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Effects of ATP and Na^+ on a K^+-activated phosphatase from red blood cell membranes

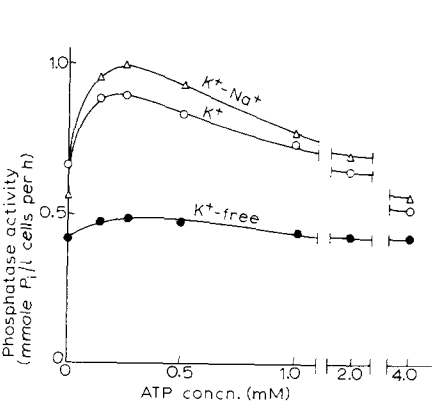
A *p*-nitrophenylphosphatase activated by Mg^{2+} and K^+ at neutral pH and inhibited by ouabain has been reported to be present in cell membrane preparations from several tissues since JUDAH, AHMED AND MCLEAN[1] described it in human red blood cell ghosts. Most authors relate this activity to a desphosphorylation step in the over-all ($Na^+ + K^+$)-dependent ATPase activity of cell membranes[1-4]. This communication deals with some aspects of the interaction of Na^+ and ATP with the *p*-nitrophenylphosphatase present in human red blood cell membranes. Fragmented membranes were obtained by freezing and thawing "hemoglobin-free" human red blood cell ghosts prepared by successive washes in hypotonic solutions buffered with Tris–HCl.

Fig. 1 shows that, both in the presence and absence of Na^+, low concentrations of ATP increase the rate of hydrolysis, but as ATP concentration is raised, the activity progressively falls. This effect is exerted mainly on the K^+-dependent activity as shown by the very small effect of ATP in the absence of K^+. The activating effect of ATP at low concentrations is in marked contrast to the inhibitory action of this nucleotide on K^+-sensitive phosphatases from other tissues[2-5]. In order to elucidate the mechanism of activation, the reaction rate was measured as a function of *p*-nitrophenylphosphate concentration in media containing non-limiting amounts of K^+ (100 mM). Under these conditions the addition of 0.25 mM ATP resulted in a 79% increase in maximum velocity and in a 56% rise in the K_m for *p*-nitrophenylphosphate. The activating effect of ATP seems, therefore, to be due to an increase in the rate of turnover that is large enough to overcome the drop in apparent affinity. The decrease in the apparent affinity for the phosphatase substrate, induced by ATP, has been reported by other authors in enzyme preparations in which ATP did not affect the maximal rate of hydrolysis and, for this reason, this effect has been interpreted in terms of competitive inhibition[2,4]. The finding that when red blood cell membrane phosphatase is combined with ATP the rate of hydrolysis increases is not compatible with the above view and necessarily demands the existence of separate sites for ATP and *p*-nitrophenylphosphate. The drop in apparent affinity for *p*-nitrophenylphosphate remains unexplained and needs further investigation.

Another interesting point shown in Fig. 1 is the effect of Na^+. In the absence of ATP, Na^+ acts as an inhibitor, but if ATP is added in increasing concentrations the inhibitory effect of Na^+ first disappears and is then reversed. Thus in the presence of adequate amounts of ATP, Na^+ acts as an activator. A rather similar effect has been reported by NAGAI AND YOSHIDA[5] in an enzyme preparation from guinea-pig brain. Maximal activation is reached at about 20 mM Na^+ when 15 mM K^+ and 0.25 mM ATP are present. Further increases in Na^+ concentration lead to a progressive decline in activity, suggesting that its inhibitory action has not disappeared but that at low concentrations it is masked by the more prominent activating effect. Na^+ has no effect in the absence of K^+.

Fig. 2 shows the phosphatase activity as a function of K^+ concentration in the presence and absence of Na^+ or ATP. The results show that: (1) When ATP is absent

Na^+ acts as a competitive inhibitor for K^-. Na^+ (20 mM) increases from 8 to 17 mM the K^+ concentration giving half-maximal activation. (2) ATP (0.25 mM) increases the K^+ concentration from 8 to 14 mM giving half-maximal activation. (3) When Na^+ (20 mM) and ATP (0.25 mM) are present together, the effect they exert singly is reversed and the half-maximal value for K^+ drops to 3 mM, showing that the activating effect of Na^+ is indirect and mediated through an increase in the apparent affinity of the enzyme for K^+.



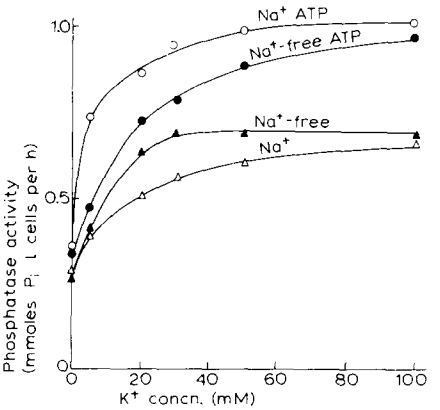


Fig. 1. *p*-Nitrophenylphosphatase activity as a function of ATP concentration. The "K^+-free" (●—●) incubation medium contained: 5.0 mM $MgCl_2$, 6.3 mM Tris–*p*-nitrophenylphosphate and 160 mM Tris–HCl (pH 7.8) at 37°. In the "K^+ medium" (○—○), 15 mM KCl replaced an equivalent amount of Tris–HCl. In the "K^+–Na^+ medium" (△—△), 15 mM KCl and 20 mM NaCl replaced an equivalent amount of Tris–HCl. Tris–ATP was added together with equimolar amounts of $MgCl_2$. The quantity of fragmented ghosts in the reaction mixture was that which gave a hematocrit of 15%, calculated on the original volume of the cells. The incubation was carried out at 37° for 30 min.

Fig. 2. *p*-Nitrophenylphosphatase activity as a function of K^+ concentration. ATP concentration was 0.25 mM. Other conditions were as described in Fig. 1.

It is known that, in the presence of Na^+ and Mg^{2+}, ATP is able to phosphorylate membrane protein[1,6]. Since the increase in the apparent affinity for K^+ induced by Na^+ and ATP could conceivably be due to such a phosphorylation, enzyme activity was measured in the presence of hydroxylamine. This compound is known to accelerate the splitting of phosphate bound to membrane protein[6]. Addition of 10 mM hydroxylamine to the incubation media completely abolished the activating effect of Na^+. Hydroxylamine did not significantly affect the enzyme activity under other conditions. The results mentioned above show that the ability of the enzyme to interact with Na^+ and K^+ is altered by ATP in a way that can be summarized as follows. Combination with ATP decreases the selectivity of the enzyme for K^+. In this condition, apart from combining with the K^+ site, Na^+ is also able to react with the system in such a way that the selectivity of the enzyme for K^+ is increased beyond that found in the absence of Na^+ or ATP. The effects of hydroxylamine suggest rather strongly that the reaction induced by Na^+ is the phosphorylation of the enzyme by ATP. These findings seem to be consistent with the close relationship claimed by most authors between the K^+-activated phosphatase and the cation transport system.

The fact that ATP affects the interactions of the enzyme with Na^+ and K^+ is

interesting because it suggests that this nucleotide, apart from providing the necessary energy for active transport, also plays a role in promoting the cyclic changes in selectivity for these ions required by most active transport schemes.

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Na^+ transport across the isolated skin of *Ambystoma mexicanus*

In contrast to the numerous studies of electrolyte transport across anuran skin, little work has been done on the skin of urodeles[1,2]. We describe the Na^+ transport of isolated skins of adult *Ambystoma mexicanus*, a system with some important differences from the frog skin.

Metamorphosis of larval Ambystoma (60–120 g) was induced by the intramuscular injection of 100 μg of thyroxine. After metamorphosis their skins were mounted between two lucite half chambers (area 3.14 cm^2)[3]. Symmetrical calomel half cells, connected through 3 M KCl–agar bridges to the chambers, were used for potential recording and current delivery. The main solutions employed were Cl^- Ringer (115 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 3 mM Tris–maleate buffer, pH 7.5) and SO_4^{2-} Ringer (77 mM Na_2SO_4, 1.25 mM K_2SO_4, 8 mM $CaSO_4$, 3 mM Tris–maleate buffer, pH 7.5).

The outer surface of the skin immersed in Cl^- Ringer was 59.5 $\pm$ 8.2 mV ($\pm$ S.E., $n = 14$) negative with respect to the inner surface. Short circuit current (s.c.c.) mean value was 26.2 $\pm$ 9.7 $\mu A/cm^2$. To study the dependence of the potential on the cations bathing the skin, we tried to reduce the short circuiting effect of Cl^- by replacing it with SO_4^{2-}. Contrary to what has been observed in frog skin, SO_4^{2-} markedly depressed s.c.c. and potential. In 12 experiments, complete substitution of

Abbreviation: s.c.c., short circuit current.