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Basic characterization of an ouabain-resistant, bumetanide-sensitive K^+ carrier-mediated transport system in J774.2 mouse macrophage-like cell line and in variants deficient in adenylate cyclase and cAMP-dependent protein kinase activities *

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$^{86}Rb(K^+)$ transport across the plasma membrane of macrophage-like cells was studied. The cells used were the wild-type J774.2 and its two variants, CT2 cells, deficient in adenylate cyclase, and J7H1 cells, deficient in cAMP-dependent protein kinase. In the three cell lines about 15% of the total $^{86}Rb(K^+)$ influx is transported by the K^+ carrier-mediated transport system. The $^{86}Rb(K^+)$ efflux carried by the same transporter is negligible when measured in the absence of ouabain in the medium. Therefore this carrier conducts a net inward flux of K^+ under the experimental conditions used. The transporter is sensitive to extracellular Na^+ and inhibited by 'loop' diuretics; bumetanide inhibits ouabain-resistant $^{86}Rb(K^+)$ influx with IC_{50} of 0.1, 5.0, and 0.05 μM for J774.2, CT2 and J7H1 macrophages, respectively. The membrane potential of the three cells was measured, using the distribution of [^{3}H]tetraphenylphosphonium ([^{3}H]TPP^+) across the plasma membrane, and found to be −80.1, −108.5 and −105.1 mV for J774.2, CT2 and J7H1 cells, respectively. The addition of bumetanide to the cell medium does not alter [^{3}H]TPP^+ uptake indicating that the transporter is electrically silent. It is concluded that despite the differences in cAMP metabolism by the three macrophages, the basic characteristics of K^+ carrier-mediated transport system of the three cells are very similar.

Introduction

An ouabain-resistant, K^+ carrier-mediated transport has been described in the plasma membrane of number of cells. Under certain conditions, it has been shown to be a linked, bidirectional, co-transport of Na^+, K^+ and Cl^- with a probable [Na^+, K^+, Cl^-] stoichiometry of 1 : 1 : 2 (for review, see Refs. 1–4). It was postulated that the movement of either cation is coupled to, and can be driven by, the gradient of its co-ion [2,4]; it thus does not require any energy [1]. It also has a specific ion requirement satisfied only by Na^+, K^+, Rb^+, Cl^- and Br^- [1,2,4,5] and is saturable by Rb^+ [6]. This transport system is resistant to

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Abbreviations: TPP^+, tetraphenylphosphonium ion; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

ouabain and amiloride, but inhibited by 'loop' diuretics such as bumetanide, furosemide and ethacrynic acid [3,4,6–8] and also by K^+ ionophores (valinomycin, dicyclohexyl-18-crown-6) probably by competition [6,9,10].

Extensive studies have been made on the physiological role of this transport system in living cells. It was postulated that it is involved in the modulation of cell volume [4,11–13] and in cell differentiation and division [4,6,8,14,15]. Moreover, it has been shown that this transport system is controlled by different regulatory mechanisms [16]. In avian erythrocytes it is stimulated by β-adrenergic agonists, cholera enterotoxin, adenosine, cAMP analogs, all agents that raise intracellular cAMP levels [4,13,17–20]; this stimulation involves probably protein phosphorylation [4,21,22]. It is also activated by several treatments which do not act through cAMP, e.g. hypertonicity, and deoxygenation [4,20], the mechanism of action of which is still unknown. In contrast with these data, it has been found that cAMP and sometimes cGMP inhibit K^+ carrier-mediated transport in epithelial tissues such as rabbit gallbladder, flounder intestine, rabbit ileum, and in human eythrocytes [23–27]. In most of those epithelia the direct effect of catecholamines has not been yet studied; however, Garay [24] showed that isoproterenol does not affect human erythrocytes which do not exhibit any β-adrenergic receptors. Moreover, we have found that adrenaline and isoproterenol inhibit this transport system through the β-adrenergic receptors in NIH 3T3 cells, but that cAMP does not seem to be involved in this phenomenon (unpublished data). Consequently, it appears that the regulation of K^+ carrier-mediated system by catecholamines and second messengers does not involve the same mechanism in different tissues. Therefore it was of interest to characterize this transport system in cells in which the adenylate cyclase-protein kinase system was described in details. The cells chosen for this study are J774.2 macrophages and two variants derived from these cells, CT2, deficient in adenylate cyclase and J7H1, deficient in cAMP-dependent protein kinase. Those three macrophage-like cell lines were isolated and characterized by Bloom, Muschel, Rosen and their colleagues [28,29]. They were previously used for studying three of the functions affected by cAMP in J744.2 cells, i.e. growth inhibition and two properties considered to be markers for macrophage activation: Fc-mediated phagocytosis and plasminogen activator secretion [29]. They were also used to study cell volume, regulation by cytoskeleton and cAMP [30].

This paper describes the characteristics of the K^+ carrier-mediated transport in these macrophages with respect to the net flux of potassium ions, its sensitivity to extracellular Na^+ and its possible effect on the electrical membrane potential.

Materials and Methods

Materials

^{86}Rb was purchased from the Radiochemical Centre, Amersham, U.K. [^{3}H]TPP$^+$ (tetraphenylphosphonium) (5670 mCi/ml) was obtained from the Nuclear Research Centre, Negev, Israel. Furosemide was purchased from Hoechst AG, Frankfurt am Main, F.R.G. Bumetanide was provided from laboratoire Leo B.P. 9-28500, Vernouillet, Denmark.

Cell culture

The J774.2 mouse macrophage cloned cell line [28] and two of its variants [29]: CT2, deficient in adenylate cyclase, and J7H1, deficient in cAMP-dependent protein kinase, were grown and maintained in modified Eagle's medium (MEM) supplemented with kanamycin 0.01%, penibrin 0.01%, glucose 0.35%, glutamine 4 mM, non essential amino acids and 20% heat inactivated horse serum on non tissue culture plastic dishes (Falcon 1005) to which the cells scarcely adhere. Harvesting was accomplished by gently scraping the cells with a rubber policeman, shaking them off culture dishes and centrifugation at room temperature ($1500 \times g$, 5 min). Then the cells were washed once with the appropriate medium (described below) and resuspended in the same medium at $(3.5–4.5) \cdot 10^6$ cells per ml.

^{86}Rb influx

In order to obtain identical experimental conditions for the influx and for the efflux (see below),

cells were held for approximately one hour at 37°C after being washed. Reactions were initiated by adding 250 μl of the cell suspension to 250 μl of a solution containing 2 μCi of ^{86}Rb in one of the following media: (I) complete MEM; (II) Na^+ and choline$^+$ media, the compositions of which are in mM (final concentrations): 5 glucose, 0.5 $MgCl_2$, 1 $CaCl_2$, 5 Hepes-Tris (pH 7.0), 145–0 NaCl/0–145 choline chloride, 5 KCl, and additional drugs (ouabain, bumetanide, furosemide, etc.) when indicated.

Reaction mixtures containing these solutions were equilibrated at 37°C prior to addition of the cells. After initiating the reactions, incubations were continued at 37°C for given times, and stopped either (a) by addition of 500 μl of ice-cold 125 mM $MgCl_2$ and centrifugation of the reaction vessels in a Brinkmann Eppendorf microcentrifuge (model 5912) for 15 s. The supernatants were immediately aspirated and the same operation repeated with 1 ml of ice-cold 125 mM $MgCl_2$. Or (b) by addition of 100 μl of di-*n*-butylphthalate. The reaction vessels were centrifuged as described above and the supernatants were immediately aspirated. Both methods gave similar results which did not vary by more than 5%. The bottom of the tube containing the cell pellet was then sliced off and transferred to counting vials containing 4 ml of toluene-Triton scintillation medium (1 liter toluene, 300 ml Triton X-100, 4 g PPO and 50 mg POPOP) and radioactivity was determined by liquid scintillation spectrometry at a counting efficiency of approx. 90%. Ouabain-sensitive ^{86}Rb influx (^{86}Rb influx due to the $(Na^+ + K^+)$-ATPase) and ouabain-resistant, bumetanide-sensitive ^{86}Rb influx (^{86}Rb influx carried by K^+ carrier-mediated transport) were determined respectively by subtracting the values obtained in the presence of 1 mM ouabain (ouabain-resistant ^{86}Rb influx) from the ones obtained with no ouabain (total ^{86}Rb influx), and by subtracting the values obtained in the presence of ouabain 1 mM and bumetanide 100 μM (ouabain-resistant, bumetanide-resistant ^{86}Rb influx or ^{86}Rb influx by diffusion) from those obtained in the presence of ouabain 1 mM only. Isotope trapping in the extracellular space of the pellet was corrected for by subtraction of zero-time counts taken immediately after addition of the cells to the media.

^{86}Rb efflux

Cells collected in their own growth medium were loaded with ^{86}Rb (1 μCi per 10^6 cells) for approximately one hour at 37°C, and washed three times with ice-cold complete MEM (2 ml per 10^6 cells). Reactions were initiated by adding 250 μl of washed cells resuspended at $(3.5–4.5)\cdot 10^6$/ml of MEM to 250 μl of prewarmed (37°C) MEM. After given times, 500 μl of ice-cold 125 mM $MgCl_2$ were added and the reaction vessels were centrifuged for 15 s. 500 μl of the supernatants were transferred to counting vials containing 4 ml of toluene-Triton scintillation and radioactivity counted as described above for ^{86}Rb influx. The K^+(^{86}Rb) specific activity was determined by counting total ^{86}Rb and measuring total K^+ content on cells submitted to the same treatments, but in the absence of ^{86}Rb, by Perkin-Elmer atomic absorbance spectrophotometry. Bumetanide-sensitive ^{86}Rb efflux (efflux due to K^+ carrier-mediated transport) was determined by subtracting the values obtained in the presence of bumetanide 100 μM from the ones obtained without it. Activation of bumetanide-sensitive ^{86}Rb efflux by ouabain was monitored under the same conditions, except that 1 mM ouabain was added to the solutions. Loss of isotope was measured by removal of cell aliquots at specific intervals following resuspension in isotope free medium.

[^{3}H]TPP$^+$ uptake

The accumulation of [^{3}H]TPP$^+$ was measured as previously described [31]. Briefly, the reactions were started by adding 250 μl of washed cells previously equilibrated at 37°C to 250 μl of a solution containing 2 μM ^{3}H[TPP$^+$] in one of the following reaction mixtures: (I) 'high-Na^+ medium', 132 mM NaCl, 5 mM KCl, 5 mM glucose, 50 mM Hepes-Tris (pH 7.0), 0.5 mM $MgCl_2$, 1 mM $CaCl_2$; (II) 'high-K^+ medium', same as high-Na^+ medium except that 132 mM KCl was used in place of NaCl; and (III) 'high-choline$^+$ medium', same as high-Na^+ medium except that 132 mM choline chloride was used in place of NaCl and no KCl was present. After given incubation times, 100 μl of di-*n*-butylphthalate were added and the reaction vessels were centrifuged for 15 s. The rest of the procedure was the same as in ^{86}Rb influx. The radioactivity was determined by

liquid scintillation spectrometry at counting efficiency of 50 to 60%. Corrections for the radioactivity trapped in the extracellular space of the pellets were determined by measuring [^{3}H]TPP$^+$ uptake at time zero. The concentration of [^{3}H]TPP$^+$ accumulated by the cells was obtained by using values for intracellular volume previously published [30], i.e. in μl/10^6 cells: 2.1 for J774.2, 1.5 for CT2 and 1.32 for J7H1. These values were determined by isotope dilution of markers for total water ([^{3}H]H_2O) and extracellular space ([^{14}C]inulin) [30]. It is noteworthy that we obtained almost the same results by a modification of the method of Kletzien et al. using 3-O-methyl-D-[^{14}C]glucose as an intracellular space marker [32]. The concentrations of TPP$^+$ were then used to calculate $\Delta\psi$ by the Nernst equation as reported [31]:

$$\Delta\psi = (2.3\ RT/F)\log([TPP^+]_{in}^{corrected}/[TPP^+]_{out})$$

in which $2.3\ RT/F = -61$ at 37°C and

$$[TPP^+]_{in}^{corrected} = [TPP^+]_{in}^{high\ Na^+} - [TPP^+]_{in}^{high\ K^+}.$$

Results

(1) Effect of ouabain on total ^{86}Rb influx

The total ^{86}Rb influx in growing J744.2 macrophages suspended in complete MEM, when measured as a function of time (Fig. 1), is linear for the first ten minutes. The addition of 1 mM ouabain to the incubation medium decreases the initial rate of ^{86}Rb influx by 76.6% indicating that ouabain-sensitive influx, due to $(Na^+ + K^+)$-ATPase activity, is the main portion of total ^{86}Rb influx by those cells. These results are in total agreement with those found for other eukaryotic cells [33–35]. The ouabain-sensitive ^{86}Rb influx is inhibited by the cardiac glycoside with an IC_{50} of approx. 40 μM. This requirement for a relatively high concentration is probably due to the known resistance of mouse cells to cardiac glycosides. Comparable results (IC_{50} about 35 μM) were obtained with growing CT2 and J7H1 macrophages (Table I).

(2) Sensitivity of ouabain-resistant ^{86}Rb influx to 'loop' diuretics

The inhibitory effect of bumetanide and furosemide was tested on ouabain-resistant ^{86}Rb influxes of the three cell lines. As can be seen in Fig. 1, the addition of 100 μM bumetanide in the presence of 1 mM ouabain causes an inhibition of 91.2% of the initial rate of ^{86}Rb influx. Ouabain-resistant, bumetanide-sensitive ^{86}Rb influx participates therefore in 14.8% of the total ^{86}Rb influx in J744.2 macrophages. Very similar results were

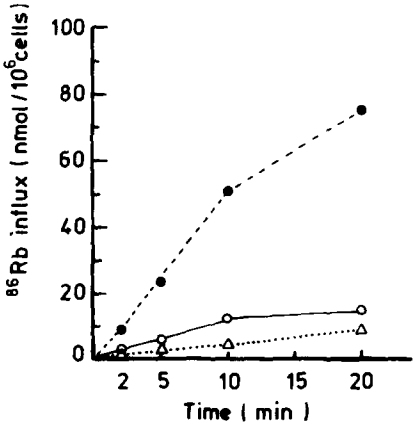

Fig. 1. Time-courses of ^{86}Rb influx to J774.2 macrophages. J774.2 macrophages were suspended in complete MEM in the absence (●) and the presence of 1 mM ouabain (○) or 1 mM ouabain plus 100 μM bumetanide (△), and incubated at 37°C for the indicated times. (A representative experiment out of three performed is demonstrated).

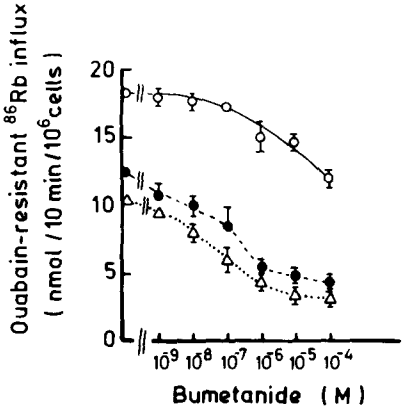

Fig. 2. Bumetanide dose-response on ouabain-resistant ^{86}Rb influx to J774.2, CT2 and J7H1 macrophages. J774.2 (●), CT2 (○) and J7H1 (△) macrophages were suspended in complete MEM containing 1 mM ouabain with the indicated concentrations of bumetanide, and incubated for 10 min at 37°C. Each point represents the mean ± S.E. of 2 to 23 experiments.

TABLE I

COMPONENTS OF UNIDIRECTIONAL ^{86}Rb INFLUX TO J774.2, CT2 AND J7H1 MACROPHAGES

J774.2, CT2 and J7H1 macrophages were suspended in complete MEM in the absence (1) and in the presence of 1 mM ouabain (3) or 1 mM ouabain plus 100 μM bumetanide (5). After 10 min incubation intracellular radioactivity was determined and ouabain-sensitive ^{86}Rb influx (2) as well as ouabain-resistant, bumetanide-sensitive ^{86}Rb influx (4) were calculated as described in Materials and Methods. The data are presented in nmol ^{86}Rb/10 min per 10^6 cells, mean ± S.E. The number in parenthesis represents the number of separate experiments on different cell populations.

Cell lines	J774.2 (WT)		CT2 (AC^-)		J7H1 (PK^-)	
	^{86}Rb influx	% of control	^{86}Rb influx	% of control	^{86}Rb influx	% of control
(1) Total ^{86}Rb influx	51.3 ± 0.7 (16)	100.0	45.8 ± 0.4 (23)	100.0	42.0 ± 0.5 (10)	100.0
(2) Ouabain-sensitive ^{86}Rb influx (($Na^+ + K^+$)-ATPase)	39.3 ± 0.7 (11)	76.6	26.9 ± 1.0 (5)	58.7	32.5 ± 0.7 (5)	77.4
(3) Ouabain-resistant ^{86}Rb influx	12.5 ± 0.4 (17)	24.4	18.2 ± 0.4 (5)	39.7	10.4 ± 0.6 (5)	24.8
(4) Ouabain-resistant, bumetanide-sensitive ^{86}Rb influx (K^+ carrier-mediated transport)	7.6 ± 0.3 (9)	14.8	6.4 ± 0.6 (4)	14.0	7.4 ± 0.5 (3)	17.6
(5) Ouabain-resistant, bumetanide-resistant ^{86}Rb influx (diffusion)	4.5 ± 0.5 (10)	8.8	12.0 ± 0.7 (4)	26.2	3.3 ± 0.4 (3)	7.9

obtained in the two variant lines (Table I).

The dose-response curves of the ^{86}Rb influx to bumetanide in the presence of 1 mM ouabain for the three cells are shown in Fig. 2. In J774.2 and J7H1 cells 100 μM bumetanide inhibits almost totally ouabain-resistant ^{86}Rb influx while in CT2 macrophages it remains still high, indicating that diffusion and/or other transport mechanisms are relatively larger in those cells. The IC_{50} for bumetanide are found to be 0.1, 5.0 and 0.05 μM for J774.2, CT2 and J7H1 cells, respectively. J774.2 and J7H1 macrophages seem therefore to be more sensitive to bumetanide than CT2 cells. Furosemide also inhibited the ouabain-resistant ^{86}Rb influx of the three cells (data not shown). In agreement with previous studies [7], it was approximately 10-times less efficient compared to bumetanide. It can be concluded that about 15% of the total ^{86}Rb influx is transported through the K^+ carrier-mediated transport system in those macrophages and that under these experimental conditions, CT2 cells are less sensitive to 'loop' diuretics than the two other cell lines.

(3) Evidence for bumetanide-sensitive ^{86}Rb efflux

The total ^{86}Rb efflux in growing J744.2 macro-

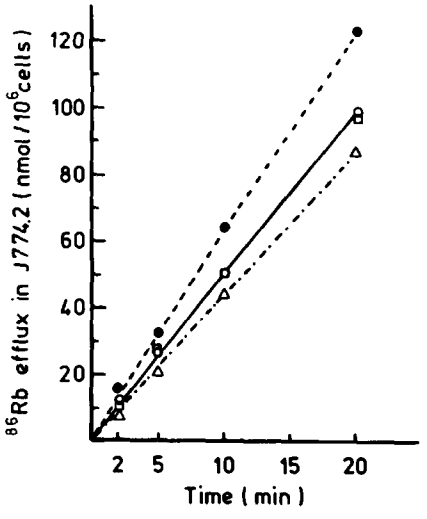

Fig. 3. Time-courses of ^{86}Rb efflux from J774.2 macrophages. J774.2 macrophages, preloaded with ^{86}Rb, were suspended in complete MEM in the absence (○) and the presence of 1 mM ouabain (●) or 1 mM ouabain plus 100 μM bumetanide (△) or 100 μM bumetanide (□) and incubated at 37°C for the indicated times. Radioactivity contained in the extracellular medium was then determined as described in Materials and Methods. (A representative experiment out of three performed is demonstrated).

TABLE II

EFFECT OF OUABAIN AND BUMETANIDE ON UNIDIRECTIONAL ^{86}Rb EFFLUX IN J774.2, CT2 AND J7H1 MACROPHAGES

J774.2, CT2 and J7H1 macrophages, preloaded with ^{86}Rb, were suspended in complete MEM in the absence (1) and in the presence of 1 mM ouabain (2) or 1 mM ouabain plus 100 μM bumetanide (3) or 100 μM bumetanide (4). After 10 min incubation extracellular radioactivity was determined and ^{86}Rb efflux due to K^+ carrier-mediated transport (5) was calculated as described in Materials and Methods. The data are presented in nmol ^{86}Rb/10 min per 10^6 cells, mean $\pm$S.E. The number in parenthesis represents the number of separate experiments on different cell populations.

Cell lines	J774.2 (WT)		CT2 (AC^-)		J7H1 (PK^-)	
	^{86}Rb efflux	% of control	^{86}Rb efflux	% of control	^{86}Rb efflux	% of control
(1) Total ^{86}Rb efflux	50.2 $\pm$ 0.8 (7)	100.0	43.7 $\pm$ 2.0 (2)	100.0	41.7 $\pm$ 1.0 (4)	100.0
(2) ^{86}Rb efflux in presence of 1 mM ouabain	65.8 $\pm$ 1.2 (4)	131.1	56.5 $\pm$ 2.4 (2)	129.3	57.4 $\pm$ 1.8 (2)	137.6
(3) ^{86}Rb efflux in presence of 1 mM ouabain and 100 μM bumetanide	45.6 $\pm$ 0.9 (4)	90.8	45.6 $\pm$ 1.3 (2)	104.3	43.7 $\pm$ 3.8 (2)	104.8
(4) ^{86}Rb efflux in presence of 100 μM bumetanide	49.6 $\pm$ 2.1 (2)	98.8	42.4 $\pm$ 1.1 (2)	97.0	41.7 $\pm$ 2.9 (3)	100.0
(5) ^{86}Rb efflux due to K^+ carrier-mediated transport	0.4 $\pm$ 0.3 (2)	0.8	1.3 $\pm$ 0.8 (2)	3.0	1.0 $\pm$ 0.6 (3)	2.4

phages as a function of time was measured in the same conditions as the total ^{86}Rb influx (Fig. 3). It was seen to be linear for at least 20 min. Interestingly the addition of 1 mM ouabain causes an increase of 27% of total ^{86}Rb efflux. Since ouabain inhibits ($Na^+ + K^+$)-ATPase activity which transports K^+(^{86}Rb) into the cells, a direct effect of ouabain on ^{86}Rb efflux is not expected. A possible mechanism for this phenomenon is that the inhibition of ($Na^+ + K^+$)-ATPase by ouabain results in an increase of extracellular Na^+ concentration which in turn induces an activation of the transporter. When ^{86}Rb efflux was monitored in presence of 1 mM ouabain and 100 μM bumetanide, it was seen to undergo a decrease of 9% compared to the control and of 31% compared with ^{86}Rb efflux measured in the presence of 1 mM ouabain.

The same measurements were performed on the two variants. On these cells, as for the wild type, ouabain caused a stimulation of total ^{86}Rb efflux (table II). Moreover, when 100 μM bumetanide was added to the solution containing 1 mM ouabain, a slight activation of ^{86}Rb efflux was still measured. There results are consistent with those observed in ascites cells [36,37], in SV-3T3 cells [38], in MDCK renal epithelial cells and in Hela carcinoma cells [32] where ouabain was shown to cause an increase in K^+ efflux, stimulation which is inhibited by 'loop' diuretics. In the absence of ouabain in the reaction medium, 100 μM bumetanide did not, or scarcely, affect total ^{86}Rb efflux (Fig. 3 and Table II), which means that ^{86}Rb efflux under normal conditions in these three cell lines is essentially a leak efflux.

(4) Determination of the net ouabain-resistant, bumetanide-sensitive ^{86}Rb flux

As mentioned previously, total ^{86}Rb influx and efflux were determined under similar experimental conditions. The total ^{86}Rb influx rates were almost equal to the total efflux rates (Tables I and II), in agreement with what one would expect from cells in steady state. On the other hand because of the ouabain-induced activation of ^{86}Rb efflux mentioned above, ^{86}Rb efflux through the K^+ carrier-mediated transport system was measured in solutions free of ouabain. ^{86}Rb influx carried by the same system in our cells was insensitive to ouabain-induced activation under the experimental conditions used (unlike those measured in MDCK cells and Hela cells [32]). The same conditions for measuring ^{86}Rb net fluxes were used in

other laboratories for Ehrlich ascites tumor cell [33,36]. Thus the net flux could be calculated by subtracting the bumetanide-sensitive ^{86}Rb efflux from the ouabain-resistant, bumetanide-sensitive ^{86}Rb influx. The values obtained for the net flux were 7.2, 5.1 and 6.4 nmol/10 min per 10^6 cells for J774.2, CT2 and J7H1 cells, respectively. Therefore it can be concluded that K^+ carrier-mediated transport system carries a net inward K^+ flux in the three cell lines.

(5) Extracellular Na^+ dependence of ouabain-resistant, bumetanide-sensitive ^{86}Rb influx

In other experimental systems the ouabain-resistant, bumetanide-sensitive ^{86}Rb influx was shown to be sensitive to extracellular Na^+ concentration [35]. As can be seen in Fig. 4 the ouabain-resistant, bumetanide-sensitive ^{86}Rb influx to J774.2 macrophages is also dependent on the presence of Na^+ in the extracellular medium. The reduction of Na^+ to 10 mM decreases the ouabain-resistant, bumetanide-sensitive ^{86}Rb influx by 81.6% while its elevation over 100 mM does not influence the carrier-mediated transport. At low Na^+ concentrations a slight decrease in ouabain-resistant, bumetanide-sensitive ^{86}Rb influx is observed when the extracellular Na^+ content is increased (between 0 and 10 mM). This

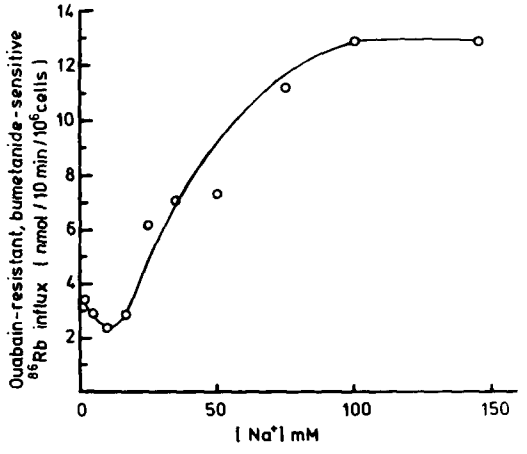

Fig. 4. Sodium dependence of ouabain-resistant, bumetanide-sensitive ^{86}Rb influx to J774.2 macrophages. Ouabain-resistant, bumetanide-sensitive ^{86}Rb influx to J774.2 macrophages, as a function of Na^+ concentration, was measured for 10 min and calculated as described in Materials and Methods. (A representative experiment out of three performed is demonstrated).

effect may reflect a true change in the transporter activity or may result from an indirect effect of the Na^+ concentration on bumetanide binding since Na^+ requirement for bumetanide inhibition of carrier-mediated transport has been previously demonstrated [41]. The K_m for Na^+ calculated from analysis of $1/V$ versus $1/[Na^+]$ (plot not shown) is about 83.3 mM, a value that is close to the one (68.9 mM) determine for NIH 3T3 cells [35].

(6) Electroneutrality of ouabain-resistant, bumetanide-sensitive ^{86}Rb flux

In order to determine whether the ouabain-resistant, bumetanide-sensitive ^{86}Rb flux is electrogenic or electroneutral, [^{3}H]TPP$^+$ accumulation was monitored to establish the electrical membrane potential of the three macrophages. J774.2, CT2 and J7H1 cells suspended in high-Na^+ and high-choline$^+$ media take up [^{3}H]TPP$^+$ rapidly for about 20 min and reach a steady-state level of accumulation at approx. 40 min (Fig. 5). The time curves obtained in those two media are almost identical indicating that Na^+ does not contribute significantly to the resting membrane potential of these cells. In contrast, when the cells are suspended in a medium containing a high K^+ concentration the steady-state level of [^{3}H]TPP$^+$ accumulation is decreased by approx. 70%, 80% and 85% in J774.2, J7H1 and CT2 macrophages, respectively. These results are similar to those obtained with neuroblastoma-glioma NG108-15 hybrid cells [31], human peripheral lymphocytes [42] and mouse spleen lymphocytes [43], and show that the $\Delta\psi$ across the plasma membrane of these macrophages is also due substantially to a K^+ diffusion potential ($K^+_{in} > K^+_{out}$). By using the differences between the results obtained in high-Na^+ and high-K^+ media, $\Delta\psi$ of -80.1 ± 0.31, -108.5 ± 0.14 and -105.1 ± 0.12 mV can be calculated from the data shown in Fig. 5 and Table III for J774.2, CT2 and J7H1 cells, respectively. The first value is in good agreement with the resting potential of -87 mV obtained on macrophages from mouse spleen cultures by measurements using intracellular microelectrodes [44]. It should be noticed that the two variants present higher resting membrane potentials than J774.2 macrophages.

As can be seen in Table III, the addition of the protonophore carbonyl cyanide *m*-chlorophenyl-

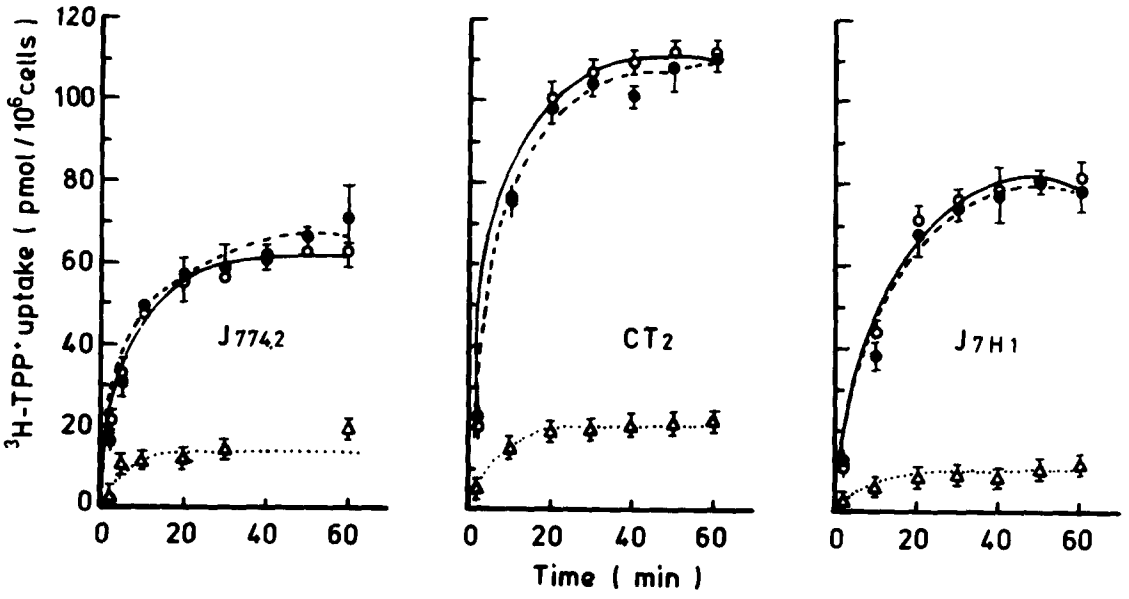

Fig. 5. Time-courses of [^{3}H]TPP$^+$ accumulation. J774.2, CT2 and J7H1 macrophages were suspended in high-Na$^+$ (○) or high-choline (●) or high-K$^+$ (△) medium with 1 μM [^{3}H]TPP$^+$ and incubated at 37°C for the times indicated. Each point represents the mean ± S.E. of 2 to 8 experiments.

hydrazone (CCCP 10 μM) to the extracellular medium results in a marked decrease in [^{3}H]TPP$^+$ accumulation by the three cell lines. Since CCCP generates an increase in proton permeability it causes a dissipation of $\Delta\psi$ and depolarization. This perturbation in [^{3}H]TPP$^+$ uptake by CCCP offers additional proof that [^{3}H]TPP$^+$ accumulation serves as a good measure of the plasma membrane $\Delta\psi$ as has been observed in other cells [31]. The addition of bumetanide (100 μM) to the medium does not affect at all [^{3}H]TPP$^+$ accumulation in the three cells indicating that the K$^+$ carrier-mediated transport system is electroneutral.

Discussion

This study has shown that J774.2 mouse macrophages and its two variant lines, CT2, deficient in adenylate cyclase, and J7H1, deficient in cAMP-dependent protein kinase, contain a K$^+$ carrier-

TABLE III

EFFECT OF CCCP AND BUMETANIDE ON STEADY-STATE LEVELS OF [^{3}H]TPP$^+$ ACCUMULATION

J774.2, CT2 and J7H1 macrophages were washed and suspended in high-Na$^+$ and high-K$^+$ media with 1 μM [^{3}H]TPP$^+$. After 30 min accumulation the indicated compounds were added, and incubations were continued for 30 more minutes. Results are presented as mean ± S.E. with number of experiments in parenthesis.

Cell lines		J774.2 (WT)		CT2 (AC$^-$)		J7H1 (PK$^-$)	
Media	Additions	[^{3}H]TPP$^+$ uptake (pmol/10^6 cells)	$\Delta\psi$ (mV)	[^{3}H]TPP$^+$ uptake (pmol/10^6 cells)	$\Delta\psi$ (mV)	[^{3}H]TPP$^+$ uptake (pmol/10^6 cells)	$\Delta\psi$ (mV)
High Na$^+$	–	63.3 ± 4.1 (3)	−80.1	112.2 ± 1.7 (4)	−108.5	81.6 ± 1.2 (4)	−105.1
High K$^+$	–	20.0 ± 0.1 (2)		22.0 ± 1.0 (4)		11.9 ± 0.6 (2)	
High Na$^+$	CCCP 10 μM	8.9 ± 0.6 (2)		2.9 ± 0.2 (2)		4.1 ± 0.3 (2)	
High Na$^+$	bumetanide	63.4 ± 1.9 (2)	−80.2	110.6 ± 3.4 (4)	−108.1	80.9 ± 4.3 (4)	−104.8

mediated transport system in their plasma membrane. The characterization of this transporter indicates that it conducts a net inward flux of K^+ under the experimental conditions used. This result is in agreement with those obtained on Ehrlich ascites tumor cells [1,34] but differs from those obtained on NIH 3T3 cells [35] and on rabbit reticulocytes [6] (net flux outward), or on human red cells [24,39,40] and on Ehrlich ascites tumor cells [33,36] (zero net flux). It should be observed that the obtained results (net flux inward or outward, or no net flux at all) are dependent on the experimental conditions. This is particularly obvious in the case of the Ehrlich ascites tumor cells where two different results where observed: a net influx, when K^+ carrier-mediated transport system was measured on K^+ depleted cells in K^+ free medium [1,34] and no net flux when it was determined under physiological conditions [33,36].

The K^+ carrier-mediated transport system requires extracellular Na^+ for transporting K^+ into the cells, as demonstrated for J774.2 cells (fig. 4) and is electrically silent, as shown for the three cell lines (Table III). These observations are in agreement with the characteristics of this transport system in other eukaryotic cells [1,34].

Despite these similarities, differences between the three cell lines have been detected. The two variant cells have a resting membrane potential higher than the J774.2 macrophages (Table III). These increased $\Delta\psi$ may result from either an increased permeability to potassium ions or an increase of the electrogenic Na^+/K^+ pump activity. Since the ouabain-sensitive ^{86}Rb influx is percentagewise approximately the same in the three cells (Table I), the later possibility seem to be ruled out. The CT2 cells show a relatively large K^+ diffusion, compared to the two other cells, which indeed may account for the increased $\Delta\psi$ of these cells. On the other hand K^+ diffusion percentagewise seems to be similar for J774.2 and J7H1 cells despite their difference in $\Delta\psi$. This difference appears, therefore, to be the result of a decreased Na^+ permeability in J7H1 cells compared with J774.2 macrophages. Interestingly a significant difference in the sensitivity of the ouabain-resistant ^{86}Rb influx to bumetanide of the CT2 cells as compared to the two other cell lines was observed. This adenylate cyclase deficient cell line is at least ten times less sensitive to bumetanide than the two other macrophages. Whether this difference is due to the decreased level of cAMP in these cells is not known at the present.

Following this basic characterization, it seems suitable to use these cells for studying the effects of several perturbations (such as changes in the media or addition of hormones) on the K^+ carrier-mediated transport system. These studies hopefully will lead to a better understanding of the action of the transporter and its physiological role.

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References

1 Geck, P., Pietrzyk, C., Burckhardt, B.-C., Pfeiffer, B. and Heinz, E. (1980) Biochim. Biophys. Acta 600, 432–447
2 Haas, M., Schmidt, W.F., III, and McManus, T.J. (1982) J. Gen. Physiol. 30, 125–147
3 McRoberts, J.A., Erlingert, S., Rindler, M.J. and Saier, M.H. (1982) J. Biol. Chem. 257, 2260–2266
4 Palfrey, H.C. and Greengard, P. (1981) Ann. N.Y. Acad. Sci. 372, 291–307
5 Bakker-Grunwald, T. (1981) Biochim. Biophys. Acta 641, 427–431
6 Panet, R. and Atlan, H. (1980) J. Membrane Biol. 52, 273–280
7 Palfrey, H.C., Feit, P.W. and Greengard, P. (1980) Am. J. Physiol. 258, C139–148
8 Panet, R., Fromer, I. and Atlan, H. (1982) J. Membrane Biol. 70, 165–169
9 Panet, R. and Atlan, H. (1979) FEBS Lett. 103, 172–175
10 Panet, R. and Atlan, H. (1979) Biochem. Biophys. Res. Commun. 88, 619–626
11 Kregenow, F.M. (1978) In Transport Processes in Red Cells (Ellory, J.C. and Lew, V., eds.), pp. 383–426, Academic Press, New York
12 McManus, T.J. and Schmidt, W.F., III. (1978) in Membrane Transport Processes (Hoffman, J.F., ed.), Vol. 1, pp. 79–106, Raven Press, New York
13 Rudolph, S.A., Schaefer, D.E. and Greengard, P. (1977) J. Biol. Chem. 252, 7132–7139
14 Panet, R., Fromer, I. and Alayoff, A. (1983) J. Membrane Biol. 75, 219–224
15 Wacholtz, M., Chan, L.N. and Sha'afi, R.I. (1978) J. Cell Physiol. 94, 1–12

16 Palfrey, H.C. and Rao, M.C. (1983) J. Exp. Biol. 106, 43–54
17 Riddick, D.H., Kregenow, F.M. and Orloff, J. (1971) J. Gen. Physiol. 57, 752–766
18 McManus, T.J. and Haas, H. (1981) Fed. Proc. 46, 484 (Abstr.)
19 McManus, T.J. and Schmidt, W.F. (1978) J. Supramol Struct. 7, 212 (Abstr.)
20 Kregenow, F.M., Robbie, D.E. and Orloff, J. (1976) Am. J. Physiol. 231, 306–312
21 Rudolph, S.A. and Greengard, P. (1974) J. Biol. Chem. 249, 5684–5687
22 Palfrey, H.C., Alpen, S.L. and Greengard, P. (1980) J. Exp. Biol. 89, 103–115
23 Frizzel, R.A., Dugos, M.C. and Schultz, S.G. (1975) J. Gen. Phys. 65, 769–795
24 Garay, R.P. (1982) Biochim. Biophys. Acta 688, 786–792
25 Frizzell, R.A., Smith, P.L., Vosburgh, E. and Field, M. (1979) J. Membrane Biol. 46, 27–39
26 Rao. M.C., Nash, N.T. and Field, M. (1983) Fed. Proc. Fed. Am. Soc. Exp. Biol. 42, 1280
27 Guandalini, S., Rao, M.C., Smith, P.L. and Field, M. (1982) Am. J. Physiol. 243, G36–G41
28 Muschel, R.L., Rosen, N. and Bloom, B.R. (1977) J. Exp. Med. 145, 175–186
29 Rosen, N., Piscitello, Schreck, J., Muschel, R.J., Bloom, B.R. and Rosen, O.M. (1979) J. Cell Physiol. 98, 125–136
30 Melmed, R.N., Karanian, P.J. and Berlin, R.D. (1981) J. Cell Biol. 90, 761–768
31 Lichtstein, D., Kaback, H.R. and Blume, A.J. (1979) Proc. Natl. Acad. Sci. USA 76, 650–654
32 Aiton, J.F. and Simmons, N.L. (1983) Biochim. Biophys. Acta 734, 279–289
33 Tupper, J.T. (1975) Biochim. Biophys. Acta 394, 586–596
34 Geck, P., Heinz, E., Pietrzyk, C. and Pfeiffer, B. (1978) in Cell Membrane Receptors for Drugs and Hormones: A multidisciplinary Approach (Straub, R.W. and Bolis, L., eds.) pp. 301–307, Raven Press, New York
35 Atlan, H., Snyder, D. and Panet, R. (1984) J. Membrane Biol. 81, 181–188
36 Bakker-Grundwald, T. (1978) Biochim. Biophys. Acta 513, 292–295
37 Bakker-Grundwald, T., Andrews, J.S. and Neville, M.C. (1980) J. Membrane Biol. 52, 141–146
38 Bakker-Grundwald, T., Ogden, P. and Lamb, J.F. (1982) Biochim. Biophys. Acta 687, 333–336
39 Dunn, M.J. (1970) J. Clin. Invest. 49, 1804–1814
40 Lubowitz, H. and Whittam, R. (1969) J. Physiol. (Lond.) 202, 111
41 Forbush, B., III and Palfrey, H.C. (1983) J. Biol. Chem. 258, 11787–11792
42 Deutsch, C.J., Holian, A., Holian, S.K., Daniele, R.P. and Wilson, D.F. (1979) J. Cell. Physiol. 99, 79–94
43 Kiefer, H., Blume, A.J. and Kaback, H.R. (1980) Proc. Natl. Acad. Sci. USA 77, 2200–2204
44 Gallin, E.K. (1981) Science 214, 458–460