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EFFECTS OF CYSTEINE AND POTASSIUM ON THE ATP-DEPENDENT RETENTION OF SODIUM IONS BY ERYTHROCYTE MEMBRANES*

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SUMMARY

The ATP-dependent retention of Na^+ by a preparation of human erythrocyte membrane fragments required the presence of either cysteine or K^+. The activation by cysteine was specific when compared with various cysteine analogues. The K^+ activation could be substituted by other alkali metal ions in the following order of efficiency: $K^+ = Rb^+ > Cs^+ > Li^+$. Ouabain completely inhibited the K^+-activated ATP-dependent retention of Na^+ and partially inhibited the cysteine-activated retention. ATP-dependent Na^+ retention in intact ghosts and in membrane fragments was similar. Possible mechanisms for ATP-dependent Na^+ retention are discussed.

INTRODUCTION

Studies by JÄRNEFELT[1] and JÄRNEFELT AND VON STEDINGK[2] on rat brain microsomes, and CHARNOCK, ROSENTHAL AND POST[3] on a microsomal preparation from guinea pig kidney cortex have demonstrated that binding of Na^+ to these microsomal preparations could be stimulated by ATP. A previous report[4] has also shown a similar activity in an erythrocyte membrane preparation. This ATP-dependent Na^+ retention by erythrocyte membrane fragments was correlated with $(Na^+ + K^+)$-activated ATPase activity in nucleotide specificity, Mg^{2+} requirement, pH dependence curves and species differences in man, rat, cat and dog.

The present report is a continuation of this study. It shows that the ATP-dependent Na^+ retention has a requirement for either K^+ or cysteine. Attempts to elucidate the nature of retained Na^+ in the membrane preparation are also presented.

* The data given in this manuscript are based in part upon the thesis submitted by FREDERICK G. WALZ, Jr. in partial fulfillment for the degree of Doctor of Philosophy, State University of New York Downstate Medical Center.

METHODS

Preparation of erythrocyte membrane fragments

Fragments of human erythrocyte ghosts, free of hemoglobin, were prepared as previously reported[4].

Na^+ retention assay

The standard reaction in a final volume of 2.5 ml contained 60 mM imidazole–HCl (pH 7.5), 6 mM cysteine, 2 mM $MgCl_2$, 1 mM ATP (as Tris salt), 4 mM NaCl containing $5 \cdot 10^5$ counts/min of $^{22}NaCl$, and membrane fragments with an equivalent protein concentration of 1.0–1.5 mg/ml. The reaction was initiated by the addition of NaCl. After the mixture was incubated for 30 min at 37°, the reaction was terminated by the addition of 37 ml of ice-cold, 0.17 M Tris–HCl buffer, pH 7.5. The membrane fragments were sedimented at 35 000 × *g* for 20 min at 4°, and washed 3 times with the same buffer. The sediment from the final wash was quantitatively transferred to a planchet. After drying under an infrared lamp, the sample was counted in a gas flow counter. Loss of protein in the initial centrifugation was 30–35%. There was no further loss in subsequent washing. ATP, cysteine or K^+ did not show detectable effect on protein loss.

ATPase assay

The standard reaction mixture in a final volume of 2.5 ml contained 2.5 mM $MgCl_2$, 1.25 mM ATP (as Tris salt), 5 mM cysteine, 60 mM imidazole–HCl (pH 7.5), and membrane fragments equivalent to a protein concentration of 0.25–0.30 mg/ml. The ($Na^+ + K^+$)-activated ATPase assay mixture contained all the above components *plus* 75 mM NaCl and 15 mM KCl. The mixture was incubated for 60 min at 37°. Orthophosphate produced was determined by the method of HORWITT[5] with some modifications as described previously.

The concentration of the protein was determined according to the method of LOWRY *et al.*[6].

Materials

^{22}Na was obtained from New England Nuclear Corp., ^{42}K from IsoServe Inc., and [8-^{14}C]ATP from Schwarz BioResearch Inc.

RESULTS

Monovalent cation stimulation

As reported previously, in the absence of ATP the retention of Na^+ by erythrocyte membrane fragments was similar to that of either K^+ or Rb^+, whereas, in the presence of ATP, Na^+ was retained much more than either of the other two ions[4]. It was of interest to examine whether the lower ATP-dependent retention of K^+ or Rb^+ represented a loose specificity of the same binding process for Na^+ retention. If the ATP-dependent retention of these three cations operated by the same mechanism, addition of K^+ or Rb^+ to a standard Na^+ retention assay medium should competitively inhibit ATP-dependent Na^+ retention. Results to the contrary are shown in Fig. 1. In this experiment Na^+ retention was determined in the presence of varying

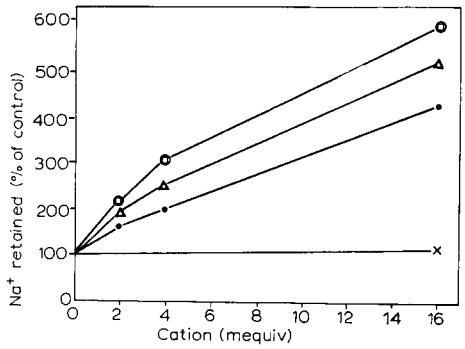

Fig. 1. Effect of different concentrations of alkali metal ions on ATP-dependent Na^+ retention. ○, K^+; □, Rb^+; △, Cs^+; ●, Li^+; X, Tris. The cations were added as their chloride salts. Other conditions of the assay were as described under METHODS. ATP-dependent Na^+ retention in the absence of added cation was arbitrarily designated 100%.

concentrations of K^+, Rb^+, Cs^+ or Li^+. The activities are presented as per cent of the ATP-dependent Na^+ retention in the absence of added second cation. Addition of a second alkali metal ion caused considerable stimulation on ATP-dependent Na^+ retention. 16 mequiv of either K^+ or Rb^+ stimulated the Na^+ retention almost 6-fold. The efficiency for the stimulation was in the following order: $K^+ = Rb^+ > Cs^+ > Li^+$. In the absence of ATP, Na^+ retention was slightly decreased by an addition of any of these cations (results not shown).

As a comparison the effect of Tris ions was also tested. Addition of 16 mequiv Tris ions (pH 7.5), did not cause significant change as compared with the other cations.

TABLE I

SPECIFICITY OF CYSTEINE ACTIVATION OF ATP-DEPENDENT Na^+ RETENTION

Membrane fragments were prepared in the usual manner except that cysteine was omitted from the final wash solution. Conditions of the assay were as described under METHODS.

Addition (6 mM)	*ATP-dependent Na^+ retention (% L-cysteine activation)*
L-Cysteine	100*
D-Cysteine	103
N-Acetyl-L-cysteine	40
L-Cysteine ethyl ester	38
DL-Homocysteine	29
S-Methyl-L-cysteine	7
β-Mercaptoethylamine	4
β-Mercaptopropionic acid	−8
L-Serine	−6
L-Alanine	3
Glycine	−3
Glutathione	4
1-Mercaptoglycerol	−4

* Arbitrarily designated as 100%.

Requirement of either cysteine or K^+

Cysteine was included in the reaction mixture of the standard Na^+ retention assay as a general sulfhydryl-group protecting agent. During the course of this study it was found that ATP-dependent Na^+ retention was not observed if cysteine was deleted. As shown in Table I, both D and L configurations of cysteine had a similar effect in the activation. *N*-Acetyl-L-cysteine and L-cysteine ethyl ester were approximately 40% as effective as cysteine, indicating that the free amino and carboxyl groups of cysteine are necessary for optimal activity. When both the amino and carboxyl groups were blocked, as in glutathione, no activation was observed. Likewise, when either the carboxyl group (β-mercaptoethylamine) or the amino group (β-mercaptopropionic acid) was absent, there was no ATP-dependent Na^+ retention. Another obligatory characteristic of the activator is a free sulfhydryl group; *S*-methyl-L-cysteine and L-serine were ineffective. A relatively strict steric requirement for activation was indicated by the comparatively much smaller effect of DL-homocysteine. The lack of activation in the presence of L-alanine, glycine and 1-mercapto-L-glycerol provided further evidence for the general specificity of the cysteine activation.

It was also observed that the cysteine activation of ATP-dependent Na^+ retention was partially inhibited by low concentrations of ouabain[4]. This inhibition was maximal at 0.1 mM ouabain. The ouabain-sensitive portion of this Na^+ retention could be due to an undetectable trace amount of K^+ in the reaction mixture. The effect of different concentrations of cysteine on Na^+ retention in the presence and absence of ouabain is seen in Fig. 2. Cysteine did not have a significant effect on Na^+ retention in the absence of ATP. When ATP was present, increasing cysteine concentration progressively activated the Na^+ retention. A maximal effect was not attained within the range of cysteine concentrations tested. At different concentrations of cysteine, additions of ouabain inhibited ATP-dependent Na^+ retention from 15 to 40%. It is of interest to note that in the absence of cysteine, the addition of either ATP or ATP *plus* ouabain had no effect on Na^+ retention.

The effect of cysteine on the ATPase was also tested. A comparison of ATPase activity in incubation mixtures with and without cysteine is shown in Table II, Expt. 1. Cysteine stimulated the ($Na^+ + K^+$)-activated ATPase to a lesser extent (10%) than the ATPase in the absence of Na^+ and K^+ (77%). Expt. 2 was intended to determine whether cysteine could substitute for K^+ in activating the ATPase in conjunction with Na^+. As usual, the presence of Na^+ *plus* K^+ activated the ATPase and this activation was inhibited by ouabain. When 25 mM cysteine was substituted for K^+, the addition of either Na^+ alone or Na^+ *plus* ouabain showed no effect on the ATPase. The results indicated that the activating effect of cysteine on the ATPase was involved mainly with the ATPase observed in the absence of added Na^+ *plus* K^+, and that cysteine could not substitute for K^+ in the ($Na^+ + K^+$)-activated ATPase.

Cysteine is not an absolute requirement for ATP-dependent Na^+ retention. It was found that additions of K^+, which activated ATP-dependent Na^+ retention in the presence of cysteine, could also stimulate the retention in the absence of cysteine. The ATP-dependent Na^+ retention activated by K^+ alone was completely inhibited by 0.1 mM ouabain. The effect of different concentrations of K^+ on Na^+ retention, in the absence of cysteine, is shown in Fig. 3. It was observed that in the absence of

TABLE II

THE EFFECT OF CYSTEINE ON THE ATPASE

Experimental conditions were as described under METHODS. When added, ouabain was 0.1 mM, NaCl was 75 mM, and KCl was 15 mM in Expt. 1 and 25 mM in Expt. 2.

Expt. No.	L-*Cysteine (mM)*	*Additions*	*ATPase (mμmoles P_i per mg protein per h)*	
			Observed	*$(Na^+ + K^+)$-activated*
1	6	None	237	—
	6	Na^+, K^+	567	330
	6	Na^+, K^+, ouabain	234	—
	0	None	134	—
	0	Na^+, K^+	434	300
	0	Na^+, K^+, ouabain	160	—
2	0	None	65	—
	0	Na^+, K^+	183	113
	0	Na^+, K^+, ouabain	83	—
	25	None	112	—
	25	Na^+	110	(−2)
	25	Na^+, ouabain	109	—

K^+ the addition of either ATP or ATP *plus* ouabain had no effect on Na^+ retention. Without ATP, Na^+ retention was slightly inhibited as the concentration of K^+ was increased. When ATP was present Na^+ retention was progressively stimulated by increasing concentrations of K^+. This stimulation achieved a maximum at about 12 mM KCl. At every concentration of K^+ tested, ouabain completely inhibited the ATP-dependent retention of Na^+.

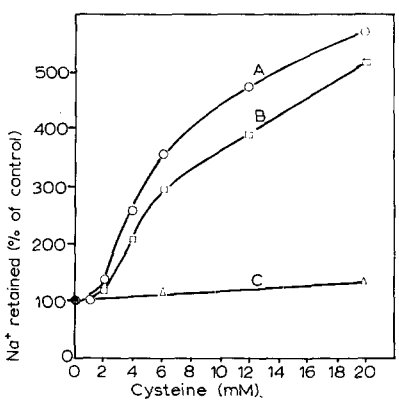

Fig. 2. Effect of cysteine concentration and ouabain on Na^+ retention. Curve A, *plus* 1 mM ATP; Curve B, *plus* 1 mM ATP and 0.1 mM ouabain; Curve C, without ATP. Other conditions of the experiment were as described under METHODS. Na^+ retention in the absence of cysteine and ATP was arbitrarily designated 100%.

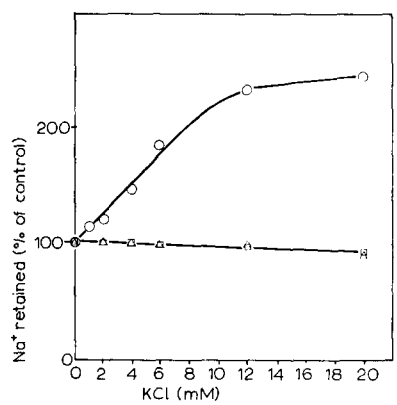

Fig. 3. The effect of K^+ concentration and ouabain on Na^+ retention in the absence of cysteine. □, without ATP; ○, *plus* 1 mM ATP; △, *plus* 1 mM ATP and 0.1 mM ouabain. Other conditions of the experiment were as described under METHODS. Na^+ retention in the absence of K^+ was arbitrarily designated 100%.

TABLE III

EFFECT OF OUABAIN, CYSTEINE AND K^+ ON Na^+ RETENTION

Experimental conditions were as described under METHODS. When added, ATP was 1 mM, cysteine 6 mM, ouabain 0.1 mM, and KCl 8 mM.

Tube No.	*Additions*	*Na^+ retained (mμmoles/mg protein)*	
		Total	*ATP-dependent*
1	ATP, cysteine	1.63	1.17
2	ATP, cysteine, ouabain	1.29	0.83
3	Cysteine	0.46	—
4	ATP, KCl	0.81	0.35
5	ATP, KCl, ouabain	0.43	−0.03
6	KCl	0.46	—
7	ATP, cysteine, KCl	4.70	4.24
8	ATP, cysteine, KCl, ouabain	1.26	0.80

A quantitative comparison of the effects of cysteine and K^+ on ATP-dependent Na^+ retention is presented in Table III: in the presence of cysteine, ATP activated Na^+ retention 254% (Tubes 1 and 3); in the presence of K^+, ATP activated this retention 76% (Tubes 4 and 6); whereas in the presence of both cysteine and K^+, ATP activated Na^+ retention 922% (Tubes 7 and 3). Either Tube 3 or Tube 6 is an adequate control for Tube 7, since it was observed that addition of either K^+ or cysteine in the absence of ATP had no significant effect on Na^+ retention (see Figs. 2 and 3). It should be noted that the effects of cysteine and K^+ on ATP-dependent Na^+ retention are not additive.

The effect of cysteine on K^+ retention was also investigated. As shown in Table IV, cysteine is also a necessary component of the reaction mixture for ATP-dependent K^+ retention. However, Na^+ could not substitute for cysteine in stimulating K^+ retention. This is in contrast to the fact that K^+ stimulated Na^+ retention in the absence of cysteine.

ATP retention

HEINZ AND HOFFMAN have observed binding of intact ATP to the erythrocyte

TABLE IV

EFFECT OF CYSTEINE AND Na^+ ON K^+ RETENTION

Experimental conditions were similar to those for a standard Na^+ retention assay except that 4 mM ^{42}KCl ($5.85 \cdot 10^5$ counts/min per μmole) was substituted for labeled NaCl.

Additions	*K^+ retained (mμmoles/mg protein)*	
	Total	*ATP-dependent*
None	0.61	—
1 mM ATP	0.61	0.0
20 mM NaCl	0.53	—
20 mM NaCl + 1 mM ATP	0.50	−0.03
6 mM cysteine	0.75	—
6 mM cysteine + 1 mM ATP	1.84	1.09

TABLE V

COMPARISON OF ATP AND Na^+ RETENTION

ATP retention reaction mixture contained: 60 mM imidazole–HCl buffer (pH 7.5), 2 mM $MgCl_2$, 6 mM cysteine, 1 mM [8-^{14}C]ATP ($1.69 \cdot 10^5$ counts/min per μmole), membrane fragments equivalent to 1.5 mg protein and additions in a final volume of 2.5 ml. Other conditions were as described for a standard Na^+ retention assay.

Additions	*ATP retained (mμmoles/mg protein)*	*ATP-dependent retention of Na^+* (mμmoles/mg protein)*
None	0.81	—
4 mM NaCl	0.81	2.72
4 mM NaCl + 8 mM KCl	0.90	5.40

* Na^+ retention in the presence of ATP *minus* retention in the absence of ATP.

membrane[7]. The experiment in Table V was intended to examine any relation between ATP retention and ATP-dependent Na^+ retention in erythrocyte membrane fragments. It was observed that addition of either Na^+ or Na^+ and K^+ did not have a significant effect on ATP binding. In contrast, addition of 8 mM KCl stimulated ATP-dependent Na^+ retention about 2-fold.

Na^+ retention by erythrocyte ghosts

An attempt was made to investigate the relation between the integrity of the membrane and ATP-dependent Na^+ retention. The Na^+ retention by intact ghosts was compared with that by membrane fragments. Although there was a quantitative difference between the two preparations, both qualitatively exhibited an ATP-dependent Na^+ retention and this activity was stimulated by K^+ addition in both the ghost and the membrane fragment preparations (see Table VI).

TABLE VI

Na^+ RETENTION BY GHOSTS AND FRAGMENTS

Intact ghosts and membrane fragments were prepared simultaneously using the same blood sample. The final wash solution in the intact ghost preparation contained 0.1 M Tris–HCl buffer, pH 7.5, and that for the fragment preparation contained 2 mM imidazole–HCl buffer, pH 7.5. Incubation mixture contained 60 mM Tris–HCl buffer, pH 7.5, instead of imidazole buffer. Other conditions were as described for a standard Na^+ retention assay.

Preparation	*Additions*	*Na^+ retained (mμmoles/mg protein)*
Ghosts	None	0.82
	1 mM ATP	2.31
	1 mM ATP + 10 mM KCl	5.39
Fragments	None	1.04
	1 mM ATP	1.73
	1 mM ATP + 10 mM KCl	9.06

DISCUSSION

Comparative studies on the retention of Na^+, K^+, or Rb^+ by erythrocyte membrane fragments showed that in the presence of ATP, Na^+ was retained to a much greater extent than either K^+ or Rb^+, while K^+ and Rb^+ were retained equivalently[4]. The Na^+ retention appeared to be of a different process from the retention of K^+ or Rb^+. Neither K^+ nor Rb^+ competed for Na^+ retention. Na^+ retention could actually be stimulated several fold by the addition of either K^+ or Rb^+. On the other hand ATP-dependent K^+ retention was neither activated nor inhibited by Na^+ addition.

In a study on the spatial asymmetry in monovalent cation activation of the erythrocyte membrane ATPase, WHITTAM AND AGER[8] found that the extracellular K^+ could be substituted by Rb^+, Cs^+ or Li^+ and that the order of efficiency for the extracellular component to stimulate the transport ATPase was as follows: $K^+ = Rb^+ > Cs^+ > Li^+$. The ATP-dependent Na^+ retention could also be stimulated by any of these four cations in a similar order of efficiency (see Fig. 1). This indicates that the site for K^+ activation in ATP-dependent Na^+ retention is either identical or similar to that for K^+ in the ($Na^+ + K^+$)-activated ATPase.

A prominent feature of the ($Na^+ + K^+$)-activated ATPase reaction is its inhibition by low concentrations of ouabain[9]. Although the mechanism of this inhibition is not completely understood, ouabain appears to have an effect on the K^+ site of the ATPase activation[10]. This cardiac glycoside was also found to be inhibitory on the ATP-dependent retention of Na^+: 0.1 mM ouabain completely abolished the activating effect of K^+ on Na^+ retention.

A property of ATP-dependent Na^+ retention that does not present an obvious correlation with the ($Na^+ + K^+$)-activated ATPase is the activating effect of cysteine. The ($Na^+ + K^+$)-activated ATPase in various microsomal preparations has been shown to be inhibited by a variety of sulfhydryl inhibitors[11,12]. DUNHAM AND GLYNN[13] used cysteine as a non-specific sulfhydryl group-protecting agent in their earlier work on the erythrocyte membrane ATPase. In the present study cysteine has also been added as a common constituent in all assay mixtures except when specified otherwise. Although cysteine stimulates the membrane ATPase in the absence of added Na^+ and K^+ and to a lesser extent, the ($Na^+ + K^+$)-activated ATPase, these effects of cysteine are not immediately reconciliable with the requirement of cysteine for ATP-dependent Na^+ retention in the absence of added second alkali metal ion. However, it seems that cysteine does not directly substitute for K^+ in activating these processes, since it cannot replace K^+ in the ($Na^+ + K^+$)-activated ATPase reaction.

In a recent report AHMED AND JUDAH[14] found that either ATP or EDTA could stimulate Na^+ retention in a rat brain microsomal preparation. They postulated that these activators served as chelating agents to remove indigenous Mg^{2+} in the preparation, and thereby expose additional non-specific cation binding sites for the retention of Na^+. This interpretation of ATP-dependent Na^+ retention was similar to that proposed by SANUI AND PACE[15] in their studies with rat liver microsomes. However, this hypothesis for the mechanism of ATP-dependent Na^+ retention is not compatible with the present investigation for the following principle reasons: (a) addition of Mg^{2+} is necessary for the ATP-dependent Na^+ retention by erythrocyte membrane fragments[4], (b) the ATP-dependent Na^+ retention requires the presence of either

cysteine or K^+ and (c) ouabain shows a marked inhibitory effect on ATP-dependent Na^+ retention.

POST and his co-workers[3,16] and ALBERS, FAHN AND KOVAL[17] have reported phosphorylated intermediates as part of the membrane ($Na^+ + K^+$)-activated ATPase system. The turnover rate of these phosphorylated intermediates was in the order of seconds. On the other hand the ATP-dependent retention of Na^+ was not saturated after 90-min incubation[4]. This seems to preclude such phosphorylated intermediates as the binding sites for ATP-dependent Na^+ retention.

Binding of ATP to the membrane fragment has also been ruled out as the cause for Na^+ retention, because addition of K^+ did not have significant effect on ATP binding while it stimulated ATP-dependent Na^+ retention about 2-fold (see Table V). If the retention were mainly due to the electrostatic association of cations with negative charges of bound ATP, addition of K^+ should have decreased the Na^+ retention instead.

The possibility of concentrating Na^+ in some aqueous enclosures formed by membrane fragments was also excluded, since the ATP-dependent Na^+ retention activity was also observed in intact erythrocyte ghosts. As demonstrated by WHITTAM AND AGER[8] the ATP-dependent transport of Na^+ was from the interior of the ghost to the extracellular medium. This appeared to indicate that the retention of Na^+ was within the matrix of the membrane.

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