SIDEDNESS OF THE INHIBITORY ACTION OF DISULFONIC ACIDS ON CHLORIDE EQUILIBRIUM EXCHANGE AND NET TRANSPORT ACROSS THE HUMAN ERYTHROCYTE MEMBRANE

Jack H. KAPLAN*, Kay SCORAH, Hugo FASOLD and Hermann PASSOW

Max-Planck-Institut für Biophysik, Frankfurt/M, and Institut für Biochemie der Universität, Frankfurt/M West Germany

Received 28 November 1975

1. Introduction

The characterization of membrane transport systems requires a study of the sidedness of the effects of modifying agents. Previous work with phlorizin showed that although this agent inhibits sugar transport at either surface of the red cell membrane, the effects on anion transport – and acceleration of Cl^- net movements [1] and an inhibition of Cl^- or SO_4^{2-} equilibrium exchange [2,3] – can only be observed if the agent is added to the external medium. Intracellular phlorizin was without effect except for a slight acceleration of Cl^-/Cl^- equilibrium exchange at low pH [4]. Similar studies on the sidedness of the effects of two disulfonic acids, 4,4′-diacetamido stilbene-2,2′-disulfonic acid (DAS) and 2-(4′-amino-phenyl)-6-methylbenzene-thiazole-3′,7-disulfonic acid (APMB), revealed that the former compound inhibits SO_4^{2-} equilibrium exchange only at the outer membrane surface while APMB produced a strong inhibition at both surfaces [5]. The present work characterizes the actions of these two inhibitors on Cl^- transport as measured under conditions of Cl^- equilibrium exchange, and net flow. The sidedness was the same under both experimental conditions. This indicates that, although Cl^- equilibrium exchange proceeds about 10^5 times faster than SO_4^{2-} equilibrium exchange the transport processes for both ion species may share part of the same transport pathway. The fact that APMB acted on both surfaces while DAS only acted on one surface suggests that two different types of binding sites are involved in the inhibition of anion transport by the two agents and that only one of these binding sites could possibly be located on a mobile carrier which is capable of crossing the erythrocyte membrane.

*Present address: Dept. of Physiology, Yale Univ. Medical School, New Haven, Conn. 06510, USA.

2. Materials and methods

Resealed ghosts were prepared as described previously [6] from the blood of healthy donors which had been stored in acid-citrate-dextrose buffer for no more than 5 days. Rate constants for valinomycin-induced K^+/K^+ equilibrium exchange and net KCl movements were measured as reported by Kaplan and Passow [1] and for Cl^-/Cl^- equilibrium exchange as reported by Schnell et al. [2] using the filtration technique of Dalmark and Wieth [7]. The concentrations of internally trapped DAS and APMB were determined photometrically at 330 and 390 nm, respectively, after precipitation of the ghosts with 7% trichloroacetic acid. The intracellular concentrations were calculated using the hematocrit and making appropriate corrections for trapped extracellular medium and intracellular dry matter. The recovery of the agents inside the resealed ghosts varied between 89% and 103%.

3. Results and discussion

3.1. *Cl^- equilibrium exchange*

The rate constant for $^{36}Cl^-$ efflux at Donnan equilibrium at 0°C was 1.24 ± 0.04 min^{-1} (S.E.M. n = 15) and agreed with that previously obtained by Schnell et al. [2] of 1.22 ± 0.09 (S.E.M. n = 5). When present in the extracellular medium both DAS and APMB inhibited Cl^-/Cl^- exchange. Inhibition was half-maximal at 1 X 10^{-4} M for DAS (fig.1) and at 5 X 10^{-4} M (fig.2) for APMB. These results are similar to those for inhibition of sulfate exchange (half-maximal inhibition at 2 X 10^{-4} M and 1 X 10^{-3} M, respectively [5]). Internal DAS present up to a concentration which produces a maximal effect at the outer membrane surface produced no inhibition: The efflux in the presence of 2.0 mM internal DAS was 102.8 ± 3.45% of that in the control without incorporated DAS (S.E.M. n = 4). In contrast to DAS, APMB inhibited at either surface. However, the concentration required for half-maximal inhibition was about 5.5 times higher at the inner surface than at the outer surface (fig.2). The described findings are similar to those observed previously with sulfate [5] except that with sulfate the effectiveness of APMB was virtually identical at both membrane surfaces.

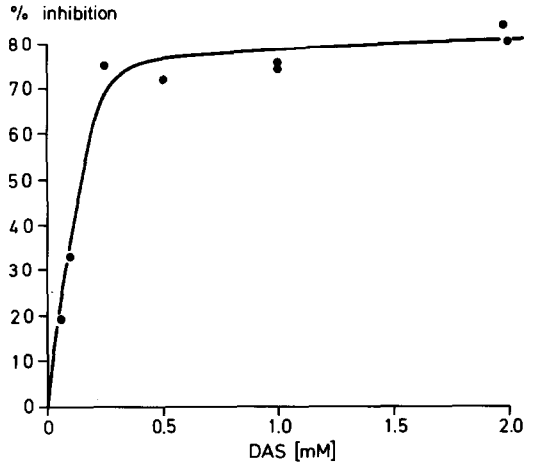

Fig.1. Concentration-dependence of inhibition of Cl^-/Cl^- exchange by extracellular DAS. $^{36}Cl^-$ efflux measured at 0°C, 1% hematocrit, from ghosts containing 140 mM KCl, 20 mM Tris-Cl, pH 7.2 and a trace of $^{36}Cl^-$, into media of the same composition without isotope, and with varying concentrations of DAS.

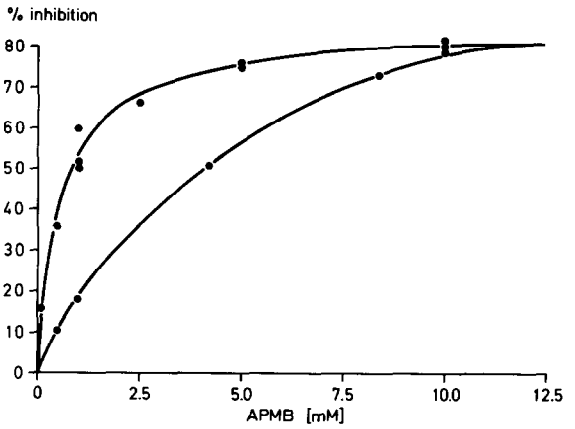

Fig.2. Concentration-dependence of inhibition of Cl^-/Cl^- exchange by APMB. Determination of rate of $^{36}Cl^-$ efflux as in legend to fig.1. Upper curve refers to extracellular APMB, lower curve to intracellular APMB.

3.2 Cl^- net movements as determined from valinomycin-induced net KCl loss and K^+/K^+ exchange

Addition of valinomycin to KCl loaded ghosts in a NaCl containing medium induces a net efflux of both K^+ and Cl^- which is accompanied by shrinkage. The rate constant for KCl efflux measured in the presence of 1 X 10^{-6} M valinomycin and in the absence of APMB or DAS was 0.125 ± 0.005 min^{-1} (S.E.M. n = 18, 37°C). Both external DAS and external APMB caused an inhibition of KCl loss, although neither agent affected the slow rate of leakage occurring in the absence of valinomycin (not shown). If applied at the inner membrane surface, only APMB caused inhibition (table 1).

The rate of the valinomycin-induced KCl loss depends on the permeabilities for both K^+ and Cl^-. For a separate determination of the permeability for Cl^-, P_{Cl}, it is necessary, therefore, to obtain an independent estimate of the permeability for K^+, P_K. Such independent estimate can be made (using $^{42}K^+$) if K^+/K^+ exchange is measured in the absence of KCl net movements at identical KCl concentrations in ghosts and external medium. When no inhibitors were present, the rate constant for K^+/K^+ exchange was 1.58 ± 0.09 min^{-1} (S.E.M. n = 20). Neither APMB nor DAS produced a measurable inhibition at either membrane surface (table 2). This suggests that the

Table 1
Effects of intracellular and extracellular DAS and APMB on valinomycin-induced KCl efflux from ghosts

Inhibitor (mM)	Membrane surface	% Inhibition on KCl efflux mean ± SEM
APMB (10 mM)	External	38.8 ± 3.5 (11)
APMB (10 mM)	Internal	37.6 ± 2.7 (7)
DAS (2 mM)	External	45.4 ± 2.6 (12)
DAS (2 mM)	Internal	1.2 ± 4.1 (8)

Ghosts containing 140 mM KCl, 20 mM Tris-Cl pH 7.2 added to media containing 140 mM NaCl, 20 mM Tris-Cl pH 7.2, $1 \cdot 10^{-6}$ valinomycin. Temperature 37°C. Hematocrit 1%. The figures in brackets indicate the numbers of experiments.

Table 2
Effects of intracellular and extracellular DAS and APMB on valinomycin-induced K^+/K^+ exchange

Inhibitor (mM)	Membrane surface	Rate constant ± SEM (min^{-1})
APMB (10 mM)	Internal	1.56 ± 0.11 (10)
APMB (10 mM)	External	1.33 ± 0.16 (6)
DAS (2 mM)	Internal	1.67 ± 0.13 (14)
DAS (2 mM)	External	1.29 ± 0.08 (6)

Ghosts containing 140 mM KCl, 20 mM Tris-Cl pH 7.2 and a trace of $^{42}K^+$ were prepared and the rate of efflux of $^{42}K^+$ into a medium containing 140 mM KCl, 20 mM Tris-Cl pH 7.2, $1 \cdot 10^{-6}$ M valinomycin, 37°C determined. Hematocrit 1%. The figures in brackets indicate the numbers of experiments.

effects on net KCl loss of these agents are due to alterations of P_{Cl} rather than P_K.

A quantitative estimate of the changes of P_{Cl} can be made if one assumes that the Goldman equation can be applied to the diffusion of the valinomycin–K^+ complex within the membrane and to the concomitant net Cl^- movements through the physiological chloride pathway [8]. Using the rate constant of K^+/K^+ equilibrium exchange indicated above as a measure of P_K, the application of the Goldman equation to the data on K^+ efflux into K^+ free media yields the P_{Cl} values indicated in table 3. It is apparent that APMB inhibits net chloride movements to about the same extent at either membrane surface while DAS is effective only at the outer membrane surface. These findings are qualitatively similar to those observed for Cl^-/Cl^- exchange.

Table 3
Analysis of effects of inhibitions on Cl^- ion permeability, P_{Cl}

Inhibitor	Membrane surface	${}^0k_K$ (min^{-1}) observed	P_{Cl} (min^{-1})	${}^0k_K$ (min^{-1}) calculated	% Reduction of P_{Cl}
None	–	0.125	0.0300	0.126	–
APMB	External	0.0765	0.0155	0.076	48.3
APMB	Internal	0.078	0.0160	0.078	46.7
DAS	External	0.068	0.0135	0.068	55.0
DAS	Internal	0.124	0.0292	0.123	2.7

A P_K value of 158 min^{-1} which is unaltered by the presence of inhibitors (see text) was employed and values for P_{Cl} have been calculated to give the best agreement between observed and calculated rate constants (${}^0k_K$) for KCl efflux under the experimental condition described in the legend to table 1. The Goldman equation in the form employed in [1] was used where $[K_i^+] = 0$, $[K_i^+] = 140$ mM, $[Cl_i^-] = [Cl_0^-] = 150$ mM.

No attempt was made to determine P_{Cl} from measurements of KCl efflux at varying K^+ concentrations in the medium as described by Hunter [8]. The Goldman equation predicts that with 140 mM intracellular K^+, at extracellular K^+ concentrations exceeding 10 mM, reduction of P_{Cl} by 90% is associated with changes of K^+ efflux of 13% or less which are barely detectable by our experimental method. The greatest changes and hence the highest sensitivity of the estimates for P_{Cl} are predicted for zero K^+ in the medium, i.e. for those conditions to which we have confined our measurements.

The evaluation of the data on net KCl movements in terms of the Goldman equation implies that net movements of Cl^- ions across the membrane proceed independent of and parallel to the Cl^-/Cl^- exchange which does not contribute to the conductance of the membrane. Although widely used, this assumption is rather arbitrary since a carrier mediated Cl^-/Cl^- exchange could easily account for the difference between the rates for net and exchange movements if one postulates that the flow of the loaded Cl^- carrier in one direction is compensated for by the flow of the unloaded carrier in the opposite direction. However, the qualitative conclusions concerning the sidedness of the effects of APMB and DAS on the Cl^- net movements associated with KCl efflux induced by valinomycin are independent of the use of the Goldman equation. In fact, the similarity of the sidedness of the effects of APMB and DAS on net and exchange permeability would suggest that both processes are mediated by a single transport system.

The finding that APMB inhibits Cl^-/Cl^- exchange at either surface is clearly compatible with the assumption that the inhibitory site in the membrane is associated with a mobile carrier whereby the quantitative difference between the inhibitory power at the inner and outer surface could easily be attributed to differences of the environment in which the carrier reacts with the agent. On the other hand, the existence of fixed anion permeability controlling sites in either surface would equally well account for our observations. The findings with DAS are not easily reconcilable with the assumption of a reaction with a mobile site which can penetrate across the membrane. The complete absence of an inhibitory effect at the inner membrane surface at a concentration which produces nearly maximal inhibition at the outer surface could reflect a difference in accessibility rather than affinity and points to a reaction with a fixed site in the outer membrane surface. In this respect, the results obtained with DAS reinforce those of our previous work with phlorizin which showed the same sidedness of its effect on Cl^- net and exchange permeability as DAS.

References

[1] Kaplan, J. H. and Passow, H. (1974) J. Membrane Biol. 19, 179–194.
[2] Schnell, K. F., Gerhard, S., Lepke, S. and Passow, H. (1973) Biochim. Biophys. Acta 318, 474–477.
[3] Lepke, S. and Passow, H. (1973) Biochim. Biophys. Acta 298, 529–533.
[4] Schnell, K. F. (1975) Habilitationsschrift, Regensburg.
[5] Zaki, L., Fasold, H., Schuhmann, B. and Passow, H. (1975) J. Cell. Physiol., in the press.
[6] Schwoch, G. and Passow, H. (1973) Mol. Cell. Biochem. 2, 197–218.
[7] Dalmark, M. and Wieth, J. O. (1972) J. Physiol. 224, 583–610.
[8] Hunter, M. J. (1971) J. Physiol. 218, 49P.