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Thiol-Dependent Passive K/Cl Transport in Sheep Red Cells: VII. Volume-Independent Freezing by Iodoacetamide, and Sulfhydryl Group Heterogeneity

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Summary. The sulfhydryl (SH) reagent iodoacetamide (IAAM) inhibits stimulation of Cl-dependent K transport in low K (LK) sheep red cells by another SH reagent, N-ethylmaleimide (NEM), without itself activating this transport pathway (*J. Membrane Biol.*, 1983, **73**:257–261). We now report that IAAM alone, acting with a kinetic slower than NEM, sharply reduced the capability of the Cl-dependent K transport system to regulate its activity in response to cell volume changes. This effect of IAAM did not depend on the cell volume maintained during chemical treatment, a fact ruling out that the reactivity of the SH groups with IAAM was a function of the volume-dependent turnover rate of the transporter. On the other hand, the prevention of the NEM-stimulatory effect on Cl-dependent K transport was found to be volume-dependent since 1) the rate with which IAAM blocked the subsequent NEM action was twice as fast in cells swollen in 250 mOsM as opposed to cells shrunken in 370 mOsM media, and 2) the dose response of the IAAM effect was different in swollen and shrunken cells. The action of IAAM with or without subsequent treatment with NEM seemed to be independent of cellular ATP which is required for full expression of the stimulatory modification of Cl-dependent K transport by NEM (*Am. J. Physiol.*, 1983, **245**:C445–C448). Clusters of SH groups on the Cl-dependent K transporter apparently react differently with IAAM and NEM when separately applied but, used in combination, reflect a complex volume-dependent effect that may reveal a "volume-sensing" component of the transport molecule.

Key Words electroneutral KCl transport · sheep red cells · sulfhydryls · iodoacetamide · N-ethylmaleimide

Introduction

A large portion of ouabain-resistant (OR) K fluxes in LK sheep red cells is volume-dependent [9, 12], inhibited by replacing Cl with other monovalent anions [9], and stimulated by treatment with NEM [17, 19, 25] or removal of cellular bivalent cations through the ionophore A23187 [21]. These K fluxes occur also in other cells such as nucleated red cells [16], human red cells [11, 23, 24, 32] and tumor epithelial cells [15, 31], perhaps with the physiologic assignment of cell volume-reduction during maturation [6] and are presently much investigated, in particular, since large activities have been described for certain human diseases [4, 5].

Over the last years we studied the molecular relations between the volume- and the chemically activated Cl-dependent K pathway [20]. Most of the evidence gathered by us [22] suggests that, at least in the sheep red cell, the NEM-activated pathway is a particular active state of the volume-sensitive Cl-dependent K transporter already present in the membrane. However, for human red cells it has been claimed that NEM activates a latent [23] or *de novo* [8] Cl-dependent K transport.

One of our early observations remained unexplained: why did IAAM, another SH reagent, prevent the stimulatory effect of NEM and yet did not activate OR K transport in LK cells [2]? In the present study we have addressed this problem by investigating in detail the IAAM action on Cl-dependent K transport in shrunken and swollen LK red cells, and also in combination with subsequent NEM transport. The data reveal that IAAM sharply diminished the volume-responsive activity of the OR and Cl-dependent K flux component. The effect of IAAM on Cl-dependent K transport was not volume and hence not turnover dependent. However, in combination with NEM a volume-sensitive aspect of the SH groups, chemically modified, became apparent suggesting that SH groups may be playing a role in the volume-sensing mechanism of the cell membrane.

Materials and Methods

Blood was drawn into heparinized tubes by venipuncture of LK sheep from two different sources: genetically purebred Dorset sheep kept at Duke University Animal Resource Facilities in Durham, N.C., and mixed type, mainly Hampshire/Suffolk sheep maintained by the Laboratory Animal Resources at Wright

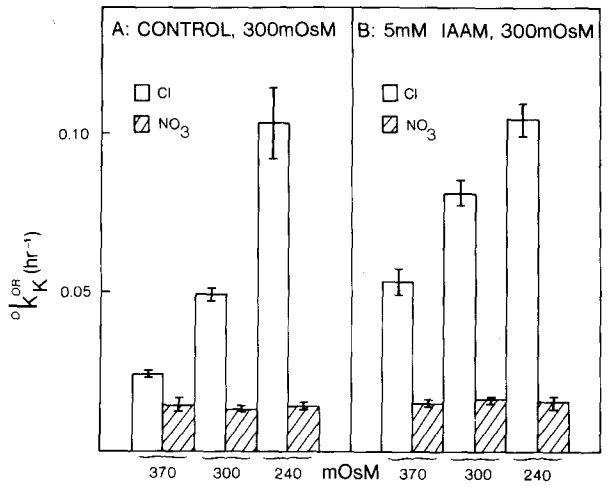

Fig. 1. Effect of iodoacetamide (IAAM) pretreatment of LK cells in 300 mOsM media on ouabain-resistant (OR) K efflux in media of varying osmolarities. The rate constants of OR K efflux, $^{o}k_K^{OR}$ (hr^{-1}), are given for cell measured in Cl (open columns) and NO_3 (hatched columns) with bars representing SE values for $n = 5$. (*A*) Control preincubation for 30 min in 300 mOsM media; (*B*) pretreatment for 30 min with 5 mM IAAM at a hematocrit of 10% (vol/vol). Note that K efflux of IAAM-treated cells was significantly higher than controls only in 300 and 370 mOsM but not in 240 mOsM solutions (Sheep LK(LL)259)

State University, Dayton, Ohio. Red cells were washed and chemically treated in a solution of the following composition (mM) with a final osmolarity of ~295 mOsM: 145 $NaNO_3$, 10 glucose, 10 Tris/MOPS [(hydroxymethyl)-amino-methane/2-(N-morpholine)-propane sulfonic acid], pH 7.4 at 0°C. When the cells were chemically treated in 240 or 370 mOsM media, only the $NaNO_3$ concentration was varied. In our hands SH group modification by the chemicals used was identical in Cl and in NO_3 media as assessed by subsequent flux studies. Usually, the cells were packed in these solutions and kept on ice prior to chemical treatment or flux analysis.

For chemical treatment, iodoacetamide (IAAM, Sigma Chemical Co., St. Louis, Mo.) was freshly dissolved in one of the above media up to concentrations of 10 mM, with equivalent $NaNO_3$ deletion. The osmolarities were checked with a Precision Osmometer by freezing-point depression. In contrast NEM was dissolved first as 1 M solution in dimethysulfoxide in which it was stored at 4°C and then, after warming, transferred by dilution into the treatment media. These media as well as the flux media contained in addition 10^{-4} M ouabain. Treatment media were brought to 37°C and cells were added at $t = 0$ to yield a hematocrit of 4 to 5% which was found optimal for the alkylation experiments. At the end of the exposure period in IAAM, cells were washed three times to remove any reagent. In the kinetic studies with IAAM and in the fast time study with NEM, the alkylating reaction was stopped by injecting a concentrated solution of dithiothreitol (DTT, Sigma Chemicals, St. Louis, Mo.) to yield a fivefold excess of DTT over that of NEM or IAAM. The cells were spun at the end of the time study and washed in their respective buffered media. This treatment with DTT immediately stopped the alkylation reaction as ascertained by constant K transport rates (*data not shown*).

Ouabain-resistant K transport was assessed mainly by the zero-*trans* K efflux method described in detail earlier [17]. Only in the fast time experiments with NEM (*see* Fig. 9) OR K influx was determined using Rb as K congener as reported before [17]. We have previously shown that OR Rb influx and OR K efflux stimulated by NEM have similar characteristics [17]. In general, the pretreated or control cells were injected at $t = 0$ into the K efflux or Rb influx media to yield hematocrits close to 4%. K efflux media were identical to the wash media with NaCl or $NaNO_3$ as the major salts. Rb-influx media contained in exchange for Na either 10 mM RbCl or $RbNO_3$, both salts being of analytic grade and obtained from Alfa Products, Danvers, Mass.

The fractional K loss into the cell-free supernatants was quantitatively measured at five time points by atomic absorption spectrophotometry using a Perkin Elmer 5000. From the K loss and the total K concentration in the suspension, the first-order rate constant, $^{o}k_K^{OR}$, was calculated using a regression line analysis program as reported earlier [17]. The Cl-dependent rate of K loss, $(^{o}k_K^{OR})_{\Delta Cl}$, is the difference between the total K loss in Cl and the K loss in NO_3 media [17]. OR Rb influx was measured under initial velocity conditions usually at one time point in triplicates as described before [17].

Relative cell volumes were determined at the end of the flux experiment. The optical density at 527 μm (OD_{527}) of each experimental suspension diluted with a hemolyzing detergent was measured, and together with the experimental hematocrit used to calculate the OD_{527} for packed cells which, when compared to the OD_{527} of unperturbed original cells, provided the relative volume ratio. Cellular ATP was assayed by the firefly method [3].

Results

Isosmotic IAAM Treatment and OR K Fluxes in Anisosmotic Media

Earlier we reported that treatment of LK sheep red cells with IAAM increased only slightly Cl-dependent K fluxes when chemical reaction *and* fluxes were carried out in isosmotic solutions [2]. However, the volume-sensitive, Cl-dependent K flux of LK cells, treated with IAAM near 300 mOsM, responded differently in anisosmotic flux solutions as shown in Fig. 1: In LK controls (Fig. 1*A*) the $^{o}k_K^{OR}$ values increased in Cl, but remained unchanged in NO_3 as the medium osmolarity was lowered from 370 to 240 mOsM. In contrast, cells pretreated with 5 mM IAAM for 30 min at 300 mOsM had $^{o}k_K^{OR}$ values higher in 370 and 300 mOsM Cl media (Fig. 1*B*) than controls, although again no effect of IAAM on OR K efflux in NO_3 was seen. Hence, pretreatment with IAAM stimulated the Cl-dependent K flux component when the cells were placed into 370 or 300 mOsM flux media.

In our earlier report we also found that treatment of LK red cells with 5 mM IAAM for 30 min was sufficient to prevent the subsequent action of NEM to stimulate Cl-dependent K fluxes [2]. Because IAAM also seemed to activate OR K trans-

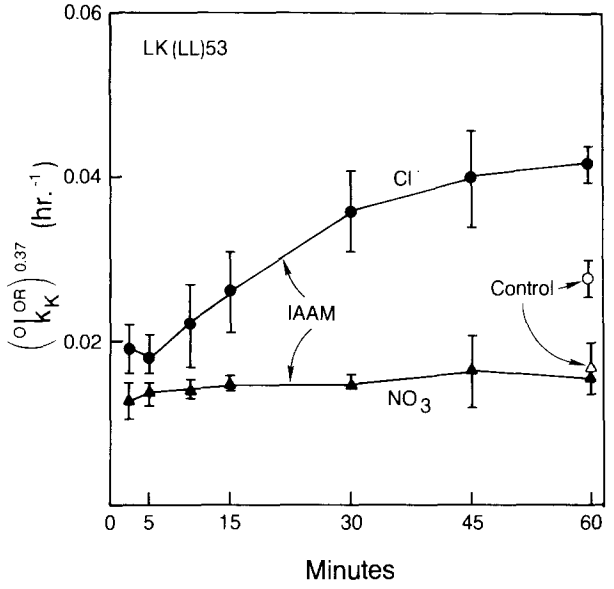

Fig. 2. Time course of the action of 2 mM iodoacetamide (IAAM) on K efflux in Cl and NO_3 media. Cells with a 4% suspension hematocrit (vol/vol) were first treated with 2 mM IAAM in isosmotic NO_3 media for the time intervals indicated. OR K efflux was then measured in 370 mOsM Cl or NO_3 media. Plotted are the rate constants (ordinate) of ouabain-resistant K efflux, $(^{\circ}k_K^{OR})$, as function of incubation time in min (abscissa). Filled symbols for IAAM-treated cells and open symbol for controls

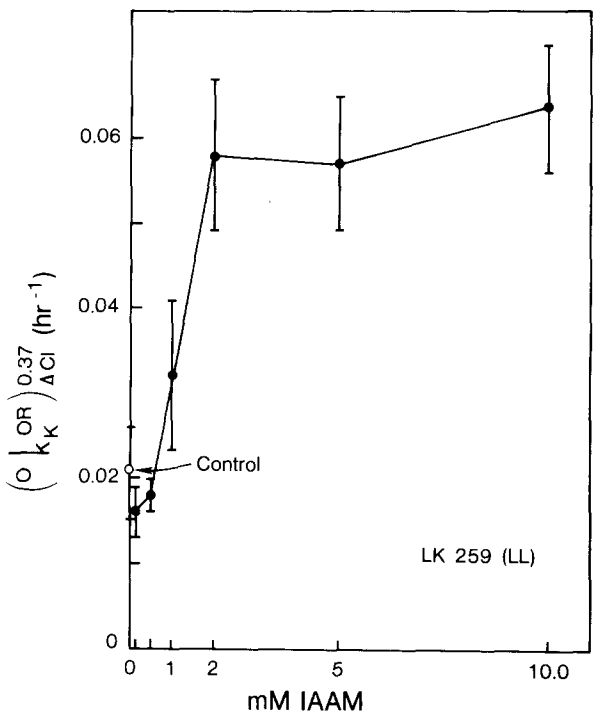

Fig. 3. Concentration dependence of iodoacetamide (IAAM) effect on Cl-dependent K efflux in 370 mOsM media. Shown are the rate constants of OR, Cl-dependent K efflux, $(^{\circ}k_K^{OR})_{\Delta Cl}$ per hr, in 370 mOsM media as function of IAAM concentrations (mM) applied for 30 min to a 5% (vol/vol) LK red cell suspension in isosmotic media. Maximum stimulation of Cl-dependent K efflux occurred at and beyond 2 mM IAAM, which is equivalent to ~40 mmol IAAM/liter packed cells (Sheep LK(LL)259). Bars indicate ± SE for $n = 3$. Filled symbols for IAAM-treated cells and open symbol for controls

port, particularly, at higher osmolarities (*see* Fig. 1*B*), we studied time and dose dependence of the IAAM action in isosmotically suspended cells on the Cl-dependent K effluxes subsequently measured in 370 mOsM solutions, i.e., when the cells were shrunken. Figure 2 shows that near maximum stimulation of OR K efflux in Cl was observed after about 30 min in 2 mM IAAM while there was no effect in NO_3. Figure 3 shows the Cl-dependent K efflux in untreated controls and LK cells exposed to IAAM concentrations ranging from 0.1 to 10 mM in solution (i.e., from 2 to 200 mM IAAM/liter packed cells). At 2 mM IAAM (i.e. 40 mM IAAM/liter packed cells) maximum stimulation was obtained and no further effect occurred at higher IAAM concentrations. Thus in further experiments 5 mM IAAM, 5% (vol/vol) hematocrit, and 45-min incubation time where chosen.

After these early studies with Dorset sheep red cells we continued to measure both Cl-dependent K flux *and* cell volume in controls and IAAM-treated LK cells obtained from blackface sheep. Figure 4 shows the $^{\circ}k_K^{OR}$ values of Cl-dependent K effluxes, $(^{\circ}k_K^{OR})_{\Delta Cl}$, as function of relative cell volumes during the flux experiment. Note the nonlinear, clearly sigmoidal behavior of $(^{\circ}k_K^{OR})_{\Delta Cl}$ of control cells as cell volume was varied between 85 to 110% of its normal value. After IAAM treatment at a relative cell volume of unity, i.e. in isosmotic solutions, the response of $(^{\circ}k_K^{OR})_{\Delta Cl}$ to cell volume changes was significantly altered: In shrunken cells $(^{\circ}k_K^{OR})_{\Delta Cl}$ was increased and decreased in swollen cells, so that the steep S-shape of the control response was virtually abolished. The "activating" effect of IAAM on Cl-dependent K flux was most pronounced at reduced cell volume, while the slightly inhibitory effect observed in swollen cells was statistically not significant in these experiments. It may be pointed out that at relative cell volume of unity, i.e. in 295 mOsM media, no stimulation of $(^{\circ}k_K^{OR})_{\Delta Cl}$ was found which contrasts with the data in Fig. 1 at 300 mOsM, where IAAM still was stimulatory. If the cells used in Fig. 1 were slightly shrunken (*see* slope of control in Fig. 4) stimulation by IAAM would have been the result. However, this possibility cannot be ascertained since cell volume was not determined in Fig. 1.

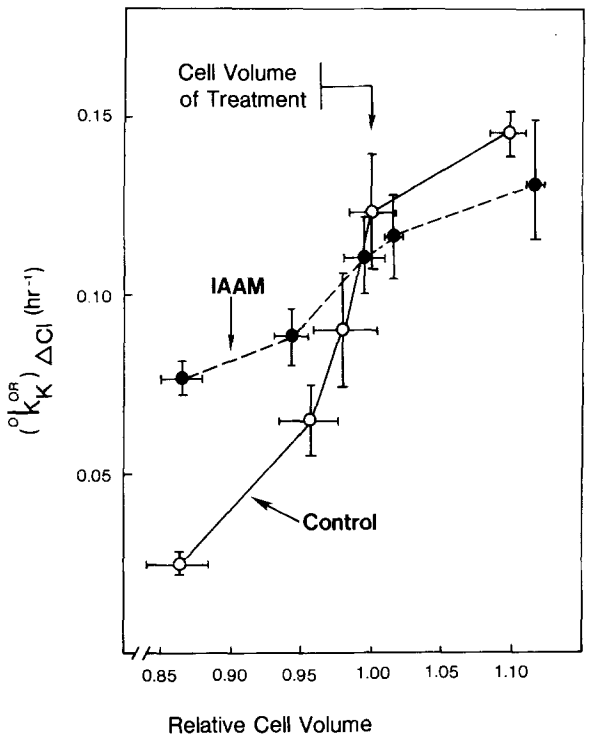

Fig. 4. Effect of pretreatment of LK erythrocytes with iodoacetamide (IAAM) at normal cell volume on Cl-dependent K effluxes subsequently measured at different cell volumes. Plotted are the rate constants of OR and Cl-dependent K efflux, $(^{o}k_K^{OR})_{\Delta Cl}$ per hr, as function of the relative cell volume during flux incubation in hyperosmotic, isosmotic and hyposmotic media. Data are from three experiments with bars indicating the SE for both rate constants (vertical bars) and actually measured cell volume (horizontal bars). The dotted line connects the data points (filled symbols) for the IAAM (5 mM, 5% hematocrit) experiments, the solid line with open circles stands for controls (Sheep LK(LL)8309)

IAAM Treatment and OR K Fluxes in Anisosmotic Media

The data shown above suggested that the "activating" effect of IAAM on $(^{o}k_K^{OR})_{\Delta Cl}$ apparent in shrunken cells may in fact be due to a chemically diminished ability of the Cl-dependent K transport system to be inactivated upon cell shrinkage. In the experiments of Fig. 4 the IAAM treatment was done at relative cell volume of unity. The early experiment in Fig. 1 as well as the data of Fig. 4 revealed that in hyposmotic solutions, i.e. when swollen, IAAM-treated LK cells exhibited rate constants of K efflux either unchanged or slightly below control values, respectively. Hence it was of interest to know, whether IAAM-treatment in 370 or 240 mOsM media would reveal a particular volume dependency of the SH group(s) reacting with IAAM as evidenced by studying OR K effluxes at all osmolarities. In such a case one would expect that the slope of the lines connecting the IAAM data points at the two extreme osmolarities should be different. Alternatively, in the case of volume independence of the alkylating reaction the slope would remain unaffected. In order to answer this question, we used all flux data obtained on the same sheep in Cl media including the experimental data points in Cl from which $(^{o}k_K^{OR})_{\Delta Cl}$ of Fig. 3 was calculated. The absolute magnitudes of the OR K effluxes varied between experiments perhaps due to experimental as well as in vivo differences in the mean age of the cell population studied. Therefore, the data of Fig. 5 are expressed in rate constants of K efflux normalized with respect to data obtained from cells which served as isosmotic controls or were treated with IAAM at 300 mOsM, both being subsequently used for K effluxes in 290 mOsM flux media. Furthermore, since we determined cell volumes only in the experiments of Fig. 4, all normalized OR K efflux rate constants were plotted versus the actually measured medium osmolarities.

Figure 5 reveals first that the relationship between OR K efflux of control cells and medium osmolarity was nonlinear, concave toward the ordinate. Furthermore, it did not matter whether the cells were exposed first to 370 or to 240 mOsM solutions and then to decreasing or increasing osmolarities during the efflux experiment. This means that control LK cells behaved as perfect osmometers. However, the relationship between OR K efflux and medium osmolarity became linear in IAAM-modified red cells, and this was the case at osmolarities greater than 290 mOsM as well as in more dilute solutions. In fact OR K efflux in IAAM-treated cells was significantly higher in 370 ($P < 0.001$) and 300 ($P < 0.005$) mOsM and significantly lower ($P < 0.001$) in 265 and 240 mOsM solutions, i.e. when the cells were shrunken or swollen, respectively. Calculations of these significance values included data from shrunken (370 mOsM), normal (300 mOsM), and swollen (240 mOsM) control and IAAM-treated cells exposed to different osmolarities during the K efflux experiment. This observation means that IAAM treatment of LK red cells at any osmolarity diminished to a similar degree the response of OR K transport to changes in medium osmolarity or cell volume.

The data compiled in Fig. 5 seem to corroborate much of those shown in Fig. 4 with perhaps the major exception that at 240 mOsM, i.e. when the cells were about 10% swollen (*cf.* Fig. 4), there was a statistically significant difference between IAAM-treated and control cells. Interestingly, although at 370 mOsM the difference between controls and IAAM-treated cells was significant, it was smaller

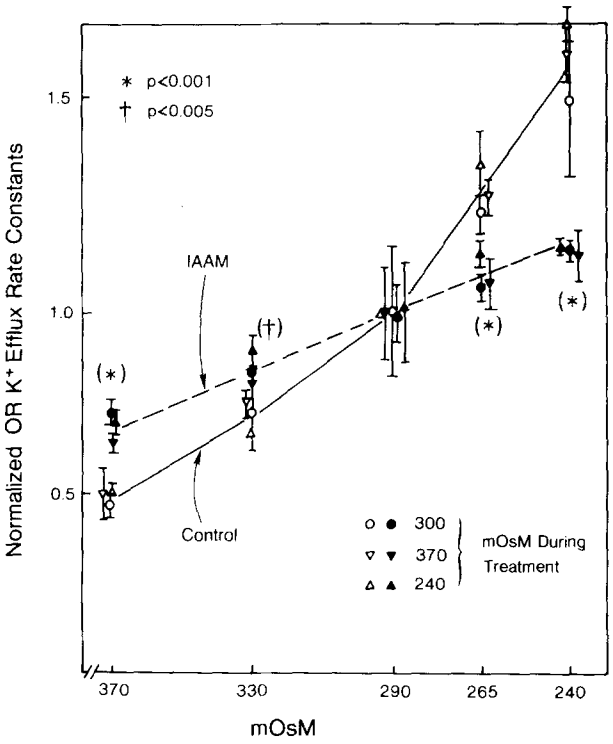

Fig. 5. Effect of pretreatment of LK erythrocytes with iodoacetamide (IAAM) at normal (300 mOsM), shrunken (370 mOsM) and swollen (240 mOsM) cell volumes on K effluxes subsequently measured at five different osmolarities. Plotted are the normalized rate constants of all OR K effluxes measured in Cl with values obtained under near isosmotic conditions (290 mOsM) equal to unity. Solid line connecting open symbols denotes controls *not* treated with IAAM. Broken line with filled symbols are for IAAM-treated cells. Both filled and open circles as well as the two types of filled and open triangles stand for the three total media solute concentrations (300, 370 and 240 mOsM) used during pretreatment for 30 min with (filled symbols) or without (open symbols) 5 mM IAAM, respectively. The bars denote SE values for four to six experiments per osmolarity. The stars and crosses indicate that the flux values obtained after IAAM treatment at the indicated medium osmolarities were statistically different from controls with $P < 0.001$ and $P < 0.005$, respectively (Sheep LK(LL)8309)

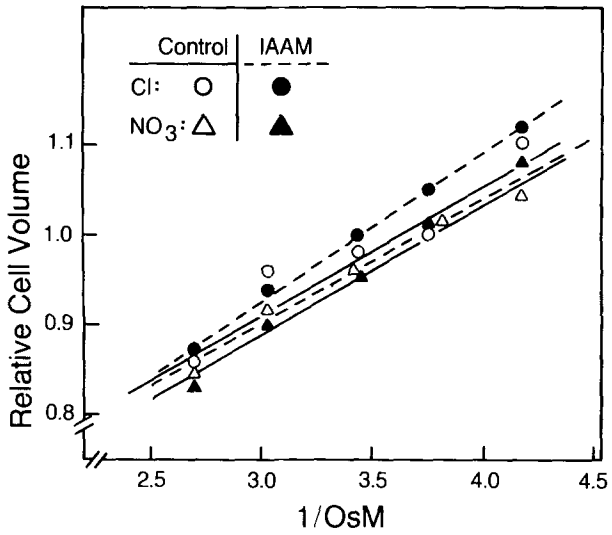

Fig. 6. Relative cell volume as function of medium osmolarity during the flux experiment of Fig. 4. Plotted are the measured relative cell volumes (ordinate) versus 1/OsM determined prior to the flux experiment in all experimental media. Open symbols and solid lines for controls, and interrupted line and filled symbols for IAAM-treated cells. The mean slope of all four conditions was 0.14 which is typical for sheep red cells

when the $(^{o}k_{K}^{OR})_{\Delta Cl}$ values were directly plotted as functions of the measured relative cell volume (*cf.* Fig. 4). This effect was not due to major incongruities derived from plotting fluxes versus relative cell volume or medium osmolarities. From Fig. 6 it can be seen that the relationship between relative cell volume and the inverse values of the medium osmolarities was generally linear for controls and IAAM-treated cells alike, and independent of the anion used. Thus other factors must have been responsible for the relatively more pronounced response of OR K efflux in hyposmotic Cl controls of Fig. 5 as opposed to the data of Fig. 4 and 1. Since changes in the base fluxes in NO_3 are ruled out (*cf.* Fig. 1) the only option left is the normalization of the data with respect to 290 mOsM. Indeed a comparison of the spread of the data points of Fig. 5 with that of Fig. 4 confirms this supposition.

Together, the data shown in Figs. 1–6 strongly imply that IAAM treatment *per se* was volume-independent. However, unlike the NEM effect reported earlier [19], IAAM "stimulated" OR- and Cl-dependent K efflux under hyperosmotic conditions, i.e. when the cells were shrunken, and inhibited when the cells were swollen in hyposmotic media.

Rate Studies of the NEM Effect

In contrast to the slow time course of modification of OR Cl-dependent K transport by IAAM (Fig. 2), NEM reacted strikingly fast. This fact has to be considered for any experimental setup involving both SH reagents. Figure 7(*A*) shows the percent stimulation of OR K transport for very short time intervals up to 3 min and at NEM concentrations comparable to the experiments carried out by others [26]. In these experiments the action of NEM was quenched with DTT. In Fig. 7 the fractional stimulation of OR Rb influx after subtracting the control fluxes in presence of DTT is plotted as a function of time. Note that in contrast to the finding

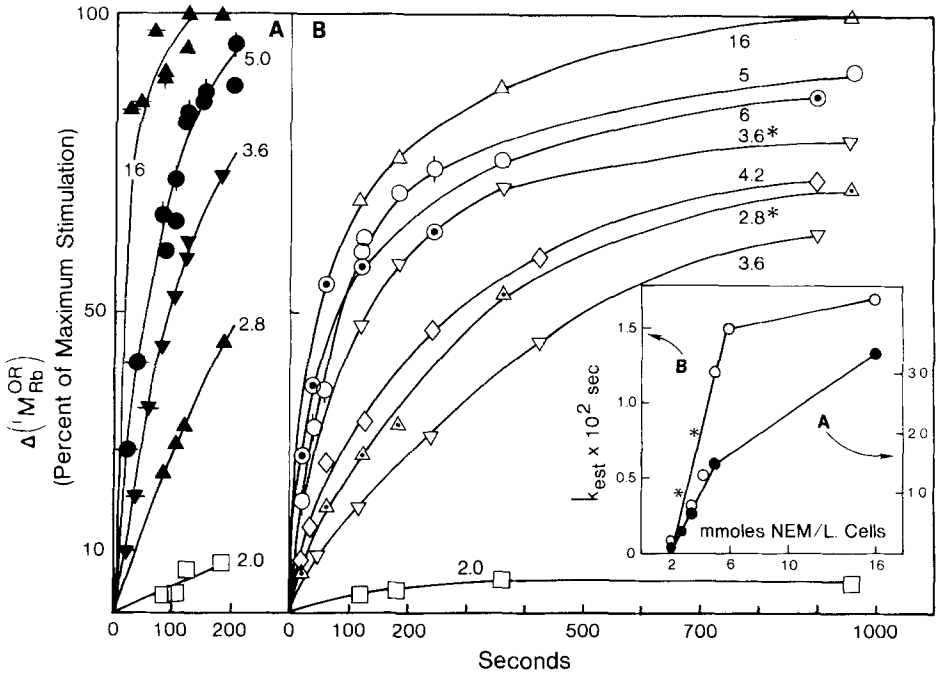

Fig. 7. Rates of stimulation of OR Rb influx by N-ethylmaleimide (NEM). Plotted are the percent values of the maximum stimulation of the NEM-activated OR Rb influx, $\Delta(^{i}M_{Rb}^{OR})$, (at 16 mmol NEM/liter packed cells) as function of time in seconds for each lower NEM concentration. Panels (*A* and (*B*) depict two sets of experiments where either more early time points (*A*) or points spread over a longer time period were taken (*B*). As noted in Materials and Methods, the reaction with NEM was carried out at 37°C and stopped by addition of a four- to fivefold excess of DTT. The numbers on the lines indicate the NEM concentration calculated in mmol/liter cells. The hematocrit for all experiments was 5% (vol/vol). In (*B*), the stars behind two numbers indicate that these data derived from a different experiment. The insert contains a plot of k_{est}, the rate constants estimated from initial velocities in (*A*) and from $t_{1/2}$ determinations in (*B*) as function of the NEM concentration calculated per liter cells (Sheep LK(LL)1894)

of others [26] there is no inhibition of OR Rb influx (at 5% hematocrit) at 0.1 mM NEM equivalent to 2 mmol NEM/liter packed cells but rather a small degree of stimulation. As the NEM concentration in the medium was raised from 0.1 to 0.8 mM (i.e. to 16 mM/liter packed cells), an even faster rate of activation was observed reaching maximum stimulation within less than 100 sec in agreement with others [26]. From the experiments of Fig. 7(*A*) one could not decide whether the activation of OR Rb influx would become 100% at longer incubation periods and lower NEM concentrations. Separate experiments (Fig. 7*B*) show that the stimulation by NEM leveled off at NEM concentrations lower than 0.8 mM (16 mM/liter packed cells). This was to be expected as the concentration of the chemical used to titrate the cellular SH groups became limiting. However, also at 0.8 mM NEM the initial fast activation rate of OR Rb influx was superseded by a second much slower rate process. Consequently, attempts to calculate reaction rate constants yielded a nonlinear function of the NEM concentration as shown in the insert of Fig. 7(*B*). Note also that there was an intercept at the abscissa at about 2 mmol NEM/liter cells, for both data sets derived from panel (*A*) and (*B*), suggesting the presence of at least one additional compartment with which NEM reacted prior to or simultaneously with activation of OR Rb influx. Based on these studies it was expected that NEM would react much faster with the functional SH groups of the Cl-dependent K transport system than IAAM.

Modification of the NEM Effect by IAAM, and the Role of Cell Volume

The experiments of Figs. 8–10 were carried out primarily to investigate the presence of any volume dependence of Cl-dependent K transport chemically modified first by IAAM and then by NEM. To study the reverse order of chemical treatment would be less informative due to the widely different reaction rates of IAAM and NEM. Figure 8 displays a kinetic study of LK cells pretreated with 5 mM IAAM for different time periods at two media osmolarities, either before or after exposure to 2 mM NEM. At each time point the reaction of IAAM

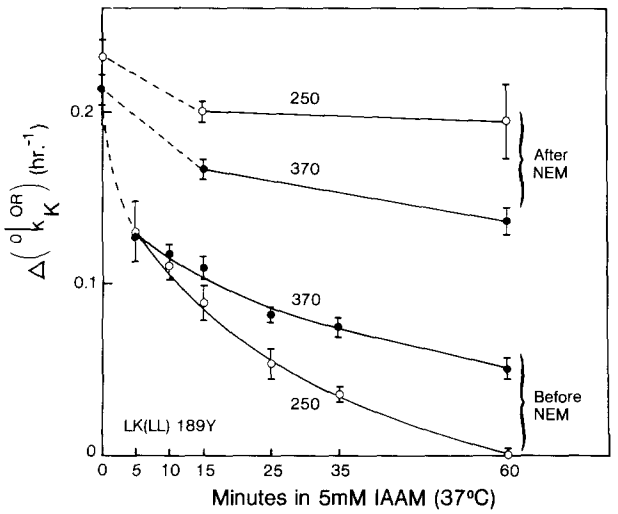

Fig. 8. Effect of iodoacetamide (IAAM) on ouabain-resistant (OR) K efflux of LK cells before or after treatment with N-ethylmaleimide (NEM). In order to test for the possible volume dependence, both chemical treatments and subsequent K efflux measurements were done in 250 and in 370 mOsM media. Shown are the rate constants of the chemically modified K efflux components $\Delta(^{\circ}k_K^{OR})$ per hr, after subtracting the values obtained from untreated controls. Cells were either first exposed to 2 mM NEM for 15 min and then to 5 mM IAAM for up to 60 min (upper curve pair) or first to 5 mM IAAM and then to 2 mM NEM (lower curve pair). Bars indicate SE of three experiments on the same animal (LK 189Y)

was terminated by washing in 250 and 370 mOsM NO_3 media. Ouabain-resistant K efflux was measured into 250 and 370 mOsM Cl media by determining $^{\circ}k_K^{OR}$ as described in Materials and Methods. The units on the ordinate of Fig. 8 are the differences between $^{\circ}k_K^{OR}$ of treated and untreated control cells, $\Delta(^{\circ}k_K^{OR})$. When LK cells were exposed first to NEM ($t = 0$) and then to IAAM (upper curve pair) a slight inhibition of the NEM-activated component was seen in both 250 and 370 mOsM media with the final level (about 25% lower than at $t = 0$) depending on and corresponding to the initial activities of the NEM-stimulated K pathway in swollen (250 mOsM) and shrunken (370 mOsM) cells (*see* ordinate at $X = 0$). A different picture was obtained with LK cells treated first with IAAM and then with NEM (lower curve pair in Fig. 8). Note that the longer the pretreatment with IAAM the greater the loss of the "NEM-effect" which, at 60 min, was complete in 250 mOsM, however, only 75% in 370 mOsM. These data mean that the time course of the IAAM interference with the NEM effect was faster at 250 than at 370 mOsM, and hence suggest volume dependence of those SH groups which are chemically reacting with both IAAM and NEM.

The effect of cell volume on the NEM effect in IAAM-treated cells was also investigated at various IAAM concentrations. Figure 9 shows the rate constants of Cl-dependent OR K efflux, $(^{\circ}k_K^{OR})_{\Delta Cl}$, in controls, and in cells treated with IAAM either before or after exposure to a saturating concentration (2 mM) of NEM. Chemical treatments and fluxes were carried out in 240 (*A*) and 370 (*B*) mOsM media. Note that the basal fluxes were greater in swollen (*A*) and smaller in shrunken (*B*) controls and NEM-treated cells (data points at IAAM = 0). Subsequent exposure of NEM-treated cells to IAAM concentrations up to 10 mM reduced the NEM-activated flux by only 20% in 240 mOsM media (*A*) and had no effect in 370 mOsM solutions (*B*). IAAM-treatment prior to reaction with NEM gradually diminished the capacity of NEM to enhance $(^{\circ}k_K^{OR})_{\Delta Cl}$ abolishing it at 5 mM in 240 mOsM (*A*) and most of it in 370 mOsM.

Both SH group reagents are known to interfere with the metabolism of the erythrocyte [14]. In addition, the NEM-effect on OR Cl-dependent K transport cannot be elicited in ATP-depleted sheep and human red cells [18, 24]. Hence, it was imperative to establish cellular ATP levels, in particular where treatment with IAAM prior to NEM fully inactivated the NEM-stimulated K flux. Figure 10 shows the ATP levels in cells chemically treated in the presence and absence of the Na/K pump inhibitor ouabain, the latter being added to evaluate the role of pump-mediated ATP hydrolysis over the period of incubation with 5 mM IAAM (30 min) and/or 2 mM NEM (15 min). Note that cellular ATP was reduced by 40 to 50%, however, independent of the sequence of addition of SH reagents, suggesting that the abolition of the NEM effect by IAAM treatment (*cf.* Figs. 8 and 9) cannot be attributed to metabolic depletion *per se*.

Discussion

The following observations were made: 1) With a slower kinetic than that of NEM (*cf.* Figs. 2 and 7) IAAM apparently stimulated OR Cl-dependent K flux in shrunken and inhibited in swollen LK red cells (Figs. 4 and 5). 2) This effect of IAAM was independent of cell volume during chemical treatment (Fig. 5). 3) Iodoacetamide exposure of LK cells prior to treatment with NEM prevented the "NEM effect" of stimulating OR Cl-dependent K transport and this effect was time and concentration dependent (Figs. 8, 9). 4) The interference of IAAM with the NEM effect was cell volume dependent (Figs. 8 and 9) and 5) and was apparently not related to a 40 to 50% drop in cellular ATP observed under all permutations of chemical treatments (Fig. 10).

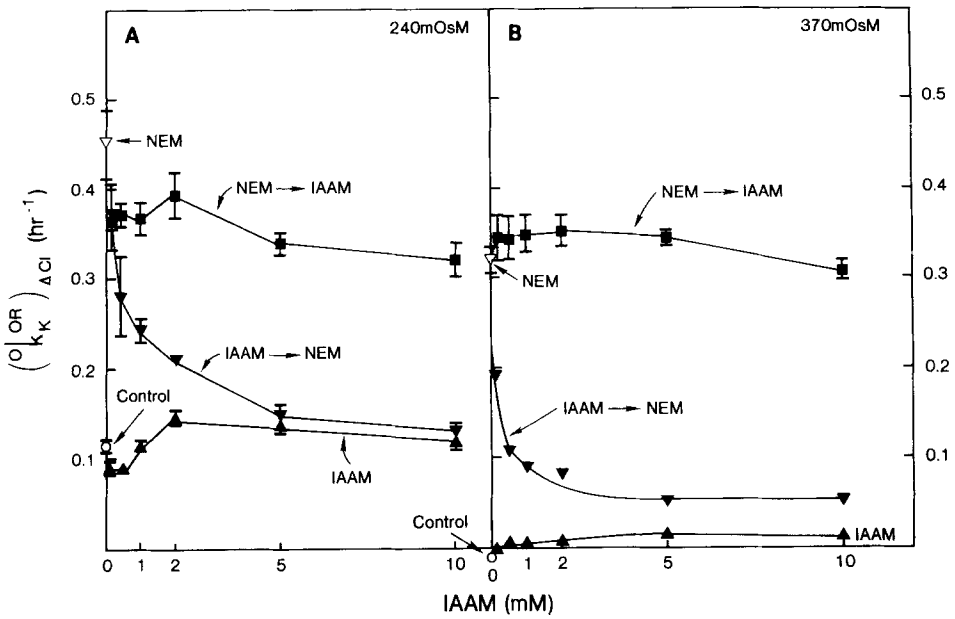

Fig. 9. Reduction of the N-ethylmaleimide-induced stimulation of OR and Cl-dependent K efflux in LK red cells pretreated with increasing iodoacetamide (IAAM) concentrations. Plotted are the rate constants of Cl-dependent K efflux, $(^{\circ}k_K^{OR})_{\Delta Cl}$ per hr, as function of IAAM concentrations used prior to (triangles) or after (squares) treatment with 2 mM NEM for 15 min in hyposmotic (*A*) and hyperosmotic (*B*) media. Note that the control K efflux is, as expected, higher in 240 than in 370 mOsM media thus contributing to generally higher flux values in chemically treated cells. Bars indicate SE of three experiments on the same animal (Sheep LK(LL)321)

The reaction kinetics between SH group-containing amino acids, mainly cysteine, and the alkylating chemicals of the type used in the present study has been well characterized for solutions [13]. An analysis of the effects of IAAM and NEM on intact cells is complicated by a number of factors. First, the reactive SH groups seem to be located within the plasma membrane or at its cytoplasmic aspect as a nonpermeating maleimide [1] did not effect stimulation of K fluxes in Cl [19]. Second, as shown by Rothstein a long time ago [29], the permeability of the membrane to various SH reagents may be quite different, a fact borne out from the very different reaction rates at similar concentrations of IAAM and NEM (Figs. 2 and 7). Third, we have tried earlier to explain the different reaction rates of IAAM and NEM based on the possibility that the latter chemical may complex alkali ions [2]. Fourth, in comparing the effects of IAAM and NEM one has to note that from a cell physiologic point IAAM may have caused a number of secondary changes which may have affected interaction of NEM with specific SH groups.

Secondary changes may involve the glutathion (GSH) pool of the erythrocyte which is ~3 mM/liter of packed cells in sheep. For the slower penetrating IAAM it may take minutes to exhaust the cellular GSH while this process may take only seconds or less with the highly permeable NEM. Indirect evidence for the participation of a cellular SH pool of the magnitude of GSH comes from the plot of the estimated rate constants of the NEM-induced activation of Cl-dependent K transport as function of the NEM concentration/liter of packed cells (Fig. 7). Aside from the fact that the plot failed to yield a second-order reaction rate constant (because the time resolution of our technique was too low) an intercept of about 2 mmol NEM/liter cells was found suggesting that GSH may have reacted with NEM (or IAAM) either immediately before or simultaneously with the effect of these compounds on membrane SH groups which can be calculated to be about less than 10% of the total SH groups available [2]. Work on GSH-depleted red cell ghosts has shown that the NEM-activated K pathway either lost its Cl-dependence [30] or seems to be already there without further stimulation by NEM [10].

Nevertheless, being aware of the above and many other factors playing a role in the activation of Cl-dependent K transport by SH group reagents, the experiments have shed light on the previous finding that IAAM alone did not affect Cl-dependent fluxes, but inhibited the NEM effect when applied to LK red cells prior to NEM [2]. Carboxymethylation of the functional SH groups in the K/Cl transporter was also achieved with iodoacetic acid

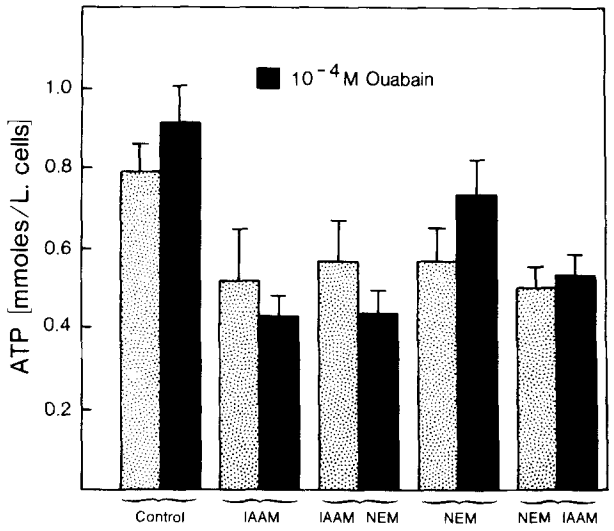

Fig. 10. Cellular ATP concentrations measured by firefly assay as function of 45-min pretreatment of LK red cells with the sulfhydryl group reagents iodoacetamide (IAAM) and/or N-ethylmaleimide (NEM). The time was chosen on basis of the time-course experiments shown in Figs. 2 and 8. Ouabain (10^{-4} M) was present (black columns) or absent (white columns) during the chemical treatment. Bars ± SE, n = 4 to 5

which like IAAM blocked the "NEM effect" [26]. From the data in Figs. 1 and 4 it is clear that the effect of IAAM became most evident when, subsequent to chemical treatment at ~300 mOsM, the flux was carried out in shrunken cells. Compared with controls this important finding can only be interpreted as a sharp reduction of the capability of the Cl-dependent K transport system to respond to volume changes of the cell, in particular to be inhibited in shrunken cells, and not as a stimulatory phenomenon as seen in swollen, NEM-treated cells [19]. This finding then is in agreement with a conclusion derived on different grounds [2] and by others [26], that at least two different SH groups must be involved in regulating the activity of the Cl-dependent K transporter: One group that reacts preferentially with IAAM, an effect apparent in shrunken cells, *and* with NEM, since NEM stimulated at all cell volumes; and at least a second group through which IAAM inhibits (Fig. 4) or NEM stimulates K fluxes [19]. The fact that IAAM inactivated the volume sensitivity of Cl-dependent K fluxes in hyperosmotic solutions supports further our concept that chemical modification of the original and not, as proposed by others [8], creation of a new, volume-sensitive K transport pathway occurred.

In concluding that the volume-sensitive K transporter possesses functionally crucial SH groups, the question is justified whether such SH groups are directly part of the volume-sensing mechanism or belong to the actually transporting moiety of the system. Such a distinction has been made for other transport systems with respect to the "permissive" role of Cl ions [7, 28]. Figure 5 shows that the reactivity of the SH groups with IAAM was independent of the cell volume kept during the alkylation reaction. This fact means that IAAM affected the K transport system similarly at any volume or independent of the turnover rate. Hence, IAAM does not "lock" Cl-dependent K fluxes in the "on" or "off" position as certain dimaleimides have been reported to do for the Na/H exchanger of dog red cells [27]. This suggests that either cross-linking of two SH groups is required or that these IAAM-alkylated SH groups are not part of a putative separate volume-sensing apparatus.

We concluded above that IAAM reacted with at least one subfraction of SH groups which also is attacked by NEM. Hence, we should see competition between IAAM and NEM for such sites. Because the reaction rates/permeabilities of IAAM and NEM for intact cells are so different, direct competitive studies are not feasible. Furthermore, it was very difficult to reproduce the same degree of NEM-induced K flux activation at high NEM concentrations due to the fast reaction rates and the extreme concentration dependency of the reaction for the NEM concentration range shown in Fig. 7. However, the combinations shown in Figs. 8 and 9 revealed well-reproducible data and a volume-dependence of SH group modification suggesting that indeed some SH groups may be part of a volume-sensing portion of the Cl-dependent K transport system. When the cells had been incubated with NEM first, all of the SH groups including those reacting with IAAM were alkylated and no further significant reduction of the "NEM effect" occurred. Conversely, the longer the cells were exposed to IAAM, the less NEM caused stimulation of K transport. The IAAM either reacted through those SH groups which also bind NEM or affected a subset of SH groups that determined the subsequent reactivity with NEM. This effect was more pronounced in swollen (250 mOsM) than in shrunken (370 mOsM) cells. Further evidence for a complex volume-dependent effect of IAAM on the reactivity of the Cl-dependent K transporter is based on the observation that it mattered whether the IAAM pretreatment was done in 370 or 240 mOsM medium.

Different SH-group reagents are often used to elucidate further the nature and operation of a transport system present in the plasma membrane at low density. Reactions with the two reagents used seem to indicate at least two major clusters, one of which after alkylation with IAAM partially but significantly reduced the ability of OR K trans-

port to be inactivated upon cell swelling. However, this effect of IAAM was *not* volume and hence *not* turnover dependent and also could not be explained on the basis of metabolic depletion known to abolish the NEM effect (*see* Fig. 10 and ref. 18). On the other hand, a volume dependence became apparent when both IAAM and then NEM were applied suggesting the presence of several SH clusters at the basis of a volume-sensing mechanism in the plasma membrane of LK red cells.

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