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A CHLORIDE DEPENDENT K^+ FLUX INDUCED BY N-ETHYLMALEIMIDE IN GENETICALLY LOW K^+ SHEEP AND GOAT ERYTHROCYTES*

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Summary

In genetically low K^+ but not in high K^+ red cells of sheep and goat N-ethylmaleimide induced a ouabain insensitive K^+ flux as measured by tracer influx or net efflux methods. The augmented K^+ flux was observed in Cl^- or Br^- but not in NO_3^-, SO_4^{2-} or PO_4^{2-} media. The action of N-ethylmaleimide was distinct from that of parachloromercuribenzoate or its sulfonic acid derivative which increased both passive K^+ and Na^+ movements across the red cell membrane. The instantaneous selective action of N-ethylmaleimide suggests that sulfhydryl groups control a K^+/Cl^- transport system which, associated with the low K^+ gene, is apparently functionally silent in adult ruminant red cells.

Ruminants such as sheep, goats and cattle have red cells with either high K^+ (HK) or low K^+ (LK) content (1, 2). The LK property is controlled by a dominant gene (3). The different cation steady state compositions are maintained by kinetically (4, 5) and quantitatively (6, 7) different Na^+K^+ pumps, and by ouabain insensitive Na^+K^+ leak fluxes of distinct magnitudes. In LK sheep red cells the passive K^+ permeability is greater than in HK cells, while the passive Na^+ permeability behaves inversely proportional (8). As there has been much effort devoted to characterizing the biophysical parameters of the Na^+K^+ pumps in these cells, very little is known about the properties of the Na^+K^+ leak pathways. In human red cells amino- and sulfhydryl-(SH) groups have been shown to be functionally involved in passive transfer of monovalent cations (9, 10), and organomercurials are known to indiscriminatorily increase passive permeabilities to both Na^+ and K^+ ion in these cells (11) as well as in sheep (4) and goat red cells (12).

Although N-ethylmaleimide (NEM) is widely used in studies of membrane SH groups and has been shown to affect the Na^+K^+ ATPase (13) and the Na^+/Na^+ or

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Na^+/Li^+ counter transport in sheep red cells (14, 15) without altering the Na^+ ground permeability (14), no studies on the action of NEM on the passive K^+ permeability in these cells have been reported. Here we show that NEM selectively increases an apparently Cl^- ion dependent K^+ flux in LK but not HK red cells of adult sheep and goat suggesting that NEM reactive membrane SH-groups reside in a funcationally silent K^+/Cl^- transport system which is under the control of the LK gene in these cells.

Material and Methods

Blood was obtained from Dorset sheep and Nubian goats whose red cell K^+ and Na^+ ion concentrations had been determined previously by atomic absorption spectrophotometry (Perkin Elmer, Model 460). Chemicals were of analytic grade. NEM and the organomercurials parachloromercuribenzoate (PCMB) and its sulfonic acid derivative (PCMBS) were purchased from Sigma Chemical Co., St. Louis, Mo. and dissolved in the experimental solutions immediately prior to the start of the experiment.

Efflux Experiments: Prior to each experiment, cells were washed in 280 mOsm Tris/Cl buffered (pH 7.4) choline-Cl containing 10^{-4}M ouabain to exclude the contributions by the Na^+K^+ pump. When Cl^- ions were replaced by Br^-, NO_3^-, SO_4^{2-} or PO_4^{2-} ions their Na^+ salts were used and the pH adjusted to 7.4 with Tris base titrated with the respective acid. At zero time packed cells were injected into incubation flasks with either of the above media ± 1 mM NEM, 0.2 mM PCMB or PCMBS, to give a final suspension hematocrit of about 5% (v/v). Incubation was carried out at 37°C in a shaker bath and samples were withdrawn at various time intervals, centrifuged for 1 min in a Sorval RC5B centrifuge, and the cell free supernatants were analyzed for hemoglobin (OD_s^{527}) and monovalent cation concentrations, $[Cat]_s$. The $[Cat]_s$ values were corrected for cation release due to spontaneous hemolysis (less than 1% hr^{-1}) using the ratio of OD_s^{527} to OD_{pc}^{527}, the theoretical optical density at 527 nm of 1 ml hemolyzed packed red cells. An aliquot of each suspension was hemolyzed with a detergent to provide the equilibrium concentration of hemoglobin, (OD_e^{527}), and the suspension hematocrit (OD_e^{527}/OD_{pc}^{527}). The equilibrium cation concentration, $[Cat]_e^{t=\infty}$, was measured in the same sample. For very low hematocrits $[Cat]_e^{t=\infty}$ approximates $[Cat]_o^{t=\infty}$, the cation concentration in the extracellular medium. Hence for K^+ and Na^+ release the ratios of $[K]_o^t/[K]_o^{t=\infty}$ and $[Na]_o^t/[Na]_o^{t=\infty}$ were calculated from which the efflux rate constants (corrected for the time interval required for removal of supernatants) were computed using the first order rate equation

$$-\ln\left(1 - \frac{[Cat]_o^t}{[Cat]_o^{t=\infty}}\right) = {}^{o}k_{cat} \cdot t.$$

Influx Experiments: K^+ influx was measured with $^{42}[K]$ as tracer (Burlington Research Facilities, Raleigh, N.C.) in Tris/Cl buffered NaCl media (280 mOsm, pH 7.4 at 37°C containing 5 mM K^+/Cl^- and 10^{-4}M ouabain ± 1 mM NEM. Experimental details of this technique have been published elsewhere (6, 7, 16). Influxes were computed in mmoles K^+/L. cells x hr and converted into influx rate coefficients by dividing the ouabain insensitive K^+ influx by the intracellular K^+ concentration.

Table 1

EFFECT OF NEM ON OUABAIN INSENSITIVE ^{42}K INFLUX RATE COEFFICIENTS IN LK AND HK SHEEP RED CELLS

Cells (n=3)	$[K]_c$ mM/L. Cells	$^{i}k_K$ (Hr^{-1}) Control	$^{i}k_K$ (Hr^{-1}) NEM (1 mM)	Ratio: $\frac{NEM}{Control}$
LK(LL)	16.5 ± 1.0	0.052 ± 0.008	0.254 ± 0.066	4.88
HK(MM)	80.9 ± 6.8	0.028 ± 0.005	0.035 ± 0.006	1.25

Values ± SEM

Results and Discussion

We first noticed the effect of 1 mM NEM in the tracer analysis of the K^+ influx rate coefficients, $^{i}k_K$, in 3 LK and 3 HK sheep red cell specimens. Table 1 shows that NEM increased $^{i}k_K$ almost 5 fold in LK cells while there was no statistically significant effect on $^{i}k_K$ in HK cells. This finding was corroborated by K^+ net efflux measurements. Figure 1 shows that 2 mM NEM increased the K^+ efflux rate constant, $^{o}k_K$, in LK cells by 8 fold above the control $^{o}k_K$ which is similar to control $^{i}k_K$. The larger NEM effect shown in Figure 1 may be due to differences between sheep as well as to the freshness

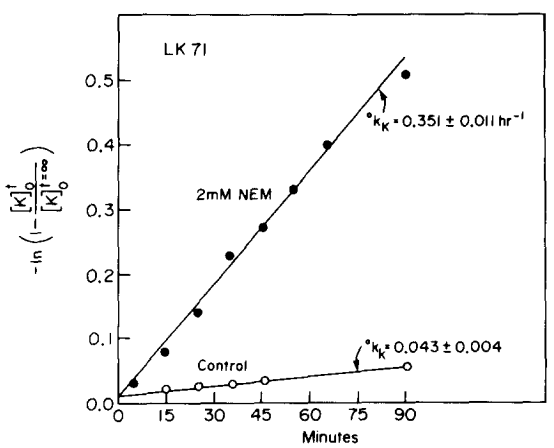

Figure 1: Effect of 2 mM N-ethylmaleimide on K^+ efflux rate constants. LK sheep red cells were washed in isotonic choline chloride medium, Tris/Cl-buffered to pH 7.4 and containing 10^{-4} M ouabain. At zero time packed cells were injected into the same medium ± 2 mM NEM to give a final suspension hematocrit of about 5% (v/v). Samples were withdrawn after given time intervals of incubation at 37°C and analyzed for released K^+ ions. Calculation of data points and of the $^{o}k_K$ values as given in Methods.

Table 2

EFFECT OF 1mM NEM ON K^+ AND Na^+ EFFLUXES IN RED CELLS FROM 8 GOATS

Cells (n)	$[K]_c$ mM/L.	$[Na]_c$ Cells	$^{o}k_K(Hr^{-1})$ Control	NEM	Ratio: NEM/Control	$^{o}k_{Na}(Hr^{-1})$ Control	NEM	Ratio: NEM/Control
HK (5)	75.7 ± 4.8	13.5 ± 1.4	0.022 ±0.003	0.033 ±0.008	1.50	0.145 ±0.024	0.159 ±0.031	1.10
LK (3)	28.5 ± 1.4	65.9 ± 1.7	0.064 ±0.009	0.176 ±0.027	2.75	0.052 ±0.006	0.054 ±0.004	1.04

Values ± SEM

of the NEM lot used and not to the different NEM concentrations used since saturation effects were achieved at 1 mM NEM. In comparison, the $^{o}k_K$ values (± SEM) for 3 HK red cells in absence and presence of 1 mM NEM were 0.036 ± 0.006 and 0.036 ± 0.005, respectively. As expected (14), there was no significant effect on $^{o}k_{Na}$ in LK nor in HK sheep red cells (data not shown).

Table 2 reveals that 1 mM NEM also increased selectively the K^+ permeability in 3 LK goat red cell specimens while there was no significant alteration of K^+ efflux in HK red cells from 5 goats. Also, in both LK and HK goat red cells no significant change in $^{o}k_{Na}$ occurred in presence of NEM. The data shown support the hypothesis that NEM reacts with some membrane SH groups which appear to be genetically and functionally associated with part of the K^+ permeability in LK sheep and goat red cells.

The putative SH groups reacting with NEM may be buried within the membrane and different from those SH groups binding organomercurials. PCMBS did not prevent the action of NEM (data not shown), and as shown in Table 3, both PCMBS and PCMB increased K^+ and Na^+ effluxes indiscriminately as opposed to NEM which affected $^{o}k_K$ only. The dual effect of the two organomercurials is not surprising in light of their known usefulness for cation exchange experi-

Table 3

EFFECT OF NEM AND ORGANOMERCURIALS ON K^+ AND Na^+ EFFLUXES IN LK SHEEP RED CELLS

Treatment	$^{o}k_K(Hr^{-1})$	Ratio	$^{o}k_{Na}(Hr^{-1})$	Ratio
Choline Cl	0.058 ± 0.006	--	0.037 ± 0.005	--
1 mM NEM	0.227 ± 0.031	3.91	0.042 ± 0.005	1.14
0.2 mM PCMB	0.121 ± 0.016	2.09	0.086 ± 0.009	2.32
0.2 mM PCMBS	0.156 ± 0.026	2.70	0.174 ± 0.014	4.70

(n=4) ± SEM

Table 4

EFFECT OF NEM ON OUABAIN INSENSITIVE K^+ EFFLUX OF LK SHEEP RED CELLS IN Cl^- SUBSTITUTED Na^+ MEDIA

	$^{o}k_K$ (Hr^{-1})		Ratio:
Anion	Control	NEM	NEM/Control
† Cl^-	0.073 ± 0.012	0.270 ± 0.047	3.70
* Br^-	0.073 ± 0.007	0.303 ± 0.072	4.15
† NO_3^-	0.061 ± 0.020	0.068 ± 0.017	1.11
* SO_4^{2-}	0.081 ± 0.010	0.100 ± 0.014	1.23
* PO_4^{2-}	0.067 ± 0.019	0.083 ± 0.013	1.24

n ± SEM

† n=6, * n=4, ± SEM

ments (4, 11, 12). Kinetically, their proposed mode of attack is first on SH groups at the surface and later within the membrane (9).

Since the passive permeability of Cl^- ions in red cells is much greater than that of K^+ ions (17), we were surprised to find that the NEM augmented K^+ efflux required the presence of external Cl^- (or Br^-) ions. Table 4 shows that the NEM effect on $^{o}k_K$ was not observed when NO_3^-, SO_4^{2-} and PO_4^{2-} ions substituted for Cl^- or Br^- ions in the incubation media. This anion substitution did not affect the ouabain insensitive ground permeability for K^+ ions as

shown in the controls of the table. The NEM induced Cl^- activated K^+ efflux is independent of the fast Cl^-/Cl^- self exchange system (17) since 0.1 mM 4-acetamido-4'-isothiocyano-stilbene-2, 2'disulfonic acid (SITS) did not reduce the augmented K^+ efflux. Furosemide known to inhibit Na^+/K^+ cotransport in human (18) and avian red cells (19) reduced the NEM effected K^+ efflux by about 25-30% (data not shown). A cotransport for K^+ and Cl^- ions has been shown to be present in duck red cells (20) and also has been proposed for Ehrlich Ascites tumor cells (21) and human red cells (22). Although detailed studies are required concerning the linkage and stoichiometry of transport of K^+ and Cl^- ions in these cells, our findings suggest the vestigeal presence of a K^+/Cl^- transport system in sheep and goat red cells which is under the control of the LK gene. It is possible that NEM forms an adduct with an SH group in the putatively Cl^- dependent K^+ transport system which may also be operative in osmotically swollen LK sheep red cells (23).

The presence of a K^+/Cl^- transport system functionally silent in adult sheep and goat red cells and its association with the LK gene raises the question of its possible role. The precursor cell of the adult LK cells are HK type reticulocytes which have a tenfold higher K^+ leak flux as they enter peripheral circulation (24). Perhaps in the adult LK cells NEM reacts with SH groups which are functionally absent in their immature precursor cells. One is tempted to speculate that the K^+/Cl^- transport system reported here, perhaps evolutionary old, may play an important role as the reticulocyte precursor cell down-regulates its K^+ steady state concentration to that of an adult LK cell.

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