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THE INTERACTION OF THE (Na^+,K^+)-ATPase OF ERYTHROCYTE GHOSTS WITH OUABAIN

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SUMMARY

The influence of P_i and K^+ on the reaction of ouabain with the (Na^+,K^+)-ATPase system of the erythrocyte membrane and resealed ghosts has been investigated. With Mg^{2+}, P_i increased the rate of inhibition. This effect of P_i was found only when orthophosphate was inside the ghosts. It was not active when applied to the outside of the membrane. K^+ (inside as well as outside the cells) decreased the P_i-induced inhibition of the (Na^+,K^+)-ATPase by ouabain. K^+ also reduced the ouabain inhibition in the absence of P_i. In this case a maximal effect was observed when K^+ attacked the erythrocyte membrane on both sides simultaneously. The results are discussed in terms of the hypothesis that the affinity for ouabain is increased when the carrier is situated on the outside of the cell membrane.

Recent work in this and other laboratories has shown that the phosphorylation of the active sites of the (Na^+,K^+)-ATPase system promotes the interaction of the enzyme with ouabain[1,2]. With Mg^{2+}, P_i phosphorylates the (Na^+,K^+)-ATPase and increases the rate of inhibition by ouabain[3]. It was suggested that such an effect of P_i results from the reversibility of the last phosphatase stage[4,5]. In view of the finding that after hydrolysis of the phosphorylated intermediate P_i was liberated inside the cells[6], we presumed that only intracellular orthophosphate would be active as regards stimulating the (Na^+,K^+)-ATPase–ouabain interaction.

The experiments were performed with human erythrocyte membranes and with resealed ghosts. Erythrocyte membranes were prepared from stored human blood. The blood was centrifuged at 3000 × *g* for 10 min and the plasma and buffy coat were removed. The remaining erythrocytes were washed 4 times with 150 mM NaCl in 10 mM Tris buffer (pH 7.4) and then haemolysed in water. Membranes were washed 4 times with 1 mM EDTA–Tris (pH 7.4).

Resealed ghosts were prepared from erythrocytes exhausted by prolonged incubation at 37 °C in a solution containing 100 mM NaCl, 20 mM KCl, and 40 mM Tris–HCl (pH 7.4). During this procedure endogenous P_i is partly removed from the cells. Exhausted erythrocytes were haemolysed in a solution containing 2 mM $MgCl_2$, 2 mM PO_4^{3-}, 50 mM Tris–HCl (pH 7.4), followed by centrifugation for 5 min at 20000

$\times g$. Haemolysis was repeated in the same system, but with 10 mM Tris–HCl. Reconstitution was carried out by incubating the ghosts with 2 mM $MgCl_2$, 2 mM PO_4^{3-} in 150 mM choline chloride (10 min, 37 °C). Resealed cells were washed with 150 mM choline chloride containing 2 mM $MgCl_2$. For the preparation of the control samples, P_i was excluded from all solutions mentioned above.

Restored erythrocytes with different concentrations of K^+ were prepared as described, but the choline chloride of the incubation medium was partially replaced by KCl.

In pretreatment experiments, the enzyme preparation (membranes or resealed ghosts) was preincubated at 37 °C with ouabain in a medium of the following composition: choline chloride (150 mM), $MgCl_2$ (2 mM), histidine (10 mM, pH 7.4), ouabain ($2 \cdot 10^{-6}$–10^{-5} M) and varying concentrations of PO_4^{3-} and K^+ if needed. Total volume of the sample was 0.2 ml. The reaction was stopped by the addition of 1.5 ml 2 mM EDTA–Tris (pH 7.5). The suspension was centrifuged (5 min, 10000 $\times g$) and the membranes were washed with 5 mM histidine buffer, pH 7.5.

ATPase activity was assayed by measuring the inorganic phosphate produced, using the method of Fiske and SubbaRow[7]. Incubation was carried out in 0.15 ml of the reaction system containing 2 mM $MgCl_2$, 2 mM ATP, 100 mM NaCl, 20 mM KCl and 40 mM Tris buffer (pH 7.8). For the determination of Mg^{2+}-ATPase activity ouabain (10^{-4} M) was added. After 15 min incubation at 37 °C the reaction was stopped by adding 0.15 ml of 6 % trichloroacetic acid.

Fig. 1 shows the percentage of inhibition of the (Na^+,K^+)-ATPase by ouabain with and without P_i. In agreement with observations by others[8,9] it was found that (Na^+,K^+)-ATPase of erythrocyte membranes reacts with ouabain more effectively in the presence of orthophosphate. With Mg^{2+}, P_i and ouabain the system was phos-

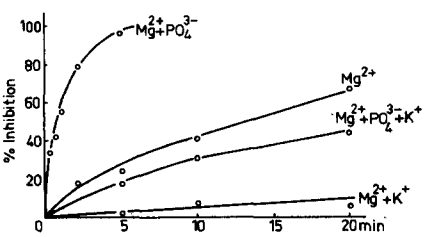

Fig. 1. The influence of PO_4^{3-} and K^+ on the inhibition of the erythrocyte membrane (Na^+, K^+)-ATPase by ouabain. The enzyme was preincubated with ouabain (10^{-5} M) in 150 mM choline chloride (37 °C, pH 7.5). Concentrations of other components: Mg^{2+}, 2 mM; PO_4^{3-}, 1 mM; K^+, 20 mM.

phorylated, in a way which seems to be identical with the phosphorylation found with ATP[10,11]. This may mean that P_i sensitizes (Na^+,K^+)-ATPase to ouabain due to the formation of phospho-enzyme. This conclusion agrees well with the effect of K^+ on the P_i-induced inhibition of the (Na^+,K^+)-ATPase. It is known that K^+ activates the dephosphorylation of the intermediate. Consequently, it must reduce the concentration of the phospho-enzyme which has a higher affinity for ouabain. Indeed, the addition of K^+ to the Mg^{2+}–P_i–ouabain system decreased the rate of inhibition significantly (see Fig. 1).

The protective effect of K^+ was found also in the nonphosphorylating system.

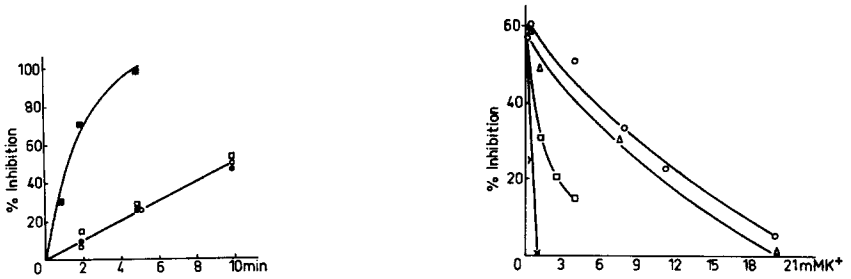

Fig. 2. The influence of PO_4^{3-} and K^+ on the ouabain inhibition of the resealed ghost (Na^+,K^+)-ATPase. The ghosts with 1.5 mM PO_4^{3-} per l of cells (■, ●) or without PO_4^{3-} (□, ○) were pretreated with ouabain ($2 \cdot 10^{-6}$ M) at 37 °C in 150 mM choline chloride containing 5 mM histidine (pH 7.5) and 2 mM $MgCl_2$ (□—□, ■—■). Concentrations of other components in the medium: PO_4^{3-}, 2 mM (○—○) K^+, 20 mM (●—●). Concentration of Mg^{2+} in ghosts, 2 mM per l of cells.

Fig. 3. The influence of K^+ on the ouabain inhibition of the membrane and ghost (Na^+, K^+)-ATPase. Pretreatment with ouabain for 5 min at 37 °C in a medium containing 2 mM Mg^{2+}, 150 mM choline chloride and different concentrations of K^+ (pH 7.5). The concentrations of ouabain were 10^{-5} M for membranes and $2 \cdot 10^{-6}$ M for ghosts. ○—○, inhibition as a function of intracellular K^+; △—△, extracellular K^+. The ghosts contained 3.6 mM of K^+ per l of cells while K^+ in the medium was varied (□—□). ×—×, samples with erythrocyte membranes.

With Mg^{2+} in the preincubation medium, K^+ (20 mM) prevented the decrease in activity. Thus, the (Na^+,K^+)-ATPase of erythrocyte membranes, as those from other tissues[8,9], is protected by K^+ from ouabain action both with and without P_i.

The same experiments were then carried out with resealed ghosts. Fig. 2 shows that, in this case, P_i only increased the rate of ouabain inhibition when applied to the inside of the membrane. When present in the medium surrounding the ghosts, P_i did not have any effect at all or a slight effect only. The latter may be accounted for by slow penetration of P_i into the cells during incubation.

One way of explaining the asymmetrical action of orthophosphate is to suggest that the last stage of the (Na^+,K^+)-ATPase reaction, which obviously proceeds on the inner cell surface, is reversible.

One would expect that K^+ only reduces the rate of P_i-induced ouabain inhibition when included in the external medium. But experiments showed that extracellular K^+ was active (Fig. 2) as well as intracellular K^+ (not shown). Additional data are necessary for the interpretation of this observation.

The same results were obtained when the effect of K^+ on ghosts without P_i was studied. In this case, K^+ also reduced the inhibition on both surfaces of the membrane (Fig. 3). However, higher concentrations of K^+ were required for adequate effects on ghosts as compared with erythrocyte membranes. Half-maximal protection was obtained at concentrations around 7–8 mM when K^+ was inside or outside the ghosts, and at 0.5 mM K^+ in the case of the erythrocyte membrane preparations. The difference between these experiments is that K^+ attacked both surfaces of the membrane fragments and only one surface of the resealed ghosts. It is interesting that the protection of the ghost (Na^+,K^+)-ATPase from ouabain inhibition is most effective in conditions when K^+ acts synergistically on opposite sides of the membrane (see Fig. 3).

The interpretation of the present results has to be rather hypothetical but it

may be suggested that ouabain binds to the phosphorylated and unphosphorylated enzyme in the conformational state reached after carrier transition to the outer surface of membrane. In view of the traditional notions about the mechanism of ionic transport the phosphorylation step would promote such a transition.

Membrane fragments are always in a symmetrical ionic medium. Thus, a carrier in the absence of concentration gradients may move spontaneously without energy consumption. This explains why ouabain reacts slowly with (Na^+, K^+)-ATPase in dephosphorylated form. If the carrier–potassium complex preferably stays at or moves to the inner cell surface, then a synergistic effect of K^+ localized on opposite sides of the membrane becomes intelligible.

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