Biochimica et Biophysica Acta, 979 (1989) 53–61
Elsevier

BBA 74303

Interaction of inhibitin with the human erythrocyte $Na^+(Li^+)_i/Na^+_o$ exchanger

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(Received 7 June 1988)
(Revised manuscript received 26 October 1988)

Key words: Inhibitin; Bumetanide; Ouabain; Promyelocyte; Sodium ion efflux; (Human erythrocyte)

The kinetic interactions of inhibitin, a peptide isolated from cultured leukaemic promyelocytes, with erythrocyte Na^+/Na^+ and Na^+/Li^+ exchanges have been investigated. Inhibitin (1 μM) reduced the ouabain- and bumetanide-resistant sodium efflux and influx by equivalent amounts indicating an inhibitin-sensitive exchange component of 0.52 mmol/l per h. This value was not significantly different from that measured as the difference in sodium-rich (140 mM) and sodium-free media (0.49 mmol/l per h). Similarly, the inhibitin-sensitive lithium efflux was equivalent to the sodium/lithium countertransport component (0.36 vs. 0.34 mmol/l per h), indicating that both exchanges were mediated by the same transport process, which is inhibitin-sensitive. The dose–response curve revealed the presence of a single inhibitin binding site per exchanger with a K_i of $2 \cdot 10^{-7}$ M. In kinetic inhibition studies, inhibitin (0.1 μM) decreased the V_{max} of ouabain- and bumetanide-resistant sodium efflux with no effect on the K_m for external sodium, i.e., inhibitin displayed a non-competitive mechanism of action. These findings indicate that inhibitin interacts with the $Na^+(Li^+)_i/Na^+_o$ exchanger at a site distinct from the sodium binding site.

Introduction

The sodium transport processes of the human erythrocyte can be divided into several components depending on their sensitivity to various inhibitors. The major route for sodium efflux is through Na^+/K^+-ATPase (the sodium pump), which requires ATP to catalyse the exchange of internal sodium for external potassium and is inhibited by ouabain [1]. The remaining pathways are ouabain-insensitive and have remained a subject for intense research and much debate; without specific inhibitors it has proved difficult to distinguish the various components from each other and 'leak'. It is now known that there is a chloride-dependent Na^+/K^+ cotransport system that is diuretic-sensitive [2] and a carrier-mediated sodium/sodium exchange system [3], which has been demonstrated to be inhibitable by inhibitin [4], a peptide isolated from leukaemic promyelocytes [5]. Whilst in the past there was some debate regarding these two systems [2,3,6–10], it is now agreed that they are distinct. The remaining sodium efflux which occurs in the presence of all these inhibitors is due to the ground permeability properties of the membrane and has been termed electrodiffusional 'leak', but other residual carrier mediated fluxes may yet be identified.

In 1975, Haas and co-workers described a Na^+/Li^+ countertransport system that exchanged Li^+, against its electrochemical gradient, for Na^+ [11] and reported that this system was inhibited by phloretin [12]. Under physiological circumstances, the Na^+/Li^+ countertransport system was assumed to operate as Na^+/Na^+ exchange because Li^+ is present only in trace quantities. There is now sufficient evidence from ion and kinetic studies [12–14] indicating that lithium is transported by the same mechanism that mediates Na^+/Na^+ exchange, but the specificity of phloretin for this exchange system has been questioned [4]. In this study we have used the newly characterised sodium transport inhibitor, (inhibitin) [4,5], to obtain additional experimental evidence in support of the contention that Na^+/Na^+ exchange and Na^+/Li^+ countertransport are conducted through the same pathway. We have also investigated the kinetics of inhibitin interaction with the human erythrocyte Na^+/Na^+ exchanger.

Methods

Reagents

All reagents were of AnalaR grade (BDH Chemicals Ltd., Poole, U.K.) and were prepared in double-glass-distilled water. ^{22}Na for erythrocyte transport experi-

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ments was purchased from Amersham International (Amersham, U.K.).

Isolation of inhibitin

Inhibitin was purified to apparent homogeneity from leukaemic promyelocyte (HL60 cell) culture medium, using a previously described method [15]. Briefly, aliquots of culture medium were injected onto a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA) which was washed with 0.1% trifluoroacetic acid (Spectrosol TFA from Sigma, St Louis, MO) and eluted with 30% acetonitrile (MeCN) containing 0.1% TFA (HPLC grade S MeCN was obtained from Rathburn, Walkerburn, U.K.). The retentiate was then dried under vacuum (Savant Speed-Vac Concentrator, Louisville, MI), resuspended in 0.1% TFA and subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on a Hypersil ODS 5μ column (Jones Chromatography, Llanbradach, U.K.). The column was processed at 1 $ml \cdot min^{-1}$ using an increasing gradient of MeCN in 0.1% TFA (1% MeCN to 30% MeCN over 45 min after 5 min isocratic at 1% MeCN) and the fractions around the peak of sodium transport inhibitory activity (retention time 30–36 min) were pooled and rechromatographed on the same column using 0.2% heptofluorobutyric acid (HFBA from Pierce UK, U.K.) in MeCN. The column was run at 1 $ml \cdot min^{-1}$ isocratically at 1% MeCN for the first 5 min then up to 12% MeCN at 15 min and 35% MeCN at 1 h (linear gradients between time points). Inhibitory activity eluted in a single sharp peak with a retention time of 28–29 min, which was used in the sodium and lithium transport experiments as outlined below. This protocol is a more convenient and faster procedure for the isolation of inhibitin compared with the one previously described [5]. The concentration of inhibitin was determined using quantitative amino-acid analysis employing dabsyl chloride derivitisation. Inhibitin was acid hydrolysed (6 M HCl, 120 °C, 24 h) under nitrogen and the derivatives formed were identified by following the method of Chang et al. [16].

Erythrocyte cation transport

Blood samples

Human red cells, obtained on the morning of study from normal healthy volunteers by venepuncture (100 U heparin/ml blood), were used in all experiments. In order to minimise intersubject variation, blood from a single donor was used for each set of experiments. The plasma and buffy coat were removed by aspiration after centrifugation (5000 × *g*, 3 min, 4 °C) and the cells were washed three times in Ringer's solution containing (mM): NaCl, 131; KCl, 8; $MgSO_4$, 1; Na_2HPO_4, 7.2; NaH_2PO_4, 1.8; $CaCl_2$, 2; glucose, 10; bovine serum albumin 0.05% w/v (pH 7.4). After the final wash the packed cell pellet was used immediately.

Sodium efflux

This method has been described in detail previously [4]. Washed erythrocytes in Ringer's solution (50% haematocrit) were loaded with ^{22}Na (3 $\mu Ci/ml$ erythrocytes) at 37 °C for 2 h. After incubation the cells were washed three times with ice-cold iso-osmotic choline chloride containing (mM): choline chloride, ISI; $MgCl_2$, 1; $CaCl_2$, 2.2 (pH 7.4) and then added to prewarmed (37 °C) Ringer's solution to give a final haematocrit of 5%. Samples were taken at 30, 60 and 90 min and the radioactivity was counted in portions of the suspension and supernatant after centrifugation at 5000 × *g* for 1 min at 4 °C. The values of 1 − (supernatant counts/suspension counts) were plotted semilogarithmically and the half-time ($t_{1/2}$) was calculated from the slope of the 'best-fit' line. The sodium-efflux rate constant, ($^oK_{Na}$) was calculated from the equation: $^oK_{Na} = 0.693/t_{1/2}$. To determine internal sodium (Na_i^+), erythrocytes were washed three times with choline chloride solution. The haematocrit of a 2 ml sample was measured (Coulter Counter, Coulter Electronics, Hialeah, FL) and 20 μl of 20% (w/v) saponin solution and 20 μl 2 M lithium bromide (internal standard) were added. The sodium concentration was measured by flame photometry using standards containing 2.4 mM Na^+, 36 mM K^+ and 20 mM Li^+. Sodium efflux (mmol/l per h, $^oM_{Na}$) was obtained by multiplying the sodium efflux rate constant ($^oK_{Na}$) by the internal sodium (mmol/l erythrocytes). Ouabain (Sigma) was used at a final concentration of 0.1 mM, as was bumetanide (Burinex, Leo Laboratories, Princes Risborough, U.K.). Phloretin (Sigma) was used at 0.2 mM final concentration.

Sodium influx

This method has also been described in greater detail elsewhere [4]. 1 ml aliquots of washed erythrocytes were incubated in Ringer's solution (haematocrit 5%) containing 8 μCi of ^{22}Na. After equilibration (15 min) at 37 °C, a 4 ml sample was removed into an ice-cold tube, centrifuged at 5000 × *g* for 1 min at 4 °C and washed three times in 5 vol. of choline chloride solution. Erythrocytes were then haemolysed in deionized water (2 ml volume) and the radioactivity was counted. Haemoglobin was measured spectrophotometrically at 541 nM using Drabkin's reagent to estimate the volume of erythrocytes in each tube. The amount of labelled sodium uptake (U) was calculated from $U = (Na'^{75} - Na'^{15})/SA$, where Na'^{75} and Na'^{15} represented the cpm per litre of erythrocytes at 75 and 15 min and SA the radioactive counts per mmol of the external medium. Sodium influx in mmol/l per h ($^iM_{Na}$) was calculated from the uptake in 60 min using $^iM_{Na} = UK/1 - e^{-Kt}$, where U was the uptake of labelled sodium in time t

and K the sodium efflux rate constant. This calculation corrects for back-diffusion due to efflux which occurs during the time-course of the experiment. Both ouabain and bumetanide were used at 0.1 mM final concentration.

Sodium / sodium exchange

In addition to measuring ouabain- and bumetanide-resistant sodium efflux and influx, a direct estimate of sodium/sodium exchange was obtained by measuring external sodium-stimulated sodium efflux in the presence of ouabain and bumetanide. Sodium efflux was measured, as described above, in Ringer's solution (140 mmol/l external sodium) and choline chloride solution (0 mmol/l external sodium). Sodium/sodium exchange was calculated as the difference in sodium efflux between the 140 mM sodium solution ($Na_o^+ = 140$) and the choline chloride solution ($Na_o^+ = 0$). Where indicated, solutions with different external sodium were prepared by direct substitution of choline for sodium.

Sodium / lithium countertransport

This process was measured following the procedure of Canessa et al. [17]. Erythrocytes were washed three times in $MgCl_2$ solution which contained (mM): $MgCl_2$, 75; sucrose, 85; Tris-HCl, 10 (pH 7.4). The cells were then incubated (37°C) in LiCl solution containing (mM): LiCl, 150; glucose, 10; Tris-HCl, 10 (pH 7.4). After 3 h, the lithium-loaded erythrocytes (approx. 6 mmol/l cells) were washed five times in $MgCl_2$ solution (4°C) and resuspended in both $MgCl_2$ solution and in NaCl solution which contained (mM): NaCl, 145, glucose, 10; Tris-HCl, 10 (pH 7.4). After 15 and 75 min, 2 ml aliquots of the cell suspension were centrifuged at 5000 × g for 1 min at 4°C and the supernatants analysed for lithium by flame photometry. The relative cell volume was calculated after haemolysis by haemoglobin determination. Ouabain-resistant (final concentration 0.1 mM) erythrocyte lithium efflux in both solutions was calculated as the difference between lithium concentration of the supernatant at 75 min and 15 min and, after taking into account the volume of cells used, was expressed as mmol/h per l of erythrocytes. Sodium/lithium countertransport (i.e., external sodium-stimulated lithium efflux) was calculated as the difference between lithium efflux in the NaCl and $MgCl_2$ solutions.

Statistical analysis

All results are presented as mean ± S.D. Cation transport in the presence of inhibitin was compared with that measured in the presence of other inhibitors, under various experimental conditions, using the unpaired t-test to determine significance. Regression lines were computed by the method of least squares.

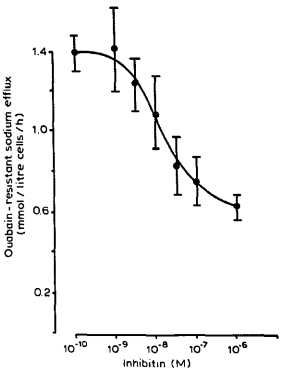

Fig. 1. The effect of inhibitin concentration on ouabain-resistant sodium efflux. ^{22}Na efflux was measured, as described in the Methods, in the presence of ouabain and varying concentrations of inhibitin. The inhibitin-sensitive component was derived by subtracting Na^+ efflux in the presence of ouabain plus different inhibitin concentrations from that obtained in the presence of ouabain alone. Data shown are means ± S.D. of three separate experiments.

Results

Inhibitin-sensitive sodium transport in erythrocytes

Dose–response curve for inhibitin. The effect of varying concentrations of inhibitin on ouabain-resistant sodium efflux in Ringer's solution (external sodium 140 mM) is shown in Fig. 1. The internal sodium concentration of the erythrocytes was approx. 8 mmol/l cells. Na^+ efflux in the presence of 0.1 mM ouabain was subtracted from Na^+ efflux in the presence of varying concentrations of inhibitin (plus ouabain) to obtain the inhibitin-sensitive component. No inhibition was detectable at 10^{-9} M inhibitin, but as the concentration increased, there was progressive inhibition of ouabain-resistant sodium transport reaching a maximum at 10^{-6} M. Inhibitin-insensitive pathways (Na^+ efflux in the presence of maximal concentrations of ouabain and inhibitin) contributed to 42% of sodium efflux in the presence of inhibitin; this is the sum of the components of sodium efflux achieved by $Na^+/K^+/Cl^+$ cotransport and Na^+'leak'. Fig. 2 shows the relative contribution of each of these pathways to total sodium efflux. When the data are corrected for this residual movement, a plot of 1/ouabain-resistant sodium efflux vs. inhibitin concentration (Dixon plot, Fig. 3) yields a straight line ($r = 0.99$) consistent with the presence of a single inhibitin binding site having an inhibitory constant (K_i) of $2 \cdot 10^{-7}$ M. A Hill plot of these data (log[inhibitin]

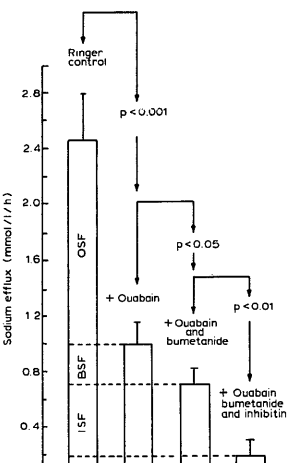

Fig. 2. Components of erythrocyte sodium efflux. Ouabain was used at a final concentration of 0.1 mM, bumetanide at 0.1 mM and inhibitin at 1 μM to distinguish transport pathways mediated by Na^+/K^+-ATPase (OSF), $Na^+/K^+/Cl^-$ cotransport (BSF) and Na^+/Na^+ exchange (ISF), respectively. Sodium transport in the presence of these three inhibitors is due to the ground permeability of the membrane and termed as residual flux (L). Results are shown as mean ± S.D. and the number of determinations is shown under each column.

vs. $\log[v/(V_{max} - v)]$) gives a slope (i.e., n_{app}) of 1, which is again consistent with the presence of a single inhibitin binding site.

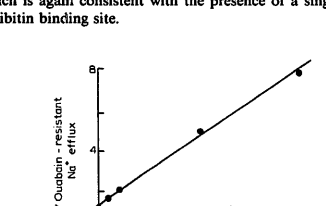

Fig. 3. A Dixon plot of 1/ouabain-resistant sodium efflux (values shown in Fig. 1) vs. inhibitin concentration after subtraction of the inhibitin-insensitive component from the inhibitin-sensitive sodium efflux values.

Components of sodium efflux. Sodium transport in erythrocytes was measured using a combination of ouabain, bumetanide and inhibitin to determine the percentage of sodium efflux sensitive to each particular inhibitor. The results of these experiments showing the breakdown of total sodium efflux into its different components are depicted in Fig. 2. Ouabain (0.1 mM) was used to specifically inhibit active sodium transport, i.e., sodium transport mediated by Na^+/K^+-ATPase. The remaining sodium efflux, collectively termed passive, can be further subdivided using bumetanide (0.1 mM) to inhibit $Na^+/K^+/Cl^-$ cotransport and inhibitin (1 μM) to block Na^+/Na^+ exchange. The sodium efflux occurring in the presence of all three inhibitors has been termed residual flux. Total sodium efflux, i.e., that measured in Ringer's solution (Fig. 2, column 1) was 2.47 ± 0.33 mmol/l per h ($n = 4$). In the presence of ouabain (Fig. 2, column 2), this was significantly reduced to 0.99 ± 0.09 ($n = 4$; $P < 0.001$) giving an ouabain-sensitive fraction of 60% equating to 1.48 mmol/l per h. Bumetanide (Fig. 2, column 3) had an additional effect in the presence of ouabain, reducing sodium efflux to 0.71 ± 0.11 ($n = 4$; $P < 0.05$), indicating a bumetanide-sensitive fraction of 0.28 mmol/l per h equivalent to 11% of total sodium efflux. Inhibitin, in the presence of ouabain and bumetanide (Fig. 2, column 4), further reduced sodium efflux to show a residual flux of 0.19 ± 0.12 mmol/l per h ($n = 4$; $P < 0.01$), indicating an inhibitin-sensitive fraction of 0.52 mmol/l per h (21% of total sodium efflux); inhibitin-sensitive Na^+/Na^+ exchange represents 53% of passive erythrocyte sodium efflux (0.99 mmol/l per h). Thus, using this combination of inhibitors, it is possible to divide total erythrocyte sodium efflux into components which are mediated by Na^+/K^+-ATPase, $Na^+/K^+/Cl^-$ cotransport, Na^+/Na^+ exchange and residual Na^+ flux with the ratio of 60 : 11 : 21 : 8, respectively.

Effect of inhibitin on passive erythrocyte sodium efflux and influx

Bidirectional passive sodium transport was measured using 1 μM inhibitin in the presence of 0.1 mM ouabain and 0.1 mM bumetanide. The results are summarized in Table I. Inhibitin displayed a significant inhibitory effect, decreasing sodium efflux from 0.72 ± 0.13 mmol/l per h ($n = 3$) to 0.20 ± 0.10 mmol/l per h ($n = 4$; $P < 0.01$). These results indicate an inhibitin-sensitive component of sodium efflux of 0.52 mmol/l per h. Sodium influx was also decreased in the presence of inhibitin falling from 1.5 ± 0.20 mmol/l per h ($n = 4$) to 0.98 ± 0.11 mmol/l per h ($n = 5$; $P < 0.005$). This gives an inhibitin-sensitive component of sodium influx of 0.53 mmol/l per h. The differences between the inhibitin-sensitive fluxes was small (0.01 mmol/l per h) and not statistically significant. Intracellular sodium content was estimated at the end of the efflux/influx

TABLE I

Effect of inhibitin (1 μM) on ouabain (0.1 mM)- and bumetanide (0.1 mM)-resistant sodium fluxes of normal human

All data are shown as mean ± S.D. of fluxes measured in erythrocytes obtained from one subject.

	mmol/l per h	Difference and significance
Sodium efflux in the presence of ouabain and bumetanide ($n = 3$)	0.72 ± 0.13	0.52 $P < 0.01$
ouabain, bumetanide and inhibitin ($n = 4$)	0.20 ± 0.10	
Sodium influx in the presence of ouabain and bumetanide ($n = 4$)	1.51 ± 0.20	0.53 $P < 0.005$
ouabain, bumetanide and inhibitin ($n = 5$)	0.98 ± 0.11	

experiments and it was found that addition of 1 μM inhibitin had not resulted in any changes (data not shown). These data indicate that the inhibitin-sensitive components of sodium efflux and sodium influx are equivalent and that the process on which inhibitin acts does not achieve net transport under the experimental conditions, i.e., Na^+/Na^+ exchange with a coupling ratio of 1.

Effect of inhibitin on erythrocyte Na^+/Na^+ exchange measured as external sodium-stimulated sodium efflux

The data presented in Fig. 2 and Table I indicate that inhibitin has a distinct site of action, which is Na^+/Na^+ exchange. In addition to measuring inhibitin-sensitive sodium efflux and influx (Table I) to confirm this site of action, the effect of inhibitin was studied on Na^+/Na^+ exchange measured directly as external sodium-stimulated sodium efflux. Sodium efflux was measured in the presence of ouabain (0.1 mM) and bumetanide (0.1 mM) in Ringer's solution (140 mM external sodium) and choline chloride solution (0 mM external sodium). Under these conditions, in Ringer's solution, Na^+/Na^+ exchange will proceed, as will sodium 'leak' (residual flux) due to the permeability of the erythrocyte membrane. In the choline chloride solution, however, Na^+/Na^+ exchange will be inhibited (since no external sodium will be available for exchange), but sodium 'leak' will continue. The difference in sodium efflux between these two solutions thereby represents a measure of Na^+/Na^+ exchange. Inhibitin (1 μM) was used under these conditions and the results are summarized in Table II. The Na^+/Na^+ exchange was 0.47 mmol/l per h, which is the difference in sodium efflux between the 140 mM (0.65 ± 0.10 mmol/l per h) and 0 mM (0.18 ± 0.04 mmol/l per h) external sodium solutions. In the presence of inhibitin, sodium efflux fell from 0.65 ± 0.10 mmol/l per h ($n = 4$) to 0.16 ± 0.08 mmol/l pr h ($n = 4$; $P < 0.001$), a value in close agreement with that observed in the choline medium (0.19 ± 0.09 mmol/l per h); the inhibitin-sensitive component was 0.49 mmol/l per h (similar to the value observed in Table I). Addition of inhibitin, therefore, reduced sodium efflux to the value observed in the absence of external sodium, i.e., complete inhibition of Na^+/Na^+ exchange. In the choline medium, inhibitin had no effect, indicating that it is specific for the external sodium-stimulated Na^+/Na^+ exchange.

Effect of inhibitin on erythrocyte Na^+/Li^+ countertransport

Other workers [12–14] have demonstrated that lithium can substitute for sodium in the sodium/sodium exchange mechanism to give rise to sodium/lithium countertransport. Since the above data had indicated that inhibitin was a specific inhibitor of Na^+/Na^+ exchange, a series of experiments was conducted to investigate if inhibitin would also inhibit Na^+/Li^+ countertransport. If both processes were mediated by the same transport mechanism, Na^+/Li^+ countertransport would be predicted to be inhibitin-sensitive. The effects of inhibitin on Na^+/Li^+ countertransport were studied using the procedure outlined in Methods. In the sodium medium (145 mM) and in ouabain Na^+/Li^+ countertransport will be maximal, but other processes for passive Li^+ transport will also proceed. On the other hand, in the magnesium medium (i.e., sodium-free), lithium transport will only occur by pathways other than Na^+/Li^+ countertransport. This makes it possible to obtain an estimate of Na^+/Li^+ countertransport by the difference in lithium loss from erythrocytes into these two solutions, since in the sodium medium external sodium will stimulate lithium efflux. Bumetanide or frusemide does not need to be included in the efflux media, since under these experimental conditions Li^+ is not appreciably transported by the $Na^+/K^+/Cl^+$

TABLE II

Effect of inhibitin (1 μM) on external sodium-stimulated (Na_o) sodium efflux (Na^+/Na^+ exchanger) in the presence of ouabain (0.1 mM) and bumetanide (0.1 mM)

Results are shown as mean ± S.D. of measurements obtained from erythrocytes of one donor. $n = 4$ for both effluxes.

Sodium efflux (mmol/l per h)	Ouabain and bumetanide	Ouabain, bumetanide and inhibitin	Difference and significance
$[Na^+]_o$ 140 mM	0.65 ± 0.10 [a]	0.16 ± 0.08	0.49 [c] $P < 0.001$
$[Na^+]_o$ 0 mM	0.18 ± 0.04 [b]	0.19 ± 0.09	

a–b = sodium-stimulated sodium efflux = 0.47 mmol/l per h.

c Inhibitin-sensitive sodium efflux = 0.49 mmol/l per h.

cotransporter. The results of experiments in which inhibitin (1 μM) was tested on Na^+/Li^+ countertransport confirmed our previous observations [4]. External sodium-stimulated lithium efflux (Na^+/Li^+ countertransport) was 0.34 mmol/l per h; the difference in Li^+ efflux between Na_o^+ 145 mM (0.56 ± 0.09, $n = 5$) and Na_o^+ 0 mM (0.22 ± 0.03, $n = 5$) media. In the presence of inhibitin, lithium efflux decreased from 0.56 ± 0.09 ($n = 5$) to 0.20 ± 0.04 ($n = 5$; $P < 0.001$), giving an inhibitin-sensitive fraction of 0.36 mmol/l per h. As observed with Na^+/Na^+ exchange (Table II), addition of inhibitin had the same effect as the removal of external sodium, i.e., complete inhibition of Na^+/Li^+ countertransport. These results show that a specific inhibitor of Na^+/Na^+ exchange, inhibitin, also inhibits Na^+/Li^+ countertransport, confirming that both processes are indeed mediated by the same transport mechanism. Thus, these studies provide additional evidence in agreement with the results obtained from ion and kinetic data.

Interaction of inhibitin of with the Na^+/Na^+ exchanger

To evaluate the kinetic mechanism underlying the inhibition of Na^+/Na^+ exchange, the rate of ouabain (0.1 mM)- and bumetanide (0.1 mM)-resistant sodium efflux was measured as a function of external sodium concentration, in the presence and absence of 0.1 μM inhibitin. The data presented in Fig. 2 show that under these conditions approx. 75% of sodium efflux is sensitive to inhibitin. Tables I and II demonstrate that inhibitin-sensitive sodium efflux occurs via electroneutral Na^+/Na^+ exchange, which can be stimulated by external sodium. The dose-dependent inhibition of sodium efflux (Fig. 1) achieved with inhibitin, indicates that this peptide inhibits Na^+/Na^+ exchange with a concentration of 0.2 μM giving half-maximal inhibition. Experiments to determine the kinetics of interaction were therefore performed with 0.1 μM inhibitin to ensure submaximal concentrations of the inhibitor, a prerequisite for meaningful analysis of the data. The results obtained are representated as Hanes-Woolf plots in Fig. 4 and demonstrate that inhibitin reduced V_{max} with no effect on K_m for external sodium. The slope of the plot increased, reflecting a decline in V_{max} from 0.52 to 0.32 mmol/l cells per h, while the negative x-intercept did not change resulting in K_m for external sodium of 19 mM (control) and 21 mM (in the presence of inhibitin). Thus, inhibitin behaves as a noncompetitive inhibitor of ouabain- and bumetanide-resistant sodium efflux, implying that the binding of inhibitin and the binding of sodium occur at separate sites. The linearity of the plot is consistent with the presence of a single class of external sodium transport sites with affinity for Na_o^+ of 20 mM.

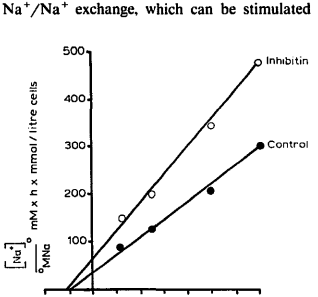

Fig. 4. Effect of inhibitin on the kinetics of ouabain- and bumetanide-resistant sodium efflux. Abscissa, extracellular sodium concentration (Na_o^+ mM); ordinate, Na_o^+ divided by the sodium flux (mmol/l cells per h). The external sodium concentration was varied by replacing choline chloride with sodium chloride in the efflux media. Each point represents the mean of at least triplicate determinations. The 'best-fit' lines were obtained by linear regression analyses.

Effect of inhibitin on erythrocyte sodium transport in the presence of phloretin

Phloretin has been credited as an inhibitor of Na^+/Li^+ countertransport [12]. For this reason, the effect of inhibitin on sodium efflux was studied in the presence of phloretin. If both inhibitors had the same locus of action, their inhibitory effects would not be additive. Phloretin (0.2 mM) was found to have an effect on ouabain-insensitive sodium transport, reducing sodium efflux from 0.93 ± 0.13 mmol/l per h to 0.71 ± 0.04 mmol/l per h ($n = 3$; $P < 0.05$). However, the inhibitory effect of inhibitin was found to persist in the presence of phloretin, further reducing sodium efflux to 0.38 ± 0.14 mmol/l per h ($n = 3$); $P < 0.02$) and indicating separate sites of action. In this subject inhibitin-sensitive Na^+/Na^+ exchange, in the presence of ouabain and phloretin, was thus 0.33 mmol/l per h (which is less than that seen in other subjects, 0.5 mmol/l per h (Tables I and II), in the presence of ouabain alone) indicating that phloretin can also partially inhibit the Na^+/Na^+ exchanger. In an attempt to clarify the site of action of phloretin further, experiments were conducted (in red cells from another donor) in which phloretin was studied alone and in various combinations with ouabain and bumetanide. These results are presented in Table III. As can be seen, the effect of phloretin overlaps with that of both ouabain and bumetanide (i.e., inhibition is not additive, suggesting similar loci of action). Phloretin also inhibited vesicular Na^+/K^+-ATPase activity, measured spectrophotometrically by enzymatically linked ATP hydrolysis to NADH oxidation [18], but did not displace ^{3}H-ouabain from its binding site (data not shown). Collectively,

TABLE III

Effect of phloretin (0.2 mM) on erythrocyte sodium efflux in the presence of ouabain (0.1 mM) and bumetanide (0.1 mM)

All data shown as mean ± S.D. of four determinations in red cells from a single donor. The ouabain-sensitive fraction was 1.10 mmol/l per h, the bumetanide-sensitive fraction was 0.48 mmol/l per h and the phloretin-sensitive fraction was 0.73 mmol/l per h. However, the combined inhibition achieved with ouabain and phloretin together (1.39) was less than the sum of ouabain-sensitive fraction (1.10) and phloretin-sensitive fraction (0.73), as was the combined inhibition with bumetanide and phloretin (0.61, compared with a bumetanide-sensitive fraction of 0.48 and phloretin-sensitive fraction of 0.73). These results indicate that phloretin does not have a specific site of action as its effect overlaps with that of ouabain and bumetanide.

Sodium efflux (mmol/l/h)		
Ringer	1.92 ± 0.25	
Ouabain	0.82 ± 0.12	ouabain-sensitive fraction 1.10, $P < 0.001$
Bumetanide	1.44 ± 0.07	bumetanide-sensitive fraction 0.48, $P < 0.02$
Phloretin	1.19 ± 0.10	phloretin-sensitive fraction 0.73, $P < 0.005$
Ouabain and phloretin	0.53 ± 0.06	combined inhibition 1.39, $P < 0.001$
Bumetanide and phloretin	1.31 ± 0.19	combined inhibition 0.61, $P < 0.01$

these results suggest that the effects of phloretin are nonspecific, as has been previously reported.

Discussion

Inhibitin appears to be a specific inhibitor of carrier-mediated, external sodium (Na_o^+)-stimulated Na^+/Na^+ exchange and Na^+/Li^+ countertransport, since its inhibitory effect is demonstrable only in the presence of Na_o^+ and the inhibitin-sensitive transport (Na^+/Na^+ exchange) remains a distinct measurable component in addition to that inhibited by ouabain and bumetanide. Using inhibitin as an inhibitor of Na^+/Na^+ exchange, the results of this study show that the carrier that transports Na_o^+ for Na_i^+ (internal Na^+) can also exchange Li_i^+ for Na_o^+ (Na^+/Li^+ countertransport) – both processes are dependent on Na_o^+ and are inhibitible by inhibitin. Inhibitin achieves its maximal inhibition at $1 \cdot 10^{-6}$ M concentration and has a K_i of $2 \cdot 10^{-7}$ M (Fig. 1). Using this recently characterised inhibitor of Na^+/Na^+ exchange [4–5], inhibitin, we have provided experimental evidence to establish that Na^+/Na^+ exchange and Na^+/Li^+ countertransport are mediated by the same transport process, confirming the results obtained in ion and kinetic studies [12–14]. We have also explored the contribution of Na^+/Na^+ exchange to basal sodium transport in normal human erythrocytes. The results presented in Table I clearly show that inhibitin inhibits equivalent amounts of ouabain- and bumetanide-resistant sodium efflux and sodium influx (0.52 and 0.53 mmol/l per h, respectively). The fact that there is no contribution of this process to net flux (no detectable change in intracellular sodium) pinpoints the site of action on Na^+/Na^+ exchange in accord with our previously published observations [5]. Inhibitin-sensitive transport represents 21% of total sodium efflux (Fig. 2) and the largest proportion (53%) of passive (ouabain-insensitive) sodium movement with diuretic-sensitive $Na^+/K^+/Cl^-$ cotransport and residual Na^+ 'leak' constituting the remainder.

Kinetic analysis of the inhibitory effects of inhibitin (Fig. 4) indicates that external sodium and inhibitin do not compete for the same site, i.e., inhibition is strictly noncompetitive. This probably means that each Na^+/Na^+ exchanger that binds inhibitin is completely inactivated. A linear Dixon plot (Fig. 3) demonstrates the presence of a single binding site. Preliminary results (data not shown) have demonstrated that the inhibitory effect is reversible. It would seem that inhibitin must interact with the human erythrocyte Na^+/Na^+ exchanger at a single site that is distinct from the external sodium transport sites.

Using an alternative method to estimate Na^+/Na^+ exchange (external Na^+-stimulated sodium efflux), the inhibitin-sensitive component was found to be the same as the difference between sodium efflux in the high (140 mM) and 0 mM sodium solutions (0.49 and 0.47 mmol/l per h; Table II). The value of Na^+/Na^+ exchange measured by this method is almost identical to the inhibitin-sensitive fraction of ouabain- and bumetanide-resistant sodium efflux and influx (Table I). These results indicate that Na^+/Na^+ exchange measured by two independent methods can be totally blocked by 1 μM inhibitin. Measurement of Na^+/Li^+ countertransport showed that inhibitin-sensitive Li^+ efflux (0.36 mmol/l per h) was equal to the portion of lithium efflux stimulated by external Na^+ (0.34 mmol/l per h). These data demonstrate that Na^+/Li^+ countertransport is achieved by Li^+ being transported in the Na^+/Na^+ exchange mechanism and that it is not an independent transport system. In these experiments, the intracellular Li^+ concentration (6 mmol/l cells) was above saturation for the internal Li^+ site (K_m for Li_i^+ is approx. 1 mM [14]). However, the amount of Li^+ efflux (0.34 mmol/l per h) was less than Na^+ efflux (0.47 mmol/l per h), which is compatible with the Na^+/Na exchanger being asymmetric for Na^+ and Li^+, as recently reported by Hannaert and Gary [19].

Phloretin has been proposed as an inhibitor of Na^+/Li^+ countertransport and Na^+/Na^+ exchange but, whilst the results in Table III indicate that it can indeed inhibit erythrocyte sodium transport, its effect overlaps with those of ouabain and bumetanide, suggesting that it is not specific for any transport process. Furthermore, the inhibitory effect of inhibitin is detectable in the presence of phloretin, albeit not as large an effect as in

its absence. Thus, a residual fraction of Na^+/Na^+ exchange remains in the presence of phloretin which is inhibitin-sensitive. Duhm [20] has observed that Na^+/Na^+ exchange in rabbit red cells is only partially inhibited by phloretin. It would appear, therefore, that phloretin exerts its inhibitory effect on various transport pathways by nonspecific interference with membrane components. Other data [21] have shown that phloretin binds to membrane lipids adjacent to carrier proteins and thus achieves its inhibitory effects on transport processes. Phloretin also inhibits the anion exchanger and the sugar and nucleoside transporters. These results indicate that phloretin is a nonspecific inhibitor of erythrocyte transport processes capable of blocking different pathways, presumably by interfering with membrane structure adjacent to carrier proteins. Alternatively, these data could be interpreted to suggest that human red blood cells have two different components of Na^+/Na^+ exchange.

Inhibitin is released not only by leukaemic promyelocytes [5] but also by a variety of other neoplastic cells [15], and could have a role in ion regulation in these cells during replication. Many workers have shown that addition of growth factors to quiescent cells activates sodium influx, and the availability of amiloride, as a specific inhibitor of Na^+/H^+ exchange [22–24], has focused attention on sodium uptake by this antiport [25–29]. Evidence has been provided for a Na^+/H^+ exchange system in human red blood cells which can be activated by acid cell pH [30]. At $pH_i = 7.4 = pH_o$ the system is silent and so could feasibly function as a Na^+/Na^+ exchanger. Experiments examining the effect of pH gradients on the activity of the Na^+/H^+ and Na^+/Na^+ exchanger in rabbit red blood cells have been performed and show that both exchanges can occur through the same transport molecule [31–33]. In renal microvillus membrane vesicles, Aronson and co-workers [34] have shown that the amiloride-sensitive exchanger can function in multiple modes and bind Na^+, NH_4^+, Li^+ or H^+ on one side of the membrane and exchange it for any one of these on the opposite side. More recent studies [35] have also demonstrated that the human red blood cell Na^+/H^+ exchanger is capable of operating in a Na^+/Li^+ exchange mode. Preliminary studies with cultured human fibroblasts have shown that inhibitin kinetically modifies Na^+/H^+ exchange in these cells [36]. The availability of inhibitin as a probe should help further elucidate these pathways and improve our understanding about cation transport and its possible role in cell proliferation; it is also possible that inhibitin interacts with the Na^+/H^+ exchanger.

Recent studies have indicated that the rate of Na^+/Li^+ countertransport is elevated in erythrocytes of essential hypertensive patients [17]. The availability of a specific inhibitor of this process will allow a direct comparison to be made by measuring the inhibitin-sensitive component in erythrocytes of normals and patients with various disorders. Also, labelled inhibitin (preliminary experiments have shown that it is possible to iodinate inhibitin using Bolton and Hunter reagent [37]) will be useful in identifying and determining the number of Na^+/Na^+ and Na^+/Li^+ exchangers in various cell types in much the same way as [^{3}H]ouabain has been used in radioreceptor assays [37]. The membrane protein responsible for these ion movements has yet to be identified, but the availability of inhibitin could prove useful in these endeavours.

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