FUNCTIONAL ORGANIZATION OF THE PARTIAL REACTIONS OF $Na^+ + K^+$ - ACTIVATED ATPase WITHIN THE RED CELL MEMBRANE

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Summary: Effects of false substrates and modifiers of the partial reactions of the Na^+, K^+-ATPase complex on Na^+ transport in resealed red cell ghosts were studied. A ouabain-sensitive efflux of Na^+ into a Na^+-free medium was initiated when p-nitrophenylphosphate (NPP) and K^+ were added to the medium. Na^+-efflux into a Na^+-containing medium required the simultaneous presence of K^+, NPP, and a nucleotide in the medium. When Na^+-efflux was stimulated, NPP hydrolysis was increased. We conclude that the K^+-dependent phosphatase facing the medium-side of the membrane is the primary ion translocator, and that an enzyme on the matrix-side of the membrane supplies a physiological substrate (the "phosphorylated intermediate") for the translocator-phosphatase.

The discovery of the membrane-bound $Na^+ + K^+$-activated adenosinetriphosphatase (Na^+, K^+-ATPase) by Skou[1], and the subsequent studies of many laboratories[2,3], have indicated that this enzyme complex is related to the energy-dependent translocation of Na^+ and K^+ through the plasma membrane. The mechanism of the intimate relation of this enzyme activity to ion movements, however, remains to be determined. The following two groups of studies have been relevant to the proposed mechanisms for this relationship:

1. Experiments with "resealed" red cell ghosts[4-6] and internally perfused squid axon[7-9] have shown that ATP, only when supplied from the inside of the membrane, can be hydrolyzed and lead to an active extrusion of Na^+.

2. The works of several investigators[3] with partially purified Na^+, K^+-ATPase have shown that ATP hydrolysis consists of at least two steps: One a Na^+-dependent phosphorylation of the enzyme, and the other a K^+-dependent hydrolysis of the resulting acylphosphate ($E\sim P$). On the basis of the above studies most of the proposed mechanisms for the active transports of Na^+ and K^+ relate the ion movements to the turnover of $E\sim P$ within the membrane.[10]

So far, however, it has not been possible to demonstrate a correlation between this turnover and the movements of ions in a functionally intact membrane.

Recent studies[11] have given strong support to the assumption that the K^+-dependent hydrolysis of some simple organic phosphates (e.g. acetylphosphate and p-nitrophenylphosphate) by the Na^+, K^+-ATPase preparations is equivalent to the K^+-dependent hydrolysis of E∿P within the ATPase complex. It occured to us that if this assumption were correct, it may be possible to supply the Na^+, K^+-ATPase of a functionally intact membrane with one of these false substrates, instead of E∿P, and determine the correlation between ion movements and at least part of the reactions catalyzed by the ATPase. In this report we present the results of our initial studies in this direction.

Isotonically "resealed" red cell ghosts, labeled with Na^{22}, were prepared as described before[12]. It has been demonstrated that these ghosts are deficient in substrates and that the active extrusion of Na^+ will not commence unless either lactate production is initiated by the addition of appropriate substrates to the medium[12,13], or ATP is placed inside the ghosts prior to resealing[4,6]. Figure 1 shows the time-course of the release of Na^{22} from the ghosts into various Na^+-free media. It is evident that when K^+ is present in the medium, the addition of p-nitrophenylphosphate (NPP) to the medium causes an increase in Na^+ efflux[a]. The sensitivity of this K^+-dependent efflux to ouabain indicates that the efflux is due to Na^+, K^+-exchange through the carrier of the classical coupled Na^+, K^+-pump. Part of the data of Figure 2 show, however, that if the incubation medium contains Na^+ the addition of NPP is not sufficient to cause an increase in Na^+ efflux regardless of the presence or absence of K^+. When NPP hydrolysis was also measured in the course of the above experiments it became apparent that in the

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a. Our preliminary studies showed that organic phosphates other than ATP when placed inside the ghosts had no effects on Na_o^+ efflux. This is in agreement with the findings of Brinley and Mullins[8] who showed that acetylphosphate perfused through the squid axon did not affect Na^+ efflux.

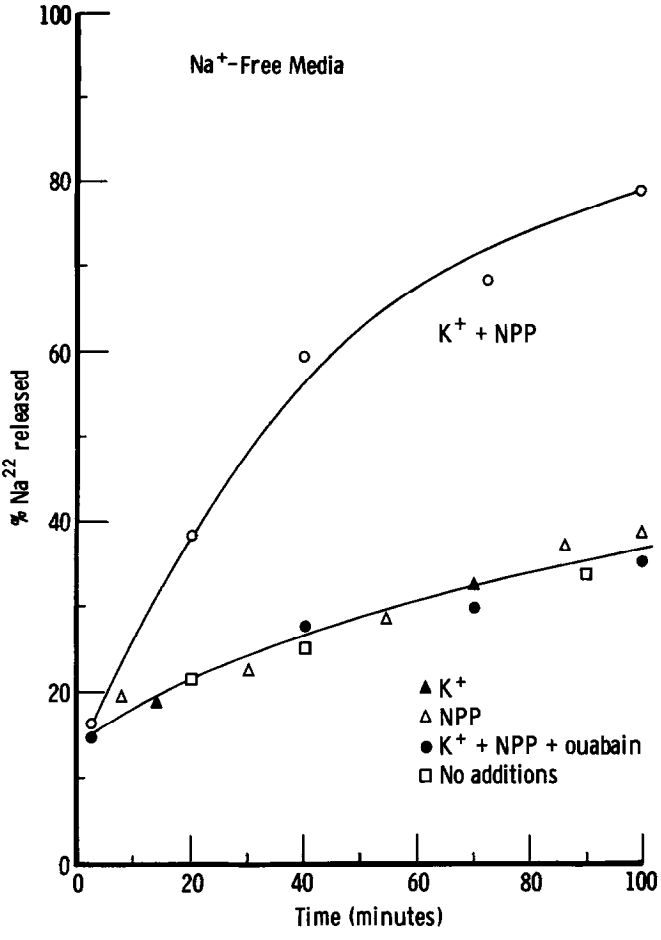

Figure 1 STIMULATION OF Na^+ EFFLUX INTO Na^+-FREE MEDIUM BY K^+ AND NPP.

The Na^+-free medium contained 160 mM choline chloride, 10 mM Tris-HCl (pH 7.4). The indicated compounds were added to this medium at the following final concentrations: KCl, 6 mM; Tris salt of NPP, 3 mM; ouabain, 0.1 mM. In each experiment 1 ml of ghosts was added to 50 ml of the medium, and the release of Na^{22} at 37° was measured by the procedure described before[12].

Na^+-free medium the presence of K^+ caused an increase in hydrolysis, but that no K^+-dependent hydrolysis could be detected in the Na^+-containing medium (Table 1). This correlation between the existence of Na^+, K^+-exchange and K^+-dependent NPP hydrolysis suggested that the absence of exchange in the Na^+-containing medium was perhaps due to the inhibitory effects of Na^+ on the K^+-dependent phosphatase[14,15]. Since nucleoside triphosphates are able not only

Table 1. CORRELATION BETWEEN THE STIMULATION OF Na^+ EFFLUX AND HYDROLYSIS OF NPP. Composition of the media, and the concentrations of added compounds were the same as indicated in legends to Figures 1 and 2. p-nitrophenol was measured by the colorimetric method[16] after the deproteinazation of aliquots of the reaction mixtures.

	Additions to media	% of Na^{22} released in one hr.	Nitrophenol (μ moles/ml/hr)
Na^+-free medium:	NPP	35	0.12
	NPP + K^+	70	0.31
	NPP + K^+ + ouabain	31	0.13
Na^+-containing medium:	NPP	33	0.14
	NPP + K^+	36	0.12

to overcome this inhibitory effect of Na^+ but also cause an extra activation of the K^+-dependent phosphatase[11,15], we determined the effects of addition of nucleotides to the medium on Na^+ efflux and NPP hydrolysis. The results of Figure 2 clearly show that in a Na^+-containing medium where neither NPP alone nor ATP alone affect Na^+ efflux, the simultaneous presence of both compounds initiates a ouabain-sensitive and K^+-dependent efflux of Na^+ from the ghosts. From the data of Table 2 the following additional points are evident: 1. In all cases there is a direct correlation between the stimulation of Na^+ efflux and an increase in the production of p-nitrophenol. 2. It is the hydrolysis of NPP, and not the presence of the products of this hydrolysis, which is related to the stimulation of Na^+ efflux. 3. CTP, which is not a substrate for Na^+, K^+-ATPase but has the same modifying effect on the K^+-dependent phosphatase as ATP[11], can also stimulate Na^+ efflux and NPP hydrolysis in the Na^+-containing medium. 4. Iodoacetate has no effect on the stimulated efflux of Na^+, indicating that the efflux is not due to the metabolism of traces of substrates that may have remained within the ghosts[12].

The data of this paper show that when a membrane-bound phosphatase, which is apparently oriented toward the medium-side of the membrane, is

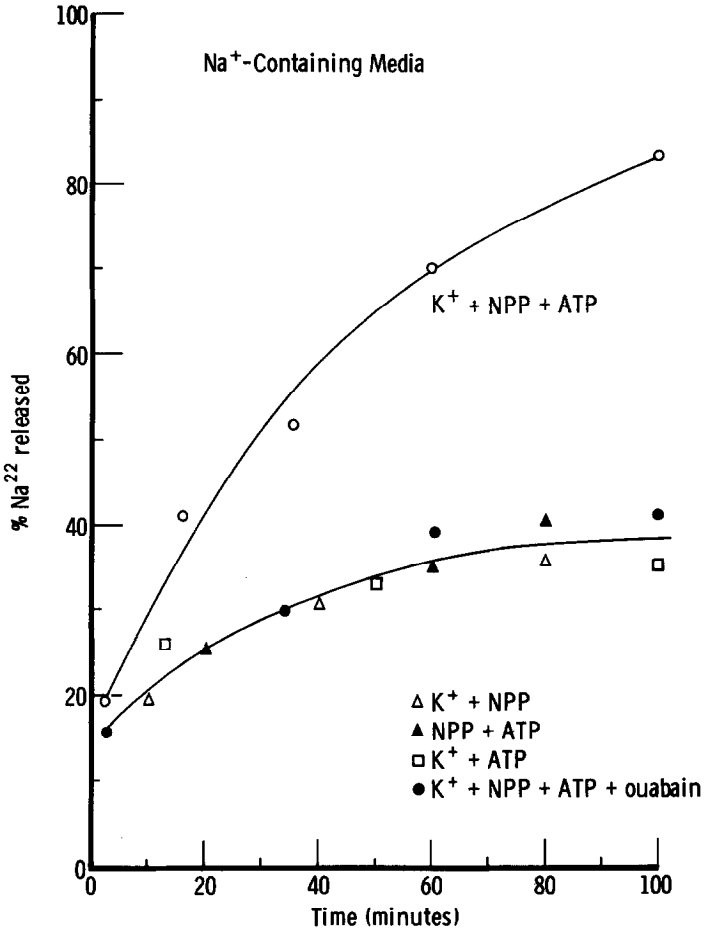

Figure 2 STIMULATION OF Na^+ EFFLUX INTO Na^+-CONTAINING MEDIUM BY THE SIMULTANEOUS PRESENCE OF K^+, NPP, AND ATP IN THE INCUBATION MEDIUM. Each medium contained 160 mM NaCl, 10 mM Tris-HCl (pH 7.4) and the other added components as described for figure 1. ATP concentration was 0.5 mM.

supplied with a substrate and a nucleotide modifier (both from the medium) it is capable of catalyzing the extrusion of Na^+ against a concentration gradient. This extrusion in its dependence on external K^+ and its sensitivity to cardiac glycosides is similar to that caused by the presence and hydrolysis of ATP on the matrix side of the membrane[4,6].

The following two important questions are raised by these findings:

1. Under the conditions described in these experiments, which are not likely

Table 2. STIMULATORY EFFECTS OF NUCLEOTIDES ON Na^+ EFFLUX AND NPP HYDROLYSIS IN A Na^+-CONTAINING MEDIUM. Concentrations of ATP, NPP and ouabain were the same as described in Figures 1 and 2. Other compounds were added as follows: CTP, 0.5 mM; p-nitrophenol, 1 mM; orthophosphate (Na_2HPO_4-HCl, pH 7.4), 1 mM; iodoacetate, 1 mM.

Incubation Medium:	Additions to the medium	% of Na^{22} released in one hr.	Nitrophenol (μmoles/ml/hr)
	None	32	---
NaCl, 160 mM;	NPP	37	0.10
KCl, 6 mM;	ATP	31	---
Tris-HCl (pH 7.4)	NPP + ATP	84	0.40
10 mM	NPP + ATP + ouabain	30	0.12
	NPP + ATP + IA	81	0.36
	NPP + CTP	78	0.39
	p-nitrophenol + Pi	29	0.06

to be duplicated under physiological conditions, how is the pump operating? The simplest way of explaining the data would be to assume that the anisotropic phosphatase is the "primary translocator"[10], and that there is no distinction between the chemical reaction catalyzed by this enzyme and the translocation of its activator cation (K^+) from outside to inside. The sufficiency of NPP hydrolysis for the occurance of Na^+, K^+-exchange in the absence of external Na^+ suggests that when the enzyme (or the active center region of the enzyme) is freed of substrate and products, it can move Na^+ from inside to outside. In the presence of relatively high concentrations of Na^+ in the medium the ion exchange does not occur because Na^+ inhibits the phosphatase and the chemical reaction does not proceed. In this case the occupation of a modifying site by a nucleotide becomes necessary. The similar effects of ATP and CTP, both in the experiments described in this paper and those dealing with the partially purified phosphatase[11], and the apparent accessability of the modifying site from the medium-side of the membrane, give further support to the idea that this site is different from that involved in the hydrolysis

of internal ATP. 2. From the findings of this paper what conclusions may be drawn about the physiological mode of operation of the pump? If there is validity to our interpretation of the NPP effects, it is perhaps more logical to consider the over-all Na^+, K^+-ATPase activity as a combination of two distinct enzyme activities: One involved in the formation of phosphorylated protein (or proteins) from ATP, and the other the translocator-phosphatase discussed above. The function of the first enzyme, which is facing the matrix side of the membrane, would be to supply a phosphoprotein substrate for the translocator-phosphatase. Since under physiological conditions (high external Na^+) the modifying site of the phosphatase must be occupied by a nucleotide, one must assume that this site, in contrast to the active site of the phosphatase, can be reached from both sides of the membrane; and that an additional physiological role of ATP is the occupation of this modifying site.

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