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Thiol-Dependent K : Cl Transport in Sheep Red Cells: VIII. Activation through Metabolically and Chemically Reversible Oxidation by Diamide

P.K. Lauf
Department of Physiology and Biophysics, Wright State University, School of Medicine, Dayton, Ohio 45401-0927

Summary. The sulfhydryl (SH) oxidant diamide activated in a concentration-dependent manner ouabain-resistant (OR), Cl-dependent K flux in both low potassium (LK) and high potassium (HK) sheep red cells as determined from the rate of zero-*trans* K efflux into media with Cl or Cl replaced by NO_3 or methane sulfonate (CH_3SO_3). Diamide did not alter the OR Na efflux into choline Cl. The diamide effect on K efflux appeared after 80% of cellular glutathione (GSH) was oxidized to GSSG, its disulfide. The stimulation of K efflux was completely reversed during metabolic restitution of GSH, a process that depended on the length of exposure to and the concentration of diamide. The action of diamide on both the K : Cl transporter and GSH was also fully reversed by the reducing agent dithiothreitol (DTT). Diamide apparently oxidized the same SH groups alkylated by N-ethylmaleimide (NEM) (Lauf, P.K. 1983. *J. Membrane Biol.* 73:237–246). Like NEM, diamide activated K : Cl transport several-fold more in LK cells than in HK cells, and the effect on LK cells was partially inhibited by anti-L_l, the allo-antibody known to inhibit OR K fluxes.

Key Words: sheep red cells · ouabain-resistant K fluxes · sulfhydryl groups · diamide · glutathione · oxidation

Introduction

Monofunctional sulfhydryl (SH) group reagents like N-ethylmaleimide (NEM), iodoacetamide (IAAM) and methane methyl thiosulfonate (MMTS) stimulate irreversibly OR Cl-dependent K fluxes (K : Cl transport or flux) in sheep red cells, particularly in low K (LK) cells [22, 31, 32], in human red cells [18, 29, 30, 39] and in other, nucleated cells [21, 36]. The same transport pathway is also activated by cell swelling in human red cells [17, 39], and in addition by removal of intracellular bivalent cations through the ionophore A23187 in sheep red cells [26]. Although in the ovine system anti-L_l, the allo-antiserum prepared in HK sheep against LK red cells [24, 32], partially inhibits OR K : Cl flux stimulated by either of the three major interventions (chemical, swelling and A23187), the identities of the K : Cl fluxes as part of one and the same transport pathway need to be further established to support the unifying functional model proposed by us [27]. One way to relate, for example, the flux mode activated by irreversible mono-functional SH reagents to that responding to volume changes or A23187 treatment would be the use of a reversible SH group reagent.

This paper presents, for the first time, a chemical approach to stimulate reversibly K : Cl fluxes in sheep red cells. The GSH oxidant diamide, introduced 20 years ago by Kosower's group [19], was found to stimulate OR K : Cl fluxes, without altering the Na permeability. This effect was metabolically and chemically fully reversible. The actions of diamide and NEM were mutually exclusive, suggesting that diamide oxidized the same chemical groups which form an adduct with NEM [22, 31], are carboxymethylated by IAAM [1, 28], and oxidized by MMTS [25]. The diamide effect was largest in LK red cells, and anti-L_l partially inhibited the stimulated K : Cl flux.

Materials and Methods

Blood was drawn into heparinized tubes by venipuncture of LK and HK sheep of mainly mixed Western type maintained by the Laboratory Animal Resources at Wright State University, Dayton, Ohio. Unless specified otherwise, red cells were generally washed in ice-cold 300 mOsM unbuffered NaCl or choline-Cl prior to chemical treatments or changes in medium anions. The composition of the isosmotic 295 mOsM flux media were (mM): 158 NaCl or Na-methane sulfonate (CH_3SO_3Na) or $NaNO_3$, buffered with 5 Na phosphate to pH 7.4 plus 1 ouabain. For Na efflux isosmotic (295 mOsM) media with 150 mM choline-Cl and 10 mM Tris/Mops [(hydroxymethyl)-amino-methane/2-(N-morpholine)-propanesulfonic acid] were used instead. The composition of the isosmotic reconstitution medium (rejuvenation cocktail) was (mM): 153 NaCl or CH_3SO_3Na or $NaNO_3$, 10 glucose, 5 $NaPO_4$, pH 7.4. All final osmolarities were adjusted/verified with an Advanced Digimatic Osmometer (Model 3D II, Advanced Instruments, Needham Heights, MA).

Treatment with diamide (diazenedicarboxylic acid bis

Table 1. Cellular cation and glutathione concentrations of HK and LK sheep red blood cells

Sheep (antigen)	*n* (sheep)	Cell cations (μmol/ml cells) ± SE		Glutathione (μmol/ml cells) ± SE
		[K]	[Na]	
HK				
(MM)	3	85.5 ± 1.8	16.0 ± 0.3	3.49 ± 0.09
LK				
(6 LL, 1 LM)	7	18.1 ± 2.3	85.6 ± 2.2	3.25 ± 0.10

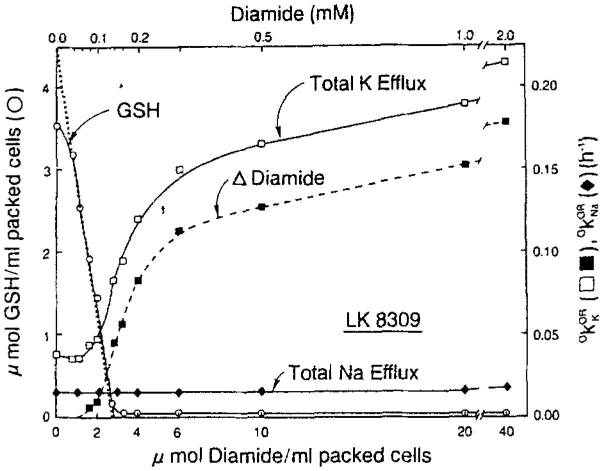

Fig. 1. The effect of increasing diamide concentrations on cellular GSH levels and zero-*trans* K and Na effluxes in the presence of ouabain in LK sheep red cells. *Left hand ordinate:* GSH concentrations in μmol/ml packed cells (open circles). The dotted line intercepts at 4.5 μmol GSH/ml packed cells and at 2.4 μmol diamide/ml packed cells. Hence 1.9 moles GSH mol/diamide were oxidized. *Right hand ordinate:* Rate constants (hr^{-1}) of ouabain-resistant (OR) K (open squares) or Na effluxes into K free or Na free (zero-*trans*) flux media. The interrupted line connecting the filled squares represents the difference between total K efflux in the presence and absence of diamide. The diamide concentrations are given for solution (mM, upper abscissa) or in μmol/ml packed cells (lower abscissa). Sheep LK8309LL

[N,N-dimethylamide] II, ref. 19, Sigma Chemicals, St. Louis, MO) was carried out at 37°C usually by adding the chemical to yield the desired concentration to the flux media prior to addition of cells. The hematocrit was always 5% (vol/vol). The diamide concentrations are given for solution (mM) or in μmol per ml packed cells. The reaction with diamide was stopped by washing the cells in ice-cold diamide-free flux media of desired anion composition. Reversal of the diamide action was achieved by incubating the cells in the rejuvenation cocktail for at least one, usually two hours with CH_3SO_3 as Cl replacement to prevent leakage of K through the K : Cl pathway. For the chemical restitution experiments cells were incubated with glucose-free, CH_3SO_3Na flux medium containing dithiothreitol (DTT, Sigma Chemical Co., St. Louis, MO) at the desired concentrations. Treatment with N-ethylmaleimide (NEM, Sigma Chemical Co., St. Louis, MO) followed published procedures [22] with a hematocrit of 5% (vol/vol) instead.

Table 2. Cellular GSH concentrations of LK sheep red cells as function of diamide concentration and time of incubation

Diamide concentration		Cellular GSH (μmol/ml packed cells)			
mM (solution)	μmol/ml cells	2 min	20 min	40 min	60 min
0	0	2.76	2.62	2.64	2.69
0.05	1.0	1.88	1.89	1.92	1.98
0.10	2.0	0.50	0.43	0.49	0.58
0.15	3.0	0.16	0.064	0.060	0.067
0.20	4.0	0.11	0.086	0.062	0.062

Ouabain-resistant K transport was assessed mainly by the zero-*trans* K efflux method described earlier [22] where the rate constants, $^{o}k_{K}^{OR}$, were calculated by regression analysis of K released into zero-*trans* flux media at five time points with 10-min intervals. The same was done for zero-*trans* Na efflux into choline Cl media. The anti-L$_1$ inhibition of zero-*trans* K efflux was studied as described earlier using a 3% final dilution of an antiserum kindly donated by Dr. Philip Dunham, University of Syracuse. Cellular GSH was measured photometrically by a standard procedure developed by Beutler [3] using 5,5′dithiobis-(2-nitrobenzoic acid). All GSH determinations were calculated per volume original packed cells. Cellular cations were determined with a Perkin Elmer 5000 Atomic absorption Spectrophotometer as described before [22].

Results

RELATION BETWEEN CELLULAR CATIONS AND GLUTATHIONE

Since the present study is concerned with the rapid oxidation of thiols, especially of GSH constituting more than 90% of the total cellular thiol pool, we determined first the cellular GSH concentrations in relation to the cation phenotype of these cells. Table 1 shows that the cellular GSH level was 3.33 μmol/ml for red cells from a total of 10 sheep, 3 being of HK, the majority, however, of LK type.

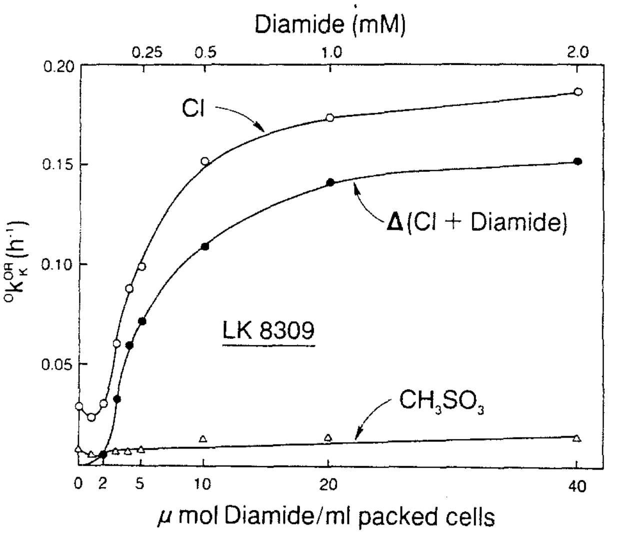

Fig. 2. Stimulation of Cl-dependent K efflux in LK sheep red cells by increasing diamide concentrations. *Ordinate:* Rate constant of zero-*trans* ouabain-resistant (OR) K efflux ($^{o}k_K^{OR}$, hr^{-1}) in Cl (open circles), methane sulfonate (CH$_3$SO$_3$, open triangles), and the Cl-specific K efflux (filled circles), which is the difference between the two former. The diamide concentrations are given either for solution (mM, upper abscissa) or in μmol/ml packed cells (lower abscissa). Sheep LK8309LL

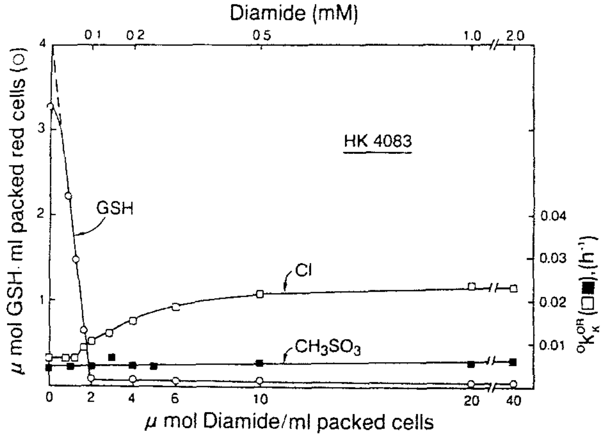

Fig. 3. Oxidation of cellular GSH and stimulation of Cl-dependent K efflux in HK sheep red cells by increasing diamide concentrations. *Left hand ordinate:* Cellular GSH levels in μmol/ml packed cells (open circles). *Upper abscissa:* mM diamide solution concentration; *lower abscissa:* μmol diamide/ml packed cells. The interrupted line intercepts at 4 μmol GSH/ml packed cells and at 2 μmol diamide/ml packed cells, yielding oxidation of 2 mol GSH/mol diamide. *Right-hand ordinate:* Rate constants of ouabain-resistant, zero-*trans* K efflux, $^{o}k_K^{OR}$ (hr^{-1}), into Cl (open squares) or methane-sulfonate (CH$_3$SO$_3$, filled squares). Sheep HK4083MM

Clearly, the mean GSH levels for all HK and LK cells tested did not significantly differ from the mean of the entire population and are in the range reported [11]. None of the individual sheep belonged to the low GSH genotype described [38]. The standard error of measurements per individual sheep varied between 3 and 8% over several months, indicating a reasonable stability of the cellular GSH levels over the time of observation.

EFFECT OF DIAMIDE ON CELLULAR GSH AND OUABAIN-RESISTANT CATION EFFLUXES

Figure 1 shows an experiment in which LK red cells were exposed to increasing diamide concentrations during the K efflux experiment. Cell GSH was determined immediately after taking the last sample point for K efflux, at less than 1 hr. As the diamide concentration was raised to 0.14 mM (upper abscissa), corresponding to 2.8 μmol/ml packed cells (lower abscissa), cell GSH became oxidized (open circles) and remained so for the entire flux period (*see* Table 2). The negative slope of 1.9 suggests the oxidation to the disulfide GSSG of 2 mol of GSH/mol diamide, in complete agreement with the first report on human red cells [19]. When about 60% of GSH was oxidized, a significant activation of OR zero-*trans* K efflux into Cl media occurred, which began to level off at about 0.3 mM diamide (open squares). Thus the diamide-stimulated K efflux component (Δ Diamide, filled squares), i.e., the difference between the total K efflux and the K efflux component in absence of diamide became significant when, at ca 2.2 μmol diamide/ml packed cells, some 80% of the cellular GSH was oxidized. In sharp contrast, there was no diamide effect on the rate constants of OR zero-*trans* Na efflux into choline Cl media (filled rhomboids), not even at 40 μmol/ml packed cells. Hence, diamide affected only OR K transport.

Figure 2 depicts an experiment similar to that of Fig. 1 on the same LK sheep red cells, however, suspended in Cl or in CH$_3$SO$_3$ media during the exposure to increasing concentrations of diamide. Note that in CH$_3$SO$_3$ (open triangles) diamide did not effect any significant change in the K efflux rate constants while in Cl (open circles) an activation of K efflux was seen at 3 μmol diamide/ml packed cells (the points at 1 and 2 μmol/ml packed cells were statistically not different from the 0 point) which was very similar to that of Fig. 1. After corrections for the basal K efflux rate constants in CH$_3$SO$_3$ and in Cl in the absence of diamide (0 point on the lower abscissa) the diamide-specific and Cl-dependent K efflux [Δ (*Cl + Diamide*)] assumed a sigmoidal shape with a plateau above 20 μmol diamide/ml packed cells (= 1 mM diamide), suggesting a complex reaction mechanism involving perhaps more than one SH group.

Figure 3 shows that diamide also stimulated albeit a tenfold smaller Cl-dependent K efflux in HK

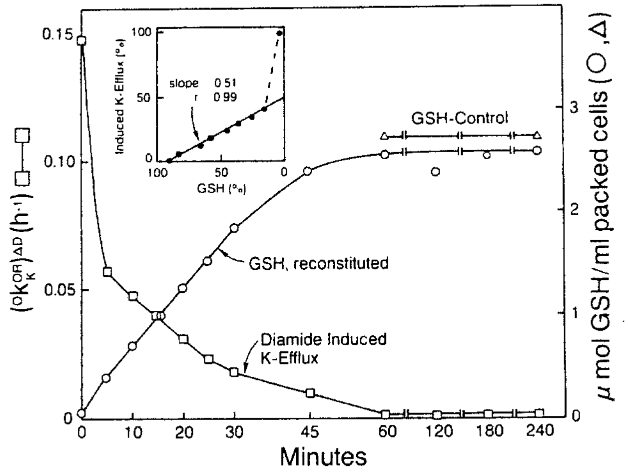

Fig. 4. Metabolic reconstitution of cellular GSH and reversal of K flux activation by diamide as function of time in LK sheep red cells. *Left-hand ordinate* (open squares): Rate constants for diamide-specific (ΔD), ouabain-resistant, zero *trans* K efflux, (${}^{o}k_K^{OR}$) ΔD. *Right-hand ordinate:* Cellular GSH levels [μmol/ml packed cells for controls (open triangles) and diamide-treated and reconstituted cells (open circles)]. After exposure to 2 mM diamide (40 μmol/ml packed cells) for 10 min, both treated cells and untreated controls were washed as described in Materials and Methods and kept for up to 4 hr (abscissa) in the rejuvenation cocktail. At intervals indicated by the symbols, samples were removed for determination of GSH and zero-*trans* K efflux. *Insert:* Diamide-induced K flux (%) versus % GSH (filled circles). Sheep LK6LL

cells. Note that like in LK cells K flux stimulation occurred at about 2 μmol diamide/ml packed cells, i.e., when about 90% of the cellular GSH was oxidized. No diamide effect was seen in CH_3SO_3.

METABOLIC RECONSTITUTION

It is known that the oxidation of GSH to GSSG by diamide can be reversed by substrate incubation of red cells [19] effecting the reduction of GSSG through the pentose-phosphate shunt. Although there is evidence for low pentose-phosphate shunt activity (T.J. McManus, *personal communication*), substrate incubation restores GSH also in sheep red cells. Figure 4 shows that cellular GSH, which was almost completely oxidized to GSSG after treatment of LK cells with 2 mM diamide for 10 min (open circle at zero time point on abscissa), returned to about 93% reduced levels after 1 hr incubation with our rejuvenation cocktail (*compare* with controls = open triangles). The metabolic reversal of the diamide-activated OR K efflux (open squares) was also complete by 1 hr. Interestingly, the relationship between the fractional reversal of the diamide-activated K flux and the reconstitution of GSH was linear for eight out of nine experimental points with a slope of about 0.5 (insert of Fig. 4).

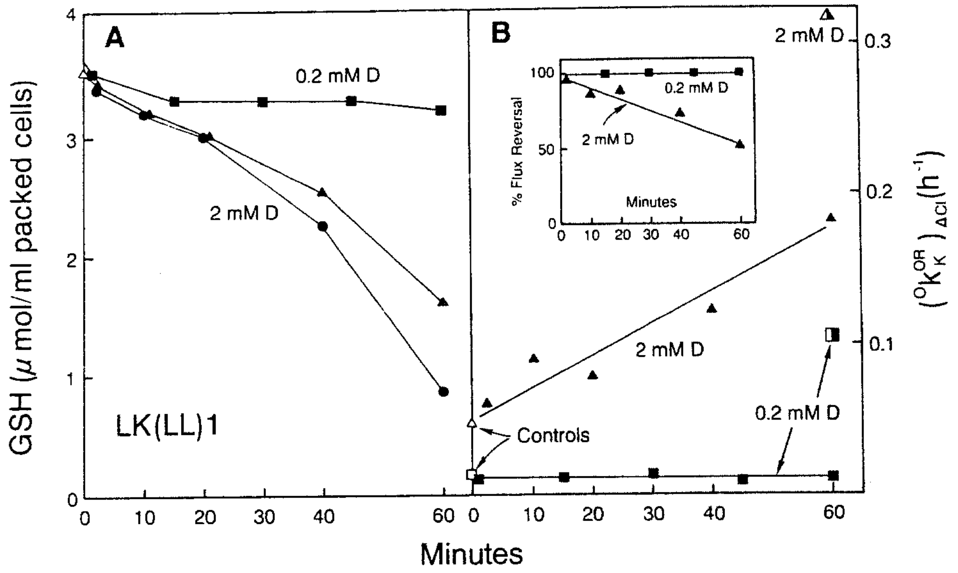

Fig. 5. Metabolic reconstitution of cellular GSH (*A*) and reversal of K flux activation (*B*) after treatment of LK sheep red cells with 0.2 and 2 mM diamide for various time periods. *Left-hand ordinate* and *A:* Cellular GSH levels in μmol/ml packed cells with filled squares for 0.2 mM diamide and filled circles and triangles (2 experiments) for 2 mM diamide. Reconstitution time, 2 hr. *Right-hand ordinate* and *B:* Rate constants of ouabain-resistant, Cl-dependent zero-*trans* K efflux, ${}^{o}k_K^{OR}$ (hr^{-1}). Samples treated with 0.2 mM (filled squares) and 2 mM diamide (filled triangles correspond to those in *A*). Open symbols denote K efflux rate constants for controls of the 2 experiments with diamide, and half-filled symbols at 60 min designate K efflux rate constants of cells activated by 0.2 and 2 mM diamide but not yet reconstituted (100% stimulation). The insert in *B* shows the percent flux reversal as function of exposure time in 0.2 and 2 mM diamide, respectively. Sheep LK1LL

The relationship between metabolically reconstituted GSH levels and reversed K flux is of complex nature. This is borne out in the experiments in which the exposure time to fixed concentrations of diamide (Fig. 5) or the concentrations of diamide were varied at constant exposure time (Fig. 6). In Fig. 5*A* it can be seen that the GSH levels of LK cells first exposed to 0.2 mM diamide up to 60 min (filled squares) were restored to more than 90% at all time points (*compare* with untreated control = open triangle). In contrast, exposure to 2 mM diamide for time periods longer than 10 min led to a decline of GSH restitution to less than 50% by 60 min even though a 2 hr metabolic repletion period was used (filled triangles and circles). Figure 5*B* shows that 0.2 and 2 mM diamide increased $({}^{o}k_{K}^{OR})_{\Delta Cl}$, i.e., the rate constants of ouabain-resistant and Cl-dependent K efflux, from 0.010 hr^{-1} (open square at $t = 0$) to 0.12 hr^{-1} (semifilled square at 60 min), i.e., tenfold, and from 0.06 hr^{-1} (open triangle at $t = 0$) to 0.32 hr^{-1} (semifilled triangle at 60 min), respectively. While OR K efflux was fully reconstituted in cells that had been exposed to 0.2 mM diamide (filled squares), the reversal, following 2 hr metabolic depletion, of OR K efflux activated by exposure to 2 mM diamide for 60 min (filled triangles), was only 50% effective (*see* insert in Fig. 7*B*).

For the experiment of Fig. 6 we chose exposure of LK red cells to diamide concentration ranging from 0.15 to 1.0 mM for 45 min when the difference in the metabolic restitution after incubation in 0.2 and 2 mM diamide was clearly significant (*cf.* Fig. 5). The treatment with diamide as well as the subsequent metabolic repletion were done in phosphate-buffered CH_3SO_3 media to assure maintenance of cellular K levels. Figure 6*A* shows that the oxidation of GSH was complete at all diamide concentrations used (open circles) while the restitution of cellular GSH was almost 100% up to 0.4 mM diamide, after which there was a decline to only 80% at 1.0 mM diamide. Figure 6*B* shows the rate constants of the diamide-specific, Cl-dependent K efflux only after oxidation (open circles) and reconstitution (filled circles). Note that K efflux stimulated by diamide concentrations exceeding those required for GSH oxidation (*cf.* Fig. 1) could not be fully reversed beyond 0.3 to 0.4 mM diamide with only 75% recovery at 1 mM diamide.

These studies permit the conclusion that the oxidation of both GSH to GSSG and of the SH groups of the Cl-dependent K transporter were metabolically fully reversible as long as at high diamide concentrations the exposure time did not exceed 10 min or at long incubation times the diamide concentrations were not greater than 6 μmol/ml packed cells.

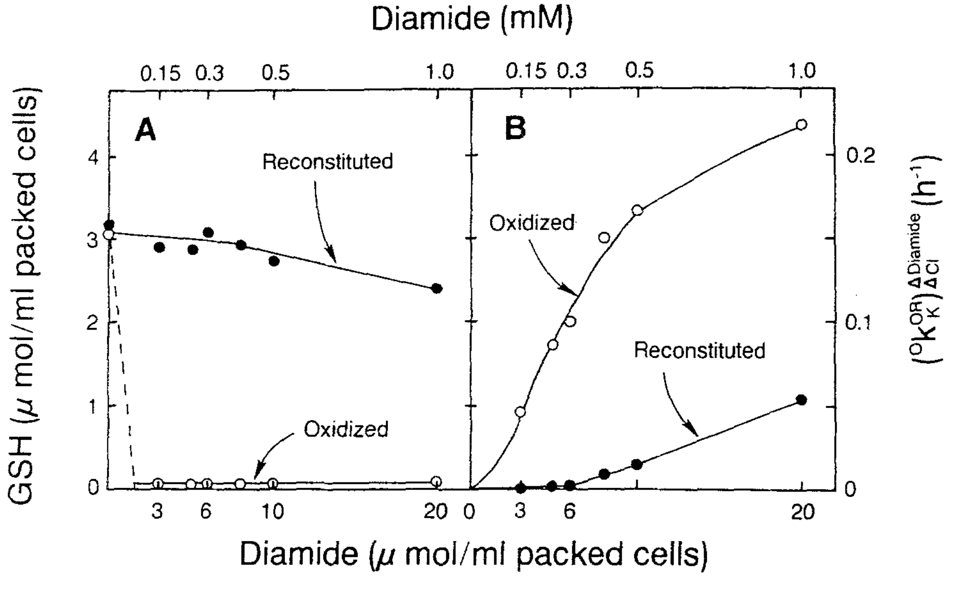

Fig. 6. Metabolic reduction of cellular GSSG to GSH (*A*) and reversal of K flux activation (*B*) in LK sheep red cells after 10 min oxidation by varying diamide concentrations. *Left-hand ordinate* and *A*: Cellular GSH (μmol/ml packed cells) determined at the end of the K efflux experiment. *Right-hand ordinate* and *B*: Rate constants of diamide-specific (ΔD) and Cl-dependent (ΔCl) zero-*trans* K efflux, ${}^{o}k_{K}^{OR}$ (hr^{-1}). Open circles: samples kept oxidized with diamide also during flux period. Filled circles: samples oxidized for 10 min by diamide (at concentrations indicated on upper and lower abscissas) and subsequently reconstituted for 2 hr in the rejuvenation cocktail prior to flux measurements. Diamide concentrations are given for solutions (mM, upper abscissa) or packed cells (μmol/ml, lower abscissa). Sheep LK1LL

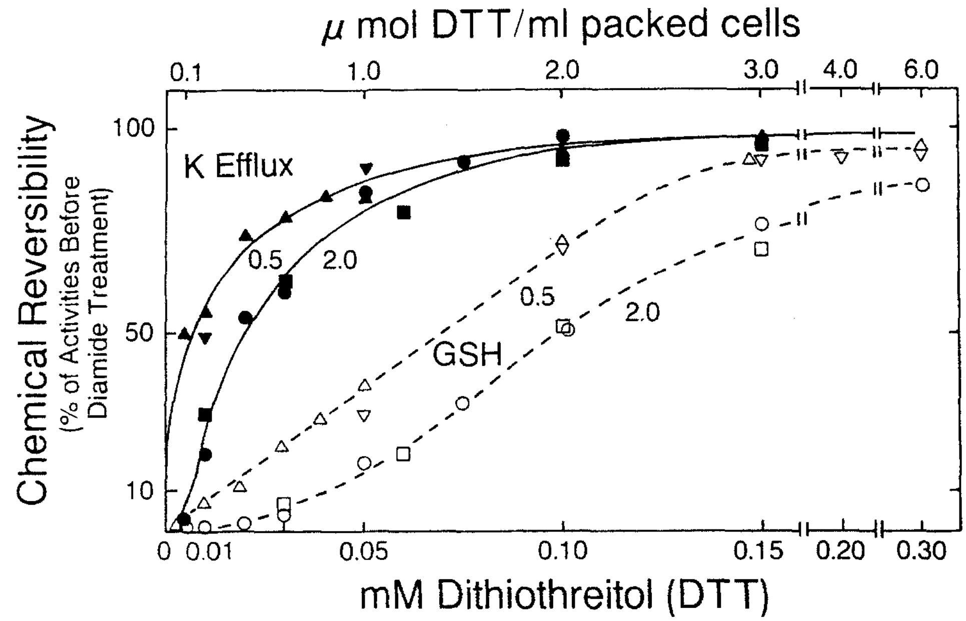

Fig. 7. Chemical reversal by dithiothreitol (DTT) of diamide-induced K efflux and GSH-oxidation in LK sheep red cells. Plotted are the percent activities before diamide treatment (ordinate) as function of the DTT concentration in solution (mM, lower abscissa) or in μmol/ml packed cells (upper abscissa). Filled symbols for K efflux and open symbols for cellular GSH levels. Data from four experiments on red cells from sheep LK8309 (▲△); 321 (▼▽), and LK 5 (● ■,○,□). Cells were first exposed to 0.5 or 2.0 mM diamide (as indicated in the figure) for 10 min in phosphate-buffered CH_3SO_3Na, then washed and resuspended in phosphate-buffered NaCl media containing varying DTT concentrations (as indicated on the lower abscissa) for determination of K efflux and GSH as described in Materials and Methods. Thus 0% chemical reversibility means fully diamide-stimulated K efflux minus the control basal flux (i.e., diamide-specific K efflux) and total GSH depletion by 0.5 and 2 mM diamide for 10 min, and 100% reversibility means return of the diamide-specific K efflux component and cellular GSH levels to control (basal) levels

Chemical Reversibility

As chemical reversibility we define the reduction of disulfides by means of reducing agents such as mercaptoethanol or its dimer, dithiothreitol (DTT). Based on our observation with metabolic repletion of LK red cells, incubated with and oxidized by 2.0 and 0.5 mM diamide (*cf.* Figs. 5 and 6), we first exposed LK cells for 10 min to these two diamide concentrations in phosphate-buffered CH_3SO_3 media to minimize K efflux. Cells were then washed in the same medium and finally transferred into Cl-flux media with DTT concentrations ranging from as low as 0.005 up to 0.3 mM. Figure 7 plots the chemical reversibility in percent of GSH levels before oxidation to GSSG by diamide and in percent of reversal of the diamide-activated K flux (measured as $({}^{o}k_{K}^{OR})^{\Delta D}$) as function of the DTT concentrations employed. Note that the curves for reversal by DTT of K effluxes activated by 0.5 and 2.0 mM diamide were of different shape, the former being hyperbolic, the latter sigmoidal and requiring about three times more DTT to reach the 50% reversibility midpoint. Both reversal curves coalesced to almost complete restitution of K efflux to baseline level, near 0.1 mM DTT. In sharp contrast, the chemical reduction of GSSG to the full level of GSH required some tenfold and fivefold more DTT for 0.5 and 2.0 mM diamide-treated cells, respectively, both curves tending to merge at DTT concentrations of greater than 0.3 mM. The data suggest that at least 50% of K efflux stimulated by diamide was restored by DTT before any significant reconstitution of cellular GSH occurred.

Relationship between Diamide- and NEM-Stimulated K Fluxes, the Role of Anions and the L_l-Antigen

In order to ascertain whether or not diamide acted through SH groups which also are the target for NEM, a kinetic study was performed in which 37 min into the efflux experiment diamide *or* NEM were added to either controls, or to NEM- or diamide-treated red cells. Figure 8 shows the fractional K release into zero-*trans* Cl flux media as function of time. Diamide and NEM identically activated OR K release in controls but did not affect further the shape of the K release curves of previously NEM- or diamide-treated cells which, a priori, were significantly different at late time

Table 3. Stimulation of ouabain-resistant K efflux by diamide and/or N-ethylmaleimide (NEM) in LK sheep red cells in different anionic media

Treatment (n = experiments)	Cl	${}^{o}k_{K}^{OR}$ (hr^{-1}) NO_3	(± SEM) ΔCl	CH_3SO_3	ΔCl
Control (8)	0.025 ± 0.001	0.018 ± 0.002	0.007	0.010 ± 0.001	0.015
2 mM diamide (4)	0.325 ± 0.018	0.046 ± 0.001	0.279	0.025 ± 0.001	0.300
2 mM NEM (4)	0.205 ± 0.008	0.022 ± 0.001	0.183	0.016 ± 0.001	0.189
2 mM diamide plus 2 mM NEM (4)	0.203 ± 0.005	0.027 ± 0.002	0.176	0.018 ± 0.002	0.185

points: the slope of the diamide-activated K release was much steeper compared to that of the NEM-activated. Thus, although the kinetics of K release seem to differ, both diamide and NEM appear to exert their effects through the same SH groups. This fact is also borne out in the data of Table 3: There is a greater activation of K efflux by diamide than by NEM: When both diamide and NEM were added together no additional stimulation occurred; rather diamide seemed to be unable to prevent NEM from its irreversible reaction with the common SH target groups.

Table 3 further indicates that diamide, like NEM, activated primarily Cl-dependent K transport. This effect exceeded by almost 60% that of NEM as seen from the difference in the rate constants of K effluxes in Cl versus NO_3 or CH_3SO_3. However, contrasting with NEM, diamide *per se* did double the rate of K loss in NO_3 or CH_3SO_3 and also had a small effect in the presence of NEM. It is of interest to note that the stimulation of K:Cl transport by diamide and NEM, in combination, was equal to that by NEM alone (Table 3). This indicates that NEM must have reacted much faster with the thiol(s) than diamide, which is consistent with a recent report by us on the kinetics of the NEM effect [28]. It is less likely that NEM acted through inhibitory groups as proposed by others [32], since we did not find evidence for their existence in our recent study [28].

Table 4 reveals that anti-L reduced K loss in both controls and diamide-treated LK cells by 40%, while there was no significant effect on the diamide-induced K efflux in HK cells. Thus diamide, like NEM shown previously [24, 31, 32], stimulated an anti-L_l-sensitive, passive K transport mechanism.

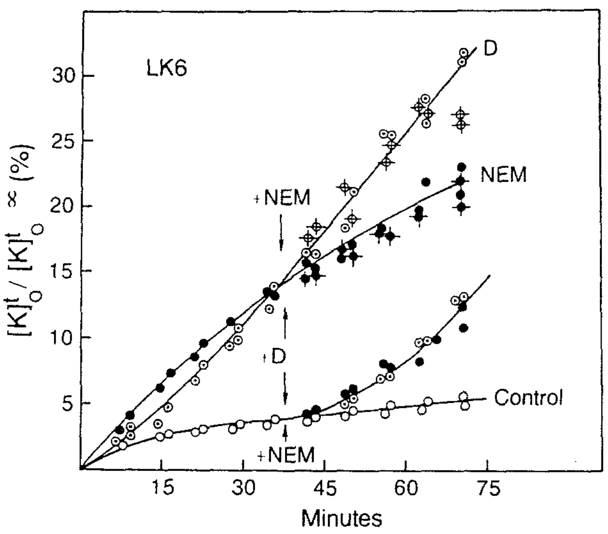

Fig. 8. Fractional K loss in Cl media of LK sheep red cells exposed to 2 mM N-ethylmaleimide (*NEM*) in combination with or without 2 mM diamide (*D*). Plotted is the fractional (%) ratio of the extracellular K concentrations at time t (min on abscissa) to that at equilibrium $[K]^t_o/[K]^{t=\infty}_o$. At t = 0, packed cells washed in phosphate-buffered NaCl were incubated in K efflux medium (same as wash medium) at 37°C for the time intervals indicated. Control (○), no addition at anytime. (⊙) 2 mM diamide and (●) 2 mM NEM were present throughout the entire experiment or added to the controls at 37 min. At about 37 min after the start of the experiment highly concentrated NEM or diamide solutions were added (arrows) to duplicate controls (●, NEM; ⊙, diamide) or to diamide-treated (⧫ for data with NEM added) and NEM-treated (ϕ for data with diamide added) cells, respectively, to yield final concentrations of 2 mM each. Data from two experiments with 10 time points per each condition on sheep LK6LL

Discussion

During the past years the SH group oxidant diamide has been used to alter cation permeabilities in human red cells [4, 5]. In general, large activations of anion-independent passive K *and* Na fluxes have been seen [4, 5] and explained in terms of diamide-induced reversible cross linking of spectrin underlying the plasma membrane as cytoskeleton [2, 10, 15]. In human red cells the effect of oxidative cross-linkage is believed to lead to phospholipid distribution changes with massive membrane permeability

Table 4. Effect of anti-L_l serum on diamide-activated K fluxes in sheep red cells

Sheep cation/ antigen type	Additions		$^{o}k_K^{OR}$ (hr^{-1}) ± SD (n = 4)	Inhibition (%)
	Diamide	Anti-L_l		
LK8309/LL	–	–	0.038 ± 0.0005	48
	–	+	0.020 ± 0.0006	
	+	–	0.133 ± 0.0007	
	+	+	0.079 ± 0.0012	41
LK189y/LL	–	–	0.049 ± 0.0004	
	–	+	0.033 ± 0.0016	33
	+	–	0.218 ± 0.0051	
	+	–	0.123 ± 0.0035	44
LK4/LM	—	—	0.010 ± 0.0001	
	–	+	0.007 ± 0.0002	30
	+	–	0.070 ± 0.0016	
	+	+	0.040 ± 0.0020	43
HK4083/MM	–	–	0.006 ± 0.004	
	+	–	0.008 ± 0.0003	
	+	+	0.008 ± 0.0001	0
HK0162/MM	—	—	0.007 ± 0.0003	
	+	–	0.014 ± 0.0004	
	+	+	0.012 ± 0.0005	0

alterations [4–6] or to a destabilization of the bilayer [12]. Deuticke's group [5] noted the refractoriness of ion leaks in ox red cells to diamide, a fact attributed to the sphingomyelin composition of the lipid layer [5] which also prevails in sheep red cells. The data of the present work clearly speak for a much more discrete effect of diamide on ion fluxes in sheep red cells and have to be analyzed in terms of the already known activation of OR K fluxes by other monofunctional SH reagents, and cell swelling.

These were the findings in sheep. Diamide activated Cl-dependent K transport in LK and, to a small extent, in HK sheep red cells without affecting OR Na fluxes (Figs. 1, 2 and 3, Table 3). Stimulation of OR K efflux by diamide occurred when about 80% of cellular GSH was oxidized (Figs. 1 and 3). The diamide-induced K efflux disappeared upon metabolic reconstitution devised to restore cellular GSH levels to normal (Fig. 4). The relationship between the diamide-oxidized K flux and the cellular GSH levels was linear for about 80% with a slope of 0.5, suggesting that at least 2 GSH are needed to return the activated K flux to base level (insert, Fig. 6). The success in achieving restoration of baseline K fluxes and GSH levels depended on the concentrations and length of exposure to diamide (Figs. 5 and 6). Both diamide-induced K efflux stimulation and GSH oxidation were chemically reversible by DTT (Fig. 7). However, the dependence of the chemical reversibility on the diamide concentration was different for K efflux and GSSG reduction to GSH. The effect of diamide was neither augmented by NEM nor vice versa, although the kinetics of OR K transport stimulation differed for diamide- and NEM-treated LK cells, in particular, at longer time periods of incubation. Anti-L_l known to reduce volume- and NEM-activated Cl-dependent K fluxes also inhibited by 40–50% diamide-stimulated K fluxes (Table 4).

The foremost finding of this study is the stimulation of Cl-dependent K flux in diamide-oxidized sheep red cells and its reversal by metabolic restitution or by chemical reduction with DTT. As with NEM [13, 22, 31] the effect of diamide on $^{o}k_K^{OR}$ was roughly ten times greater in LK than in HK red cells (Figs. 2 and 4). Since the apparent $K_{0.5}$ of Cl-dependent K efflux for cellular K is close to the actual K concentrations in LK cells [24], the maximum zero-*trans* K effluxes of LK cells may be estimated to be at least four to sixfold greater than those of HK cells where K-saturated K effluxes presumably existed after diamide oxidation. Thus the ratio of diamide-activated K fluxes in LK versus HK red cells is close to the "permeability-ratio" calculated for K ions by Tosteson and Hoffman 27 years ago [37]. This suggests that LK cells have substantially more K : Cl transport units or a higher turnover per unit than HK cells. This conclusion is consistent with our hypothesis that the K : Cl transport activity is quantitatively reduced during cellular maturation of HK red cells but due to the presence of the L_l-antigen [27], much less in LK cells. Indeed, anti-L_l did inhibit the diamide-stimulated K efflux (Table 3) to a degree known to inhibit the NEM-stimulated and volume-sensitive K : Cl fluxes [24, 33].

In light of the fact that diamide is known to oxidize SH groups to disulfides [4, 10, 20] and based on our earlier finding that the monofunctional MMTS also stimulated K : Cl transport [25], it is inviting to conclude that formation of at least one if not more (mixed) disulfide(s) was functionally involved with the K flux stimulation by diamide. It is most likely that the disulfide formation involves at least some of those SH groups which we earlier have shown to be alkylated by NEM or IAMM [28], since NEM was unable to alter the effect of diamide and vice versa (Fig. 8 and Table 3). Thus, any modification of the crucial SH groups in the K : Cl transporter by either alkylation (NEM, IAAM) or oxidation to intramolecular disulfides or to intermolecular (mixed) disulfides (for example, with MMTS, *see* ref. 25, and GSH *see* refs. 20, 33, 34) leads to K : Cl transport activation.

The relationship between diamide-mediated K flux stimulation and oxidation of GSH to GSSG is of interest in light of its metabolic/enzymatic linkage to the redox potential of NADP/NADPH$_2$ or NAD/NADH, which ultimately may control the activity of membrane cation transport through crucial SH groups. Such mechanism has been proposed for Ca-dependent K fluxes in the human red cell [14], and its maintenance of membrane thiol status [20], for the mitochondrial K permeability [16], and as redox cycling for the function of certain membrane carriers [35]. However, only when the GSH concentration was lowered by about an order of magnitude, K flux stimulation by diamide commenced. This fact may indicate that disulfide bond formation is caused by initial diamide oxidation of neighboring protein SH groups and does not involve GSH (mixed disulfide). On the other hand, activation of K : Cl transport through oxidation with the monofunctional SH reagent MMTS [25] supports the notion that mixed disulfide bond formation, known to occur elsewhere [20, 33, 34], seems to be an effective activation mechanism for K : Cl transport.

Information about the relationship between diamide-induced K : Cl transport and cellular GSH levels should come from the metabolic reconstitution experiment of Fig. 4 indicating a slope of 0.5 (*see* insert). This finding is consistent with the hypothesis that metabolic reversal of diamide-oxidation of the K : Cl transporter may require at least several GSH molecules, i.e., metabolic repletion leads to restoration of the basal K : Cl transport activity via direct reduction of the protein disulfide(s) by GSH. Nevertheless, when high diamide concentrations were used for longer times, only a partial restoration of cellular GSH and reversal of diamide-activated K : Cl flux occurred (Figs. 5 and 6). Reduction of the diamide-caused disulfide bond(s) was also achieved chemically by DTT, however, at much lower DTT concentrations than required for restoration of cellular GSH. This finding argues against a simple dependence of the K : Cl transport thiols on the cellular glutathione status.

We have recently proposed a model [27] to account for and integrate the various mechanisms of K : Cl transport activation such as cell swelling [7–9], SH group alkylation [22, 28, 31, 39], and bivalent metal ion removal [26]. We also have shown that there may be several SH-group clusters reacting differently with NEM and IAAM [28]. At least one of these SH groups has a low pKa [1], which we think is the result of the close proximity of another ionized group, such as in imidazols of histidine [27]. We postulated that ionization of such a group and perhaps ion pair formation may (during swelling) activate K : Cl transport [27]. Indeed, we have recently put forward further support for this hypothesis by showing a protonizable group present at the cytoplasmic face of the K : Cl transporter [40]. We conceive that the activation of K : Cl transport by alkylating or oxidizing SH reagents causes the same distant effect on K : Cl transport as cytoplasmic protonation, i.e., ion pair formation with the low pKa SH group. From the point of activation it does not matter then whether, instead of ion pair formation (cell swelling), alkylation by NEM or IAAM, or oxidation by MMTS or diamide occur. Future studies have to decide whether the low pKa of the SH group(s), which we discovered years ago in the K : Cl transporter, determines their reactivity with either neighboring SH groups or those of the cytoplasmic GSH pool to form disulfides. Whatever the outcome may be, the metabolic or chemical reversibility of the SH group modification by diamide may be a serendipidous tool toward finding means to molecularly identify and isolate the K : Cl transport molecule.

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