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Investigation of monovalent cation influxes of diamide-treated human erythrocytes in solutions of different ionic strength

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Total and residual i.e. (ouabain + bumetanide + EGTA)-insensitive K^+ as well as Na^+ influxes were investigated in human erythrocytes before and after treatment with diamide (5 mM). In physiological and in low ionic strength solution these influxes were increased after diamide treatment. Diamide-treated cells do not exhibit significant differences between the total and residual influxes for both Na^+ and K^+. The diamide-induced cation fluxes in low ionic strength solution are significantly higher compared with the fluxes in physiological ionic strength solution. The diamide-induced K^+ influx is not chloride-dependent, and replacement of NaCl by sodium methylsulfate does not significantly reduce this flux. A subsequent incubation of diamide-treated erythrocytes with dithioerythritol which restores the cellular glutathione level to its original value only partly decreases the enhanced K^+ influx. From these results it can be concluded that electrodiffusion and K/Cl cotransport are not involved in the diamide-induced stimulation of the residual K^+ influx of human erythrocytes.

Introduction

Deuticke et al. explained their results of diamide-induced permeability changes of erythrocytes for different non-electrolytes and ions by cross-linking of spectrin [1]. The disturbance of membrane barrier function after 5 mM diamide treatment has been interpreted as the formation of aqueous leaks [2].

Lauf [3] explained enhanced cation fluxes in sheep erythrocytes caused by diamide treatment (0–2 mM) as a stimulation of the K/Cl cotransport. In sheep erythrocytes the diamide-induced cation flux is specific for K^+ (no stimulation of the Na^+ flux) and is Cl^--dependent. Furthermore, HK sheep (high-potassium type sheep) red blood cells show only 10% of this diamide-induced increase of the K^+ flux as compared with LK sheep (low-potassium type sheep) red blood cells [3].

Ouabain, bumetanide, and EGTA inhibit the Na^+ pump, the Na/K/Cl cotransport, and the Ca^{2+}-induced K^+ transport (Gardos channel) in human erythrocytes, respectively [4]. Using these inhibitors it is possible to measure the residual cation transport (leak) [4,5]. In mature human erythrocytes the K/Cl cotransport is suppressed under normal physiological conditions [6]. It could be shown that the residual K^+influx is increased in a solution of low NaCl concentration (low ionic strength medium), which could not be explained on the basis of simple electrodiffusion [5].

The aim of the present paper was to investigate a possible participation of the K/Cl cotransport in the stimulation of the cation fluxes caused by diamide treatment in human erythrocytes. In addition, we were interested to find out if there is a true electrodiffusion pathway (aqueous leak) for monovalent cations in the erythrocyte membrane after incubating the cells with diamide. Therefore we used solutions of different ionic strength for these investigations. As a result of the Goldman flux equation one would expect a decrease of residual K^+ influxes under conditions of reduced extracellular NaCl concentration (sucrose replacement) [4].

Parts of the present paper were previously published

Abbreviations: diamide, diazine-dicarboxylic acid (bis-[*N*,*N*-dimethylamide]); DTE, dithioerythritol; DTT, dithiothreitol; GSH, glutathione; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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as an abstract at the Meeting of the European Association for Red Blood Cell Research (Dundee, 1990).

Materials and Methods

Cell preparation and chemical modifications

Blood from apparently clinically healthy men (O Rh^+ blood group, citrate preserved) were washed three times in a solution of the following composition (mM): NaCl, 145; glucose, 10; NaH_2PO_4/Na_2HPO_4, 5.8 (pH 7.4) at room temperature (Cl^--containing physiological ionic strength solution).

Diamide (Sigma) treatment of the erythrocytes was performed in accordance with the method of Haest et al. [7]. The erythrocytes were washed twice and always resuspended in the incubation medium composed of (mM): KCl, 90; NaCl, 45; sucrose, 44; NaH_2PO_4/Na_2HPO_4, 10 (pH 8.0) at 37°C. The red blood cells were pre-treated with 10 mM iodoacetate (Merck) (37°C, hematocrit 10%, 15 min). After three washes the cells were resuspended in this incubation medium containing 5 mM diamide (hematocrit 10%, 37°C, 1 h).

Additionally, parts of the erythrocyte suspensions were treated with 10 mM DTE (Fluka) in the incubation medium (37°C, hematocrit 10%, 45 min) after diamide treatment of the cells which in this case were not pre-treated with iodoacetate.

Determination of GSH content

The cellular reduced GSH level of diamide-treated and untreated erythrocytes was measured photometrically by a standard procedure according to Beutler et al. [8] using 5,5'-dithiobis-(2-nitrobenzoic acid), (Serva).

Cation influx measurements

For $^{86}Rb^+$ influx measurements diamide treated as well as untreated (control) cells were washed twice in the Cl^--containing physiological ionic strength solution (see above). After this the cells were washed once in a medium which had the same composition as the flux medium (except for K(^{86}Rb)Cl and inhibitors). In the experiments where Cl^- was replaced by $CH_3SO_4^-$, the cells were treated as described by Dunham et al. [9] in a medium consisting of (mM): $NaCH_3SO_4$, 165; glucose, 10; NaH_2PO_4/Na_2HPO_4, 5.8 (pH 7.4) at room temperature. This $CH_3SO_4^-$-containing physiological ionic strength solution as well as the low ionic strength solution of composition (mM): sucrose, 250; glucose, 10; NaH_2PO_4/Na_2HPO_4, 5.8 (pH 7.4) had the same tonicity as the Cl^--containing physiological ionic strength solution (300 mosM, measured with a vapour pressure osmometer). For $^{22}Na^+$ influx measurements the same procedure was used except that 145 mM NaCl in the wash and flux solution was replaced by 145 mM KCl, and 5.8 mM KH_2PO_4/K_2HPO_4 was used.

The cells were suspended at about 5% hematocrit in a total volume of 0.95 ml of flux medium. Influxes were measured in physiological and in low ionic strength solutions given above. For measuring the residual cation influxes ouabain (Serva), bumetanide (Lovens Kemise Fabr. Kobenhavn), and Na-EGTA were added to these flux media to a final concentration of 0.1 mM.

After a 5 min pre-incubation (37°C) 0.05 ml of a radioactive stock solution (^{86}RbCl + KCl, ^{86}RbCl + KCH_3SO_4, or ^{22}NaCl + NaCl) was added to give a final concentration of 7.5 mM. The labelled suspensions (radioactivity about 50 kBq per ml) were incubated for another 30 min at 37°C in a shaking water bath. The influxes were stopped after this period by quick centrifugation and replacing the extracellular solution with 1 ml of ice-cold $MgCl_2$ solution composed of (mM): $MgCl_2$, 107; Mops, 10 (pH 7.4). Additionally, the samples were washed four times in this $MgCl_2$ solution (15000 × *g*; 10 s).

The cell pellet was lysed with 0.5 ml of 0.1% (v/v) Triton X-100 and the protein precipitated by adding 0.5 ml of 5% (w/v) trichloroacetic acid. After 5 min centrifugation the activity of $^{86}Rb^+$ in the supernatant was determined by Cerenkov counting and $^{22}Na^+$ activity was determined by liquid scintillation counting in an Ultrabeta Liquid Scintillation Counter. The specific activity of the $^{86}Rb^+$ and $^{22}Na^+$ solutions was determined by counting a suitable sample of the radioactive stock solution. Haemoglobin content of the erythrocyte suspensions was determined as cyanomethemoglobin content using Drabkin's reagent [10].

For statistical analysis Student's or Welch's *t*-test were used ($P < 0.05$).

Results and Discussion

Diamide as a specific oxidant for sulfhydryl groups cross-links membrane proteins [1,2,7] and leads to a concomitant loss of red cell deformability [11,12], to phospholipid distribution changes [13] and to destabilization of the membrane bilayer [14]. These changes of the state of membrane sulfhydryl groups can be detected by measuring the GSH level of the cells [1,2].

The mean cellular GSH level was determined at 2.11 ± 0.50 mM ($n = 25$) and did not differ from the value reported for human erythrocytes [15]. After a pre-treatment of the erythrocytes with 10 mM iodoacetate and a subsequent incubation with 5 mM diamide the GSH content decreased to 0.09 ± 0.08 mM ($n = 14$).

The total as well as the residual K^+ influx of human erythrocytes in solutions of different ionic strength containing Cl^- or $CH_3SO_4^-$ as the main anion before and after treatment with 5 mM diamide are shown in Table I. In line with earlier results obtained on control cells suspended in Cl^--containing low ionic strength solution the total influx was found to be decreased in

TABLE I

Effects of a pre-treatment of human erythrocytes with diamide on total and residual K^+ influxes in media of variable ionic composition

Cells were suspended in solutions of physiological and low ionic strength with chloride or methylsulfate as the main anion in the presence or absence of the transport inhibitors ouabain (0.1 mM), bumetanide (0.1 mM), and EGTA (0.1 mM) before and after treatment of the cells with 10 mM iodoacetate and 5 mM diamide successively. $[^{86}RbCl+KCl]_0$ (or $[^{86}RbCl+KCH_3SO_4]_0$) was 7.5 mM. Results represent mean values ± S.D. The number of independent experiments is given in parenthesis. All values obtained on diamide treated erythrocytes are significantly different ($P < 0.05$) from control values. The total K^+ influx of control cells in physiological ionic strength solution is significantly different ($P < 0.05$) for Cl^- and $CH_3SO_4^-$ media.

Extra cellular main anion	Ionic strength	Inhibitors	K^+ influx (mmol/l cells per h)	
			control	iodoacetate + diamide
Cl^-	physiol.	-	2.97 ± 0.58 (3)	4.89 ± 1.11 (3)
Cl^-	physiol.	+	0.11 ± 0.02 (3)	5.60 ± 0.91 (3)
Cl^-	low	-	1.90 ± 0.29 (3)	7.70 ± 1.34 (3)
Cl^-	low	+	0.98 ± 0.34 (3)	8.11 ± 1.04 (3)
$CH_3SO_4^-$	physiol.	-	1.92 ± 0.11 (4)	6.82 ± 0.65 (4)
$CH_3SO_4^-$	physiol.	+	0.09 ± 0.03 (4)	7.03 ± 0.97 (4)
$CH_3SO_4^-$	low	-	1.96 ± 0.19 (4)	9.70 ± 2.83 (4)
$CH_3SO_4^-$	low	+	1.19 ± 0.27 (4)	9.72 ± 2.31 (4)

comparison to the flux in Cl^--containing physiological ionic strength solution [16], whereas the residual transport was significantly increased in Cl^- as well as in $CH_3SO_4^-$ media [5,17–20].

We now found that a 5 mM diamide treatment of the erythrocytes produced an increased total as well as residual K^+ influx in both physiological and low ionic strength media. It has been demonstrated that the low ionic strength-induced enhancement of the residual cation influx in untreated cells cannot be explained on the basis of a changed membrane potential [4,5]. Assuming electrodiffusion the residual K^+ influx in low ionic strength solution (low extracellular Cl^- concentration) should decrease three fold in comparison to physiological ionic strength solution (physiological Cl^- concentration). Such a decrease was not found. On the contrary, for untreated (control) cells and diamide-treated cells residual K^+ influxes increased. It is concluded that in both cases the measured residual K^+ influx cannot be exclusively explained by simple electrodiffusion.

In untreated (control) cells no differences between the residual K^+ influx in Cl^- and in $CH_3SO_4^-$ media were found. For these cells the total K^+ influx in Cl^--containing physiological ionic strength solution was significantly different ($P < 0.05$) from the total K^+ influx in $CH_3SO_4^-$-containing physiological ionic strength solution. This is due to a decrease of the Na/K/Cl cotransport after the replacement of Cl^- by $CH_3SO_4^-$ in physiological ionic strength solution [9]. The diamide-induced K^+ influxes were not Cl^--dependent, since no signifinant differences could be observed under conditions where Cl^- was replaced by $CH_3SO_4^-$ (Table I). These results suggest that diamide treatment does not stimulate the K/Cl cotransport pathway.

After diamide treatment no significant differences between the corresponding total and residual K^+ influxes could be measured. This would be expected when one considers a possible inhibition by diamide of the Na^+ pump as well as of the Na/K/Cl cotransport, which have been observed for other SH-reagents [21,22].

For reversibility experiments the cells were incubated with diamide without an iodoacetate pre-incubation. The GSH content reached the same level (0.09 ± 0.03 mM ($n = 10$)) as with iodoacetate pre-incubation (see above). DTE as a reducing substance [7] reconstituted the cellular GSH concentration to about 100% of the control value (2.21 ± 0.80 ($n = 3$)). Table II shows that the K^+ influx enhancement caused by diamide was only partly reversible under these conditions. This

TABLE II

Reversibility of the diamide-induced increase of total and residual K^+ influxes into human erythrocytes

Cells were treated with 5 mM diamide or 5 mM diamide and 10 mM DTE successively. $[^{86}RbCl+KCl]_0$ was 7.5 mM. Results represent mean values ± S.D. from three independent experiments. All influx values obtained from diamide or diamide and DTE treated cells are significantly different ($P < 0.05$) from control values (no additives). The results represent a set of experiments different from that in Table I.

Cells treated with	K^+ influx (mmol/l cells per h)			
	physiol. ionic strength		low ionic strength	
	total	residual	total	residual
None	2.94 ± 0.01	0.10 ± 0.01	1.83 ± 0.04	0.86 ± 0.16
Diamide	6.17 ± 0.50	5.25 ± 0.33	8.60 ± 0.22	8.94 ± 0.88
Diamide + DTE	4.75 ± 0.25	3.98 ± 0.22	6.76 ± 0.55	7.53 ± 0.44

TABLE III

Effects of a pre-treatment of human erythrocytes with diamide on total and residual Na^+ influxes in media of variable ionic strength

Cells were treated with 10 mM iodoacetate and 5 mM diamide successively. $[^{22}NaCl + NaCl]_0$ was 7.5 mM. Results represent mean values ± S.D. from three independent experiments. All influx values obtained on iodoacetate and diamide treated cells are significantly different ($P < 0.05$) from control values (no additives).

Cells treated with	Na^+ influx (mmol/l cells per h)			
	physiol. ionic strength		low ionic strength	
	total	residual	total	residual
None	0.40 ± 0.02	0.40 ± 0.01	0.92 ± 0.04	1.15 ± 0.06
Iodoacetate + diamide	3.97 ± 0.70	4.20 ± 0.59	17.75 ± 1.60	17.31 ± 2.28

result implies that not only the sulfhydryl group cross-linking is responsible for the enhancement of the K^+ influxes. It cannot be decided whether this effect is due to secondary irreversible changes in the membrane properties evoked by the cross-linking mechanism or due to diamide-induced membrane changes independent of the sulfhydryl cross-linking.

Since the K^+ influx measured by our method was independent of the duration of exposure of the cells to isotope (20–45 min) (data not shown), this method was suitable for investigating diamide-stimulated influxes. Furthermore there were no effects of iodoacetate or DTE itself on K^+ influx (data not shown).

To find out whether the diamide-induced stimulation of the ion transport is specific for K^+ we investigated the diamide effect (5 mM) on Na^+ influx (Table III). The total as well as the residual Na^+ influxes of untreated cells at low ionic strength were 2–3-fold higher than those at physiological ionic strength. For diamide-treated cells the increase was 4-fold. The diamide treatment increased the total as well as the residual Na^+ influx at physiological ionic strength about 10-fold. In low ionic strength this increase was about 17-fold. This supports the idea that the diamide-induced enhancement of K^+ influx is not due to a stimulation of the K/Cl cotransport system.

These results are different from the finding of Lauf [3] in LK sheep erythrocytes. In sheep erythrocytes the enhancement of the cation flux induced by diamide (2 mM) is specific for K^+ (not for Na^+), Cl^--dependent and fully reversible after treating the cells with dithiothreitol (DTT) which also restores the GSH level to the original value. In addition, DTT alone reduced the ouabain-insensitive, furosemide-sensitive K^+ influx of LK sheep erythrocytes by 30% [23].

The observation that there is no significant difference between the total and the residual Na^+ influx in both solutions (physiological and low ionic strength) is a consequence of the absence of any ouabain-sensitive Na^+ influx as well as an inhibition of the Na/K/Cl cotransport under the experimental conditions (low extracellular NaCl concentration).

Summarizing, the results suggest that the diamide-induced cation influxes in human erythrocytes are neither a result of a stimulation of the K/Cl cotransport system nor due to electrodiffusion.

References

1 Deuticke, B., Poser, P., Lütkemeier, P. and Haest, C.W.M. (1983) Biochim. Biophys. Acta 731, 196–210.
2 Deuticke, B., Lütkemeier, P. and Sistemich, M. (1984) Biochim. Biophys. Acta 775, 150–160.
3 Lauf, P.K. (1988) J. Membr. Biol. 101, 179–188.
4 Bernhardt, I., Hall, A.C. and Ellory, J.C. (1988) Stud. Biophys. 126, 5–21.
5 Bernhardt, I., Hall, A.C. and Ellory, J.C. (1991) J. Physiol. 434, 489–506.
6 Hall, A.C. and Ellory, J.C. (1986) Biochim. Biophys. Acta 858, 317–320.
7 Haest, C.W.M., Kamp, D., Plasa, G. and Deuticke, B. (1977) Biochim. Biophys. Acta 469, 226–230.
8 Beutler, E., Duron, O. and Kelly, B.M. (1963) J. Lab. Med. 61, 882–888.
9 Dunham, P.B., Stewart, G.W. and Ellory, J.C. (1980) Proc. Natl. Acad. Sci. USA 77, 1711–1715.
10 Drabkin, D.L. and Austin, J.H. (1935) J. Biol. Chem. 112, 51–54.
11 Kon, K., Maeda, N. and Shiga, T. (1987) Blood 69, 727–734.
12 Hessel, E., Meier, W. and Bäumler, H. (1988) Biorheology 25, 803–816.
13 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32.
14 Maeda, N., Kon, K., Imaizumi, K., Sekiya, M. and Shiga, T. (1983) Biochim. Biophys. Acta 735, 104–112.
15 Haest, C.W.M., Kamp, D. and Deuticke, B. (1979) Biochim. Biophys. Acta 557, 363–371.
16 Bernhardt, I., Erdmann, A., Ihrig, I., Seidler, G., Schmidt, G. and Zöllner, K. (1989) Stud. Biophys. 134, 133–138.
17 Davson, H. (1939) Biochem. J. 33, 389–401.
18 Donlon, J.A. and Rothstein, A. (1969) J. Membr. Biol. 1, 37–52.
19 Bernhardt, I., Donath, E. and Glaser, R. (1984) J. Membr. Biol. 78, 249–255.
20 Jones, G.S. and Knauf, P.A. (1985) J. Gen. Physiol. 86, 721–738.
21 Hart, W.M. and Titus, E.O. (1973) J. Biol. Chem. 248, 1365–1371.
22 Lauf, P.K., Adragna, N.C. and Garay, R.P. (1984) Am. J. Physiol. 246, 385–390.
23 Lauf, P.K. (1984) J. Membr. Biol. 82, 167–178.