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Chloride mediated inhibition of the phosphate and the sulfate transport by dipyridamole in human erythrocyte ghosts

M. Renner, M. Dietl and K.F. Schnell

Institut für Physiologie, Universität Regensburg, Postfach 397, D-84 Regensburg, FRG

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The inhibition of the unidirectional phosphate and sulfate flux in human erythrocyte ghosts by dipyridamole has been investigated. The inhibition of the phosphate and the sulfate flux is mediated by chloride. At zero chloride, dipyridamole was found to be completely ineffective. At 10 mM chloride, dipyridamole acts as a noncompetitive inhibitor of the phosphate and the sulfate flux and elicits an up to 95% inhibition of the phosphate and the sulfate transport. The results of our studies provide strong evidence for a cooperative binding of chloride and of dipyridamole to the non-protonated form of the band 3 membrane domain.

Anion transport; Dipyridamole; (Erythrocyte, Human)

1. INTRODUCTION

Anion transport across the erythrocyte membrane is mediated by band 3. Most of the anion transport inhibitors, as studied so far, cause a concomitant inhibition of the chloride, bicarbonate, phosphate and sulfate transport, but the molecular mechanism of the transport inhibition is not yet known. Some of the inhibitors like salicylate, DNP or DNDS act as competitive inhibitors of the anion transport while other inhibitors like phlorizin, dipyridamole or niflumic acid display either a noncompetitive or a mixed-type inhibition [1–5]. Detailed studies concerning the relation between the inhibition of chloride and sulfate self-exchange by DNDS have shown a 1:1 relation between the inhibition of the chloride and the sulfate self-exchange flux [6]. These observations clearly indicate that both the chloride and the sulfate self-exchange are mediated by the same transport system (for review see [7–9]).

The present paper is concerned with the inhibition of the sulfate and the phosphate flux in human erythrocyte ghosts by dipyridamole. Dipyridamole is a noncompetitive inhibitor of phosphate and sulfate transport [1,2]. In contrast to other inhibitors, the inhibition of phosphate and sulfate flux by dipyridamole is mediated by chloride. In the absence of chloride, the phosphate and sulfate fluxes are insensitive to dipyridamole while only small amounts of chloride suffice to cause an almost complete inhibition of phosphate and sulfate transport. The results of our studies suggest a cooperative binding of chloride and of dipyridamole to the non-protonated form of the band 3 transport domain.

Correspondence address: K.F. Schnell, Institut für Physiologie, Universität Regensburg, D-8400 Regensburg, FRG

Abbreviations: DIDS, 4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid, K-salt; DNDS, 4,4′-dinitro-2,2′-stilbenedisulfonic acid, K salt; DNP, 2,4-dinitrophenol; 5-doxylstearic acid, 5-4,4′-dimethyloxazolidine-*N*-oxyl-stearic acid; 16-doxylstearic acid, 16-4,4′-dimethyloxazolidine-*N*-oxyl-stearic acid; FDNB, 1-fluoro-2,4-dinitrophenol; NDS-TEMPO, *N*-4-(2,2,6,6-tetramethyl-1-oxyl)piperidinyl-*N*′-4,4′-nitro-2,2′-stilbenedisulfonic acid)thiourea, K salt

2. MATERIALS AND METHODS

Erythrocytes were obtained from fresh ACD blood of healthy

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adult donors. In order to remove the intracellular chloride, the red blood cells were washed and preincubated for 10 min in 20 vols of an isotonic, 132 mM K-phosphate or 132 mM K-sulfate solution (pH 7.3, 37°C). Throughout the washing procedure, plasma and buffy coat were removed. Erythrocyte ghosts were prepared by osmotic hemolysis (5 min, 0°C, pH 6.2) of fresh human erythrocytes and subsequent resealing (45 min, 37°C, pH 7.2) of the erythrocyte ghosts in isotonic (330 mosM) or double-isotonic (660 mosM) K-[^{32}P]phosphate/40 mM K-citrate/sucrose, K-[^{35}S]sulfate/40 mM K-citrate/sucrose or K-[^{36}Cl]chloride/40 mM K-citrate/sorbitol solutions. K-citrate is required for the resealing of the red blood cells, sucrose and sorbitol were used for osmotic substitution. The yield of resealed erythrocyte ghosts is approx. 90%. The cell number was counted with a Coulter counter. 1 g cells wet wt (centrifugation 5000 × *g*, 10 min, pH 7.3, 20°C) correspond to 1.10×10^{10} cells.

The unidirectional fluxes of phosphate, sulfate and chloride were calculated from the rate constant of the tracer back-exchange and from the intracellular phosphate, sulfate or chloride. The intracellular phosphate, sulfate and chloride, was calculated from the intracellular [^{32}P]phosphate, [^{35}S]sulfate and [^{36}Cl]chloride and the specific activities of phosphate, sulfate or chloride in the resealing solution. The rate constant of the tracer back-exchange was assessed by incubating the radioactively labelled erythrocyte ghosts in a nonradioactive back-exchange solution supplemented with dipyridamole and measuring the extracellular [^{32}P]phosphate, [^{35}S]sulfate and [^{36}Cl]chloride at suitable time intervals. The phosphate and the sulfate flux experiments were performed at 25°C, with 10% (w/v) suspension while the chloride flux experiments were executed with 1% (w/v) suspensions of resealed erythrocyte ghosts at 0°C. Erythrocyte ghosts and incubation solution were separated either by centrifugation or by means of a filtration technique [10]. The rate constants for the tracer back-exchange were obtained by fitting the curves of cpm versus time to an exponential function. For details see [11,12].

Tri-magnesium dicitrate, K-citrate, citric acid, KCl, NaCl, KH_2PO_4, $K_2HPO_4 \cdot 3H_2O$ and TCE and sucrose of p.a. grade were purchased from Merck, Darmstadt, FRG. Sorbitol (pure) was obtained from Serva, Heidelberg, FRG. [^{32}P]Phosphate, [^{35}S]sulfate and [^{36}Cl]chloride were supplied by Amersham-Buchler, Braunschweig, FRG. DIDS was synthesized according to [13]. Dipyridamole (charge FN 4184) was obtained from Dr Karl Thomae GmbH, Biberach, FRG.

3. RESULTS

The inhibition of the phosphate, sulfate and the chloride self-exchange in human erythrocyte ghosts by dipyridamole has been studied. All experiments were performed under self-exchange conditions, where the intracellular and the extracellular concentrations of the substrate-anion except of the tracer are at equilibrium. The unidirectional fluxes of phosphate, sulfate and chloride were calculated from the rate of the tracer back-exchange and the intracellular phosphate, sulfate or chloride as delineated in section 2. Fig.1 shows the inhibition of the unidirectional phosphate and the unidirectional sulfate flux in human erythrocyte ghosts by dipyridamole at 0 mM and at 10 mM chloride. At zero chloride, dipyridamole was found to be ineffective, but at 10 mM chloride, dipyridamole causes an inhibition up to 95% of the unidirectional phosphate and sulfate flux.

The inhibition of the unidirectional phosphate flux by dipyridamole at a fixed chloride concentration of 20 mM is shown in fig.2. The Dixon plots of $1/J_P$ versus the concentration of dipyridamole gave straight lines which intersect on the I axis. Similar results have been obtained with sulfate. This pattern of inhibition is indicative of a non-competitive type of inhibition. The apparent dipyridamole inhibition constant from the phosphate and from the sulfate flux experiments at 10–20 mM chloride is in the range of 2.5–5.0 μM (pH 7.2, 25°C).

Fig.3 shows the inhibition of the unidirectional phosphate flux by low concentrations of chloride at zero and at 10 μM dipyridamole. At zero dipyridamole, 5 mM chloride elicit an 5–10% inhibition of the phosphate and the sulfate flux (not shown), but at 10 μM dipyridamole 5 mM chloride are sufficient to induce an almost complete inhibition. The respective Dixon plots of the reciprocal phosphate flux versus the chloride concentration intersect above the I axis and close to the $1/J_P$ axis, indicating a competitive inhibition of the phosphate and the sulfate flux by chloride. In the absence of dipyridamole, the chloride inhibition constants from the phosphate and the sulfate flux experiments were 45 ± 8 mM (3 expts) and 36 ± 7 mM (3 expts), respectively. At 10 μM dipyridamole, however, the chloride inhibition constants from the phosphate and from the sulfate flux experiments amounted to 0.52 ± 0.12 mM (4 expts) and to 0.62 ± 0.18 mM (mean ± SD, 3 expts, pH 7.2, 25°C). Thus the chloride inhibition constant at zero dipyridamole is approximately 100 times higher than the chloride inhibition constant at 10 μM dipyridamole.

Correspondingly, the inhibitory effect of chloride upon the unidirectional phosphate flux is reinforced by dipyridamole (table 1). At 5 μM dipyridamole, approximately 10 mM chloride are

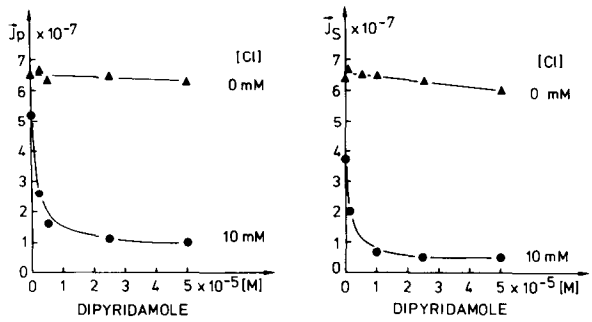

Fig.1. Inhibition of the unidirectional phosphate flux (left side) and of the unidirectional sulfate flux (right side) by dipyridamole at 0 and at 10 mM KCl. $\vec{J}_P$ and $\vec{J}_S$ [mol/(min × g cells)], unidirectional fluxes of phosphate or sulfate. (Abscissa) Dipyridamole [M]. 10% (w/v) suspension of resealed erythrocyte ghosts, pH 7.2, 25°C. Incubation solution, 90 mM K-phosphate, 40 mM K-citrate, KCl as indicated in the figure.

required for a half-maximal inhibition of the unidirectional phosphate flux while at 50 μM dipyridamole, approximately 2 mM chloride are sufficient to elicit a half-maximal inhibition. At 50 μM dipyridamole about 10 mM chloride are sufficient to induce a maximum inhibition of the unidirectional phosphate flux. In contrast, at 10 μM dipyridamole more than 100 mM chloride would be required in order to reach a maximum inhibition of the phosphate transport. The results shown in fig.3 and in table 1 suggest that chloride

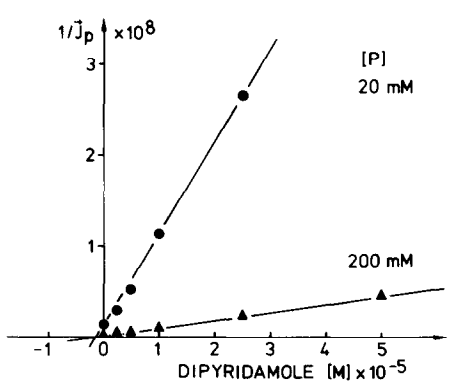

Fig.2. Inhibition of the unidirectional phosphate flux by dipyridamole at 20 mM KCl. Dixon plot of the reciprocal phosphate flux, $1/\vec{J}_P$ [min × g cells × mol^{-1}], vs the dipyridamole concentration. 10% (w/v) suspensions of erythrocyte ghosts, 25°C, pH 7.2. K-phosphate [P] as indicated in the figure, 40 mM K-citrate, 20 mM KCl, osmotic substitution up to 660 mosM was made by sucrose.

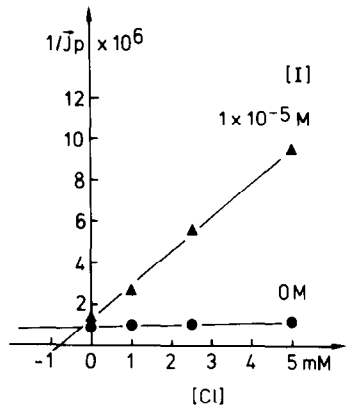

Fig.3. Inhibition of the unidirectional phosphate flux by chloride at 0 and at 10 μM dipyridamole. Dixon plot: The reciprocal phosphate flux, $1/\vec{J}_P$ [min × g cells × mol^{-1}] is plotted vs the chloride concentration, [Cl]. 10% (w/v) suspension of erythrocyte ghosts, pH 7.2, 25°C. 132 mM K-phosphate, 40 mM K-citrate, dipyridamole [I] as indicated in the figure.

and dipyridamole act as cooperative inhibitors of phosphate and sulfate transport in erythrocyte ghosts.

Concerning chloride transport in erythrocyte ghosts, dipyridamole acts as a mixed-type inhibitor. The dose response curves and the respective Dixon plots are shown in fig.4. The plots of the reciprocal chloride flux versus the concentration of dipyridamole are linear and intersect below the I axis. The Dixon plots intersect at 6.3 ± 1.5 μM dipyridamole (mean ± SD, 4 expts), but

Table 1

Effect of chloride upon the inhibition of the unidirectional phosphate flux by dipyridamole (25°C, pH 7.2)

[Cl] (mM)	Dipyridamole			
	5 μM		50 μM	
	$\vec{J}_P$ [mol/(min × g cells)]	$\vec{J}_{P_i}/\vec{J}_{P_o}$ (%)	$\vec{J}_P$ [mol/(min × g cells)]	$\vec{J}_{P_i}/\vec{J}_{P_o}$ (%)
0	2.68×10^{-7}	100.0	2.08×10^{-7}	100.0
5	1.68×10^{-7}	62.7	3.32×10^{-8}	15.9
10	1.28×10^{-7}	47.9	1.62×10^{-8}	7.8
25	8.82×10^{-8}	32.9	1.02×10^{-8}	4.9
50	4.53×10^{-8}	16.9	8.52×10^{-9}	4.1
100	3.97×10^{-8}	14.8	9.15×10^{-9}	4.4

10% suspension of erythrocyte ghosts

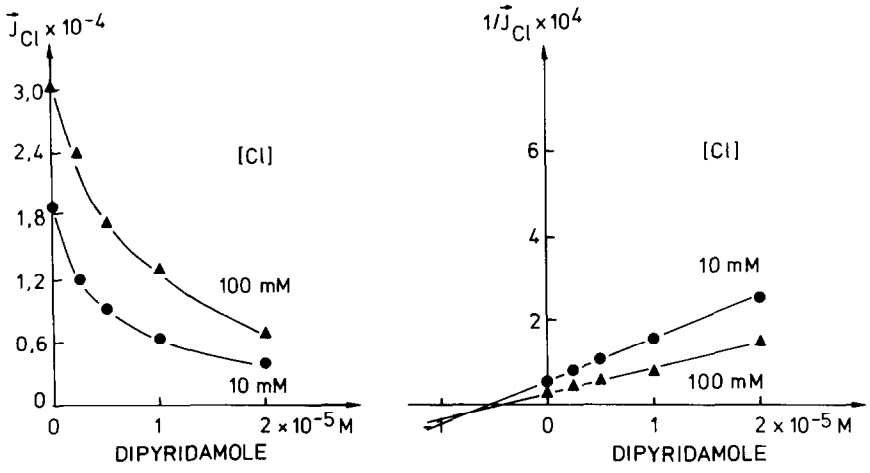

Fig.4. Inhibition of the unidirectional chloride flux by dipyridamole. (Left panel) Dose response curves; (right panel) Dixon plots. $\vec{J}_{Cl}$ [mol/(min × g cells)] is the unidirectional chloride flux. 1% suspension of resealed erythrocyte ghosts, pH 7.3, 0°C. Chloride as indicated in the figures.

the dipyridamole inhibition constant from the chloride flux experiments is in accordance with the dipyridamole inhibition constants from the phosphate and the sulfate flux experiments.

The pH dependence of $K_{i\,app}$, the apparent dipyridamole inhibition constants from the phosphate and sulfate flux experiments with erythrocyte ghosts are listed in table 2. At 10 mM chloride, the dipyridamole inhibition of the phosphate and sulfate flux experiments passes through a minimum. In contrast the apparent dipyridamole inhibition constant, at zero chloride, decreases as pH is elevated. At 10 mM chloride and pH 7.2, the apparent dipyridamole inhibition constant amounts to approx. 3.0 μM and is at least 20 times lower than the dipyridamole inhibition constant in chloride-free solutions.

Table 2

pH dependence of the apparent dipyridamole inhibition constant

pH	$K_{i\,app}$ [μM] 132 mM K-phosphate, 115 mM K-citrate, 10 mM KCl (25°C, 660 mosM)	$K_{i\,app}$ [μM] 132 mM K-phosphate, 122 mM K-citrate, 0 mM KCl (25°C, 660 mosM)
6.4	9.16 (2)	>150 (1)
7.2	2.52 ± 0.54 (3)	>100 (1)
8.0	3.44 ± 0.62 (3)	75 (2)
8.6	3.82 ± 0.42 (3)	55 (1)
	90 mM K-sulfate, 40 mM K-citrate, 10 mM KCl (25°C, 330 mosM)	90 mM K-sulfate, 40 mM K-citrate/ 20 mM sucrose, 0 mM KCl (25°C, 330 mosM)
6.4	7.5 (1)	>100 (1)
7.2	4.1 ± 1.2 (5)	>100 (1)
8.2	5.2 ± 1.3 (3)	75 (1)

10% suspensions of resealed erythrocyte ghosts, 25°C, pH 7.2. Mean ± SD, the number of experiments is given in parentheses

4. DISCUSSION

Anion transport across the erythrocyte membrane is inhibited by a great many substances. Competitive, reversible inhibitors of the anion transport like salicylate, and DNP or DNDS could interact either with the band 3 substrate-site or they could bind to an allosteric inhibitor site which is distinct from the band 3 substrate-site. Non-competitive, reversible inhibitors of the anion transport such as phlorizin, niflumic acid or dipyridamole, could induce a conformational change of the band 3 which inhibits the binding of the substrate-anions, the translocation of bound substrate-anions or both of these reactions.

Dipyridamole exhibits a specific pattern of interaction with the band 3 membrane domain. The inhibition of the phosphate and the sulfate

transport by dipyridamole is mediated by chloride. As studied so far, none of the above inhibitors require chloride for their efficacy. With regard to phosphate and to sulfate transport dipyridamole acts as a noncompetitive inhibitor, while for chloride transport dipyridamole acts as a mixed-type inhibitor. Since the pK_1 of dipyridamole in aqueous solutions is 6.3 most of the dipyridamole is protonated at physiological pH. Thus the binding of dipyridamole to the band 3 substrate-site introduces a positive electrical excess charge which probably is counterbalanced by the binding of the negatively charged chloride anion. The failure to inhibit phosphate and sulfate transport in chloride free solutions suggests that dipyridamole cannot bind to the protonated form of band 3 which is responsible for the phosphate and the sulfate transport across the erythrocyte membrane. ESR studies from our laboratory with the stilbene-disulfonic acid spin label, NDS-TEMPO, 5-doxylstearic acid and 16-doxylstearic acid indicate that dipyridamole acts on the band 3 transport protein itself and not on the lipid domain of the red cell membrane (unpublished).

The dipyridamole inhibition constants from the phosphate and the sulfate flux experiments at 10–20 mM chloride were in the range of 2.5–5.0 μM and agree fairly well with the dipyridamole inhibition constants from the chloride flux experiments which amount to approximately 6 μM. At zero dipyridamole, the chloride inhibition constant from the phosphate and from the sulfate self-exchange experiments is ~40 mM while at 10 μM dipyridamole the chloride inhibition constant is reduced to ~0.5 mM. Correspondingly, the dipyridamole inhibition constant decreases from ~100 μM at zero chloride to ~3 μM at 10 mM chloride. The increasing affinity for chloride in the presence of dipyridamole and the increasing affinity for dipyridamole in the presence of chloride provide strong evidence for a cooperative binding of chloride and dipyridamole to the anion transporter of the red cell membrane.

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