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Characterisation of a $Na^+/K^+/Cl^-$ cotransporter in alkylating agent-sensitive L1210 murine leukemia cells

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The mode of influx of $^{86}Rb^+$, a K^+ congener, to exponentially proliferating L1210 murine leukemia cells, incubated in a Krebs-Ringer buffer, has been characterised. The influx was composed of a ouabain-sensitive fraction (approx. 40%), a loop diuretic-sensitive fraction (approx. 40%) and a fraction which was insensitive to both types of inhibitor (approx. 15%). The fraction of ouabain-insensitive $^{86}Rb^+$ influx, which was fully inhibited by furosemide (1 mM) or bumetanide (100 μM), was completely inhibited when Cl^- was completely substituted by nitrate or gluconate ions, but was slightly (29 ± 12%) stimulated if the Cl^- was substituted by Br^-. The substitution of Na^+ by Li^+, choline or tetramethylammonium ions inhibited the loop diuretic-sensitive fraction of $^{86}Rb^+$ uptake. These results suggested that a component of $^{86}Rb^+$ influx to L1210 cells was mediated via a $Na^+/K^+/Cl^-$ cotransporter. $^{86}Rb^+$ efflux from L1210 cells which had been equilibrated with $^{86}Rb^+$ and incubated in the presence or absence of 1 mM ouabain, was insensitive to the loop diuretics. Additionally, efflux rates were found to be independent of the external concentration of K^+, suggesting that efflux was not mediated by K^+–K^+ exchange. The initial rate of $^{86}Rb^+$ influx to L1210 cells in the plateau phase of growth was reduced to 44% of that of exponentially dividing cells, the reduction being accounted for by significant decreases in both ouabain- and loop diuretic-sensitive influx; these cells were reduced in volume compared to cells in the exponential phase of cell growth. In cells which had been deprived of serum for 18 h, and which showed an increase of the proportion of cells in the G_1 phase of the cell cycle, the addition of serum stimulated an immediate increase in the furosemide-sensitive component of $^{86}Rb^+$ influx. Diuretic-sensitive $^{86}Rb^+$ influx was not altered by the incubation of the cells with 100 μM dibutyryl cyclic AMP, but was inhibited by 10 μM of the cross-linking agent nitrogen mustard (bis(2-chloroethyl)methylamine, HN2).

Introduction

The malignant phenotype is characterised by an aberrant control of cell proliferation and differentiation. The cell membrane is a major locus for the expression of this control, and presents itself as an attractive target for pharmacological inter-

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vention. In this, and the following paper [37], the ionic flux of potassium across the plasma membrane of murine L1210 leukemia cells is characterised and the effects of the cytotoxic drug nitrogen mustard upon this flux are presented.

Changes in the flux of ions across the plasma membrane accompany the early biochemical events which characterise the mitogenic stimulation of quiescent cells [1]. Notable among these changes is a stimulation of potassium influx modulated by the ouabain-sensitive Na^+/K^+-ATPase [2]. In certain cells, a mitogen-stimulated increase of the ouabain-resistant potassium flux has also been observed. The sensitivity of the flux to the loop diuretics, such as furosemide, identifies it, pharmacologically, as occurring via the $Na^+/K^+/Cl^-$ cotransporter [3–7]. Stimulation of the $Na^+/K^+/Cl^-$ cotransporter by growth factors in hamster fibroblasts was shown to be independent of the activation of the Na^+/H^+ antiporter, and has been suggested, possibly, to occur via the stimulation of the activity of protein kinase C [6]. The $Na^+/K^+/Cl^-$ cotransporter is also considered to play a role in the volume regulation of some cells [8–10].

There is some controversy with regard to both the role of the $Na^+/K^+/Cl^-$ cotransporter in mitogenesis and the nature and role of any possible changes in intracellular potassium concentration which may occur after mitogenic stimulation. Amsler et al. [5] and Paris and Pouyssegur [6] have suggested that activation of the cotransporter was neither necessary nor sufficient for the events which led to a stimulation of DNA synthesis, whereas Panet [4] has suggested that in NIH-3T3 mouse fibroblasts the mitogen-stimulated influx of K^+, mediated by the activation of the cotransporter, may be important in initiating DNA synthesis, and may explain an increase in intracellular potassium concentration found in the G_1 phase of their cell cycle. Mitogen-stimulated changes in intracellular potassium concentration appear to vary amongst cell types, since both Kaplan et al. [11] and Holien et al. [12] showed that there was a decrease in the intracellular K^+ concentrations of lymphocytes after phytohaemagglutinin treatment, but Amsler et al. [5] showed that in Swiss 3T3 cells no significant changes in intracellular K^+ concentration occurred during a 24-h period following mitogenic stimulation with a mixture of insulin, epidermal growth factor and arginine vasopressin.

Changes in the concentration of intracellular ions may regulate cellular volume, and the converse regulation may pertain; that is, intracellular volume may be regulated by the cell, perhaps via the cytoskeleton, and ionic concentration adjusted accordingly. How a cell determines and regulates its steady-state volume is a matter of much conjecture, but the $Na^+/K^+/Cl^-$ cotransporter is considered to play a role in the regulation of the volume of some cell types, the best defined being avian erythrocytes and Ehrlich ascites cells [8–10]. In a recent study by Levinson of Ehrlich ascites cells [13], the intracellular concentration of chloride ions appeared to be the prime regulator of cell volume. Other methods of volume control may be active in some cell types, and it is possible that the mitogen-stimulated activation of the $Na^+/K^+/Cl^-$ cotransporter is a reflection of just one of the pathways of volume regulation, perhaps associated with reorganisation of cellular architecture during proliferation [14].

We and others have been interested in the effect of antiproliferative drugs on the function of the cell membrane in malignant cells (reviewed in Ref. 15). In order to identify the mechanism whereby the cytotoxic alkylating agent nitrogen mustard (bis(2-chloroethyl)methylamine) inhibited the influx of the potassium congener rubidium into tumor cells, as described in detail in the following paper, we have characterised the pathways of rubidium influx to L1210 murine leukemia cells which were in different phases of growth, since the sensitivity of these cells to nitrogen mustard is greater in actively dividing cells in comparison to quiescent cells. In particular, we have characterised an ouabain-resistant fraction of influx as occurring via the $Na^+/K^+/Cl^-$ cotransporter and have shown its activity to be expressed in actively dividing cells. This characterisation is provided by the widely accepted criteria of interdependency of ion flux, ion selectivity and pharmacological inhibition.

Methods

Chemicals, radiochemicals and reagents

Inorganic salts and general reagents were purchased from BDH Ltd. (Poole, U.K.) or Fisher Chemical Co. (Fair Lawn, NJ, U.S.A.) and were of analytical grade. Dow-Corning 550 silicon oil was obtained from Hopkin and Williams (Romford, U.K.) and corn oil from local supermarkets. Rubidium chloride, choline chloride, sodium gluconate, ouabain, furosemide and dibutyryl cyclic 3′,5′-AMP were purchased from Sigma Ltd. (Poole, U.K. or St. Louis, MO, U.S.A.). Bumetanide was the gift of Leo Laboratories (Aylesbury, U.K.) and nitrogen mustard was the gift of the Boots Co. (Nottingham, U.K.). Radiochemicals were purchased from Amersham Plc. or from New England Nuclear (Southampton, U.K. or Boston, U.S.A.). The liquid scintillant Fisofluor 'mpc' was purchased from Fisons Plc (Loughborough, U.K.).

Cell culture

L1210 murine leukemia cells were obtained from Flow Laboratories (Irvine, U.K.). Cells were maintained in logarithmic growth in RPMI 1640 medium supplemented with 10% horse serum (Gibco Ltd, Glasgow, U.K.), and when seeded at $3 \cdot 10^4$ cells/ml were in exponential growth by 48 h, with a doubling time of approx. 14 h. These cells reached a plateau of growth by 72 h, by which time the cell density was $(1–2) \cdot 10^6$ cells/ml. The cells were grown at 37 °C in an atmosphere of 10% carbon dioxide in air. Cultures were rendered quiescent by the harvesting of cells from dense, plateau phase cultures, and after washing in serum free RPMI 1640 medium they were suspended in serum-free medium and gassed with 5% CO_2 in air. Cell number and cell volumes were estimated by a model ZB_1 Coulter Counter fitted with a Channelyzer (Coulter Electronics, Harpenden, U.K.) and short-term cell viability (< 6 h) was determined by the exclusion of a solution of 0.1% Trypan blue.

Flow cytometry

The distribution of populations of cells in the cell cycle was determined essentially by the method of Gray and Coffino [16]. Normal saline washed cells (10^6/ml) were fixed with 70% ice-cold ethanol, centrifuged at 11 600 × *g* for 1 min, then incubated with 1 mg/ml of ribonuclease A in 0.1 M phosphate buffer (pH 7.0) for 0.5 h at 37 °C. Cells were resuspended in 1 ml of 50 μg/ml propidium iodide in 0.1% sodium citrate and 0.1% Triton X-100 for 0.5 h. Flow cytometry was performed using a Becton Dickinson FACS 440 fluorescence-activated cell sorter (excitation 540 nm, emission 625 nm). Histograms to show the distribution of DNA in the cell population were constructed by the use of a Consort 40 computer.

Measurement of ion flux of L1210 cells

Experiments were conducted with $5 \cdot 10^6$ cells/ml which, unless otherwise stated, were harvested from cultures in exponential growth. These were washed twice with the appropriate medium (see below) and then suspended either in RPMI 1640 medium (Gibco, Glasgow), or in Krebs-Ringer buffer (pH 7.4): the full buffer consisted of 118 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1.18 mM $MgSO_4$, 1.17 mM KH_2PO_4 and 5.5 mM glucose with the addition of 1.27 mM $CaCl_2$. Substitution of various ions was made under conditions of the maintenance of buffer osmolarity. For estimations of ion influx, the cell suspensions were preincubated with different agents at 37 °C, with shaking, for various times, while being supplied continuously with 5% CO_2 in air, before the addition of tracer amounts of either $^{42}K^+$ Cl (2 μCi/ml), $^{86}Rb^+$Cl (5 μCi/ml), $^{22}Na^+$ Cl (1 μCi/ml) or inulin [^{14}C] carboxylic acid (1 μCi/ml) and ^3H_2O (1 μCi/ml). 200-μl samples were withdrawn at various times, in triplicate, and layered over 100 μl of a mixture of 10 parts of dow-Corning 550 silicon oil mixed with 3 parts of corn oil which was layered, in turn, over 50 μl of 90% formic acid in a 400-μl microcentrifuge tube. The tubes were rapidly centrifuged at 11 600 × *g* for 30 s in a Beckmann Microfuge B, frozen in liquid nitrogen, cut across the oil phase and the two fragments were placed in vials for counting on an ICN Tracerlab Gamma Set 500, a Packard TriCarb 2660 or a Beckman LS230 liquid scintillation counter. When fractional influxes were estimated, the radioactivity in the acid pellet was expressed as a fraction of the total in the supernatant and pellet. Initial rates of tracer $^{86}Rb^+$

influx were calculated during the initial linear phase of influx, which was shown to last 15 min (see the Results below) and, because we had found it to substitute completely for K^+, were expressed as nmol of Rb^+ and K^+.

$^{86}Rb^+$ efflux from L1210 cells was measured from $5 \cdot 10^6$ cells which were labelled with 5 μCi/ml $^{86}Rb^+$ to equilibrium over a 2-h period at 37°C in RPMI 1640 medium. Incubation with drugs, for various times, was initiated in the same medium after this loading period. The cells were then washed rapidly, twice, in an ice-cold buffer which contained 123 mM KCl, 25 mM $KHCO_3$, 1.17 mM KH_2PO_4, 1.18 mM $MgSO_4$ and 5.5 mM glucose with the addition of 1.27 mM $CaCl_2$, and efflux was initiated by resuspension of the cells in RPMI 1640 medium or a standard Krebs-Ringer (see above) at 37°C, or in a modified Krebs-Ringer with depleted potassium, in which the potassium ion was replaced by sodium ion. The volume of the cells was estimated by measurement of the cell-associated ^3H-labelled water, with a correction for the extracellular space as estimated by ^{14}C-labelled inulin carboxylic acid.

Results

In preliminary experiments, it was determined that incubation of L1210 cells in Krebs-Ringer buffers maintained the viability of the cells at greater than 96% after a 3-h incubation. The profile of the initial rate of influx of $^{86}Rb^+$ to exponentially growing L1210 cells, preincubated for 0.5 h in Krebs-Ringer or RPMI 1640 medium at 37°C, was shown, in more than 50 experiments, to be linear for 15 min, and influx up to this time was used in subsequent experiments to estimate the effects of various inhibitors on $^{86}Rb^+$ influx, none of which brought about any deviation of the influx kinetics from linearity during this time. It was also established, by the use of $^{42}K^+$, that the potassium congener $^{86}Rb^+$ was transported identically to K^+ when L1210 cells were incubated in Krebs-Ringer in which the final K^+ or Rb^+ concentration was 0.3 mM, with and without the inhibitors ouabain and bumetanide (Fig. 1). It was therefore assumed that the transport of tracer amounts of the two isotopes was equivalent, as has been reported by other workers [17].

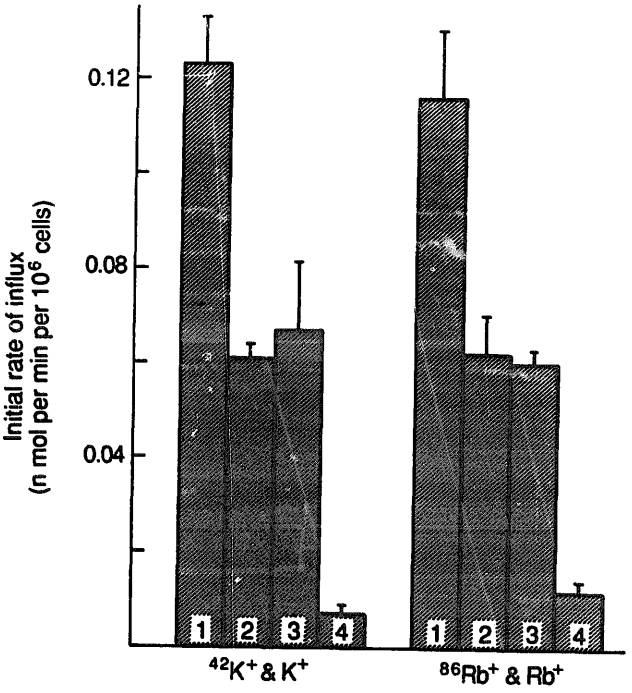

Fig. 1. Histograms to show the pharmacological sensitivity of the initial rates of $^{42}K^+ + K^+$ and $^{86}Rb^+ + Rb^+$ influxes to L1210 cells, preincubated with each drug for 5 min in Krebs-Ringer buffer. 1, control; 2, 1 mM ouabain; 3, 100 μM bumetanide; 4, 100 μM bumetanide plus 1 mM ouabain (mean ± S.D., $n = 3$).

Pharmacological definition of $^{86}Rb^+$ influx

Fig. 1 shows the effects of a 5-min preincubation, in Krebs-Ringer with 1 mM ouabain, 100 μM bumetanide and a mixture of 1 mM ouabain/100 μM bumetanide, on the initial rate of $^{86}Rb^+$ influx to exponentially growing L1210 cells.

A 3-h preincubation of L1210 cells with 10 μM of the alkylating agent bis(2-chloroethyl)methylamine (HN2) reduced by 49% the $^{86}Rb^+$ influx to L1210 cells; the inhibition was accounted for by a reduction of the diuretic-sensitive component of influx – the details of these experiments are reported in the following paper [37]. Preincubation of L1210 cells in Krebs-Ringer buffer for 1 h with 100 μM dibutyryl cyclic AMP had no effect on $^{86}Rb^+$ influx (data not shown).

Co-ion dependency of diuretic-sensitive $^{86}Rb^+ + K^+$ influx

Fig. 2 shows the effect of substituting the chloride ions of Krebs-Ringer buffer with bromide or nitrate or gluconate anions on the bumetanide-sensitive $^{86}Rb^+ + K^+$ influx to L1210 cells. In the

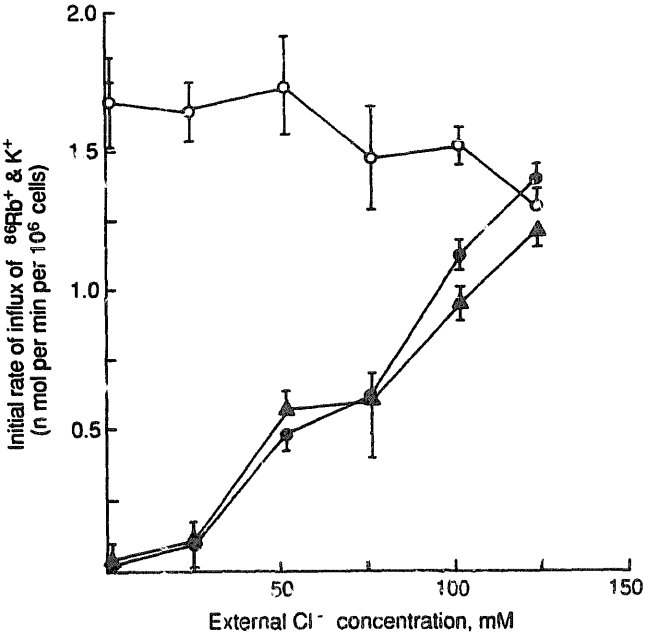

Fig. 2. The effect of substitution of external chloride ions with (○) bromide, (●) nitrate or (▲) gluconate anions on the initial rate of bumetanide-sensitive $^{86}Rb^+ + K^+$ influx to L1210 cells in Krebs-Ringer buffers. (Mean ± S.D., $n = 3$).

presence of nitrate or gluconate, an increase in the concentration of extracellular chloride increased the $^{86}Rb^+ + K^+$ influx. However, substitution of extracellular chloride ions with bromide was able to support $^{86}Rb^+ + K^+$ influx, and even to stimulate it significantly, so that in the complete absence of chloride, influx was 29 ± 12% greater than in the normal Krebs-Ringer with 123 mM chloride. Fig. 3 shows the effects of the substitution of Na^+ ions with lithium, tetramethylammonium or choline cations on the rate of $^{86}Rb^+ + K^+$ influx into L1210 cells. Substitution with Li^+ at concentrations of Na^+ below 75 mM partially supported the $^{86}Rb^+ + K^+$ influx, whereas at external Na^+ concentrations above 75 mM, the $^{86}Rb^+ + K^+$ influx was proportional to the Na^+ concentration. Neither choline nor tetramethylammonium ions were capable of supporting the $^{86}Rb^+ + K^+$ influx. Substitution of the bicarbonate anions in the Krebs-Ringer buffer with nitrate (whilst maintaining the external Cl^- ion concentration at 123 mM, and the pH at 7.4) reduced the initial rate of ouabain-resistant $^{86}Rb^+ + K^+$ influx by 28% (data not shown); this element of HCO_3-dependent $^{86}Rb^+$ influx was completely inhibited by preincubation of the cells for 15 min with 1 mM furosemide.

Because of the lack of linear relationships between the rate of $^{86}Rb^+ + K^+$ influx and the con-

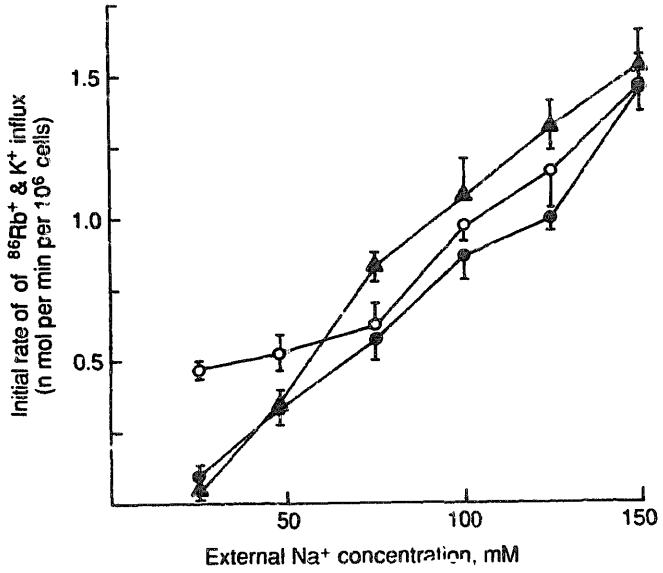

Fig. 3. The effect of substitution of Na^+ ions with: (○) Li^+, (●) choline or (▲) tetramethylammonium ions on the rate of bumetanide-sensitive $^{86}Rb^+$ influx to L1210 cells in Krebs-Ringer buffers. (Mean ± S.D., $n = 3$).

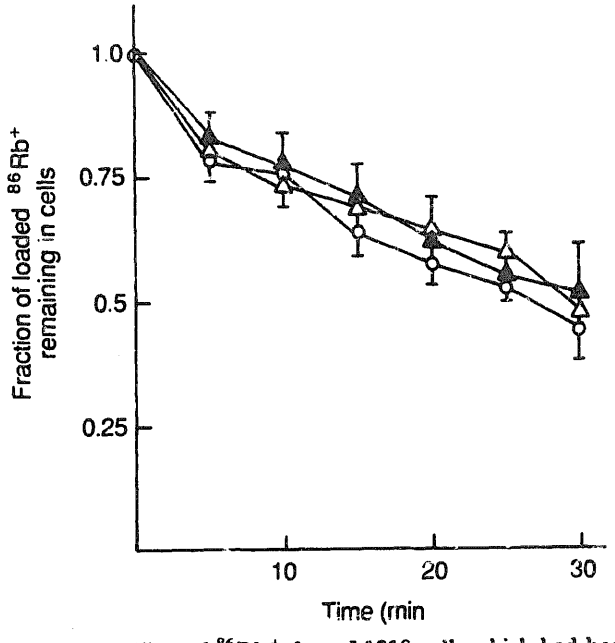

Fig. 4. The efflux of $^{86}Rb^+$ from L1210 cells which had been allowed to reach isotopic equilibrium represented as the fraction of $^{86}Rb^+$ remaining in the cells at various times after placing the cells in isotope-free media which contained 1 mM ouabain. Efflux into RPMI medium. ○, controls, and in the presence of (△) 1 mM furosemide or (▲), 100 μM bumetanide.

centrations of ions substituting for Cl^- or Na^+, and because the ions, when present at physiological concentrations, were not at saturating concentration, it was considered inappropriate to draw conclusions regarding the stoichiometry of cotransport or the K_m value for each ion, and this is discussed below.

$^{86}Rb^+$ efflux from L1210 cells

Preliminary experiments had established that the distribution of $^{86}Rb^+$ in L1210 cells had reached isotopic equilibrium within 2 h of incubation with 5 μCi/ml of $^{86}Rb^+$ (data not shown). Fig. 4 shows the efflux of $^{86}Rb^+$ into RPMI 1640 medium in the presence of 1 mM ouabain; approx. 50% of the isotope initially present in the cells was retained after 0.5 h of efflux. Furosemide, bumetanide and nitrogen mustard (data not shown), all of which inhibited influx of $^{86}Rb^+$ via the $Na^+/K^+/Cl^-$ cotransporter (see above and the following paper [37]) had no effect on the rate of $^{86}Rb^+$ efflux, after the cells had been preincubated for 3 h. Similar efflux kinetics were observed when efflux was allowed to occur in Krebs-Ringer buffer (data not shown). Efflux of $^{86}Rb^+$ into potassium-free Krebs-Ringer followed essentially the same pattern, and comparison of the $^{86}Rb^+$ efflux in K^+-repleted or -depleted medium, measured by the fraction retained after 15 min, showed no significant difference ($P > 0.05$, $n = 3$).

$^{86}Rb^+ + K^+$ influx into L1210 cells in different phases of growth

Fig. 5 shows the initial rates of $^{86}Rb^+$ influx when cells were in (a) exponential growth or were growth-limited by either harvesting them at the (b) plateau or the stationary phase (high cell density) or (c) by serum deprivation of plateau-phase cells for 18 h. This latter procedure did not reduce cell viability below 90%, as measured by the exclusion of Trypan blue, and flow cytometry showed that more than 80% of the cells were in the G_1 phase of the cell cycle (data not shown). Fig. 5d shows the effect of the readdition of 10% serum to serum-deprived cells on the initial rate of influx of $^{86}Rb^+$ and K^+ when the serum and tracer $^{86}Rb^+$ were added simultaneously.

As expected, the $^{86}Rb^+$ plus K^+ influx to 10^6 cells which were dividing exponentially (1.99 ± 0.22 nmol/min, Fig. 5a) exceeded that to quiescent, stationary-phase cells (0.88 ± 0.13 nmol/min, Fig. 5b). The increase in influx to the serum-deprived cells (Fig. 5c), compared to plateau-phase cells was presumed to be the result of placing them in fresh RPMI 1640 medium 18 h previously, albeit without serum. In the serum-deprived cells, the activity of the furosemide- and bumetanide-sensitive fraction of transport was greatly reduced. Unexpectedly, the addition of serum immediately stimulated the component of the total flux which was furosemide-sensitive (from 0.05 ± 0.14 to 0.42 ± 0.14 nmol/min, $P < 0.01$,

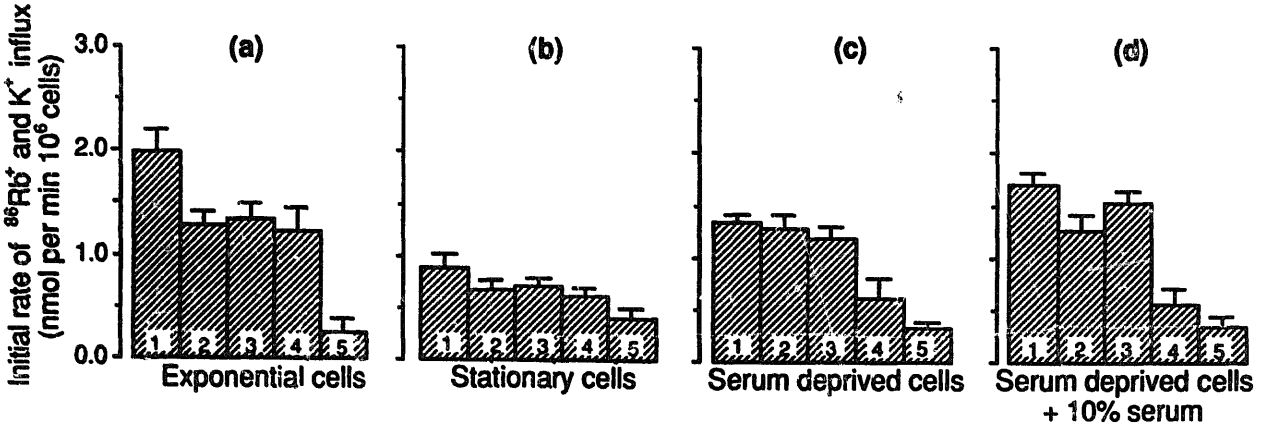

Fig. 5. The pharmacologically defined components of the initial rate of $^{86}Rb^+ + K^+$ influx to L1210 cells in different phases of cell growth (incubated in RPMI 1640 medium). a, cells in exponential growth; b, cells from stationary, dense cultures; c, cells which had been deprived of serum for 18 h. d, Cells deprived of serum for 18 h to which 10% serum had been added. All drugs were added 15 min prior to addition of $^{+86}Rb^+$: 1, control; 2, 1 mM furosemide; 3, 100 μM bumetanide; 4, 1 mM ouabain; 5, 1 mM ouabain plus 1 mM furosemide (means ± S.D. of at least three determinations).

$n = 3$), but had less effect on the bumetanide-sensitive component of influx of $^{86}Rb^+$ (from 0.15 ± 0.12 to 0.18 $\pm$ 0.12 nmol/min, $P > 0.05$, $n = 3$) although this must have ultimately risen to the level seen in exponentially growing cells (Fig. 5a). Whether this immediate change in the component of diuretic-sensitive influx represents a sodium-independent K^+/Cl^- influx, which is generally less sensitive to bumetanide than to furosemide [18], remains to be established.

Alterations of cell volume during various phases of L1210 cell growth

In exponential growth, the mean volume of 10^6 cells, harvested and placed in RPMI 1640, determined by distribution of tritiated water, was 0.812 ± 0.197 μl for exponentially growing cells and 0.622 ± 0.155 μl for cells in the plateau phase of their growth ($P < 0.05$), but when resuspended in Krebs-Ringer buffer their volume was less significantly different: 0.905 ± 0.108 μl for exponentially growing cells and 0.707 ± 0.177 μl for plateau-phase cells ($P > 0.05$, $n = 6$). Treatment of exponentially growing cells in Krebs-Ringer buffer with 1 mM furosemide or 100 μM bumetanide for 3 h reduced L1210 cell volume by 33% and 29%, respectively, and treatment with 1 mM ouabain increased cell volume by 25%. Similar treatment of cells which had been harvested in the stationary phase of growth with furosemide (1 mM) brought about no change in cell volume.

Discussion

The characterisation of the components of K^+ flux which constitute the activity of a $Na^+/K^+/Cl^-$ cotransporter in the L1210 leukemia cell line is a prelude to studies of the role that it may play in the regulation of proliferation and particularly in the pharmacological effects of the antiproliferative drug nitrogen mustard, which are described in the following paper [37]. The present studies suggest that exponentially growing L1210 cells contain an ouabain-insensitive component of transport with properties which are similar to those of the $Na^+/K^+/Cl^-$ cotransporter, as described in other proliferating cells [8–10]. Thus, potassium transport, for which rubidium proved to be a complete congener (Fig. 1), in accordance with other work [17], was found to consist of three pharmacologically defined components, one of which, in the presence of ouabain, was inhibited by the loop-diuretics furosemide and bumetanide (Fig. 1). This ouabain-insensitive fraction of potassium transport was identified as having characteristic properties of co-ion dependence on external ions, typical of the $Na^+/K^+/Cl^-$ cotransporter (Figs. 2 and 3). That is, there was absolute dependence on extracellular Na^+ ions and, when these were progressively substituted by choline or tetramethylammonium, potassium influx diminished. A partial replacement of Na^+ by Li^+ to allow K^+ transport was observed, in common with findings in cultured human HSWP fibroblasts, HeLa cells, Ehrlich ascites cells and avian erythrocytes [19–25]. With regard to anion dependency, we were surprised to discover that complete substitution of Cl^- with Br^- actually stimulated rubidium uptake; Br^- has previously been shown to partially substitute for Cl^- [19–25]. Our finding of a Br^- stimulation of K^+ influx, measured in the presence of ouabain, appears to be at variance with the findings of Aiton et al. [26], who reported that bromide ions stimulated the furosemide-insensitive influx, but inhibited the furosemide-sensitive influx to HeLa cells, possible by preventing binding of Cl^- to the Cl^--binding site(s) on the diuretic-sensitive cotransporter protein. We presume that in the L1210 cells Br^- binds to this site in a way that activates cation transport; we planned to measure Cl^- transport in the presence of Br^- to ascertain the effects on anion flux, but were unable to establish reproducible influx patterns of this ion.

The failure, under our experimental conditions, to demonstrate saturation of $^{86}Rb^+$ (K^+) influx under conditions of physiological chloride concentrations, prevented the construction of Hill plots, so as to determine the stoichiometry of influx. In addition, the almost sigmoidal nature of some of the data, particularly when Cl^- ions were being substituted (Fig. 2) suggests that the substituting co-ions may have been exerting elements of inhibition of Rb^+ influx at some concentrations. Owen and Prastein made similar observations with regard to the characterisation of the cotransporter in cultured fibroblasts [19], and defined their estimates of the stoichiometry of

cotransport as 'qualitative'. They criticise the results of other workers who, with similar data, present quantitative estimates of stoichiometry. Comparable cautions, regarding the quantitative use of such data were expressed by O'Grady et al. in a recent review [8].

In many of the cell types studied the activity of the $Na^+/K^+/Cl^-$ cotransporter has been found to be symmetric (reversible), for example in Ehrlich ascites cells [13]. The pattern of Rb^+ efflux from L1210 cells was biphasic: with an initially rapid phase (half-time approx. 13 min) followed by a slower phase (half-time of approx. 42 min) (Fig. 4). These are patterns of efflux similar to those observed in mouse fibroblasts [27,28], and probably represent loss from a rapidly exchangeable pool, perhaps in the glycocalyx, followed by a slower phase of efflux presumed to represent genuine ion flux from an intracellular pool(s). The most particular and peculiar difference between the $Na^+/K^+/Cl^-$-cotransporter of L1210 cells and that of other cell types concerns its apparent asymmetry (Fig. 4). Not only was Rb^+ efflux unaffected by the loop diuretics, but treatment of the cells with 10 μM nitrogen mustard for 3 h, prior to measurement of efflux, was also without effect (see following paper [37]); this treatment is sufficient to inhibit completely bumetanide-sensitive influx. The rate of $^{86}Rb^+$ efflux was independent of the presence of 1 mM ouabain when added just prior to efflux, which indicated that the flux of Rb^+ out of the cell was not via a separate mode of operation of the sodium pump [29].

Inhibition of the sodium pump, with the subsequent elevation of intracellular sodium concentration, may activate Rb^+ (K^+) efflux either by activation of the $Na^+/K^+/Cl^-$ cotransporter or by activation of K^+–K^+ exchange [20,30,31]. In potassium-free medium (data not shown), preincubation with ouabain for 1 h or more, prior to the commencement of efflux, led to a greater efflux of Rb^+, but the loop diuretics remained without activity, even after 3 h of incubation. The pathway of Rb^+ efflux is thus not by K^+–K^+ exchange, and it remains an enigma. The asymmetry of the $Na^+/K^+/Cl^-$ cotransporter of L1210 cells clearly distinguishes it from that of Ehrlich ascites cells [13], 3T3 cells [32], MDCK cells [33] and mouse L cells [27,28]. A recent report suggests that human erythrocytes show an asymmetry in the action of the $Na^+/K^+/Cl^-$ cotransporter, although K^+ efflux remained sensitive to furosemide [34]. Other (unpublished) results of ours suggest that the potassium efflux in L1210 cells is not mediated via a Ca^{2+}-activated K^+ channel.

There were changes in the components of pharmacologically defined potassium uptake in L1210 cells according to their state of growth (Fig. 5). As the cells moved from an exponential phase of cell growth to the plateau phase, the activity of both ouabain- and diuretic-sensitive influx diminished. Although cell viability was greater than 90%, these stationary- or plateau-phase cells were in a nutrient and serum-deprived state, and it may be that the passive component of K^+ influx was increased as cell deterioration proceeded; when these cells were resuspended in fresh nutrient medium, but without serum, a fall in the level of the rate of passive diffusion occurred (bar 5 in Fig. 5c), which may support this hypothesis. Conditions of serum deprivation in fresh nutrient medium diminished the activity of loop diuretic-sensitive K^+ transport, while the activity of the sodium pump appeared to rise marginally. In common with the results of other studies [4–6], the readdition of serum to quiescent cells immediately stimulated the potassium influx but, interestingly, the diuretic component which increased was more significantly inhibited by furosemide in comparison with bumetanide (Fig. 5d): this is a topic being further investigated by us. The mean volumes of the cells in exponential growth differed significantly from those cells which were quiescent, when determined in RPMI medium, but, for reasons which are unclear, the difference was less significant when the cells were resuspended in Krebs-Ringer buffer prior to the determination of their volume (see Results). The activity of anion-coupled transport systems has been implicated in the control of the steady-state volume [35,36] and the results presented here support this: thus, cells from stationary cultures, which show a reduced component of cotransporter activity, were smaller than those cells from the exponential phase of cell growth. Additionally, incubation with the loop diuretics decreased the volume of cells which were in the exponential phase of their growth, but not those in the sta-

tionary phase, which had exhibited a much diminished activity of the cotransporter and already had reduced their volume (Results and Fig. 5).

In summary, the modes of K^+ influx to L1210 murine leukemia cells resemble those of some other cell types, and its modulation according to conditions of growth, supports the idea that changes in ouabain-resistant, diuretic-sensitive flux might be closely associated with proliferation. Whether the $Na^+/K^+/Cl^-$ cotransporter has an important role in the control of the proliferation of L1210 cells remains to be determined unequivocally, but towards this end we have isolated a mutant which apparently lacks the cotransporter. Pharmacological evidence, presented in the following paper [37], is suggestive of a role for the cotransporter in the control of cell proliferation, in that an antiproliferative drug specifically inhibits the activity of the $Na^+/K^+/Cl^-$ cotransporter. The results presented here form the basis for these pharmacological studies.

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