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PLASMA MEMBRANE NADH DEHYDROGENASE AND Ca^{2+}-DEPENDENT POTASSIUM TRANSPORT IN ERYTHROCYTES OF SEVERAL ANIMAL SPECIES

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Ca^{2+}-dependent K^+ transport and plasma membrane NADH dehydrogenase activities have been studied in several 'high-K^+' (human, rabbit and guinea pig) and 'low-K^+' (dog, cat and sheep) erythrocytes. All the species except sheep showed Ca^{2+}-dependent K^+ transport. NADH-ferricyanide reductase was detected in all the species and showed positive correlation with the flavin contents of the membranes. NADH-cytochrome *c* reductase was very low or absent in dog, sheep and guinea pig membranes. No correlation was found between NADH dehydrogenase and Ca^{2+}-dependent K^+ channel activities in the species studied. Nor were any of the above activities correlated with $(Na^+ + K^+)$-ATPase activity.

Introduction

It has been shown that the electron donor system ascorbate + phenazine methosulphate activates the Ca^{2+}-dependent K^+ channel in the human erythrocyte [1] and in the Ehrlich cell [2]. Similar results were obtained with several other chemically unrelated reducing agents loaded into red cell ghosts [1], suggesting that the activation of the K^+ channel by these agents is the consequence of redox interactions with cellular components somehow connected with the channel function. However, the nature of the cellular redox systems implicated in these effects remains to be clarified. Atebrin, a potent inhibitor of NADH dehydrogenase activities of mammalian plasma membrane [3], inhibited the effect of electron donors and also that of other activators of the K^+ channel in the human erythrocyte [1,4]. This fact suggests that these membrane-bound oxido-reductase systems or some of their components could be involved in the process.

Plasma membrane NADH dehydrogenase activities have been found in the human erythrocyte [5,7], but similar studies in other species are lacking. On the other hand, it is known that erythrocytes of several mammalian species show differences in Ca^{2+}-dependent K^+ transport [8]. In this work we have taken advantage of the red cell polymorphism among different species to investigate whether a meaningfull correlation exists between plasma membrane oxido-reductase and Ca^{2+}-dependent K^+ transport activities.

Material and Methods

In the present study erythrocytes from the following species were used: human, rabbit, guinea pig, dog, cat and sheep (only the low K^+ type). All

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid.

the experiments were performed with fresh blood collected using heparin as the anticoagulant. Erythrocytes were sedimented by centrifugation, washed three times with 0.15 M ice-cold choline chloride, resuspended at 50% hematocrit, and divided into two halves to be used for enzymatic assays and transport experiments, respectively.

NADH dehydrogenase [5] and $(Na^+ + K^+)$-ATPase [9] activities were assayed in membranes prepared by hypotonic hemolysis [10]. Since addition of 0.005% saponin to the assay medium [10] increased the apparent activity for both enzymes and gave most regular time courses, it was systematically included into the medium for enzymatic assays.

The activity of the Ca^{2+}-dependent K^+ channel was asessed from measurements of the cell uptake of ^{86}Rb, which behaves similarly to K^+ for this transport system [11]. In other experiments net K^+ fluxes were also measured. K^+ channel activation was elicited by three different treatments: (i) 1–2 μM A23187 + 0.5 mM Ca^{2+}; (ii) 20 mM sodium ascorbate + 0.1 mM phenazine methosulphate, and (iii) 0.5 mM propranolol. Since depletion of ATP has been shown to facilitate Ca^{2+}-dependent K^+ transport [12,14], 'depleted' as well as 'fresh' cells were used. For ATP depletion red cells were incubated at 37°C with 2 mM iodoacetate and 10 mM adenosine for a period of time enough to obtain ATP levels below 0.02 mmol/l of cells (see legend of Table III). The cells were then washed twice with ice-cold 0.15 M choline chloride and resuspended at 50% hematocrit.

The uptake of ^{86}Rb was measured at 37°C during a 5-min incubation period terminated by ice-cold dilution and centrifugation, as described elsewhere [1], and expressed as an operational rate constant, k, calculated as (cpm per g of cells)/(cpm per ml of incubation medium). Net fluxes of Na^+ and K^+ were calculated from the difference between the cell contents of these ions (mequiv./kg of cells) at the beginning and at the end of the incubation period. For these experiments the composition of the incubation medium was (mM): NaCl, 140; KCl, 4.8; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$ 0.5; Hepes-NaOH buffer, 20 (pH. 7.4).

In order to investigate the net fluxes of K^+ in the low-K^+ erythrocytes (dog, cat and sheep), they were incubated in a high-K^+ medium of the following composition (mM): KCl, 100; NaCl, 50; Hepes-NaOH buffer, 10 (pH 7.4) and other additions as specified in the legends to Figs. 1 and 2. In these experiments, the incubation period was started by mixing 1 volume of 'fresh' cells with 4 volumes of medium prewarmed at 37°C. At different time intervals (up to 150 min) 0.5 ml aliquots of the cell suspension were diluted with 9 ml of ice-cold 0.113 M $MgCl_2$ solution. After centrifugation the cell pellet was washed twice and further processed as described elsewhere [1]. Na^+ and K^+ contents of the cells were calculated allowing a correction for the degree of hemolysis which was asessed from hemoglobin measurements in the supernatant solution from the centrifugation.

Spectrophotometric procedures were used for the determination of hemoglobin [15] and proteins [16]. Flavins were measured fluorometrically [17,18]. ATP was determined either fluorometrically [19] or radioenzymatically [20]. Na^+ and K^+ were measured by flame photometry, and ^{86}Rb by Cerenkov counting.

All the radiochemicals were obtained from The Radiochemical Center, Amersham. The ionophore A23187 was a generous gift from Lilly Indiana de España, S.A. Other chemicals were obtained either from Sigma London Chem. Co. Ltd. or from E. Merck, Darmstadt.

Results

NADH dehydrogenase activity

NADH dehydrogenase activity was studied using as the electron acceptor either potassium ferricyanide or ferricytochrome *c*. Table I shows that the values obtained with both acceptors were not always parallel, and also that those with ferricyanide were always higher. The highest rates were obtained with cat and human membranes. Sheep and guinea pig membranes showed high NADH-ferricyanide reductase activity and not measurable NADH-cytochrome *c* reductase activity. Dog membranes showed low rates for both activities.

It has been suggested that flavin nucleotides may be a component of plasma membrane NAHD dehydrogenase from the human erythrocyte [7]. We found flavins in the membranes of all the six specie studied (Table II). The relative contents

TABLE I

ERYTHROCYTIC NADH DEHYDROGENASE ACTIVITY

Activity is expressed as nmol of acceptor reduced per mg of protein per min. Each datum is the mean ± S.E. of at least four experiments.

Species	Electron acceptor	
	$Fe(CN)_6K_3$	Ferricytochrome *c*
Human	241 ± 33	13 ± 2
Guinea pig	76 ± 5	0
Rabbit	80 ± 12	11 ± 2
Sheep	132 ± 27	0
Cat	489 ± 45	26 ± 1
Dog	15 ± 2	3 ± 1

were grossly correlated with the NAHD-ferricyanide reductase activity (compare with Table I), except for the dog membranes which showed high flavin contents and low NADH-ferricyanide reductase activity. The type of flavin nucleotide present in the membranes was further investigated in the human cells. Essentially all the flavin extracted from these membranes was identified as FAD on the basis of the increase of fluorescence after acidic hydrolysis [17]. Digestion of the membrane with trypsin [18] did not increase the fluorescence, suggesting that the human erythrocyte membranes do not contain covalently-bound flavins.

Ca^{2+}-dependent K^+ transport

This transport system is silent in the red cell under usual conditions and it can be activated by extreme metabolic depletion with iodoacetate and adenosine (Gárdos effect [14,21]). Other circumstances that are known to activate this transport system include: (i) The increase of cell Ca^{2+} induced by the ionophore A23187 in the presence of external Ca^{2+} [22,23]. (ii) The treatment with the electron donor system sodium ascorbate + phenazine methosulphate [1,4]. (iii) The treatment with propanolol [24]. In all the cases the activation is facilitated if the cell level of ATP has been lowered.

TABLE II

FLAVIN CONTENTS OF ERYTHROCYTE MEMBRANES

These values correspond to not covalently bound (acid-extractable) total (after acidic hydrolysis) flavin and are given as mol per 10^9 g of protein.

Species	*n*	Mean ± S.E.
Human	6	12.4 ± 1.2
Guinea pig	5	6.3 ± 2.3
Rabbit	3	9.4 ± 1.2
Sheep	3	5.6 ± 0.8
Cat	8	24.6 ± 4.1
Dog	5	15.0 ± 4.0

Table III shows the results of a series of experiments where the effects of ascorbate + phenazine methosulphate, A23187 + Ca^{2+} and propranolol on the uptake of ^{86}Rb by 'fresh' and 'depleted' erythrocytes were investigated. All the three treatments increased the uptake of ^{86}Rb in the 'high-K^+' (human, guinea pig, and rabbit) erythrocytes, the effect being stronger in depleted cells. These treatments produced also a net loss of K^+ (only the results with A23187 are shown in Table VI) which was not balanced by an equimolar uptake of Na^+. The uptake of Na^+ was much more apparent in the rabbit and guinea pig erythrocytes than in the human red cell. This observation suggests that the ratio of the Cl^- permeability to the Na^+ permeability is larger in the human than in the other two 'high-K^+' erythrocytes studied.

The behavior of the 'low-K^+' erythrocytes of different species was not uniform. In the dog erythrocytes A23187 stimulated the uptake of ^{86}Rb both in 'fresh' and 'depleted' cells and ascorbate + phenazine methosulphate did so only in depleted cells (Table III). As a matter of fact, the uptake was already increased more than 10-times in the depleted dog cells, and that could be attributed to a partial activation of the Ca^{2+}-dependent K^+ transport during the depletion treatment (Fig. 1, see below). In the sheep erythrocytes none of the three treatments used stimulated significantly the uptake of ^{86}Rb. In these cells, the depletion of ATP increased the uptake of ^{86}Rb in the controls (Table III), but this increase was not Ca^{2+}-dependent nor it was found in the experiments performed in high K^+ medium, to be described below. In the cat erythrocytes A23187 increased the uptake of ^{86}Rb in both 'fresh' and 'depleted' cells while the other two treatments did

TABLE III

EFFECTS OF ASCORBATE + PHENAZINE METHOSULPHATE (Asc-PMS), A23187 AND PROPRANOLOL ON THE UPTAKE OF ^{86}Rb BY 'FRESH' AND 'DEPLETED' ERYTHROCYTES

The uptake of ^{86}Rb was studied during 5 min at 37°C, and is expressed as the operational rate constant k (see Methods) ($\times 10^3$). The concentrations of the added substances were: Asc-PMS: 20 mM sodium ascorbate (substituting for an isosmotic amount of NaCl) + 0.1 mM phenazine methosulfate; A23187: 1 μM; propranolol: 0.5 mM. Each value is the mean ± S.E. of at least three experiments. 'Depleted' cells were first incubated with iodoacetate and adenosine as described in Methods during the following periods (min): Cat, 30; human and rabbit, 45; guinea pig, sheep and dog, 60. Depletion treatment lowered ATP to less than 0.02 mmol/kg of cells without significant modifications of the cell Na^+ and K^+ contents.

Type of cells	Species	Control	+ Asc-PMS	+ A23187	+ Propranolol
'Fresh'	Human	16.9 ± 0.7	92 ± 26	1173 ± 220	
	Guinea pig	28.9 ± 0.9	655 ± 18	1420 ± 137	
	Rabbit	33.4 ± 1.0	32.9 ± 1.0	1015 ± 145	
	Sheep	3.7 ± 0.4	4.9 ± 0.4	3.7 ± 0.4	
	Cat	8.5 ± 0.9	9.5 ± 1.4	12.6 ± 0.4	
	Dog	7.9 ± 1.4	7.9 ± 1.4	65 ± 14	
'Depleted'*					
	Human	8.8 ± 0.4	1613 ± 293	2750 ± 147	2457 ± 330
	Guinea pig	19.3 ± 1.8	412 ± 46	1145 ± 92	321 ± 5
	Rabbit	41.6 ± 8.7	435 ± 145	1450 ± 145	870 ± 29
	Sheep	12.0 ± 1.1	15.0 ± 1.1	10.9 ± 0.7	4.9 ± 0.4
	Cat	4.9 ± 0.4	5.8 ± 0.4	16.2 ± 1.8	3.1 ± 0.4
	Dog	103 ± 5	163 ± 14	163 ± 19	75 ± 5

not have any effect. None of the treatments produced significant net fluxes of K^+, but it should be kept in mind that the cell K^+ concentrations were almost the same as the medium K^+ concentrations in these 'low-K^+' erythrocytes. It should be mentioned however that the treatment with ascorbate + phenazine methosulphate produced a net loss of Na^+ (9–12 mequiv./kg cells per 5 min) in both 'fresh' and 'depleted' cat erythrocytes (not shown).

TABLE IV

EFFECTS OF QUININE ON THE FLUXES INDUCED BY A23187

In the experiments type A the cells were incubated in medium containing 6 mM K (as in Table III) during 15 min. In the experiments type B the cells were incubated in medium containing 100 mM K^+ (as in Figs. 1 and 2) during 60 min. The fluxes are calculated as the difference between the levels at the begining and at the end of the experiment and are expressed as mequiv./kg of initial cells. Each datum is mean ± S.E. of three experiments. The concentrations of quinine and A23187 were 1 mM and 2 μM, respectively.

Expt. type	Species	Uptake of K^+			Uptake of Na^+		
		Control	+ A23187	+ A23187 + Quinine	Control	+ A23187	+ A23187 + Quinine
A	Human	–	−48 ± 1	−19 ± 3	–	5 ± 1	5 ± 1
	Guinea pig	–	−72 ± 2	−48 ± 2	–	29 ± 2	8 ± 2
	Rabbit	–	−69 ± 1	−44 ± 2	–	17 ± 1	9 ± 1
B	Cat	5.4 ± 0.2	22.5 ± 1.0	5.1 ± 2	3 ± 2	5 ± 5	−3 ± 2
	Dog[a]	3.1 ± 0.7	46.8 ± 1.3	7.3 ± 0.5	4 ± 7	0 ± 5	−21 ± 5

[a] With A23187 and no Ca^{2+} (1 mM EGTA present) the uptakes of K^+ and Na^+ were, respectively, 4.4 ± 0.5 and 2 ± 4.

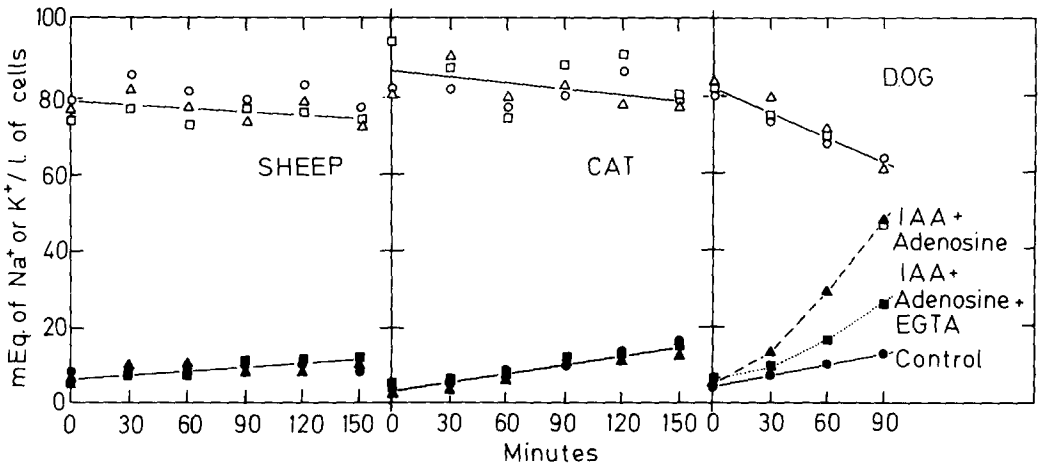

Fig. 1. Time course of the changes of the Na^+ and K^+ levels in cells incubated at 37°C in '$high\text{-}K^+$' medium (for composition see Methods). The additions to the medium were: Circles, 0.5 mM $CaCl_2$; triangles, 0.5 mM $CaCl_2$, 2 mM sodium iodoacetate and 10 mM adenosine; squares, 2 mM sodium EGTA, 2 mM sodium iodoacetate and 10 mM adenosine. Open symbols represent Na^+ levels and closed symbols represent K^+ levels. Each datum is the mean of at least two values obtained in different experiments. Variation range of individual values was under 10% for K^+ and under 25% for Na^+. In the dog erythrocyte frank (more than 15%) hemolysis was produced with incubation periods larger than 90 min.

In order to investigate the net fluxes of K^+ in the '$low\text{-}K^+$' erythrocytes, they were incubated in high-K^+ medium. This experimental design has been used previously to show the existence of Ca^{2+}-dependent K^+ transport in the dog red cell [25]. In the experiments presented here the activation of Ca^{2+}-dependent K^+ transport was attempted by either metabolic depletion with iodoacetate and adenosine (Fig. 1) or treatment with A23187 in the presence of external Ca^{2+} (Fig. 2).

Metabolic depletion produced net uptake of K^+ in the dog erythrocyte. The effect was already observed after 30 min of incubation and was partly

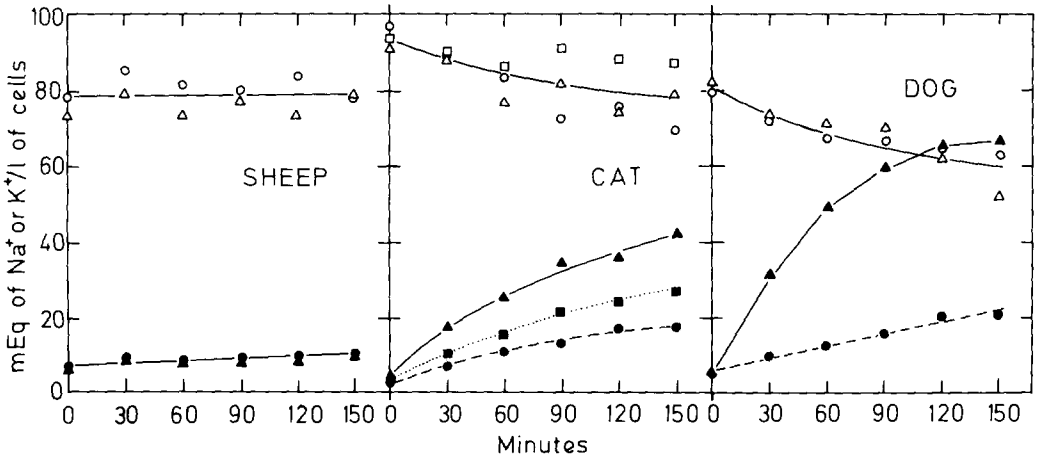

Fig. 2. Effects of A23187 on the levels of Na^+ and K^+ of '$low\text{-}K^+$' erythrocytes incubated in '$high\text{-}K^+$' medium. The additions to the medium were: Circles, 0.5 mM $CaCl_2$; triangles: 0.5 mM $CaCl_2$ and 2 μM A23187; squares: 2 mM sodium EGTA and 2 μM A23187. Other details as in Fig. 1.

prevented by the removal of Ca^{2+} from the medium. The fluxes of Na^+ were not modified. This treatment did not produce measurable effects in the cat and sheep cells (Fig. 1).

The treatment with A23187 produced net uptake of K^+ without apparent modification of the Na^+ fluxes in the dog and cat cells, and this effect was partly prevented by the removal of Ca^{2+} (Fig. 2 and Table IV). No effect was observed in the sheep erythrocyte.

Quinine has been found to inhibit the Ca^{2+}-dependent K^+ transport in the human erythrocyte and other animal cells [1,2,26]. Table IV shows the effect of quinine on the net fluxes of Na^+ and K^+ induced by A23187 in human, guinea pig, rabbit, cat and dog red cells. In all the cases quinine inhibited the K^+ fluxes and also the associated Na^+ fluxes. An unrelated and striking fact shown in Table IV is that quinine promoted a net loss of Na^+ in the dog erythrocyte. The result was observed only in the presence of A23187, quinine alone being without effect.

Discussion

Our results do not show correlation between NADH dehydrogenase activity and Ca^{2+}-dependent K^+ transport in the erythrocytes of the six species studied (Table V). All the species except sheep showed Ca^{2+}-dependent K^+ fluxes while cytochrome *c* reductase activity was lacking in sheep and guinea pig membranes and very low in dog. On the other hand, NADH-ferricyanide reductase was detected in all the species including sheep. These results do not support previous proposals that Ca^{2+}-dependent K^+ transport and plasma membrane NADH dehydrogenase activity were functionally related [1,4]. It has been also suggested that $(Na^+ + K^+)$-ATPase could be related to NADH dehydrogenase activity [27,28] or to Ca^{2+}-dependent K^+ transport [29,30]. We do not find correlation with none of them (Table V).

The two NADH dehydrogenase activities did not run parallel in the species studied suggesting that there could be differences on the electron carriers involved. Flavins were detected in all the six species and could well be engaged in the transfer of electrons from NADH to ferricyanide, as it is the case for other well-know similar activities. Cytochrome *b*, identified in red cell [33] as well as in other plasma membranes (33,34] would be a good candidate for the transfer of electrons to cytochrome *c*.

The existence of Ca^{2+}-dependent K^+ transport had been previously shown in human, guinea pig, rabbit [8] and dog [25] erythrocytes. Our results show that A23187 elicited Ca^{2+}-dependent K^+ fluxes also in the cat erythrocyte, and that these fluxes were sensitive to quinine, a known inhibitor of Ca^{2+}-dependent K^+ transport in the human

TABLE V

SUMMARY OF THE PRESENCE (+) OR ABSENCE (0) OF NADH DEHYDROGENASE, Na^+/K^+-PUMP OR Ca^{2+}-DEPENDENT K^+ TRANSPORT ACTIVITES IN ERYTHROCYTES OF DIFFERENT ANIMAL SPECIES

Species	NADH dehydrogenase; acceptor:		Ca^{2+}-dependent K^+ transport	Na^+/K^+ pump [a]
	Ferricyanide	Cytochrome *c*		
Human	++	+	+	+
Guinea pig	+	0	+	+
Rabbit	+	+	+	+
Sheep	+	0	0	0
Cat	+++	++	+	0
Dog	±	±	+	0

[a] This colum was elaborated on the basis of the existing data on $(Na^+ + K^+)$-ATPase activity [31,32], confirmed in this study. The cytoplasmic levels of Na^+ and K^+ and the ouabain-sensitive ^{86}Rb influx were also measured and found consistent with $(Na^+ + K^+)$-ATPase activities. Erytrocytes from 1-year-old Spanish Merino sheeps were used; the $(Na^+ + K^+)$-ATPase activity in these cells was not significantly different with and without 1 mM ouabain and their K^+ concentration was 11.1 ± 0.5 mequiv./l intracellular water.

erythrocyte and other cells systems [1,2,26]. Ascorbate + phenazine methosulphate and propranolol, which promoted Ca^{2+}-dependent K^+ transport in the human erythrocyte and other animals cells [1,2,24] failed, however, to do so in the cat red cell. The same can be said of extreme metabolic depletion with iodoacetate and adenosine (Fig. 1). These results suggest that: (i) ascorbate + phenazine methosulphate and propranolol do not act simply by increasing the cell calcium concentration; additional evidence for this view has been presented elsewhere [1]; and (ii) Ca^{2+}-dependent K^+ transport of the cat erythrocyte can show minor differences with that of other cells. Ca^{2+}-dependent K^+ fluxes in the dog erythrocytes differed also from those found in the 'high-K^+' species in not being activated by propranolol under the circumstances it was tested.

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