the assay mixture to give the final indicated concentrations. Total Na^+ influx in the control cultures (100%) were 12.47 pmol/min per μg protein.

TABLE I

THE EFFECT OF EXTERNAL Cl^- CONCENTRATIONS ON Na^+ INFLUXES

Na^+ influx was measured as described in Materials and Methods. The cells were washed before the assay with isotonic Na^+ nitrate, Na^+ acetate or Na^+ sulfate, and Cl^- concentrations were changed by substitution of Cl^- with the indicated anion. The furosemide-resistant Na^+ influx at each Cl^- concentration was subtracted from the total Na^+ influx to yield the furosemide-sensitive Na^+ influx.

Cl^- concn. (mM)	Main anion substituting for Cl^-	Na^+ influx (pmol/min per μg)	
		Furosemide-sensitive (pmol/min per μg)	Furosemide-resistant (pmol/min per μg)
0	NO_3^-	0	43.0
0	CH_3COO^-	0	24.3
0	SO_4^{2-}	0	43.0
40	SO_4^{2-}	0	41.0
60	SO_4^{2-}	7.0	33.5
80	SO_4^{2-}	20.1	29.3
100	SO_4^{2-}	19.5	33.5
150	SO_4^{2-}	13.5	9.6

sured the dependence of the Na^+ influx mediated by the loop diuretic-sensitive transporter on these three ions. We found a hyperbolic dose-response curve for extracellular Na^+, with saturation occurring within the physiological range of Na^+ concentration (100 mM). The calculated K_m of Na^+ for both fluxes, Na^+ influx and Rb^+ efflux was similar (Table II). In spite of limitations due to

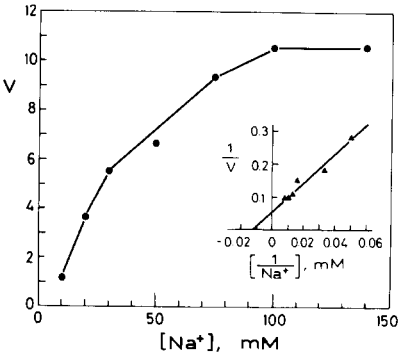

Fig. 2. The effect of external Na^+ on furosemide-sensitive Na^+ influx. Na^+ influx was measured as described in Materials and Methods except that Na^+ concentrations were changed by substitution of Na^+ with choline to keep isotonicity. The furosemide-resistant Na^+ influx at each Na^+ concentration was subtracted from the total Na^+ influx to yield the furosemide-sensitive Na^+ influx. The furosemide-resistant Na^+ influx was 12.13 pmol/min per μg protein at 150 mM Na^+ and by lowering Na^+ concentration it was almost linearly lower (not shown here). V is expressed as pmol/min per μg protein.

short assay time, the K_m determinations are accurate enough to conclude that the affinity of this transporter for the external Na^+ (expressed by K_m for Na^+) is similar for all the loop diuretic-sensitive fluxes, namely Na^+ influx, Rb^+ influx and Rb^+ efflux (Fig. 2, Table II). Titration of the Rb^+ requirement for the furosemide-sensitive Na^+ influx gave a hyperbolic relationship, with saturation occuring at 3 mM Rb^+. The absence of Rb^+ in the extracellular medium inhibited the furosemide-sensitive Na^+ influx to 11% of the maximal value (Fig. 3). On the other hand, the furosemide-re-

TABLE II

COMPARISON OF Na^+ WITH Rb^+ FLUXES IN GROWING NIH 3T3 MOUSE FIBROBLASTS

Kinetic constants for Na^+ influx were calculated from Figs. 1–3. The same kinetic constants for Rb^+ influx and efflux were taken from our previous work [19]

Kinetic constants	Furosemide-sensitive cation fluxes		
	Na^+ influx	Rb^+ influx	Rb^+ efflux
K_m for Na^+ (mM)	76.9	71.4	38.5
K_m for Rb^+ (mM)	1.05	3.14	–
Cl^- dependency	reached saturation	not saturated up to 165 mM Cl^-	not saturated up to 165 mM Cl^-
IC_{50} (M)			
Furosemide	$12 \cdot 10^{-6}$	$5 \cdot 10^{-6}$	$8 \cdot 10^{-6}$
Bumetanide	$7 \cdot 10^{-7}$	$5 \cdot 10^{-7}$	$5 \cdot 10^{-7}$

sistant Na^+ influx was unaffected in the absence of Rb^+ (results not shown). Again, the calculated K_m for Rb^+ is similar for the loop diuretic-sensitivity Na^+ and Rb^+ influx (Table II). Thus, it seems that similar concentrations of Rb^+ are necessary for the transport of both ions, Na^+ and Rb^+, by the loop diuretic-sensitive transport system. Recently, we showed that the loop diuretic-sensitive Rb^+ fluxes were Cl^--dependent. When the external Cl^- requirement for the furosemide-sensitive Rb^+ influx and efflux was titrated, a sigmoidal relationship was observed [19]. In this work, the external Cl^- requirement of the furosemide-sensitive Na^+ influx was also studied. Replacing Cl^- in the assay mixture by other anions such as NO_3^-, $CH3COO^-$, and SO_4^{2-}, resulted in a complete inhibition of the furosemide-sensitive Na^+ influx (Table I). By titrating the external Cl^- requirement of the furosemide-sensitive Na^+ influx using SO_4^{2-} as a major anion, a sigmoidal relationship was observed.

By increasing Cl^- from zero to 40 mM, the furosemide-sensitive Na^+ influx was completely inhibited. Above 40 mM, the furosemide-sensitive Na^+ influx started to increase, and apparent saturation was obtained above 80 mM Cl^- (Table I). A similar relationship was observed using acetate as a major anion (not shown here).

Thus, it seems reasonable to conclude that more than one Cl^- is necessary for one Na^+ to be transported by the loop diuretic-sensitive transport

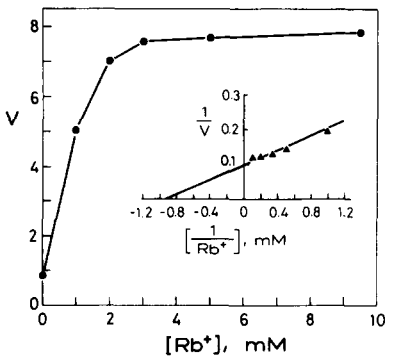

Fig. 3. The effect of external Rb^+ on furosemide-sensitive Na^+ influx. Na^+ influx was measured as described in Materials and Methods except that the Rb^+ concentration was varied. Na^+ influx in the presence of 1 mM furosemide (5.52 pmol/min per μg protein) was subtracted to yield furosemide-sensitive Na^+ influx. V is expressed as pmol/min per μg protein.

system. The same conclusion was drawn from our results obtained by measuring the Cl^- dependency of the diuretic-sensitive Rb^+ influx and efflux [19]. In addition, while replacing Cl^- by acetate and sulfate in the extracellular medium produced a complete inhibition of the furosemide-sensitive Na^+ influx, it was accompanied by a dramatic increase of the total Na^+ influx (Table I). Increasing Cl^- concentrations in the assay mixture, resulted in a reduction of the furosemide-resistant Na^+ influx, whereas the furosemide-sensitive Na^+ influx increased. This result is consistent with the finding that the amiloride-sensitive Na^+ influx was activated by replacing Cl^- in the assay mixture [7].

Discussion

In a previous work, we showed that the furosemide-sensitive K^+ transport system performs a net efflux of K^+ in growing mouse 3T3 cells [19]. This conclusion was based on the finding that under the same assay conditions the furosemide-sensitive K^+ efflux was found to be 2- and 3-fold higher than the furosemide-sensitive K^+ influx. We suggested in this study that 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 [19]. The work reported in this paper support this mechanism, it shows the presence of a loop diuretic-sensitive Na^+ transporting system in growing NIH 3T3 cells, coupled to a net K^+ efflux. A transport system which catalyzes coupled fluxes of Na^+ and K^+, is expected to exhibit several properties: (i) It should have the same ionic requirement for both cation fluxes; (ii) Its sensitivity towards specific inhibitors would be similar for the two coupled fluxes. These parameteres were examined and the results were consistent with the existence of a coupling between K^+ efflux and Na^+ influx through the diuretic-sensitive transport system. Due to difficulties in the assay of Na^+ influx, some of the early studies were conducted after cellular depletion of Na^+, and at low Na^+ concentration in the assay [4,7–10]. These conditions are toxic, and thus are expected to influence differently the various transport systems including that of Na^+. Furthermore, both the loop diuretic-sensi-

tive [15,16–20] and the amiloride-sensitive transport systems [7–9] were shown to be dependent on extracellular and intracellular K^+ and Na^+ concentrations, and also on external pH [21]. Depletion of cellular Na^+ would therefore affect differently the loop diuretic-sensitive and the amiloride-sensitive Na^+ transporters. Na^+ at normal cellular concentration was shown to inhibit almost completely the amiloride-sensitive Na^+ influx [8,9], while it is needed for the loop diuretic-sensitive Na^+ influx [8,9], while it is needed for the loop diuretic-sensitive Na^+ influx [20]. Thus, depletion of cellular Na^+ would activate the amiloride-sensitive Na^+ influx, and inactivative the diuretic-sensitive Na^+ influx.

Indeed, the amiloride-sensitive Na^+ influx is very small under physiological conditions in many cells [9]. The amiloride-sensitive transport system is also dependent on the extracellular Na^+ concentration [4,7,8]. In a few cells, saturation of the amiloride-sensitive Na^+ influx, was shown to occur at lower Na^+ concentrations than is needed to saturate the loop diuretic-sensitive Na^+ influx [4,8].

As a result, by lowering the extracellular Na^+ in the assay [4,7–10], the activity of the loop diuretic-sensitive Na^+ influx would be low, whereas that of the amiloride-sensitive Na^+ influx would be decreased also, but to a lesser extent. The loop diuretic-sensitive Na^+ influx is dependent on the presence of K^+ in the medium [15–17,19]; therefore by excluding K^+ from the assay mixture it would completely inactivate the diuretic-sensitive Na^+ influx, but on the other hand might not affect other Na^+ transporters. The same considerations would be true for changing the pH of the cell and of the extracellular medium. For all these considerations, we kept the assay under more physiological conditions (100 mM Na^+, 5 mM K^+, pH 7.0), with no change in the intracellular ionic concentrations. Under these conditions, the loop diuretic-sensitive Na^+ influx was found to be at least 50% of the total Na^+ influx. Therefore, it seems fair to conclude that the loop diuretic-sensitive transporter is a major carrier for Na^+ into the growing NIH 3T3 cells. The loop diuretic-resistant Na^+ influx was not characterized here, and it probably represents other Na^+ transport systems, such as Na^+/H^+, Na^+/Li^+, Na^+/Ca^{2+} and others.

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