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Antibodies to pig kidney (Na^++K^+)-ATPase inhibit the Na^+ pump in human red cells provided they have access to the inner surface of the cell membrane

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SUMMARY

Antisera from rabbits that had been immunized with a highly active membrane preparation of (Na^+ + K^+)-ATPase from the outer medulla of pig kidney strongly inhibited (Na^+ + K^+)-ATPase activity in various tissues. When the antiserum was incorporated into released human red cell ghosts, the ouabain-sensitive efflux of Na^+ into both 15 mM K^+ and K^+-free high Na^+ media was completely abolished. This effect was not observed when non-immune serum was used, or when the immune serum was allowed access only to the outer surface of the red cell membranes.

Antibodies to (Na^+ + K^+)-ATPase preparations might be useful in attempting to identify and localize components of the Na^+ pump, and in investigating the relations between chemical reactions catalysed by the ATPase and the movements of ions across cell membranes. Although pure (Na^+ + K^+)-ATPase has not yet been prepared, it seemed worthwhile to look at the immunological properties of recent membrane preparations in which (Na^+ + K^+)-ATPase protein accounted for more than half of the total protein[1]. The experiments reported here were designed to see whether membrane-bound (Na^+ + K^+)-ATPase was antigenic, and whether antibodies to it could interfere with (Na^+ + K^+)-dependent hydrolysis of ATP in broken membrane preparations or with the active transport of alkali-metal ions in intact cells.

Rabbits were immunized with highly active preparations of (Na^+ + K^+)-ATPase obtained from the outer medulla of pig kidney. The sera were tested for an inhibitory action on (Na^+ + K^+)-dependent hydrolysis of ATP, using preparations from rabbit kidney and ox brain as well as the preparation from pig kidney used as an antigen. To test for inhibition of ion transport, human red cells were employed, since with these cells the

process of "reversible haemolysis" gives the experimenter access to both surfaces of the cell membrane.

Membrane preparations of $(Na^+ + K^+)$-ATPase with a specific activity of 15–18 μmoles $P_i \cdot$mg protein$^{-1} \cdot$min^{-1} were obtained by isopycnic zonal centrifugation of microsomal fractions from the outer medulla of pig kidney, as previously described[1].

For the preparation of antiserum, rabbits were injected subcutaneously, in the medial side of the right posterior crural region and in the left subscapular region, with an amount of the enzyme preparation containing 4–5 mg protein dissolved in 2 ml of a solution containing 25 mM imidazole and 1 mM EDTA, pH 7.0. A total of 6 injections were given at weekly intervals. At the time of the first injection, 3 ml of Freund's complete adjuvant was injected separately in the same regions.

Blood samples were taken from the lateral ear vein before immunization and every 10 days after the first injection of antigen. The sera were tested for precipitation with antigen by immunodiffusion in a solution containing 1% agarose, 5% glycerol and 0.1 M sodium phosphate, pH 7.0 (ref. 2). 2-fold serial dilutions of serum were allowed to diffuse against antigen in a concentration of 0.8–1.2 mg protein per ml. After 3 days at 20 °C, the agar showed a prominent band of precipitation together with a faint second band. A maximal titre was obtained 6 to 9 weeks after the first injection of antigen.

To see whether the antiserum inhibited $(Na^+ + K^+)$-dependent hydrolysis of ATP, tests were first made on the pig kidney preparation that had been used as an antigen.

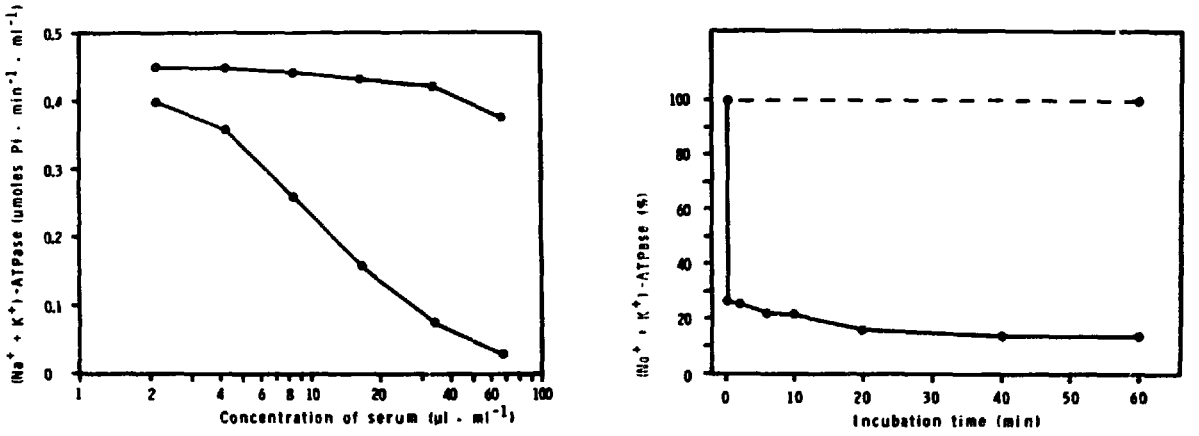

Fig. 1. Aliquots of the enzyme preparation containing 5.7 μg protein were incubated with 2-fold serial dilutions of control serum (○) or immune serum (●) in 25 mM imidazole (pH 7.5 at 37 °C), the total volume being 0.15 ml. After 1 h, 25-μl portions were transferred to test tubes each containing 1 ml of a solution containing 3 mM ATP (Tris salt), 3 mM $MgCl_2$, 130 mM NaCl, 20 mM KCl, 30 mM histidine (pH 7.5 at 37 °C) and, where appropriate, 1 mM ouabain. After 10 min at 37 °C the reaction was stopped with 100 μl of 50% trichloroacetic acid, and the P_i was measured[7]. $(Na^+ + K^+)$-ATPase activity was taken to be the difference in activity with and without ouabain.

Fig. 2. Aliquots of the enzyme preparation containing 5.7 μg protein were incubated with 66 μl control serum (○) or 66 μl immune serum (●) in a total volume of 1 ml 25 mM imidazole (pH 7.5 at 37 °C). At the intervals shown on the abscissa, 25-μl portions of each mixture were transferred to test tubes for measurement of $(Na^+ + K^+)$-ATPase activity as described in Fig. 1.

Portions of this preparation, each containing 5.7 μg protein were preincubated at 25 °C with 2-fold serial dilutions of sera from 1:15 to 1:480, in a total volume of 0.15 ml. After an hour, 25-μl portions of each mixture were transferred to test tubes for the measurement of $(Na^+ + K^+)$-ATPase activity as described in the legend to Fig. 1. That figure shows that serum from the blood of a rabbit taken 8 weeks after the start of immunization strongly inhibited the $(Na^+ + K^+)$-dependent hydrolysis of ATP by the pig kidney preparation. Some inhibition was apparent even with the 1:480 dilution, and inhibition was nearly complete with the 1:15 dilution. Control serum, from blood taken before immunization, had very little effect on the $(Na^+ + K^+)$-ATPase activity.

A study of the time course of the reaction, Fig. 2, shows that the inhibition of $(Na^+ + K^+)$-ATPase approached its maximal value within a few seconds after addition of antiserum, in a 1:15 dilution, to the enzyme suspension. This result makes it unlikely that the disappearance of enzyme activity is due to precipitation of the membrane fragments, which is seen to occur after several hours of incubation with antibody,

TABLE I

THE EFFECT OF IMMUNE SERUM ON $(Na^+ + K^+)$-ATPase ACTIVITY IN PREPARATIONS FROM THE OUTER MEDULLA OF PIG AND RABBIT KIDNEY AND FROM OX BRAIN

The activities are given relative to the activities of the same preparations incubated with control serum. The conditions of exposure to the serum and the method of enzyme assay were the same as in Fig. 1.

	$(Na^+ + K^+)$-ATPase activity (% of control)	
	33 μl serum · ml^{-1}	*66 μl serum · ml^{-1}*
Pig kidney	39	17
Rabbit kidney	59	47
Ox brain	38	28

The data of Table I show that, as well as inhibiting the ATPase activity of its own antigen, the immune serum also inhibited the $(Na^+ + K^+)$-ATPase activity of preparations from the outer medulla of rabbit kidney and ox brain. The binding of tritiated ouabain to the ox brain preparation[3] in the presence of Mg^{2+}, Na^+ and ATP was also depressed by the immune serum (not shown).

In testing the inhibitory effect of antiserum on Na^+ transport in red cell ghosts, it was important to ensure that sufficient antibody was present to inhibit most of the pump sites. Under the conditions of the experiment illustrated by Fig. 1, 50 μl of serum inhibited an amount of kidney enzyme yielding 0.4 μmole P_i per min. If the molecular activity of $(Na^+ + K^+)$-ATPase is 9000 moles P_i per min[4], this amount of enzyme represents $2.7 \cdot 10^{13}$ enzyme sites. Assuming that each red cell has 300 pump sites[5], we can calculate that $5.6 \cdot 10^{-16}$ l of serum would be necessary to inhibit all the sites in one cell. To test for an inhibitory effect at the inner surface of the cell, it is therefore necessary to lyse the cells in a solution that contains at least this quantity of serum in a volume equal to the volume of a single cell (say 10^{-13} l); for the antibody will enter the cells only at the moment of lysis,

TABLE II

INHIBITION OF OUABAIN-SENSITIVE Na^+ EFFLUX BY IMMUNE SERUM ACTING AT THE INNER SURFACE OF THE RED CELL MEMBRANE

Cells were lysed in 10 vol. of lysing solution at 22 °C. All lysing solutions contained 1.5 mM Na_2ATP, 2 mM $MgCl_2$, 5 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (sodium salt), 1 mM cysteine, 15 mM KCl; pH 7.3. The concentration of serum, when present, was 2.5 times or 5 times the concentration estimated to be necessary to combine with all the pump sites (see text). The sera for these experiments had been dialysed against 10 mM imidazole (pH 7.0) and freeze–dried. 5 min after lysis, ^{24}Na was added as isotonic NaCl, bringing the Na^+ concentration to 9 mM. Normal tonicity was restored with 3 M KCl, and the ghosts incubated at 37 °C for 40 min to allow the membranes to reseal. The resealed ghosts were washed 5 times at 0 °C with a solution containing 152 mM NaCl, 2 mM $MgCl_2$ and 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.3 at 22 °C), and were then incubated at 37 °C for 45 min in a similar solution ± ouabain ($7 \cdot 10^{-5}$ M) and with 15 mM KCl substituted for an equivalent quantity of NaCl where appropriate. The efflux measurements with each preparation of ghosts were made in triplicate, and the errors shown in the table are ± S.E. It is important to note, however, that only one preparation of each kind was made, so that small differences between the preparation may not be significant even if they exceed what would be expected from the errors shown in the table.

Expt	*Conditions of lysis*	*Rate constants for Na^+ efflux (h^{-1})*		
		Ouabain-sensitive		*Ouabain-insensitive*
		Into 15 mM K^+ medium	*Into K^+-free high-Na^+ medium*	
1	No serum	0.288 ± 0.005	0.136 ± 0.005	0.189 ± 0.004
	Non-immune serum	0.258 ± 0.006	0.121 ± 0.005	0.119 ± 0.004
	Immune serum	−0.002 ± 0.003	0.103 ± 0.003	0.105 ± 0.002
	Immune serum added after lysis	0.363 ± 0.005	0.152 ± 0.007	0.186 ± 0.004
2	No serum	0.264 ± 0.004	0.134 ± 0.004	0.137 ± 0.003
	Non-immune serum	0.236 ± 0.002	0.103 ± 0.006	0.133 ± 0.001
	Immune serum	0.001 ± 0.004	0.100 ± 0.007	0.133 ± 0.004

and only antibody trapped within the cells will be able to reach the inner surface.

Table II summarises the results of two experiments designed to discover whether Na^+ transport is inhibited by antibodies having access to the inner surface of the cell membrane. In the first experiment, four lots of resealed ghosts were prepared by lysing washed human red cells in four different lysing solutions. The first lysing solution contained no serum, the second non-immune serum, and the third immune serum at an equal concentration. The fourth lysing solution contained no serum before haemolysis, but immune serum was added to the ghost suspension after normal tonicity had been restored, *i.e.* at a time when the membranes were no longer permeable to proteins. All the lysing solutions contained ATP, ^{24}Na and suitable salts and buffer. After sufficient concentrated

KCl had been added to the ghost suspensions to bring their tonicity back to normal, the suspensions were incubated at 37 °C to allow the membranes to regain their relative impermeability to cations. The resealed ghosts were then washed thoroughly, and reincubated in suitable salt solutions with and without ouabain, so that the efflux of ^{24}Na could be measured. Immune serum was added to the salt solution used for the final incubation of the ghosts of the fourth lot; the outer surface of these ghosts was therefore exposed to immune serum both during the resealing and during the final incubation. In a second experiment a similar procedure was followed, except that the effect of immune serum added after haemolysis was not tested. Both experiments show that if immune serum was present during haemolysis, so that it could enter the cell interior, the ouabain-sensitive efflux of Na^+ was completely abolished. This was true both under conditions in which the pump functioned normally, exchanging Na^+ for K^+, and under conditions in which the pump catalysed an exchange of internal and external Na^+. Ouabain-sensitive Na^+ efflux was not abolished by non-immune serum or by immune serum that had access only to the outer surface of the cells. It is also worth noting that the ouabain-insensitive efflux of Na^+, part of which was sensitive to furosamide (not shown), was the same in the presence of immune as in the presence of non-immune serum.

The ineffectiveness of extracellular immune serum on the ouabain-sensitive cation pump was confirmed in a further experiment in which intact red cells were incubated (i) without serum, (ii) in the presence of non-immune serum, or (iii) in the presence of immune serum at the same concentration as that which completely blocked ouabain-sensitive Na^+ transport in the second experiment of Table II. After 30 min ouabain was added to half of each suspension, and 5 min later ^{42}K was added and its uptake measured over the next hour. The ouabain-sensitive uptake of K^+ was 2.16 mmoles·(l·cells)$^{-1}$·h^{-1} in the absence of serum, 2.15 mmoles·(l·cells)$^{-1}$·h^{-1} in the presence of non-immune serum, and 2.11 mmoles·(l·cells)$^{-1}$·h^{-1} in the presence of immune serum. The ouabain-insensitive uptake of K^+ was also the same under all conditions. Removal of neuraminic acid from the cell surface with neuraminidase[6] caused a very slight reduction in the ouabain-sensitive K^+ uptake, but the neuraminidase-treated cells were equally unaffected by extracellular immune serum.

Because the preparation of pig kidney used as an antigen was not a pure preparation of ($Na^+ + K^+$)-ATPase, it is likely that several antibodies were formed in response to its injection. Nevertheless, the effects of the immune serum on the hydrolysis of ATP by broken cell preparations, and on the pumping of Na^+ by resealed red cell ghosts, make it clear that among the antibodies formed there must have been one (or more) with the ability to combine with part of the ($Na^+ + K^+$)-ATPase which is exposed at the inner surface of the cell membrane. The fact that the antibody to the pig kidney enzyme inhibits rabbit kidney and ox brain enzyme as well as Na^+ transport in human erythrocytes suggests that the antigenic determinants of the Na^+ pump are the same regardless of their origin.

Why the antiserum should block transport when present at the inner surface of the membrane but be ineffective at the outer surface is not clear. It is conceivable that an antibody to an inward-facing component of the pump might merely interfere with the binding

of the large ligand ATP, and that inhibition of Na^+–K^+ exchange, Na^+–Na^+ exchange and ouabain binding could all be secondary to this interference. An antibody to a component of the pump facing outwards might not interfere with the binding of the small ligands K^+ and Na^+ and, if it had no other action, it would not have been detected in these experiments. Another possibility is that antibodies to an outward-facing component of the pump might have been absorbed onto the surface of the rabbit's own red cells and therefore not found in the rabbit's serum. The ineffectiveness of extracellular antiserum in these experiments is therefore not evidence that the external aspect of the Na^+ pump is not antigenic.

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