Selective inhibitors of KCl cotransport in human red cells

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Two analogues of the loop diuretics furosemide and bumetanide have been identified as differential inhibitors of KCl and NaKCl cotransport systems, assayed by measuring K^+ influx in 'young' human red cells. H25 inhibited both NaKCl and KCl cotransport, with $I_{50\%}$ values of 0.03 and 30 μM respectively; H74 had no effect on NaKCl cotransport, even at 0.3 mM, but inhibited KCl cotransport with an $I_{50\%}$ of 75 μM. These compounds are therefore useful for resolving the two transport systems.

K^+ transport; Red cell; HbSS

1. INTRODUCTION

The coupled KCl cotransport system has been described in the plasma membranes of a number of cell types (red cells, hepatocytes, ascites tumour cells, epithelia, thick ascending limb of the loop of Henle [1-5]), and has been shown to play a major role in regulatory volume decrease (RVD) following cell swelling. Although there is a variety of experimental manoeuvres capable of activating this pathway (*N*-ethylmaleimide treatment, ghosting, hydrostatic pressure [6-9]) studies of its properties have been hindered by the lack of specific inhibitors, although recently certain aryloxyalkenoic acid derivatives have been identified as inhibitors for this system [10].

Volume-sensitive KCl cotransport can mediate a powerful RVD response in normal (i.e. HbAA) 'young' human erythrocytes, but the activity of this pathway is dramatically attenuated with cell maturation [1,11,12]. In certain cases of haemoglobinopathy (e.g. HbSS) the KCl pathway is operational in much of the cell population, mainly because a large fraction of the total red cell population consists of reticulocytes and 'young' red cells, due to increased cell turnover. Swelling these red cells either osmotically or by acidification results in a large K^+ efflux of up to 50 mmol/l cells/h via this pathway [13-15]. This could cause cellular dehydration, an increased MCHC, and in the case of HbSS cells, an enhanced risk of sickling [16].

The 'loop' diuretics furosemide and bumetanide are poor inhibitors of KCl cotransport, but inhibit NaKCl cotransport with a considerably higher affinity [1]. The *differential* sensitivity to these diuretics has been used as a criterion to separate KCl from NaKCl cotransport activity (see [1]). We have taken advantage of the observation of partial inhibition by high concentrations (>0.5 mM) of 'loop' diuretics on KCl cotransport (e.g. [1]) to screen structural analogues of these compounds for enhanced potency against this system.

In this paper we present data on two compounds out of 165 analogues of bumetanide and furosemide which we have screened as inhibitors of the two Cl^--dependent K^+ transport systems (NaKCl and KCl cotransport). Experiments have been carried out either on 'young' cell-enriched fractions of blood (HbAA), prepared by centrifugation from haemochromatotic patients, or on red cells obtained from donors with sickle cell disease (HbSS). These red cells express normal levels of NaKCl cotransport activity, but elevated KCl cotransport [13]. We have identified two molecules designated H25 and H74, which act differentially; H25 has a high affinity for NaKCl cotransport inhibition, and a lower, but significant affinity for KCl cotransport; in contrast H74 is completely ineffective on NaKCl cotransport, but has a significant inhibitory effect on KCl cotransport.

2. MATERIALS AND METHODS

2.1. *Blood*

Blood (HbAA) from haemochromatosis patients was taken into heparin, and washed three times by centrifugation (2500 × *g*; 15 min, 5°C) with a medium containing (in mM); NaCl (150); MOPS (15); glucose (5); 300 mOsm, pH 7.4). To separate a 'young' cell enriched cell population, the loosely packed cells (about 80% haematocrit) were divided between twenty 10 ml tubes and spun at 2500 × *g* for 1 h at 20°C. The top third of cells was then carefully harvested, and put into 6 tubes for a recentrifugation. The top third of this second separation was harvested, yielding about 15 ml of 'young' cell-enriched cell suspension [17]. HbSS blood was obtained as 10 ml

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Abbreviations: MOPS, 3-[*N*-morpholino]propanesulfonic acid; TCA, trichloroacetic acid

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samples taken into lithium heparin, and stored on ice for less than 12 h before use.

2.2. *Measurement of K^+ and Na^+ fluxes*

K^+ uptake experiments, using ^{86}Rb as a congener, were performed using standard techniques as described previously [12,18]. Briefly, red cells at about 5% haematocrit, suspended in the saline described above, were pretreated with ouabain (0.1 mM) in the presence of either water or 1 M sucrose so that the cells were swollen or shrunken respectively by about 12%. The flux was started by the addition of isotonic KCl (including ^{86}Rb to give a final K^+ concentration of 7.5 mM, and radioactivity of about 2 μCi/ml cell suspension). The incubation period was 10 min, after which the cells were washed four times by centrifugation (10000 × g; 15 s), by aspiration and addition of ice-cold medium comprising (mM): $MgCl_2$ (106); MOPS (15); pH 7.4. The final cell pellet was lysed with 0.5% (v/v) Triton X-100 in water, deproteinised with 5% TCA (w/v), centrifuged and the supernatant counted for radioactivity using Cerenkov radiation. Na^+ uptake was measured in a medium containing (mM); NaCl (20); KCl (20) *N*-methyl-D-glucamine Cl (110); MOPS (10); glucose (5); and ouabain (0.1) (pH 7.4) with ^{22}Na at 5 μCi/ml. Sodium uptake was measured over 30 min, the cells washed as above, and processed for β-scintillation counting. The replacement of Cl^- by methylsulphate ($CH_3SO_4^-$) was achieved by washing cells 3 times at 5°C with (mM); $NaCH_3SO_4$ (165); glucose (5); MOPS (15) pH 7.4, incubating at 5°C for 2 h, then washing 3 more times with the same solution. It was necessary to maintain the temperature low during this period because of the rapid disappearance of KCl cotransport activity at higher temperatures [1,11]. The drugs studied were dissolved in water, or DMSO as appropriate. Controls for the effect of DMSO were included. NaKCl cotransport was defined as the fraction of K^+ uptake in shrunken cells inhibitable by bumetanide (0.1 mM). KCl cotransport was taken as the volume-sensitive component of K^+ uptake, defined as the difference in K^+ uptake between swollen and shrunken cells.

2.3. *Chemicals*

All inorganic salts were of analytical grade, and ouabain and MOPS were obtained from Sigma Chemical Co. Bumetanide was provided by Leo Pharmaceuticals, and ^{22}Na and ^{86}Rb were obtained from Amersham International. The two molecules H25 and H74 were supplied by Hoechst Pharmaceuticals and are analogues of bumetanide; the chemical name of H25 is a 3-benzylamino-4-phenoxy-5-sulphamoylbenzoic acid, and that of H74 is 3-(2-(1-naphthyl)-ethyl)amino-4-phenoxy-5-sulphamoylbenzoid acid.

3. RESULTS AND DISCUSSION

In preliminary experiments, the efficacy of the two drugs for inhibition of KCl cotransport (defined as the volume-sensitive component of ^{86}Rb uptake) was tested at a single drug concentration (0.1 mM) on both HbAA (haemochromatosis) and HbSS red cells. KCl cotransport was inhibited by H74 by 58.2 ± 8.9% (n = 6 donors; HbAA), and 54.2 ± 2.4% (n = 5; HbSS) respectively. H25 was more potent inhibiting 78.0 ± 6.9% (n = 5 donors), and 76.3 ± 0.7% (n = 3 donors) respectively. Equivalent results were obtained for inhibition of NaKCl cotransport in the two cell types: fluxes in the presence of H74 being 91.5 ± 5% (HbAA, n = 8) and 96.7 ± 5.0% (HbSS, n = 7) of the control values. From these experiments, we conclude that there is no difference in the pharmacological sensitivity of KCl or NaKCl cotransport in HbAA or HbSS cells, and that H25 and H74 have clear differential effects on the two systems. In fig.1 the effects of increasing concentrations of H25 and H74 on ouabain-insensitive K^+ uptake in 'young' red cell-enriched fractions of blood are compared at two different cell volumes. In shrunken cells, H25 inhibited K^+ uptake at all concentrations tested (3–300 μM). This inhibition was equal to that observed by adding bumetanide to shrunken cells, and represents total suppression of the NaKCl cotransport pathway (see also fig.2a). In swollen cells, the NaKCl cotransport system only represents a minor component of the total K^+ uptake which is dominated by the flux through the KCl cotransporter. It can be seen that H25 is also effective at inhibiting this component of K^+ transport, although with a lower affinity. This behaviour is similar to the published effects of bumetanide and furosemide on the two transport systems [1,19]. Fig.1b, in contrast, shows that H74 has no inhibitory effect on NaKCl cotransport at any concentration tested (3–300 μM), whilst it is an effective inhibitor of KCl cotransport.

A direct comparison of the inhibitory potency of the drugs H25 and H74 on the two transport mechanisms showed that the $I_{50\%}$ for H25 is 0.03 μM for NaKCl cotransport and 30 μM for KCl cotransport. In contrast, H74 had no effect on NaKCl cotransport, but inhibited volume-sensitive K^+ transport with an $I_{50\%}$ of 75 μM.

Although it is apparent from the results for shrunken cells (fig.1b) that these drugs do not markedly increase basal K^+ permeability, we tested H74 in cells under Cl^--free conditions (Cl^- replaced by $CH_3SO_4^-$) where no KCl or NaKCl activity was possible. Thus, in $CH_3SO_4^-$ medium, K^+ uptake was 0.219 ± 0.004 and 0.228 ± 0.006 mmol/l cells/h in swollen and shrunken cells, respectively; in the presence of H74 (0.2 mM), the respective values were 0.239 ± 0.006 and 0.234 ± 0.014 mmol/l cells/h, (results are means ± SD for triplicate determinations from one of two similar experiments). This confirms that H74 did not induce a significant increase in K^+ leak.

An important control experiment was the effect of H25 and H74 on Na^+ fluxes, where one would expect that only H25 should inhibit Na^+ uptake via the NaKCl cotransporter. In practice, measuring Na^+ influx via NaKCl cotransport is difficult since there are several parallel pathways, and its contribution to the total Na^+ flux is small. We have therefore measured Na^+ uptake under conditions chosen to minimise Na^+ flux through other pathways, in a low Na-containing solution (see section 2 for details). Thus, in one of two similar experiments, control Na^+ uptake was 0.498 ± 0.006; in the presence of H74 (0.1 mM) it was 0.468 ± 0.015, whilst the addition of bumetanide (0.1 mM) reduced the flux to 0.327 ± 0.009 mmol/l cells/h. In a further series of experiments using H25 (0.1 mM) the control Na^+ flux was 0.444 ± 0.005, this being reduced to 0.238 ± 0.011 and 0.235 ± 0.005 mmol/l cells · h in the presence of H25 or bumetanide respectively (results

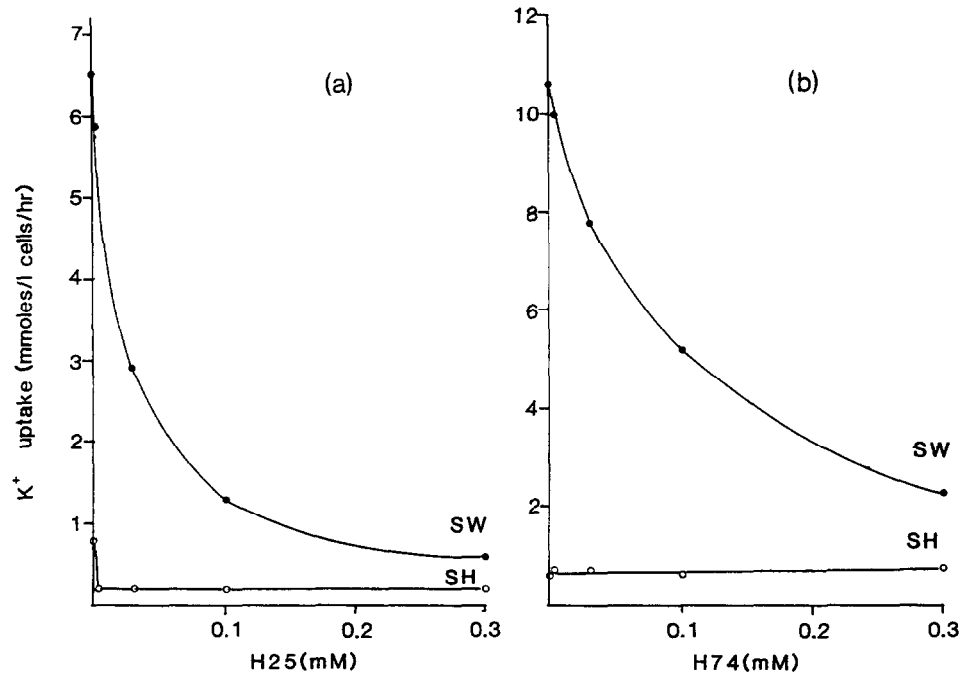

Fig.1. The effect of (a) H25 and (b) H74 on K^+ uptake in 'young' human red cells. The data for (a) were obtained from a haemochromatosis patient, and for (b) from a donor with HbSS red cells. Filled circles represent the flux measured in cells swollen (15%) hypotonically. Open circles represent the flux measured in cells shrunken by the addition of sucrose (final osmolarity 400 mOsm). The figure is representative of four similar experiments.

are one of 6 similar experiments with data given as means ± SD). Therefore, H25 completely inhibited the Na^+ flux via NaKCl cotransport (as defined as that fraction inhibited by bumetanide (0.1 mM), whereas H74 showed only a very small effect.

The present results identify two potentially useful molecules for separating NaKCl and KCl cotransport activity. H25 is a potent inhibitor of NaKCl cotransport, and a reasonable inhibitor of KCl cotransport. In contrast, H74 is without effect on NaKCl cotransport, but inhibits KCl cotransport with a modest potency. The structural component responsible for the inhibitory efficacy is presumably the naphthyl residue in position 2. This bulkier group can be tolerated by the KCl cotransporter, but not by the NaKCl cotransporter. In this context it is interesting to note that the KCl cotransporter prefers the larger Br^- ion to Cl^- (supporting a 3 × greater flux), whilst for the NaKCl cotransporter these two ions are more or less equal in their ability to support K^+ transporter [1].

The inhibitors H25 and H74 resolve the two Cl^--dependent K^+-transport pathways, providing a convenient alternative to swelling-shrinking or Cl^--replacement protocols for defining KCl cotransport in cells. This distinction provides good evidence for the separate identity of these two transporters, and should facilitate the study of KCl cotransport.

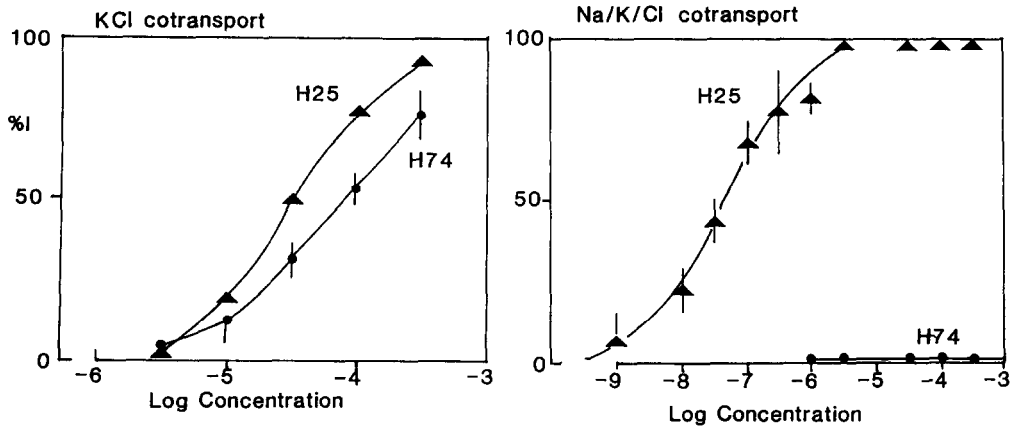

Fig.2. The effect of H25 and H74 as inhibitors of either (a) KCl or (b) NaKCl cotransport. The % inhibition of K^+ influx was calculated from four experiments similar to fig.1, but using different appropriate inhibitor concentrations. Results are pooled from data obtained from 4 separate donors (3 haemochromatosis and 1 HbSS patient for the H25 data, and 2 haemochromatosis and one HbSS patient for the H74 data).

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