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Asymmetric inhibition by phlorizin of sulfate movements across the red blood cell membrane

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SUMMARY

Measuring SO_4^{2-} exchange across the red cell membrane at Donnan equilibrium, it was found that extracellular phlorizin inhibited SO_4^{2-} movements while intracellular phlorizin did not. This asymmetric action of phlorizin on anion permeability is in contrast to the effect on the permeability to D-xylose. In accordance with previous findings of Beneš, I., Kolinská, J. and Kotyk, A. (1972) *J. Membrane Biol.* 8, 303, the penetration of the sugar was inhibited by phlorizin from either surface. It is concluded that phlorizin inhibition of anion transfer across the red cell membrane is due to interactions with binding sites which are located at the outer surface of the membrane and which do not diffuse all the way through the membrane like a sugar carrier or a lipid-soluble ionophore.

Many non-permeating agents are known to inhibit SO_4^{2-} permeability across the red blood cell membrane. These agents include proteolytic enzymes such as pronase[1] or papain (unpublished observations), amino-reactive agents such as 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS)[2], and amphiphilic substances such as phlorizin[3,4]. The fact that these various types of inhibitors act without penetrating into the interior of the cell could be explained in at least two ways. Either the inhibitors interact with immobile binding sites which are externally located and which facilitate the entry of SO_4^{2-} into the membrane, or they react with anion carriers. Since the carriers are mobile, they will diffuse to that surface of the membrane which is in contact with the inactivating agent. If this agent is present in the external medium at a sufficiently high concentration, then virtually

Abbreviations: SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid.

all of the carrier molecules should be trapped on the outer surface and hence an almost complete inhibition of anion permeability would result.

A simple way of discriminating between the inactivation of externally located fixed binding sites and an inactivation of mobile carrier molecules consists of comparing the effects which a given non-permeating inhibitor exerts from the inside and from the outside of the cell membrane. In experiments of this type, Beneš *et al.*[5] have recently demonstrated that the inhibition of sugar permeability in red cell ghosts by phloretin is symmetric. The experiments described below indicate that, in contrast to sugar permeability, SO_4^{2-} permeability can only be inhibited by phlorizin if the agent is applied from the outside, but not if it is present on the inside of the membrane.

The experiments were performed with resealed ghosts of human red blood cells. The ghosts were prepared by the method of Bodemann and Passow[6]. After hemolysis at 0 °C in the presence of 4 mmoles/l $MgSO_4$ and 1.5 mmoles/l acetic acid[7], isotonicity was restored by the addition of concentrated solutions containing sufficient KCl, K_2SO_4 and Tris–HCl (pH 7.6) to obtain final concentrations in the hemolysate of 100, 10 and 20 mmoles/l, respectively. When necessary, the ghosts were labeled by adding $^{35}SO_4^{2-}$ to the hemolysate. 5 min after reversion to isotonicity, the hemolysate was transferred to 37 °C and incubated at that temperature for 45 min. During this time period, most of the resealable ghosts actually resealed. Thereafter, the remaining leaky ghosts were separated from the resealed ones by centrifugation across a sucrose cushion, as described by Bodemann and Passow[6]. The resealed ghosts on top of the cushion were collected and washed 3 times at 0 °C. Subsequently, the ghosts were mixed with a medium of the same electrolyte composition as the hemolysate except that Na_2SO_4 was present in place of K_2SO_4. The final ghost suspension had a density of 5 vol.%. Since the medium was prewarmed to 37 °C, the mixing of the small volume of cold cells with the large volume of warm external solution initiated the $^{35}SO_4^{2-}$ exit immediately. The appearance of $^{35}SO_4^{2-}$ in the supernatant was followed and plotted as represented in Fig.1.

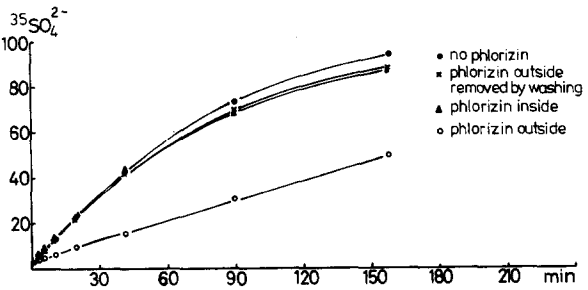

Fig.1. Effect of phlorizin on SO_4^{2-} efflux from human red blood cell ghosts. In order to make this experiment strictly comparable with that represented in Fig.2, in addition to the electrolytes listed in the text, 10 mmoles/l D-xylose were also present inside the ghosts and in the medium. Temperature, 37 °C. Ordinate, $^{35}SO_4^{2-}$ concentration in the supernatant, as a percentage of the equilibrium value. Abscissa, time in min.

For studying the effects of incorporated phlorizin immediately after restoration of isotonicity, sufficient glycoside was added to the hemolysate to give a final concentration of 2.0 mmoles/l. For studying the effects of external phlorizin, 2.0 mmoles/l glycoside were dissolved in the final medium in which the resealed and washed ghosts were suspended at the start of the efflux measurements. For determining the effect of the extracellular phlorizin which is left in the hemolysate after resealing of the ghosts, solid phlorizin was dissolved in the phlorizin-free hemolysate at the end of the resealing period (final concentration 2.0 mmoles/l), 5 min before the separation of resealed and leaky ghosts on the sucrose cushion. Subsequently, the ghosts were subjected to the same treatment as those which were loaded with phlorizin by the addition of the glycoside to the hemolysate before the start of the resealing process. Finally, one batch of ghosts served as control and was neither exposed to intracellular nor to extracellular phlorizin.

Fig.1 shows the results of a representative experiment. If extracellular phlorizin is present during the measurement of $^{35}SO_4^{2-}$ efflux, then we observe a considerable inhibition of SO_4^{2-} exit. If the glycoside is added to the medium after resealing, it can be removed by washing and the inhibitory effect disappears. If the glycoside is added before resealing there is also virtually no inhibition of efflux. This is not due to release of trapped phlorizin into the medium during washing. Direct estimates of the phlorizin concentration inside the washed ghosts yielded a value of 1.87 mmoles/l, *i.e.* about the concentration that one would expect to observe in successfully resealed ghosts which are completely impermeable to phlorizin.

Phlorizin is strongly bound to many erythrocyte proteins (unpublished observations). It therefore seemed feasible that the effect of phlorizin inside the ghosts was reduced by binding to the hemoglobin in the hemolysate to which the glycoside had been added for subsequent incorporation. However, the following experiment shows that this is not so: phlorizin was added to the hemolysate immediately after reversal to isotonicity to give the final concentration of 2.0 mmoles/l. After resealing, the ghosts and the supernatant were separated by centrifugation, and the supernatant was removed and used as a medium for suspending fresh, resealed ghosts which were free of intracellular phlorizin. $^{35}SO_4^{2-}$ efflux from these ghosts was still inhibited to the same extent as in a hemolysate-free medium containing the same phlorizin concentration. There was also no effect on $^{35}SO_4^{2-}$ exit from the phlorizin-loaded ghosts in the sediment after resuspension of these ghosts in phlorizin-free hemolysates.

Intracellular phlorizin was completely ineffective at pH 7.2 and 7.6, while extracellular phlorizin inhibited at both pH values. In a single experiment we found that phloretin (0.1 mmole/l) showed a similar behavior as phlorizin. However, we did not ascertain whether or not the incorporated aglycone remained trapped inside the resealed ghosts. The work of Beneš *et al.*[5] suggests that the ghost membrane is impermeable to phloretin.

Although the described experiments strongly suggest that, in contrast to extracellular phlorizin, intracellular phlorizin does not inhibit SO_4^{2-} permeability, it would seem desirable to show that, in principle at least, incorporated phlorizin is capable of affecting the transport process. We therefore performed experiments with D-xylose. These experiments were strictly

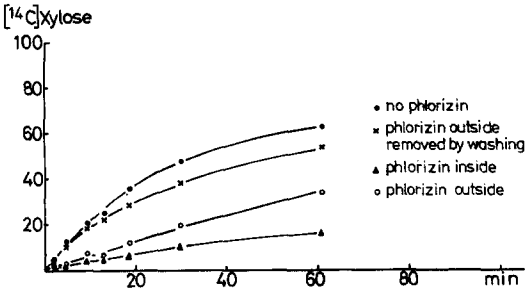

Fig.2. Effect of phlorizin on D-xylose efflux from human red cell ghosts. Temperature, 0 °C. Ghosts and media contained 10 mmoles/l D-xylose in addition to the electrolytes listed in the text. Ordinate, D-[^{14}C] xylose concentration in the supernatant, as a percentage of the equilibrium value. Abscissa, time in min.

analogous to those with SO_4^{2-} described above. D-Xylose movements had been shown by Beneš *et al.*[5] to be inhibited by intracellular as well as extracellular phloretin. Using phlorizin instead of phloretin, we found that intracellular phlorizin inhibits movements of the sugar at least as strongly as extracellular phlorizin. In fact, the inhibition seems to be slightly more powerful if the agent is applied to the inside rather than to the outside of the membrane (Fig.2).

In view of the described observations, we conclude that the inhibition by phlorizin of SO_4^{2-} permeability is asymmetric. This is in contrast to what one would expect if SO_4^{2-} movements would be blocked by interaction between phlorizin and a diffusible carrier. We suggest, therefore, that SO_4^{2-} permeability is largely controlled by binding sites which are located in the outer cell surface and which do not penetrate across the red blood cell membrane. The action on SO_4^{2-} permeability of non-penetrating modifiers like pronase, SITS and phlorizin would therefore be confined to a modification of easily accessible external fixed sites and not to a destruction of freely diffusible carrier molecules. This statement does, of course, not exclude the possibility that some anion-binding sites of a large, fixed molecule perform oscillatory movements, and that such oscillations play an important role in the transfer of the anions across the rate-determining barrier. However, if such oscillations should occur, they are confined to the outer surface of the membrane and do not lead to an anion transfer from one surface of the membrane to the other. Our findings also do not rule out the possibility that the phlorizin-sensitive fixed site located in the outer membrane surface controls the interactions between the anions to be transported and a phlorizin-insensitive mobile carrier.

In contrast to SO_4^{2-}, the rapidly penetrating halide ions seem to exchange across the membrane many orders of magnitude faster than they are capable of transferring net negative charges[8]. This suggests that an overwhelming portion of the halide flux takes place by a carrier-exchange mechanism. Nevertheless, preliminary experiments in our laboratory indicate that even in the special case of the halides, phlorizin inhibition is asymmetric. This would not support the contention that these anion species are transferred

by a lipid-soluble ionophore whose role in anion permeability would be similar to that of valinomycin in the transfer of K^+ across lipid bilayers. The details of our work on halide permeability will be published in a separate communication (Gerhard, S., Schnell, K.F., Lepke, F. and Passow, H., unpublished).

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