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INFLUENCE OF LOOP DIURETICS AND ANIONS ON PASSIVE POTASSIUM INFLUX INTO HUMAN RED CELLS

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SUMMARY

1. Passive K influx into human red cells was measured with and without Cl ions, Na ions and loop diuretics.

2. Ouabain and loop diuretics appear to inhibit specifically and respectively the Na pump and (Na+K) 'co-transport'. Inhibitors of other pathways, e.g. 4,4′-diisothiocyantostilbene-2,2′-disulphonic acid or amiloride did not inhibit passive K influx.

3. Loop diuretics inhibited with high apparent affinity in Na-containing media and with low apparent affinity in Na-free media where there was a substantial Cl-dependent component.

4. The Cl concentration dependence was measured using six anion substitutions for Cl. With NO_3, acetate and gluconate, the curves were sigmoidal and not fully saturable at 150 mM-Cl; with iodide and thiocyanate, the curves were convex; with sulphate, there was saturation at 120 mM-Cl.

5. The half-maximal K influx as a function of $[Na]_o$ was 40 mM for the Cl-dependent flux component and 12 mM for the diuretic-sensitive flux.

INTRODUCTION

In 1980, two groups of investigators showed that the furosemide-sensitive component of the passive Na and K fluxes in human red cells requires Cl ions (Chipperfield, 1980; Dunham, Stewart & Ellory, 1980). In the same year, Geck and his colleagues presented evidence for a furosemide-sensitive, (Na+K+Cl) co-transport in ascites cells which was only guessed at in human red blood cells (r.b.c.s) (Geck, Pietrzyk, Burckhardt, Pfeiffer & Heinz, 1980). The experiments on human r.b.c.s were not, however, entirely new. They were a synthesis of three earlier pieces of work. One established that passive K influx is partly dependent on Cl ions (Funder & Wieth, 1967) and the others that passive Na and K fluxes are inhibited by furosemide and that they are dependent on the other ion (Sachs, 1971; Wiley & Cooper, 1974).

The work by Funder & Wieth (1967) brought out another feature. When a range of different anions was used to replace Cl, passive Na and K fluxes were variously increased or decreased. This point was rather overlooked in the more recent work (Chipperfield, 1980; Dunham *et al.* 1980) and it seemed worthwhile to examine the effects of different anions in more detail. At the same time, it seemed advisable to

check on the specificity of the drugs usually employed to block the putative (Na + K + Cl) co-transport in human r.b.c.s. This is important because furosemide-sensitive (Na + K + Cl) co-transport is now known to be widely distributed and to serve a number of functions in a variety of tissues (for a brief review, see Palfrey & Rao, 1983).

The Cl-dependent (Na + K) co-transport in human red cells behaves as if it were a model for (Na + K + Cl) co-transport elsewhere. In this paper, only the results for K influx at low $[K]_o$ will be described. A preliminary account has been presented (Chipperfield, 1984*a*).

METHODS

Abbreviations. DIDS: 4,4′-diisothiocyantostilbene-2,2′-disulphonic acid. SITS: 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid. MOPS: 3-(*N*-morpholino)-propanesulphonic acid. Tris: tris (hydroxymethyl)-aminomethane. NMDG: *N*-methyl-D-glucamine. TEA: tetraethylammonium. $[Na]_o$: external Na concentration. $[K]_i$: internal K concentration, etc.

Isolation of human r.b.c.s. Blood was withdrawn by venepuncture from adult volunteers into heparinized tubes. Small samples were taken for determination of cell and plasma [Na] and [K], whole blood [haemoglobin], haematocrit and, at least once for each volunteer, r.b.c. and white blood cell (w.b.c.) numbers. The remainder was centrifuged at 2300 *g* for 30 min at 4 °C and then the plasma and buffy coat were removed.

R.b.c.s in Cl-containing media. The cells were washed five times in 150 mM-NaCl, 10 mM-glucose and 10 mM-Tris–MOPS pH 7·5 by resuspension and centrifugation as above for 5 min or, after the last wash, 30 min. Samples were taken for determination of r.b.c. [Na], [K] and [haemoglobin]. The cells were finally resuspended in the same medium to a final haematocrit of 10–20%. Samples of the cell suspension were taken for determinations of [haemoglobin] and haematocrit.

R.b.c.s in Na-free and Cl-free media. The cells were suspended in the appropriate Na-free and Cl-free media and incubated at 37 °C for 1 h. They were then centrifuged, washed, sampled and resuspended as described above. Most of the substitutions for Na (i.e. choline, TEA and NMDG) and for Cl (i.e. NO_3, SCN, I, acetate and gluconate) were simple one-for-one replacements. When Mg (for Na) and SO_4 (for Cl) were used, the osmolarity was made up with sucrose (see legends to Tables 4 and 6 and Fig. 4). The control Cl-prepared cells were incubated at 37 °C likewise.

K influx. A sample (0·25 ml) of the r.b.c. suspension was added to 1 ml ^{86}Rb-labelled medium (all previously cooled on ice) so that the final composition (in the standard NaCl condition) was 142 mM-NaCl, 8 mM-KCl, 10 mM-glucose, 10 mM-Tris–MOPS pH 7·5, 0·1 mM-ouabain and 1·6 μCi ^{86}Rb ml^{-1}; variations (i.e. cation and anion replacements, and additions of drugs) were made as indicated in the legends to the Figures and Tables. The assay mixture was incubated at 37 °C for 1 h and then centrifuged at 1300 *g* for 10 s (MSE Microcentaur). The supernatant was removed, the cells resuspended in 1 ml original washing solution (previously cooled on ice) and centrifuged again. The cells were washed four times in the same way, centrifuging finally for 60 s. The washed cells were haemolysed in 0·6 ml 1% Triton X-100 and 0·6 ml 10% trichloroacetic acid was added. The tubes were centrifuged at 13000 *g* for 60 s and 1 ml samples of the supernatants withdrawn into vials for scintillation counting. A sample of the ^{86}Rb-labelled medium was also taken for scintillation counting. All procedures were carried out in triplicate. An unincubated control was made up, washed etc. for each condition. With SCN substitution, all washing solutions were cooled to 20 °C because K permeability is at a minimum at this temperature (Wieth, 1970).

K influx (μmol (ml cells)$^{-1}$ h^{-1} ± S.E. of mean) was calculated from the activity inside the cells (less control) and the optical density at 540 nm of the packed cells and the cell suspensions as described previously (Chipperfield, 1981). K influxes on the 'co-transport' pathway are quantitatively and qualitatively identical with ^{42}K and ^{86}Rb as tracers for K (Chipperfield, 1981; Hall, Ellory & Klein, 1982).

Cell [Na] and [K]. Fresh r.b.c.s were washed five times in ice-cold 75 mM-$MgCl_2$, 95 mM-sucrose and 10 mM-Tris–MOPS pH 7·5 (Adragna & Tosteson, 1984), centrifuged at 13000 *g* for 10 s and finally 60 s. Triplicate samples were diluted 1000 times for analysis.

Analytical methods. [Na] and [K] were determined by atomic absorption (Unicam SP191),

haemoglobin as cyanmethaemoglobin from its optical density at 540 nm and radioactivity by liquid scintillation using the Cerenkov effect. R.b.c. and w.b.c. numbers were counted in a Coulter Counter.

Materials. Choline chloride and iodide, Trizma, TEA, MOPS, NMDG, DIDS, SITS, Drabkin's reagent and haemoglobin standards were obtained from Sigma Chemical Company, Poole, Dorset, ^{86}Rb from Amersham International, Amersham, Bucks, and ouabain and all other reagents from BDH Ltd., Poole, Dorset. Furosemide and piretanide were gifts from Hoechst (U.K.) Ltd., bumetanide from Leo Laboratories Ltd. and amiloride from Professor A. W. Cuthbert.

TABLE 1. Inhibition of K influx by ouabain and piretanide: additive effects

	K influx (μmol (ml cells)$^{-1}$ h^{-1})		
	Control	+ Piretanide	Difference
NaCl medium			
Control	2·712 ± 0·024	2·210 ± 0·012	0·502 ± 0·026
+ Ouabain	0·675 ± 0·023	0·192 ± 0·019	0·482 ± 0·029
Difference	2·037 ± 0·033	2·017 ± 0·022	
$NaNO_3$ medium			
Control	2·332 ± 0·021	2·285 ± 0·032	0·047 ± 0·038
+ Ouabain	0·157 ± 0·018	0·156 ± 0·020	0·002 ± 0·027
Difference	2·175 ± 0·028	2·129 ± 0·038	
Choline chloride medium			
Control	1·699 ± 0·047	1·624 ± 0·014	0·075 ± 0·049
+ Ouabain	0·405 ± 0·003	0·320 ± 0·016	0·085 ± 0·016
Difference	1·293 ± 0·047	1·304 ± 0·022	

K influx into fresh human r.b.c.s was measured in media containing 142 mM-NaCl (or $NaNO_3$ or choline chloride), 8 mM-KCl (or KNO_3), 10 mM-glucose, 10 mM-Tris–MOPS pH 7·5 and 1·6 μCi ^{86}Rb ml^{-1} *plus* 10^{-4} M-ouabain and 10^{-4} M-piretanide as required. Measurements were the means of three determinations (± S.E. of means).

RESULTS

Inhibitors of K influx

The aim was to verify that loop diuretics are 'specific' for the putative co-transport pathway and that other drugs, most importantly ouabain, do not act on it. The loop diuretics employed were furosemide, bumetanide and (most commonly) piretanide.

Ouabain and loop diuretics. The effects of ouabain and piretanide individually and together on K influx were tested in three media which contained mainly either NaCl, $NaNO_3$ or choline chloride (Table 1). The piretanide-sensitive component was reduced on removing external Na and abolished on removing Cl as described previously (Wiley & Cooper, 1974; Chipperfield, 1980, 1981; Dunham *et al.* 1980). In each condition, the piretanide-sensitive K influx was unaffected by ouabain and vice versa. Thus, at the concentrations employed (both 10^{-4} M) the effects of ouabain and piretanide were entirely additive. This observation does not contradict the finding that furosemide can inhibit the Na pump (Wiley & Cooper, 1974). These authors used a higher concentration of furosemide (10^{-3} M) throughout. On the other hand there is disagreement on the effectiveness of piretanide (or furosemide) in Na-free media. According to Wiley & Cooper (1974), the presence of external Na is immaterial but here piretanide did not inhibit completely in Na-free media (Table 1). This difference will be dealt with later.

The results seem to conflict with earlier work on the effect of removing external Na on active K influx. In this study the replacement of Na with choline reduced the ouabain-sensitive K influx by about 17% (Table 1). In contrast, Garrahan & Glynn (1967) and Priestland & Whittam (1968) saw no effect of choline replacement at comparable $[K]_o$. The explanation may be that here the cells were equilibrated for

TABLE 2. Inhibition of passive K influx by loop diuretics: non-additive effects

	K influx (μmol (ml cells)$^{-1}$ h^{-1})	
Drugs added	K influx	Difference from control
None, control	0·724 ± 0·008	
Furosemide	0·215 ± 0·002	0·508 ± 0·009
Piretanide	0·189 ± 0·003	0·535 ± 0·009
Bumetanide	0·192 ± 0·004	0·532 ± 0·010
Furosemide + piretanide	0·200 ± 0·003	0·525 ± 0·009
Furosemide + bumetanide	0·193 ± 0·007	0·532 ± 0·011
Piretanide + bumetanide	0·192 ± 0·000	0·533 ± 0·008
Furosemide + piretanide + bumetanide	0·185 ± 0·001	0·539 ± 0·009

K influx into fresh human r.b.c.s was measured in media containing 142 mM-NaCl, 8 mM-KCl, 10 mM-glucose, 10 mM-Tris–MOPS pH 7·5, 0·1 mM-ouabain and 1·6 μCi ^{86}Rb ml^{-1} *plus* 10^{-4} M-furosemide, 10^{-4} M-piretanide and 10^{-4} M-bumetanide as indicated. Measurements were the means of three determinations (± S.E. of means).

TABLE 3. Inhibition of passive K influx by loop diuretics: effect of external Na

	K influx (μmol (ml cells)$^{-1}$ h^{-1})	
Drugs added	Control media	Na-free media
None, control	0·921 ± 0·067	0·633 ± 0·020
Furosemide	0·124 ± 0·009	0·312 ± 0·004
Piretanide	0·084 ± 0·006	0·297 ± 0·015
Bumetanide	0·076 ± 0·003	0·277 ± 0·003

K influx into fresh human r.b.c.s was measured in media containing 142 mM-NaCl (or choline chloride), 8 mM-KCl, 10 mM-glucose, 10 mM-Tris–MOPS pH 7·5, 0·1 mM-ouabain and 1·6 μCi ^{86}Rb ml^{-1} *plus* 10^{-4} M-furosemide, 10^{-4} M-piretanide or 10^{-4} M-bumetanide as indicated. Measurements were the means of three determinations (± S.E. of means).

1 h at 37 °C before the experiment. Thus, $[Na]_i$ was lower in the choline-treated cells than the Na-treated cells: in this experiment, respectively 10·2 (±0·3 S.E. of mean, $n = 3$) and 11·1 ± 0·4 μmol (ml cells)$^{-1}$ in Mg–sucrose-washed cells. The difference is small but the Na pump is very sensitive to changes in $[Na]_i$ in this range (Garay & Garrahan, 1973).

In all, seven experiments of this kind were carried out with different combinations of Na replacements (choline, Mg–sucrose and NMDG) and the three loop diuretics (furosemide, piretanide and bumetanide). In one with furosemide, there was some evidence of interaction between this drug and ouabain but this did not occur in other experiments (e.g. Tables 2 and 3).

Loop diuretics. It is generally assumed that all loop diuretics block the same path-

way (Ellory & Stewart, 1982). If so, their effects should be non-additive. To confirm this, passive K influx was measured in the presence of furosemide, piretanide and bumetanide either alone or in combination (Table 2). As expected, once 'co-transport' was blocked by one loop diuretic the addition of one or two more was without effect.

The inhibition of passive K influx by the three loop diuretics was then tested in the presence and absence of external Na (Table 3). In this experiment, Na was

TABLE 4. Inhibition of passive K influx by loop diuretics: Na substitutions

	K influx (μmol (ml cells)$^{-1}$ h^{-1})		
Main external ions	Control	±Piretanide	Difference
NaCl	0·418±0·012	0·159±0·002	0·256±0·012
Choline chloride	0·359±0·025	0·282±0·000	0·114±0·025
$MgCl_2$	0·152±0·003	0·095±0·002	0·056±0·004
TEA chloride	0·443±0·002	0·362±0·001	0·081±0·002
NMDG chloride	0·333±0·011	0·261±0·003	0·072±0·012
NMDG nitrate	0·140±0·002	0·125±0·002	0·014±0·002

K influx into fresh human r.b.c.s was measured in media containing (except $MgCl_2$, see below) 142 mM-NaCl (or choline chloride, TEA chloride, NMDG chloride or NMDG nitrate), 8 mM-KCl (or KNO_3), 10 mM-glucose, 10 mM-Tris–MOPS pH 7·5, 0·1 mM-ouabain and 1·6 μCi ^{86}Rb ml^{-1} *plus* 10^{-4} M-piretanide as indicated. The $MgCl_2$ medium contained 75 mM-$MgCl_2$, 59 mM-sucrose and KCl, glucose, Tris–MOPS, ouabain, ^{86}Rb and piretanide as above. Measurements were the means of three determinations (±S.E. of means).

replaced with choline. The results show three features. First, all three loop diuretics inhibit in Na-containing media as usual. Secondly, removal of Na lowered K influx but not down to the level observed with loop diuretics in Na-containing media. Thirdly, in Na-free media all three loop diuretics reduced K influx but again not down to the level seen in Na-containing media. Wiley & Cooper (1974) also found that the Na-dependent K influx was less than the furosemide-sensitive flux but, as Wiater & Dunham (1983) have pointed out, they did not draw attention to it.

A similar experiment was carried out with four different Na substitutions and one NaCl substitution (Table 4). Three new conclusions can be drawn. First, piretanide inhibited in all of the Na-free media tested but less than in Na-containing media. Thus, this is not an effect unique to choline. Secondly, the absence of external Na *per se* does not create a new inward K leak. This is shown by the results with NMDG nitrate and $MgCl_2$–sucrose (notwithstanding whatever inhibitory effects Mg may have (Ellory, Flatman & Stewart, 1980)). Thirdly, the results with NMDG chloride and nitrate show that K influx is Cl dependent in the absence of external Na. This agrees with the results of Wiater & Dunham (1983) although they used a much higher $[K]_o$ of 50 mM and choline nitrate substitution. It also excludes the possibility that the effect of Na removal is due to the K leak induced by choline and NMDG at 0 °C whilst washing the cells after incubation. This leak is not Cl-dependent (Blackstock, Ellory & Stewart, 1985) and, in any case, all washing procedures were comparable (see Methods). However, TEA does appear to enhance the inward K leak; this was not explored further.

The concentration dependence of the inhibition by loop diuretics was examined in

the presence and absence of external Na. In these experiments, Na was replaced by choline and the results for furosemide, for example, are shown in Fig. 1. In the presence of Na, there was a sigmoidal relationship between log (dose) and K influx. In the absence of Na, there was again slight inhibition and it did not appear to be reaching a maximum even at 10^{-3} M. Similar results were obtained with piretanide

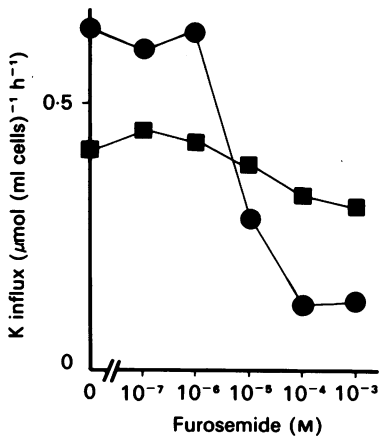

Fig. 1. Inhibition of passive K influx by furosemide: effect of external Na. K influx was measured in conditions similar to those described in the legend to Table 3 except that piretanide was varied from 0 to 10^{-4} M as indicated. All results are the mean of three determinations: error bars showing S.E. of means fall within the size of the symbols. ●, Na-containing media; ■, Na-free (choline) media.

and bumetanide. In the course of these experiments, the K_i values for inhibition in Na-containing media were estimated as 9·6 (±1·3, S.E of mean, $n = 6$) $\times 10^{-6}$ M for furosemide, 1·1 (±0·3, $n = 3$) $\times 10^{-6}$ M for piretanide and 1·7 (±0·8, $n = 3$) $\times 10^{-7}$ M for bumetanide. These values agree well with those given by Ellory & Stewart (1982): respectively 9×10^{-6}, $1{\cdot}2 \times 10^{-6}$ and $1{\cdot}6 \times 10^{-7}$ M.

Drugs other than loop diuretics. The effects of some drugs that are known to block ion transport in human r.b.c.s were tested (Table 5). None of them inhibited K influx at the concentrations employed and the removal of external Na did not disclose any unforeseen inhibitory effects. Thus, as shown previously for SITS (Dunham *et al.* 1980), SITS, DIDS and amiloride do not appear to interact with the loop diuretic-sensitive pathway. The small effects of DIDS and 10^{-4} M-amiloride shown here are not significant. They were not seen in other experiments.

Cl replacements

The recent work on Cl dependence of passive K influx into human r.b.c.s was incomplete in a number of respects (Dunham *et al.* 1980; Chipperfield, 1981). Only seven anions were tested, the Cl replacement was sometimes partial, the action of loop diuretics was not always explicit, the [Cl] dependence was tested only with NO_3 substitution, and some of the anions tested by Funder & Wieth (1967) were overlooked.

Simple substitutions. The effects of five different anion substitutions on passive K influx were examined in the presence and absence of piretanide (Table 6). Amongst the anions tested, NO_3, SCN and I are interesting because Funder & Wieth (1967) studied them previously, SO_4 and SCN because they are commonly employed in work on plasma membrane vesicles, and gluconate because it is impermeable and has been useful in work on r.b.c.s and Madin–Darby canine kidney (MDCK) cells (Cotterrell & Whittam, 1971; McRoberts, Erlinger, Rindler & Saier, 1982).

TABLE 5. Inhibition of passive K influx by loop diuretics: specificity

	K influx (μmol (ml cells)$^{-1}$ h^{-1})		
Drug added	Control	Na-free	Difference
None, control	0·867 ± 0·011	0·404 ± 0·004	0·462 ± 0·011
10^{-4} M-bumetanide	0·142 ± 0·004	0·224 ± 0·009	−0·082 ± 0·017
10^{-5} M-amiloride	0·876 ± 0·004	0·412 ± 0·019	0·464 ± 0·024
10^{-4} M-amiloride	0·841 ± 0·011	0·412 ± 0·011	0·429 ± 0·016
5×10^{-5} M-DIDS	0·819 ± 0·004	0·406 ± 0·004	0·415 ± 0·006
5×10^{-5} M-SITS	0·884 ± 0·018	0·414 ± 0·004	0·470 ± 0·018

K influx into fresh human r.b.c.s was measured in media containing 142 mM-NaCl (or choline chloride), 8 mM-KCl, 10 mM-glucose, 10 mM-Tris–MOPS pH 7·5, 0·1 mM-ouabain and 1·6 μCi ^{86}Rb ml^{-1} *plus* bumetanide, amiloride, DIDS and SITS as shown. Measurements are means of three determinations (± S.E. of means).

TABLE 6. Influence of anion substitutions on passive K influx

	K influx (μmol (ml cells)$^{-1}$ h^{-1})		
External anion	Control	+ Piretanide	Difference
Cl	0·578 ± 0·041	0·163 ± 0·010	0·414 ± 0·043
NO_3	0·140 ± 0·001	0·120 ± 0·002	0·020 ± 0·002
SO_4	0·214 ± 0·001	0·190 ± 0·015	0·025 ± 0·015
I	0·219 ± 0·003	0·216 ± 0·005	0·003 ± 0·006
SCN	0·294 ± 0·006	0·293 ± 0·011	0·001 ± 0·012
Gluconate	0·660 ± 0·013	0·668 ± 0·007	−0·008 ± 0·015

K influx was measured into fresh human r.b.c.s in media containing 142 mM-NaCl (or NO_3 etc. except SO_4 (see below), 8 mM-KCl (or NO_3 etc., except SO_4), 10 mM-glucose, 10 mM-Tris–MOPS pH 7·5, 0·1 mM-ouabain and 1·6 μCi ^{86}Rb ml^{-1} *plus* 10^{-4} M-piretanide as shown. For SO_4, the media contained 75 M-Na_2SO_4, 4 mM-K_2SO_4, 59 mM-sucrose, 10 mM-glucose, 10 mM-Tris–MOPS pH 7·5, 0·1 mM-ouabain and 1·6 μCi ^{86}Rb ml^{-1} *plus* 10^{-4} M-piretanide as required. Measurements were means of three determinations (± S.E. of means).

None of the anions tested supported a significant piretanide-sensitive K influx. The piretanide-insensitive influx was also affected by anions but in the opposite sense. It increased with all except NO_3. The increase with gluconate was so large that total passive K influx was greater than in Cl-containing media. One explanation for these results could be that Cl ions are required for piretanide binding and inhibition. This would be comparable to the effect of external Na described above. Against this, the affinity for bumetanide increases as [Cl] is reduced in avian r.b.c.s (Haas & McManus, 1983).

A direct comparison with the work of Funder & Wieth (1967) is invalid because they used partial replacements in HCO_3-containing media and they did not test loop diuretics. With these provisos, their results and ours agree in showing that NO_3 and I reduce passive K influx.

[Cl] dependence. The [Cl] dependence of passive K influx was measured with and without 10^{-4} M-piretanide using the following anions to replace Cl: NO_3 (Fig. 2),

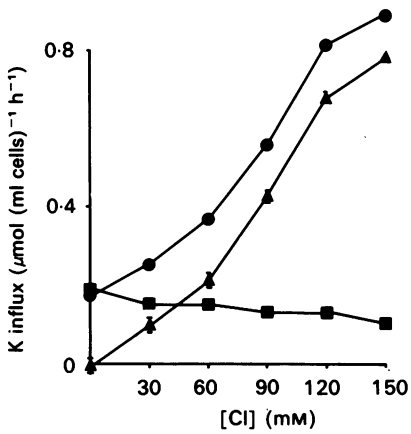

Fig. 2. [Cl] dependence of passive K influx: NO_3 substitution. K influx was measured in media containing 142 mM-Na, 8 mM-K, 0–150 mM-Cl and 150–0 mM-NO_3, 10 mM-glucose, 10 mM-Tris–MOPS pH 7·5, 0·1 mM-ouabain and 1·6 μCi ^{86}Rb ml^{-1} *plus* 10^{-4} M-piretanide as indicated. All results are the mean of three determinations: error bars show S.E. of means unless they fall within the size of the symbols. ●, control; ■, with piretanide; ▲, difference.

acetate (Fig. 3), SO_4 (Fig. 4), SCN (Fig. 5), iodide (Fig. 6) and gluconate (Fig. 7). The results obtained with each substituting anion were distinct with regard to both the piretanide-sensitive and piretanide-insensitive components. In all experiments, the cells were pre-equilibrated in media containing Cl as required and therefore, except for gluconate, which is impermeable, [Cl] may be taken to be equal inside and outside.

NO_3. There was a sigmoidal, saturable component of the passive K influx (Dunham *et al.* 1980) and this is now shown to be entirely piretanide-sensitive (Fig. 2). The half-maximal activation, here and in the results of Dunham *et al.* (1980), occurs at about 80 mM-Cl. In this experiment, the piretanide-insensitive influx appears to fall slightly as [Cl] increases. This is not significant and not consistently seen (Chipperfield, 1981 and Table 6). There is no conflict with the earlier claim that there is a linear dependence on [Cl] because [Cl] was varied only over the range 0–90 mM (Chipperfield, 1981).

Acetate. There was a sigmoidal, saturable component of the passive K influx and this was fully inhibited by piretanide (Fig. 3). The results with acetate are thus similar to those with NO_3 but the apparent affinity seems to be greater. The mean $K_{\frac{1}{2}}$ (of three) was 61 mM. The piretanide-insensitive influx was unaffected by acetate.

At concentrations as low as 30 mM, acetate almost completely inhibits diuretic-sensitive Na efflux from r.b.c.s (Chipperfield & Shennan, 1983) and K influx into

cultured cells (Aiton, Chipperfield, Lamb, Ogden & Simmons, 1981). The results with K influx are clearly quite distinct.

SO_4. The piretanide-sensitive component more obviously reached saturation than with NO_3 and acetate and there was a maximum at 120 mM-Cl. The apparent affinity ($K_{\frac{1}{2}}$ = 40 mM) was greater than with the two anions just described (Fig. 4).

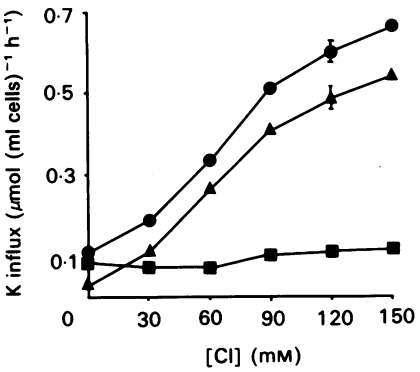

Fig. 3. [Cl] dependence of passive K influx: acetate substitution. K influx was measured in conditions similar to those described in the legend to Fig. 2 except that acetate was the replacement anion for chloride. ●, control; ■, with piretanide; ▲, difference.

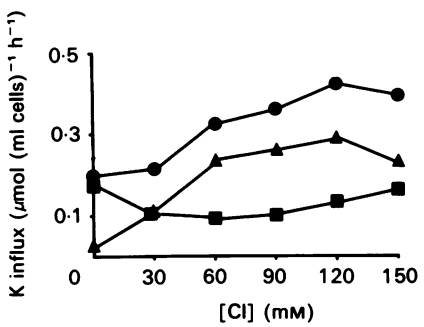

Fig. 4. [Cl] dependence of passive K influx: SO_4 substitution. K influx was measured in media containing 0–142 mM-NaCl (or 100–0 mM-Na_2SO_4), 8 mM-KCl (or 4 mM-K_2SO_4), 0–75 mM-sucrose, 10 mM-glucose, 10 mM-Tris–MOPS pH 7·5, 0·1 mM-ouabain and 1·6 μCi ^{86}Rb ml^{-1} *plus* 10^{-4} M-piretanide as indicated ●, control; ■, with piretanide; ▲, difference.

The piretanide-insensitive influx was also affected by [Cl] or [SO_4] or both, confirming the result in Table 6. In this experiment, the piretanide-insensitive influx fell to a minimum at 60 mM-Cl at which level [SO_4] was 50 mM.

SCN. The piretanide-sensitive influx increased with [Cl] but the curve curved upwards and there was no indication of saturation (Fig. 5). The results for the piretanide-insensitive influx were inconsistent amongst the three experiments done. Nevertheless, all showed maxima at [Cl] between 0 and 150 mM, in the experiment shown at 60 mM-Cl.

One reason for the inconsistency may be that SCN induces a substantial influx of

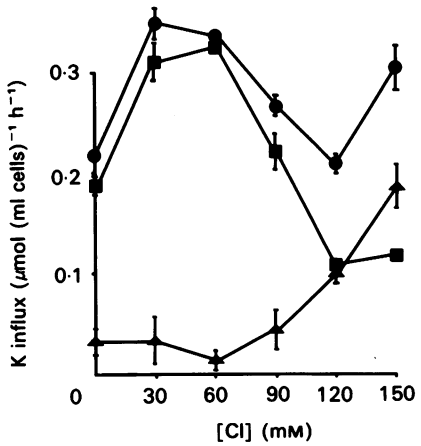

Fig. 5. [Cl] dependence of passive K influx: SCN substitution. K influx was measured in conditions similar to those described in the legend to Fig. 2 except that SCN was the replacement anion for Cl. ●, control; ■, with piretanide; ▲, difference.

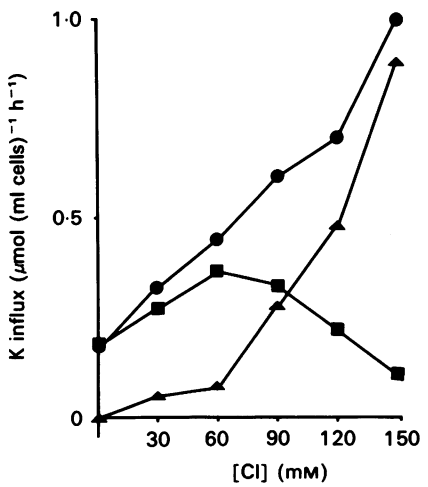

Fig. 6. [Cl] dependence of passive K influx: I substitution. K influx was measured in conditions similar to those described in the legend to Fig. 2 except that I was the replacement anion for Cl. ●, control: ■, with piretanide; ▲, difference.

Na (Funder & Wieth, 1967; Chipperfield, 1984*b*) and this might lead to haemolysis. Moreover, SCN induces a substantial efflux of K (Funder & Wieth, 1967; Shennan, 1984) and this could lead to re-efflux of tracer. Probably, these effects of SCN also explain why the errors were unusually large in all three experiments.

Iodide. The results (Fig. 6) were similar to those just described for SCN. Thus, the piretanide-sensitive influx followed an upward-curving line and the piretanide-insensitive influx a maximum at 60 mM-Cl.

Gluconate. Unlike the other anions tested, gluconate is impermeable and this causes the membrane potential (E_m) to be reversed to inside positive (Cotterrell & Whittam,

1971). Despite the opposing E_m, gluconate increases the piretanide-insensitive influx (Fig. 7). The piretanide-sensitive influx is sigmoidal and saturable as it was with NO_3 and acetate. The $K_{\frac{1}{2}} = 53$ mM appears to be lower than with these two ions. In this experiment, gluconate produced a slight fall in the total passive K influx but sometimes gluconate stimulated passive K influx over-all (Table 6).

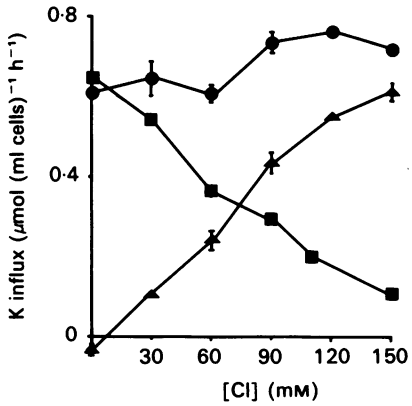

Fig. 7. [Cl] dependence of passive K influx: gluconate substitution. K influx was measured in conditions similar to those described in the legend to Fig. 2 except that gluconate was the replacement anion for Cl. ●, control; ■, with piretanide; ▲, difference.

Intracellular Na, K and haemoglobin. These were measured routinely in fresh cells, fresh $MgCl_2$–sucrose-washed cells and cells prepared into Cl-containing and Cl-free media. In addition, plasma [Na] and [K], whole blood haemoglobin and haematocrit were determined and, on at least one occasion with every individual, r.b.c. and w.b.c. numbers and mean cell volume as well. All parameters were within the normal range at all times (See Table 7).

Table 7 shows results from two experiments. In one, five different anion replacements were used and in the other acetate only. These results are representative of those obtained in the course of the anion-replacement experiments (Table 6 and Figs. 2–7).

At the $[K]_o$ employed here, passive K influx into human r.b.c.s increases by about 10% when the external osmolarity is raised so as to reduce cell volume by 10% (Poznansky & Solomon, 1972). The mean cell haemoglobin concentration gives an estimate of any cell volume changes in Cl-depleted cells (Table 7). Of the anions tested, SCN and I produced no change in mean cell haemoglobin concentration. In contrast, SO_4 and gluconate appeared to make the cells shrink and acetate to make them swell. However, the mean changes over several experiments were small and the standard errors were large. They were 6 ± 5% for gluconate, 10 ± 7% for SO_4 and 6 ± 2% for acetate.

All of the anions tested support greater diuretic-insensitive fluxes of Na inwards and, to a smaller extent, of K outwards than Cl (Funder & Weith, 1967; Chipperfield, 1984*b*; Shennan, 1984). As regards $[Na]_i$, the results confirm that it was greater than in Cl-prepared cells except for acetate but there was evidence of cell swelling with

acetate. As regards $[K]_i$ the results do not show the expected fall and this may be attributable to the smaller effect of anions on K efflux than Na influx.

Funder & Wieth (1967) made the paradoxical finding that the total [Na + K] inside Cl-prepared cells is less than that in SCN-prepared cells and yet the water content is greater in the Cl-cells. They offered no explanation. In this study, SCN did not alter

TABLE 7. Intracellular Na, K and haemoglobin in Cl-depleted cells

Expt.	Cells	Wash	Internal ions (μmol (ml cells)$^{-1}$) Na	K	Haemoglobin (g(l cells)$^{-1}$)
I	Fresh	—	20·6 ± 0·6	97·3 ± 1·4	329 ± 2
	Fresh	$MgCl_2$	7·8 ± 0·4	86·0 ± 0·7	333 ± 0
	Plasma	—	132·0 ± 4·0	4·0 ± 0·07	—
	Cl	NaCl	18·5 ± 0·5	84·9 ± 0·8	332 ± 4
	NO_3	$NaNO_3$	24·5 ± 0·8	85·2 ± 2·1	343 ± 2
	SO_4	Na_2SO_4	22·3 ± 0·7	84·1 ± 1·4	362 ± 5
	SCN	Na SCN	25·4 ± 0·8	84·9 ± 1·0	335 ± 2
	I	NaI	22·5 ± 2·8	91·0 ± 0·50	345 ± 2
	Gluconate	Na gluconate	23·2 ± 0·9	93·1 ± 1·3	384 ± 3
II	Fresh	—	22·6 ± 1·0	83·2 ± 0·8	304 ± 2
	Fresh	$MgCl_2$	6·5 ± 0·1	90·3 ± 1·7	325 ± 2
	Plasma	—	137·0 ± 1·0	3·8 ± 0·11	—
	Cl	NaCl	16·6 ± 0·3	78·3 ± 0·8	338 ± 3
	Acetate	Na acetate	16·3 ± 0·3	73·7 ± 0·3	314 ± 5

[Na], [K] and [haemoglobin] were measured after incubation at 37 °C for 1 h and washing in Na-containing, K-free and (as required) Cl-free media as described in the Methods. All results are the means of three ± S.E. of means. In all experiments blood haemoglobin and haematocrit were also measured and were 143 ± 1 g l^{-1} and 41·2 ± 0·2 % respectively in, for example, Expt. I. In this individual (female, aged 25), the r.b.c count was 4·55 (± 0·02) × 10^6 μl^{-1}, mean cell volume 91 ± 2 fl and the w.b.c. count 3·80 (± 0·02) × 10^3 μl^{-1}.

cell volume despite $[Na+K]_i$ being greater (Table 7). The unexplained dissociation between $[Na+K]_i$ and cell volume was seen also with NO_3, SO_4 and I. Whether this holds for gluconate or whether E_m and internal pH effects account for the observed shrinkage cannot be said.

External Na dependence

The furosemide-sensitive passive K influx is saturable with respect to external K (K_m = 6–8 mM) and the corresponding Na influx saturable with respect to external Na (K_m = 24 mM) (Wiley & Cooper, 1974). If the fluxes are tightly linked, then K influx should be saturable with respect to external Na with the same apparent affinity. To test this, the $[Na]_o$ dependence of passive K influx was determined as both the piretanide-sensitive flux and the Cl-dependent flux.

Piretanide-sensitive K influx. Passive K influx was measured over a range of $[Na]_o$ (0–112 mM, choline substitution) with and without piretanide (Fig. 8). The piretanide-sensitive flux showed two components, one saturable with a high apparent affinity ($K_{\frac{1}{2}}$ = 12 mM) and one either non-saturable or saturable with a very low affinity.

In interpreting this experiment, the effect of $[Na]_o$ on piretanide binding (Table 3

and Fig. 1) must be taken into account. This result reflects the Na affinity with respect to binding rather than K influx itself. Accordingly, the affinities of the loop diuretics were measured with $[Na]_o$ of 0, 16 and 112 mM in a single experiment. For furosemide, for example, the $K_{\frac{1}{2}}$ valves were estimated as $2{\cdot}8 \times 10^{-5}$, 1×10^{-5} and $5{\cdot}6 \times 10^{-6}$ M respectively and this is consistent with the $K_{\frac{1}{2}}$ for Na with respect to binding being about 10 mM. This agrees with the apparent affinity as judged by the piretanide-sensitive flux and both are lower estimates than the $K_{\frac{1}{2}}$ for Na influx.

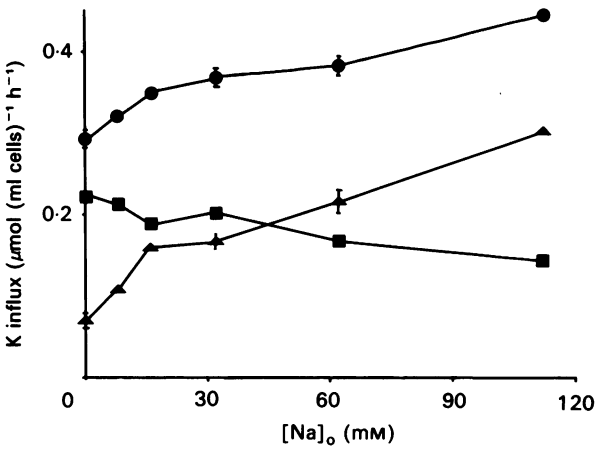

Fig. 8. $[Na]_o$ dependence of K influx: choline substitution and piretanide sensitivity. K influx was measured in media containing 0–112 mM-NaCl (or 150–38 mM-choline chloride) 8 mM-KCl, 10 mM-glucose, 10 mM-Tris–MOPS pH 7·5, 0·1 mM-ouabain and 1·6 μCi ^{86}Rb ml^{-1} *plus* 10^{-4} M-piretanide as required. All results are the mean of three (+ S.E. of mean). ●, control; ■, with piretanide; ▲, difference.

The apparent fall in the piretanide-insensitive influx is also consistent with the effect of $[Na]_o$ on binding. It does not exclude the additional possibility that external Na inhibits a 'non-co-transport' pathway (see Fig. 9).

Cl-dependent K influx. Passive K influx was measured over a range of $[Na]_o$ (NMDG substitution) with and without Cl (NO_3 substitution) (Fig. 9). The Cl-dependent fluxes showed two components. When the results were transcribed into a Lineweaver–Burk plot, the curve curved down giving two $K_{\frac{1}{2}}$ values, one of 40 mM and one about 70 mM. Neither agrees with the $K_{\frac{1}{2}}$ for Na influx of 24 mM. The Cl-independent influx decreased as $[Na]_o$ was raised. Thus external Na does appear to inhibit a 'non-co-transport' pathway.

DISCUSSION

Since (Na + K + Cl) co-transport was first described in ascites cells (Geck *et al.* 1980), a system with similar properties has been found in a number of other tissues (Palfrey & Rao, 1983; Warnock, Gregor, Dunham, Benjamin, Frizzell, Field, Spring, Ives, Aronson & Seiffer 1984). The salient properties are its absolute requirements for Cl ions and its specific inhibition by loop diuretics. The human red cell possesses a

(Na+K) co-transport system with these properties (Wiley & Cooper, 1974; Chipperfield, 1980, 1981; Dunham *et al.* 1980). The question addressed here is whether these two criteria are unfailingly reliable. Before discussing this, it must be emphasized that the results refer only to K influx at low $[K]_o$ (8 mM). Other passive fluxes, notably Na influx and K efflux (Chipperfield, 1984*b*; Shennan, 1984), can behave in quite different ways.

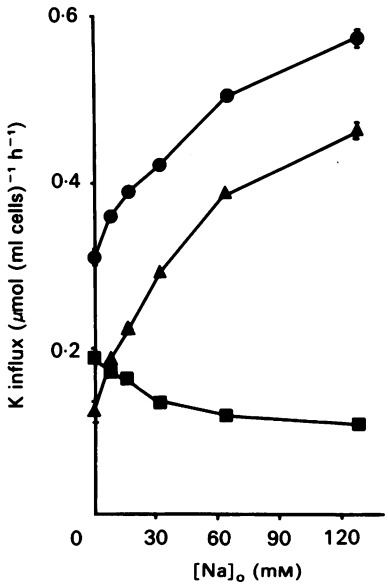

Fig. 9. $[Na]_o$ dependence of K influx: NMDG and NO_3 substitutions. K influx was measured in media containing 0–112 mM-NaCl (or 150–38 mM-NMDG chloride), 8 mM-KCl, 10 mM-glucose, 10 mM-Tris–MOPS pH 7·5, 0·1 mM-ouabain and 1·6 μCi ^{86}Rb ml^{-1} *or* $NaNO_3$, NMDG nitrate and KNO_3 as required. ●, 150 mM-Cl media; ■, Cl-free (NO_3) media; ▲, difference.

Inhibitors of K influx. Only two classes of drug inhibit K influx into human r.b.c.s namely cardiac glycosides and loop diuretics. The present confirmation is not very remarkable. On the other hand, it is important that the drugs were shown not to interact. This offers additional evidence that the Na pump does not mediate loop diuretic-sensitive passive K influx (Lew & Beauge, 1979; Karlish, Ellory & Lew, 1981). Nevertheless, interactions between ouabain and loop diuretics can arise. Loop diuretics are (or at high concentrations, especially, can be) rather non-specific (Ellory, Dunham, Logue & Stewart, 1983). They inhibit several processes including (at 10^{-3} M) the Na pump (Wiley & Cooper, 1974).

The influence of $[Na]_o$ on the inhibition of K influx by loop diuretics (e.g. Fig. 1) is in line with a number of observations on avian r.b.c.s and kidney cells. Thus, raising [Na] or [K] outside avian r.b.c.s increases the apparent affinity of loop diuretics: their binding to kidney membranes is wholly dependent on Na and K (Palfrey & Rao, 1983; Forbush & Palfrey, 1983). Moreover, the apparent affinities for Na and K with respect to binding and inhibition appear to reflect their affinities for transport. The present results on human r.b.c.s fit this pattern and show the most extreme case: lack of

substantial inhibition in the absence of external Na. The implications regarding the identification of transport mechanisms and an apparent furosemide-sensitive (K + Cl) co-transport (Wiater & Dunham, 1983) have been discussed elsewhere (Chipperfield, 1985).

Cl replacements. Qualitatively, the anion used to replace Cl is almost immaterial. Of the ten anions tested on human r.b.c.s, only Br supports a significant diuretic-sensitive K influx (Chipperfield, 1981). Quantitatively, there are substantial differences. The results with different anions fall into three groups. In the first group were three anions all giving sigmoidal activation curves with a $K_{\frac{1}{2}}$ of 50–80 mM. They were NO_3, acetate and gluconate. This form of activation curve with roughly this affinity is overwhelmingly the most common form seen in studies on (Na + K + Cl) co-transport. It has been observed, for example, in human and avian r.b.c.s, HeLa and MDCK cells (Dunham *et al.* 1980; Haas & McManus, 1983; Aiton *et al.* 1981; McRoberts *et al.* 1982). However, NO_3 was the substituting anion in every study except the last (McRoberts *et al.* 1982) where it was gluconate. In the second group are the halide and pseudohalide ions, I and SCN. They gave upward-curving activations. They resemble Cl and are thus likely to bind at the Cl site. If they do and if they are not transported, they will inhibit. The shapes of the curves are consistent with I and SCN competing at the Cl site.

In the last group, SO_4 is alone. Here, the apparent affinity is substantially higher and the influx reaches a plateau more obviously than with NO_3 etc. The activation shows evidence of being sigmoidal, and in this respect and in showing a lower $K_{\frac{1}{2}}$ it agrees with our results on Na efflux (Chipperfield & Shennan, 1983).

The parallels regarding Cl dependence of K influx and Na efflux extend to NO_3, I and SCN (Chipperfield & Shennan, 1983). However, the results with gluconate and acetate were not the same. Thus, with gluconate there was no diuretic-sensitive K influx but there was a diuretic-sensitive Na efflux. In this condition, Cl would be retained inside the cells and this must account for the diuretic-sensitive Na efflux. As for acetate, it blocks diuretic-sensitive Na efflux at concentrations as low as 30 mM and K influx likewise in cultured cells (Chipperfield & Shennan, 1983; Aiton *et al.* 1981). This may be attributable to a fall in internal pH but, whatever causes it, K influx into r.b.c.s is not inhibited by acetate.

Comparison with (Na + K + Cl) co-transport. It is not established that furosemide-sensitive (Na + K) movements in human r.b.c.s are linked to Cl movements. This is because the anion exchanger is so fast that it swamps any Cl fluxes via the co-transport pathway (Wiater & Dunham, 1983). In fact, net fluxes do conform to an obligatorily coupled co-transport with 1 : 1 : 2 Na : K : Cl stoicheiometry but, unfortunately, equally well to a 1 : 1 Na : K system (Duhm & Goebel, 1984). In the more favourable case of ferret r.b.c.s (Flatman, 1983), the linkage of Cl with (Na + K) co-transport has been demonstrated (Ellory & Hall, 1984) and there is compelling evidence for it in other tissues (Palfrey & Rao, 1983; Warnock *et al.* 1984). The Cl-dependent (Na + K) co-transport in human r.b.c.s shows many similarities with (Na + K + Cl) co-transport elsewhere. The apparent affinity changes caused by varying $[Na]_o$ and the [Cl]-dependence curves all have parallels which have been discussed above. A further set of parallels are the apparent affinities for Na and K which are comparable in HeLa, MDCK and human r.b.c.s (Aiton *et al.* 1981) and avian r.b.c.s (Palfrey & Rao, 1983).

The Cl-dependent, furosemide-sensitive (Na+K) co-transport in human r.b.c.s appears to be, therefore, a model for (Na+K+Cl) co-transport in more complex tissues.

Diuretic-insensitive K influx. The pathways other than (Na+K) co-transport are not unaffected when Cl is replaced by another anion. This phenomenon is more prominent in Na influx and K efflux (Funder & Wieth, 1967; Chipperfield, 1984*b*; Shennan, 1984) but it is discernible in K influx. Funder & Wieth (1967) were concerned with the possibility that ion permeabilities would increase in parallel with the lyotropic series, i.e. $Cl < Br < NO_3 < I < SCN$. They did not see this pattern largely because of the existence of the Cl-dependent (Na+K) co-transport. The results show that this sequence is, at least partly, followed by the diuretic-insensitive component of K influx ($Cl = NO_3 < I < SCN$). Moreover, the sequence $Cl < Br < NO_3 < I < SCN$ holds for diuretic-insensitive Na influx and K efflux (Chipperfield, 1984*b*; Shennan, 1984). The exception is Na efflux where this component is unaffected by the substituting anion. Nevertheless, Funder & Wieth's (1967) suggestion that ion permeabilities will follow the lyotropic series is more clearly borne out than they had reason to believe at the time.

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