Biochimica et Biophysica Acta, 1063 (1991) 217–225

ADONIS 000527369100155J

BBAMEM 75181

Phosphorylation of Na,K-ATPase by acetyl phosphate and inorganic phosphate. Sidedness of Na^+, K^+ and nucleotide interactions and related enzyme conformations

Graciela Berberián and Luis Beaugé

División de Biofísica, Instituto de Investigación Médica 'Mercedes y Martín Ferreyra', Córdoba (Argentina)

(Received 7 August 1990)
(Revised manuscript received 27 November 1990)

Key words: ATPase, Na^+/K^+-; Enzyme conformation; Phosphorylation; Acetyl phosphate; Inorganic phosphate; (Pig kidney)

The effects of K^+, Na^+ and nucleotides (ATP or ADP) on the steady-state phosphorylation from [^{32}P]P_i (0.5 and 1 mM) and acetyl [^{32}P]phosphate (AcP) (5 mM) were studied in membrane fragments and in proteoliposomes with partially purified pig kidney Na,K-ATPase incorporated. The experiments were carried out at 20°C and pH 7.0. In broken membranes, the P_i-induced phosphoenzyme levels were reduced to 40% by 10 mM K^+ and to 20% by 10 mM K^+ plus 1 mM ADP (or ATP); in the presence of 50 mM Na^+, no E-P formation was detected. On the other hand, with AcP, the E-P formation was reduced by 10 mM K^+ but was 30% increased by 50 mM Na^+. In proteoliposomes E-P formation from P_i was (i) not influenced by 5–10 mM K^+_{cyt} or 100 mM Na^+_{ext}, (ii) about 50% reduced by 5, 10 or 100 mM K^+_{ext} and (iii) completely prevented by 50 mM Na^+_{cyt}. Enzyme phosphorylation from AcP was 30% increased by 10 mM K^+_{cyt} or 50 mM Na^+_{cyt}; these E-P were 50% reduced by 10–100 mM K^+_{ext}. However, E-P formed from AcP without K^+_{cyt} or Na^+_{cyt} was not affected by extracellular K^+. Fluorescence changes of fluorescein isothiocyanate labelled membrane fragments, indicated that E-P from AcP corresponded to an E_2 state in the presence of 10 mM Na^+ or 2 mM K^+ but to an E_1 state in the absence of both cations. With pNPP, the data indicated an E_1 state in the absence of Na^+ and K^+ and also in the presence of 20 mM Na^+, and an E_2 form in the presence of 5 mM K^+. These results suggest that, although with some similarities, the reversible P_i phosphorylation and the phosphatase activity of the Na,K-ATPase do not share the whole reaction pathway.

Introduction

The Na,K-ATPase is a plasma membrane enzyme that catalyzes the ATP hydrolysis coupled to the active transport of Na^+ out of and K^+ into the cell. The reaction mechanism of this enzyme involves several reversible intermediate steps going through two major conformations, or family of conformations, named E_1 and E_2. In the forward direction, Na(i)–K(o) exchange accompanied by ATP hydrolysis, there are two main steps: the transfer of the terminal phosphate from ATP to the protein and the subsequent dephosphorylation [1,2]. The reverse reaction, Na(o)–K(i) exchange associated to ATP synthesis, includes the reversible enzyme phosphorylation from inorganic phosphate. The ATP- and P_i-dependent phosphoenzymes involve the same chemical group and are chemically identical (a covalent phosphoryl group incorporation leading to the formation of an aspartyl phosphate). Despite that fact, the ligands needed for their formation and their reactivity to ligands are quite different. Phosphorylation from ATP requires intracellular Na^+ and Mg^{2+} and its breakdown is accelerated by external K^+. On the other hand, the P_i phosphorylation needs only Mg^{2+}; Na^+ and nucleotides (ADP, ATP and ATP analogues) are inhibitors whereas the effects of K^+ depend on the time of its addition to the reaction mixtures: K^+ accelerates turnover and dephosphorylation if present during E-P formation, but the phosphoenzyme is completely insensitive to K^+ if it has been made in its absence [3,4]. All studies on P_i phosphorylation of Na,K-ATPase have been carried out in broken membrane preparations; under these conditions, the constituents of the incubation solutions have simultaneous access to extracellular and cytoplasmic sites of enzyme. Thus, although these types of experiments can give some insight on the

Correspondence: L. Beaugé, Instituto de Investigación Médica M. y M. Ferreyra, Casilla de Correo 389, 5000 Córdoba, Argentina.

sidedness of a ligand site, they do not provide clear cut answers on the matter; in addition, possible trans effects are missed.

Every preparation that shows Na,K-ATPase activity consistently displays a Mg^{2+}-K^+-dependent phosphatase activity, that is the hydrolysis of non nucleotide phosphate anhydrides (acetyl and *p*-nitrophenyl phosphate are the most commonly used). Acting alone, Na^+ and nucleotides are phosphatase inhibitory; still, the simultaneous presence of Na^+ and ATP increases the affinity to K^+ and usually reduces the maximal reaction rate [2,5]. The real meaning of this phosphatase activity remains under debate and its reaction cycle is far from established. Recent studies support the idea that it has similarities with the reversible binding of inorganic phosphate [6,7]. In addition, it has been shown that acetyl phosphate can phosphorylate the enzyme in the presence of Mg^{2+} and K^+ [6]; unfortunately, the interpretation of the latter finding is complicated because acetyl phosphate can also phosphorylate the enzyme (i) in the presence of Na^+ and Mg^{2+}, acting as substrate for Na,K-ATPase, and (ii) even in the absence of Na^+ and K^+ as part of a yet unidentified reaction [8]. Likewise, the Na,K-ATPase is phosphorylated by *p*-nitrophenyl phosphate, but this occurs only in the presence of ouabain [9]. Whether these phosphoenzymes belong to the reaction mechanism of the phosphatase activity remains an open question.

The aim of the work described in this paper was to look for the sidedness of the effects of Na^+, K^+ and nucleotides on the steady-state phosphorylation levels acquired with acetyl phosphate and inorganic phosphate. To this end, we used liposomes with Na,K-ATPase incorporated where the intra and extravesicular composition was varied at will. The fluorescein-labelled enzyme covalently incorporates inorganic phosphate and exhibits a K^+-dependent phosphatase activity [10]; therefore, we used this probe in broken membrane to investigate the most abundant phosphoenzyme conformation induced by the two aforementioned phosphoryl group donors. The results have determined the sidedness of Na^+ and K^+ effects in both cases; in addition, they suggest that although with some similarities, the reversible P_i phosphorylation and the phosphatase activity of the Na,K-ATPase go through different pathways.

Methods

Na,K-ATPase was partially purified from pig kidney red outer medulla by the procedure described by Jørgensen [11]. The membranes were suspended in a medium containing 25 mM imidazole (pH 7.5 at 20 °C)/1 mM EDTA-Tris/250 mM sucrose at a protein concentration of 2–3 mg/ml and stored at −85 °C. Before used about 1 ml of the suspension was dialyzed overnight against 1000 ml of ice-cold 25 mM imidazole (pH 7.0 at 20 °C)/1 mM EDTA-Tris. The Na,K-ATPase activity under standard conditions (120 mM NaCl/30 mM KCl/30 mM imidazole (pH 7.4)/3 mM $MgCl_2$/3 mM ATP and 37 °C) was between 18 and 22 units per mg protein and remained stable for months. Protein was determined by the method of Lowry et al. [12] with modifications [13], using bovine serum albumin as standard.

Proteoliposomes were prepared following the procedure described by Karlish and Pick [14]. The phospholipids were converted to Tris salt as indicated in Ref. 15. The enzyme was solubilized with Tris cholate. Immediately after solubilization, the non soluble protein was removed by centrifugation in a Beckman Airfuge (10 min at an air pressure of 20 lb/inch^2). With this technique, any enzyme fraction that eventually did not incorporate into the liposomes remained inactive [14]; this was checked by performing ATPase assay in the supernatant after the liposomes were centrifuged. We were particularly careful in this point because due to the experimental protocols we could not use either vanadate or ouabain as Na,K-ATPase inhibitors: (i) ouabain would stabilize E-P formation by any active enzyme molecule that did not incorporate into the vesicles (Ref. 16; Campos and Beaugé, unpublished) and (ii) vanadate, which acts intracellularly [17], would prevent phosphorylation of all active enzyme species, whether incorporated or not. To change the composition of the extravesicular media the liposomes were centrifuged at 1000 rpm for 3 min through a 1 ml syringe filled with Sephadex G-50 equilibrated with 140 mM Tris-HCl (pH 7.0).

K–K exchange was estimated in some experiments in parallel with enzyme phosphorylation from [^{32}P]P_i. The proteoliposomes, containing 100 mM KCl and 40 mM Tris-HCl (pH 7.0 at 20 °C), were incubated in media with 5 mM KCl, 3 mM $MgCl_2$, 130 mM Tris-HCl (pH 7.0 at 20 °C) without or with 0.1 mM ATP and/or 0.5 mM inorganic phosphate. The experiments were performed in the absence and presence of 0.5 mM vanadate using [^{86}Rb]Rb^+ as a K^+ marker; the incubation time lasted 2 min. Temperature was 20 ± 2 °C. The ^{86}Rb incorporated was estimated from the radioactivity present in the effluent of short Sephadex column as described previously [18].

Enzyme phosphorylation from 5 mM acetyl [^{32}P]phosphate, 0.5 mM or 1 mM inorganic [^{32}P]phosphate and 0.01 mM [γ-^{32}P]ATP was carried out by incubating 50 μg of broken membrane protein, or 0.2 ml of proteoliposomes suspension, in media containing 3 mM $MgCl_2$/0.1 mM EGTA-Tris/(140 − ([KCl] + [NaCl])) mM Tris-HCl (pH 7.0 at 20 °C) in a final volume of 0.35 ml. The reaction was started by the addition of enzyme (or proteoliposomes) and terminated after 10 s (acetyl phosphate and inorganic phosphate) or

2 s (ATP); the stopping solution consisted of 3.5 ml of an ice cold 15% perchloric acid/50 mM phosphoric acid/10 mM pyrophosphate; in the case of [γ-^{32}P]ATP the stopping solution contained in addition 0.5 mM unlabelled ATP. The mixture was allowed to stand for 30 min at 0°C and the denatured protein was collected and washed in glass fiber filters as indicated previously [19]. The amount of phosphoenzyme obtained, even under identical experimental conditions, varied in different preparations. A likely source for that variation could be the amount of enzyme incorporation. Therefore, we normalized the E-P values based on the maximal *p*-nitrophenylphosphatase activity observed in liposomes incubated for 5 min at 20°C in 0.2 ml of the following media: 3 mM $MgCl_2$/20 mM KCl/140 mM Tris-HCl (pH 7.0)/5 mM *p*-nitrophenyl phosphate with or without 1 mM ouabain [19]. This phosphatase activity varied between 0.78 and 1.32 nmol/min per 10 μl of liposomes. Identical rates of hydrolysis were obtained with and without ouabain; this is another indication that there was no contamination with unincorporated non soluble active Na,K-ATPase. Regarding the incubation times for phosphorylation, proper controls showed that, with the times chosen, the E-P formed with all phosphoryl donors was on steady-state conditions. The concentrations of acetyl phosphate and *p*-nitrophenyl phosphate chosen were those we have consistently used in our studies on phosphatase activity [6,18]. As we intended to compare this with previous works, we found preferable to stick to similar concentrations of the substrates.

The synthesis of acetyl [^{32}P]phosphate was done according to Stadtman [20] using [^{32}P]P_i purified as in Ref. 21. Labelled [γ-^{32}P]ATP was made following the method of Glynn and Chappell [22] as modified by De Meis [23]. The synthesized acetyl [^{32}P]phosphate may have been contaminated with inorganic [^{32}P]phosphate, but that would not go beyond the errors of P_i [24] and acetyl phosphate [20] detections. Combining the two errors in our hands the total P_i contamination could be, at the most, 2 percent. However, the fact that E-P formed from P_i and AcP have different reactivities toward K^+ and Na^+ (see Results), makes the problem of P_i contamination irrelevant.

To prepare the fluorescein labelled enzyme, membrane fragments of Na,K-ATPase were incubated with fluorescein-isothiocyanate (FITC) as indicated in Ref. 25. Steady-state fluorescence signals were followed in a mixture of 50 μg labelled enzyme suspended in 2 ml of a solution containing 100 mM Tris-HCl (pH 7.0 at 20°C)/2 mM CDTA (Mg^{2+}-free) or 3 mM $MgCl_2$ (no CDTA) and the other ligands listed in the legends to Figs. 1 and 2. A Mark I (Ferrand Optical Co.) spectrofluorometer was used. Excitation was at 495 nm and emission was measured at 520 nm with a slit width of 10 nm. As usual, the temperature was 20 ± 2°C.

All solution were made with bidistilled deionized water. NaCl and KCl were Baker Ultrex; all other chemicals were reagent grade. ADP, ATP (Na salts), *p*-nitrophenyl phosphate (Tris salt), acetyl phosphate (Li plus K salt), ouabain, cholic acid, L-α-phosphatidylcholine, Sephadex G-50-40, and fluorescein isothiocyanate (isomer I) were obtained from Sigma Chemical Co., U.S.A. Nucleotides and acetyl phosphate were transformed into Tris salts by passing them through Amberlite IR-120-P columns. 86-Rubidium, as chloride salt, was purchased from New England Nuclear, U.S.A. Inorganic [^{32}P]phosphate was provided by the Comisión Nacional de Energía Atómica, Argentina.

Experimental values are means of duplicate (phosphorylation) or triplicate (fluorescence) determinations. In addition, they were repeated and when relevant, these results are included in the tables. In no case the difference between E-P duplicate samples was larger than 4%, while the differences observed when varying the experimental conditions were at least 30%.

Results

Effects of Na^+ and K^+ on the levels of E-P obtained from ATP, inorganic phosphate and acetyl phosphate

In the first group of experiments we studied the effects of 50 mM Na^+ and 10 mM K^+, alone and in combination, on the steady-state levels of phosphoenzyme in a broken membrane preparation. This was essential in order to compare liposomes with broken membranes preparations under identical conditions including pH (7.0) and temperature (20°C). In addition, it was also indispensable to contrast, under the same conditions, the E-P levels obtained from three phosphoryl group donors: 0.01 mM ATP, 0.5 or 1.0 mM P_i and 5 mM AcP. As expected (Table I, 1, 2), in the absence of both Na^+ and K^+, phosphorylation was

TABLE I

Effects of Na^+ and K^+ on steady-state levels of phosphorylation from ATP, P_i and AcP in a broken membrane preparation of partially purified Na,K-ATPase

Membrane fragments of partially purified Na,K-ATPase from pig kidneys were incubated in solutions containing 3 mM $MgCl_2$, 150–([Na + K]) mM Tris-HCl (pH 7.0 at 20°C) with 0.01 mM [γ-^{32}P]ATP, 5 mM acetyl [^{32}P]phosphate or 0.5 mM or 1 mM inorganic [^{32}P]phosphate. Temperature was 20 ± 2°C. The Na^+ and K^+ concentrations are shown in the table. Experimental values are means of duplicate determinations; in no case the difference between the duplicate samples was larger than 2%. See Methods for details.

Na^+ (mM)	K^+ (mM)	E-P (nmol/mg)			
		0.01 mM ATP	0.5 mM P_i	1.0 mM P_i	5 mM AcP
–	–	0.01	1.16	1.73	1.52
50	–	2.20	0.03	0.04	1.98
–	10	0.01	0.56	0.70	0.61
50	10	0.14	0.05	0.09	0.76

negligible with ATP; under the same conditions, 1 mM P_i gave values close to 70 percent of the maximum phosphorylation whereas AcP did promote a sizable level of E-P that reached 63% of that maximal. In the presence of Na^+ alone, ATP rendered the maximal values whereas those obtained with AcP were even higher than that with no monovalent cations (83% of that seen with ATP); on the other hand, with P_i the E-P formation was practically nill. When K^+ was the only monovalent cation, the picture was qualitatively similar to that seen with no Na^+ or K^+ (only P_i and AcP resulted in E-P formation) but the absolute values were about halved. Finally, in the simultaneous presence of Na^+ plus K^+ E-P formation was only noticeable with AcP. Although not included in Table I, the addition of 1 mM unlabelled ADP or 0.1 mM unlabelled ATP in the presence of 10 mM K^+ reduced by 50% the P_i- and AcP-dependent ^{32}P incorporation.

Sidedness of Na^+, K^+ and nucleotides effects on E-P levels from different sources

To study the sidedness of ligands effects on E-P from different phosphoryl donors we use as a preparation Na,K-ATPase incorporated into liposomes. The first approach was to check the behavior of the liposomes and the incorporated enzyme. To this end we measured their ability to perform K–K exchange; in addition, we used these assays to gather information regarding the relationship between the levels of fluxes and those of P_i incorporation into the enzyme. In agreement with previous reports [15] the basal vanadate sensitive K–K exchanges fluxes were low (0.048 nmol/min per 10 μl liposomes) and not affected by 0.5 mM P_i alone. Still, in the presence of 0.5 mM P_i, 0.1 mM ATP increased K–K exchange by 5.7-fold (to 0.278 nmol/min per 10 μl liposomes); on the other hand, the levels of E-P were reduced by only 30% (from 1.08 pmol/10 μl liposomes to 0.73 pmol/10 μl liposomes); i.e., a large ATP stimulation of P_i-dependent K–K exchange fluxes coexisted with a small reduction in the steady-state levels of enzyme phosphorylation. It is worth mentioning at this point that these ATP effects on [E-P] were consistently smaller in proteoliposomes than in broken membrane preparations, where the reduction was systematically about 50% (not shown).

The actual experiments on sidedness are illustrated in Tables II and III. These results can be summarized as follows (note that cyt and ext refer to the cytoplasmic (extravesicular) and the extracellular (intravesicular) sides, respectively): (i) The sites at which Na^+ ions inhibited P_i-dependent phosphorylation, were required for phosphorylation from ATP and stimulated E-P formation from AcP, are all cytoplasmic. (ii) K^+_{cyt} had no effect on the levels of E-P formed from P_i whereas it increased by 30% those obtained with AcP. (iii) The values of E-P formed from Pi, with or without K^+_{cyt},

TABLE II

Effects of cytoplasmic and extracellular Na^+ and K^+ on the steady-state levels of phosphorylation from 0.5 mM inorganic phosphate of Na,K-ATPase incorporated into liposomes

Liposomes with Na,K-ATPase incorporated were incubated in solutions which, besides what is shown in the table, had the following composition: (i) intravesicular (extracellular): 140–([Na+K]) mM Tris-HCl (pH 7.0 at 20°C); (ii) extravesicular (cytoplasmic): 135–([Na+K]) mM Tris-HCl (pH 7.0 at 20°C), 3 mM $MgCl_2$, and 0.5 mM inorganic [^{32}P]phosphate. The reaction was started by the addition of the proteoliposomes while the tubes were mixed in a vortex; the reaction time lasted 10 s. Note: (i) the same letters in the last column refer to the same batch of proteoliposomes; (ii) ** indicates the presence of 0.1 mM ATP in the extravesicular solution. (iii) The E-P values were normalized based on the maximal *p*-nitrophenylphosphatase activity observed in each liposome batch. (iv) Intravesicular concentrations refer to those present in the media when the enzyme was incorporated into the liposomes. (v) Experimental values are means of duplicate determinations; in no case the difference between the duplicate samples was larger than 4%. See Methods for details.

Cytoplasmic		Extracellular		E-P (pmol/10 μl liposomes)	
Na^+ (mM)	K^+ (mM)	Na^+ (mM)	K^+ (mM)		
–	–	–	–	2.40	(a)
–	5	–	–	2.60	(a)
–	10	–	–	2.30	(b)
–	–	–	5	1.19	(a)
–	–	–	10	1.37	(b)
–	5	–	5	1.26	(a)
–	10	–	10	1.05	(b)
–	5	–	100	1.17	(c)
–	5	–	100	0.85 **	(c)
50	5	–	5	0.08	(a)
50	–	–	–	0.04	(a)
–	–	100	–	2.40	(d)
–	10	100	–	2.29	(d)
–	10	100	10	0.97	(d)

were about 50% reduced in the presence of K^+_{ext}. At 10 mM K^+_{ext} this action could not be counteracted by 100 mM Na^+_{ext}. On the other hand, in K^+-free conditions 100 mM Na^+_{ext} showed no effect by themselves. (iv) In contrast, when E-P was formed from AcP, a reduction due to K^+_{ext}, which also amounted to 50%, occurred only if these was a simultaneous presence of K^+ or Na^+ in the cytoplasmic side. (v) Another important difference between [E-P] from P_i and [E-P] from AcP, is that the maximal levels of the former came about in the absence of any monovalent cation on both membrane phases. (vi) The Na^+_{cyt}-dependent phosphorylation from ATP decreased to about 10% in the presence of K^+_{ext}. (vii) With the concentrations of phosphorylating compounds used here, and in the absence of K^+_{ext}, the maximal levels of E-P formation obtained from ATP in the presence of Na^+_{cyt} matched those seen with AcP in the presence of either K^+_{cyt} or Na^+_{cyt}. (viii) The [E-P] formed

from AcP in the absence of Na^+ and K^+ at both sides of the membrane are comparable to what was observed in broken membranes incubated in the absence of both monovalent cations. (ix) Finally, the addition of intracellular ATP or ADP, resulted in a 30–40% fall in [E-P] formed from inorganic phosphate or acetyl phosphate in the presence of K^+_{cyt} and K^+_{ext}.

Conformations of Na,K-ATPase incubated with different phosphorylating agents under varying ionic conditions

A comparison between the properties of E-P from inorganic phosphate and acetyl phosphate is relevant to the understanding of the phosphatase reaction catalyzed by the Na,K-ATPase enzyme and its relationship/s with the ATPase activity [2,6,7,26]. Therefore, it seemed important to establish the enzyme conformations associated to each phosphorylating agents in the absence and presence of Na^+, K^+ and Mg^{2+}. An ideal probe should allow binding of nucleotides to the enzyme and distinguish between phosphorylated from unphosphorylated states [27]. 5-Iodoacetamidofluorescein is good for those purposes in dog kidney enzyme, but unfortunately it does not work with enzyme from the pig [28]. So, we settle for isothiocyanate of fluorescein. This probe, although prevents nucleotide binding, has the advantages that (i) can be used without problems at 20 °C, (ii) it does not interfere, or does it very little, with the phosphatase reaction, (iii) it allows AcP to act as substrate of Na,K-ATPase and (iv) it permits phosphorylation from P_i [10,25,29,30]. With this compound, a reduction in the fluorescence signal is considered to correspond to a transition from E_1 toward an E_2 state, whereas a rise in that signal indicates an E_2 to E_1 shift (see above references). Accordingly, we followed the steady-state fluorescence changes of membrane fragments containing Na,K-ATPase labelled with fluorescein isothiocyanate in the presence of acetyl phosphate, *p*-nitrophenyl phosphate and inorganic phosphate. Another limitation of this approach, was the fluorescence quenching due to the phosphoryl group donors and to Mg^{2+} ions; there is now evidence indicating that the effect of Mg^{2+} is not on the enzyme conformation as thought before [10] but on the phospholipids order parameters [31]. At 5 mM concentration, quenching was about 40 percent with *p*-nitrophenyl phosphate and 5–7% with acetyl phosphate; 0.5 mM or 1.0 mM P_i had practically no effect, and around 20% quenching was seen with 3 mM $MgCl_2$. Therefore, for acetyl and *p*-nitrophenyl phosphate the initial base lines were established in the presence of the phosphorylating agents without or with Mg^{2+} and with no monovalent cations. The changes observed following the addition of Na^+ and K^+ were related to the signals obtained under those initial conditions. Typical experiments of this kind are illustrated in Figs. 1 and 2. We performed introductory experiments in the absence of phosphorylating substrates, with and without Mg^{2+}. Under these conditions, 2 mM K^+ produced about 6% quenching in the fluorescence, and that quenching was reversed by 20 mM Na^+; in K^+-free media, the addition

TABLE III

Effects of cytoplasmic and extracellular Na^+ and K^+ on the steady levels of phosphorylation from acetyl phosphate and ATP of Na,K-ATPase incorporated into liposomes

The general experimental procedure was identical with that described in the legend to Table II. In this case the phosphoryl group donors were acetyl [^{32}P]phosphate or [γ-^{32}P]ATP. Note: (i) the same letters in the last column refer to the same batch of proteoliposomes; (ii) ** indicates the presence of 1.0 mM ADP in the extravesicular solution. (iii) The E-P values were normalized based on the maximal *p*-nitrophenylphosphatase activity observed in each liposome batch. (iv) Intravesicular concentrations refer to those present in the media when the enzyme was incorporated into the liposomes. (v) Experimental values are means of duplicate determinations; in no case the difference between the duplicate samples was larger than 4%. See Methods for details.

Cytoplasmic		Extracellular		E-P (pmol/10 μl liposomes)	
Na^+ (mM)	K^+ (mM)	Na^+ (mM)	K^+ (mM)	5 mM AcP	0.01 mM ATP
–	–	–	–	2.40	– (a)
–	–	–	–	2.22	– (b)
50	–	–	–	3.00	3.40 (a)
–	10	–	–	3.05	– (a)
–	10	–	–	3.15	– (b)
–	–	–	10	2.46	– (a)
–	–	–	100	2.35	– (b)
–	10	–	10	1.64	– (a)
–	5	–	100	1.43	– (c)
–	5	–	100	0.96 **	– (c)
–	10	–	100	1.55	– (b)
50	–	–	10	1.80	0.42 (a)

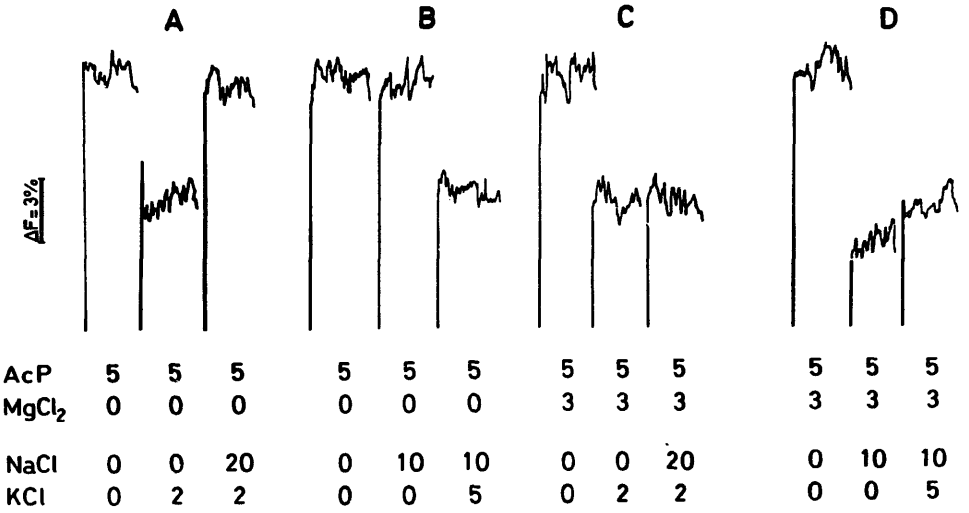

Fig. 1. Effects of Na^+ and K^+, alone or in combination, on the steady-state levels of fluorescein fluorescence of fragmental FITC-Na,K-ATPase in the presence of acetyl phosphate (AcP) without (A and B) and with (C and D) $MgCl_2$. The FITC-labelled enzyme was incubated in 2 ml of a solution containing 100 mM Tris-HCl (pH 7.0 at 20 °C). The final concentrations of added ligands (in mM) are shown at the bottom of the figure. Those concentrations were obtained by adding small volumes (not greater than 20 μl) of concentrated solutions to the fluorescence cell. The experiments were performed at 20±2 °C and at pH 7.0. When $MgCl_2$ was omitted 2 mM CDTA-Tris was included. A 3% relative fluorescence change is indicated by a vertical bar. Note: the traces shown are parts of the recorded steady-state fluorescence signals which lasted for at least 1 min. For details see Methods.

of 20 mM Na^+ did not induce any detectable change in the signal.

In the presence of acetyl phosphate and no Mg^{2+}, 2 mM K^+ reduced the relative fluorescence by 5.5 ± 0.3% ($n = 3$) and this effect was fully counteracted by 20 mM Na^+ (Fig. 1A). Without potassium, 10 mM Na^+ had no effect on the basic fluorescence; the addition of 5 mM K^+ on top of 10 mM Na^+ (a K^+/Na^+ ratio of 0.5) resulted in a fluorescence decline equivalent to that seen with 2 mM K^+ alone (compare Figs. 1A and 1B). With 3 mM $MgCl_2$, the addition of 2 mM K^+ produced a fluorescence quenching similar to that detected in Mg^{2+}-free solutions (5.6 ± 0.3% (n = 3)) (Fig. 1C); however, in this case 20 mM Na^+ did not revert the action of K^+ (Fig. 1C). Moreover, 10 mM Na^+ alone also induced a fluorescence drop (8.6 ± 0.5% ($n = 3$)) (Fig.

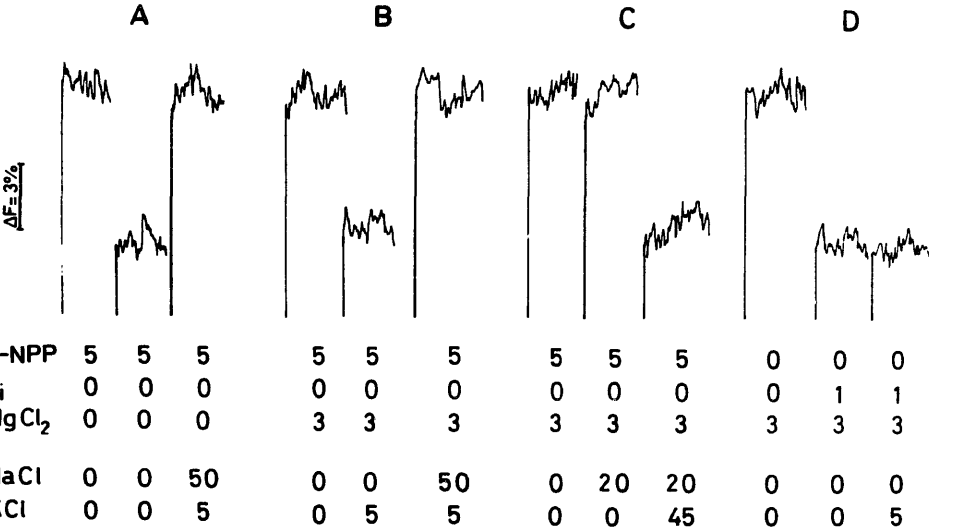

Fig. 2. Effects of Na^+ and K^+, alone or in combination, on the steady-state levels of fluorescein fluorescence of fragmental FITC-Na,K-ATPase in the presence of *p*-nitrophenyl phosphate (p-NPP), without (A) and with (B and C) $MgCl_2$. As a control, the effect of inorganic phosphate (P_i), with and without K^+, in the presence of $MgCl_2$ is included (D). The FITC-labelled enzyme was incubated in 2 ml of a solution containing 100 mM Tris-HCl (pH 7.0 at 20 °C). The final concentrations of added ligands (in mM) are shown at the bottom of the figure. Those concentrations were obtained by adding small volumes (not greater than 20 μl) of concentrated solutions to the fluorescence cell. The experiments were performed at 20±2 °C and at pH 7.0. When $MgCl_2$ was omitted, 2 mM CDTA-Tris was included. A 3% relative fluorescence change is indicated by a vertical bar. Note: the traces shown are parts of the recorded steady-state fluorescence signals which lasted for at least 1 min. For details see Methods.

1D). The fact that when either Na^+ or K^+ were added to an enzyme in the presence of AcP and Mg^{2+} the signal was systematically deflected downwards, is compatible with (i) the initial state of the enzyme was of an E_1-P type, and (ii) both Na^+ and K^+ shifted that state towards an E_2-P form.

With *p*-nitrophenyl phosphate, 5 mM K^+ reduced the steady-state fluorescence level by $6.7 \pm 0.7\%$ ($n = 3$) in the absence (Fig. 2A) and by $5.3 \pm 0.5\%$ ($n = 3$) in the presence (Fig. 2B) of Mg^{2+}. Yet, and as a difference with acetyl phosphate, the K^+-dependent deflection was fully recovered by the addition of Na^+ ions even in the presence of Mg^{2+} (Figs. 2A and 2B). Another remarkable difference is that with *p*-nitrophenyl phosphate in K^+-free condition, Na^+ ions were unable to induce a significant drop in fluorescence in solutions containing Mg^{2+} (Fig. 2C). On the other hand, as it happens with acetyl phosphate, the Na,K-ATPase incubated with *p*-nitrophenyl phosphate in the absence of any monovalent cation, with or without Mg^{2+}, is in an E_1 state (see also Ref. 32). However, we do not know if with Mg^{2+} phosphorylation takes place.

Finally, Fig. 2D is a control experiment showing that (i) the major enzyme conformation obtained in the presence of inorganic phosphate and Mg^{2+} is of an E_2-P type [10,32], and (ii) that basic conformation is not modified by K^+.

Discussion

The experiments presented in this work provide new evidence regarding the sidedness of Na^+ and K^+ effects on the steady-state levels of E-P of Na,K-ATPase carrying on ATPase and phosphatase activities and reversible phosphorylation from inorganic phosphate. In addition, they allow a comparison of that information with the predominant enzyme conformations given by the FITC fluorescence probe at the same pH (7.0) and temperature (20 °C).

In the fluorescence experiments we have looked at the prevailing enzyme conformations in situations which were not explored before with this approach. These correspond to phosphatase activity in the presence of AcP plus K^+, AcP plus Na^+ and K^+, pNPP plus K^+ and pNPP plus Na^+ and K^+. On the other hand, the effects of AcP with Na^+ (ATPase activity) coincide with those reported by Rephaeli et al. [30]. If we compare our findings with those where trypsin digestion and inactivation was applied to test for enzyme forms [8,10,32,33] we see that they concur. This coincidence is important because it has been contemplated, at least in the case of K^+ occlusion [34], that the possibility of conflicting results about enzyme conformations depends on the method used.

One rule than seems to emerge from our results is that regardless the phosphoryl group donor and the reaction taking place, the levels of E-P in the E_2 state are lower when phosphorylation occurs in the presence of external K^+. This effect could be thought to be related to an E_2 shift; however, the available experimental evidence [30,35] indicates that it is K^+_{cyt} and not K^+_{ext}, which takes the Na,K-ATPase into the $E_2(K)$ form. Still, although qualitatively identical, the response was quantitatively different. A good example is the ATPase reaction when the regulatory ATP site is out of action: 10 μM ATP and 5 mM AcP [8]. Under these conditions, K^+_{ext} rendered a 90% reduction in [E-P] when ATP was the substrate and only 44% when AcP played that role. These were steady-state measurements in an enzyme which prevailing form was E_2; the results cannot attributed to the slower E_1P-E_2P transition with AcP as compared to ATP [29,30]; in the case of Ref. 29 the incubation solutions contained 2 M NaCl, which makes a straight forward comparison with our results difficult. Plausible explanations for this distinct K^+ effects are: (i) phosphorylation is faster with AcP than with ATP, (ii) the K^+-stimulated dephosphorylation is slower with AcP, or (iii) a combination of (i) and (ii). In other experiments (not shown) we have found that, at 0 °C, the rate of K^+-stimulated dephosphorylation was indeed slower when the enzyme had been phosphorylated with AcP as compared with ATP (Campos and Beaugé, unpublished). Regarding the E-P from ATP, it is interesting to point out that the reduction due to K^+_{ext} was more noticeable in our experiments than in those of Van der Hijden and De Pont [37] and Yoda and Yoda [38]; these authors worked at lower temperatures and/or with liposomes of different lipid composition, which might account for the discrepancy.

Phosphorylation from P_i takes the enzyme into an E_2 state (Fig. 2, and Refs. 10, 33 and 36) and is maximal in the absence of any monovalent cation on both membrane surfaces or in the presence of intracellular K^+ (Tables I and II). The notion that while covalently binding inorganic phosphate the Na,K-ATPase goes into an E_2 state is in line with the fact that it is intracellular, and not extracellular, Na^+ what inhibits P_i phosphorylation (Table II). The steady state [E-P] obtained from P_i were reduced when there was K^+_{ext} both in the absence and presence of K^+_{cyt}. This suggests that the reduction due to K^+ observed in broken membranes, either at 0 °C [3] or 20 °C (Table I), is the consequence of K^+ acting on extracellular sites. In addition, also in broken membranes and at 0 °C Post et al. [3] observed that K^+ ions were ineffective if added after E-P was formed. Again, our results (Table II) indicate that it is external K^+ the responsible for that behavior; i.e., E-P formed from P_i and Mg^{2+} is sensitive to K^+_{ext} provided that cation is attached to extracellular sites during E-P formation.

Tables I and III show that it is possible to obtained enzyme phosphorylation from AcP in the presence of

Mg^{2+} and in the absence of any monovalent cation on both membrane sides (see also Ref. 39). At first sight, one would be tempted to identify this phosphorylation with the obtained from P_i under similar conditions. However, there are four major differences: (i) with AcP the E-P levels are not maximal; (ii) the addition of intracellular K^+ increases the [E-P] formed from AcP to its maximal levels; (iii) the phosphoenzyme formed from P_i and Mg^{2+} in broken membranes preparations corresponds to an enzyme in the E_2 state, whereas that obtained with AcP is in the E_1 conformation (Fig. 1; Refs. 8, 10, 33 and 36); (iv) the levels of P_i incorporation from AcP are not modified by K^+_{ext} unless intracellular K^+ is simultaneously present. A second rule to be extracted then, is that K^+_{ext} does not affect the levels of phosphoenzyme in the E_1-P state. An effect of K^+_{cyt} taking the phosphoenzyme into the E_2-P state has been suggested even in the case of phosphorylation from ATP [38].

The 50% reduction due to K^+_{ext} of the E-P formed from AcP in the presence of K^+_{cyt} and Mg^{2+} (conditions for phosphatase reaction) was an unexpected finding. The reason is that in the phosphatase activity the cytoplasmic, but not the external, K^+ is the one that seems to be involved (Refs. 40 and 41; this work). The reduction in E-P was similar with 5 mM, 10 mM or 100 mM K^+_{ext}; thus, this cannot be taken as the mechanism by which high [K^+] partially inhibits the phosphatase reaction because that inhibition takes place with very low apparent affinity [26,42].

In proteoliposomes incubated with Mg^{2+}, and in the absence of any external or internal monovalent cations, a small ouabain-sensitive hydrolysis of acetyl phosphate (about 3% of the maximal phosphatase activity) but not of *p*-nitrophenyl phosphate, was consistently detected. Similar results were observed in broken membrane preparations (Ref. 6; Beaugé and Berberián, unpublished). Whether this acetyl phosphate hydrolysis is an expression of a phosphatase or ATPase reaction remains unknown. As stated before, under those conditions the prevailing enzyme conformation is E_1-P (Fig. 1; Ref. 8). Intracellular K^+ increases the levels of phosphorylation, changes the conformation towards an E_2-P state and stimulates phosphatase activity (see also Refs. 40 and 41). In addition, the reduction in the [E_2-P] from AcP due to ADP is accompanied by an inhibition of the acetyl phosphatase reaction (not shown). Therefore, at least with acetyl phosphate (Ref. 7; this work) and with β-(2-furyl)acryloyl phosphate [42] the rate of phosphatase activity runs parallel with the levels of E_2-P. In addition, interestingly enough, this is the opposite to what happens in the Na,K-ATPase reaction where K^+_{ext} stimulates ATP hydrolysis while drastically reducing the [E-P] even at millimolar concentrations of labelled ATP [43,44,45].

The lack of correspondence between [E-P] and rate of K–K exchange (this work) and the reported absence of ADP effect on the K-dependent medium P_i-H_2O oxygen exchange [46], suggest complex biochemical events associated to the K–K exchange reaction. In addition, we have shown here that the phosphoenzymes obtained from P_i and phosphatase substrates have different reactivities towards K^+_{cyt} and K^+_{ext}; this indicates that the overall pathways of reversible P_i phosphorylation and phosphatase activity are not the same.

Acknowledgments

This work was supported by Grants from CONICET, CONICOR, Fundación Antorchas and Fundación Pérez Companc. We wish to thank Frigorífico Colonia Tirolesa for the pig kidneys supply. The technical assistance of M. Siravegna is greatly appreciated. G.B. and L.B. are established investigators of CONICET.

References

1 Glynn, I.M. (1985) in The enzyme of Biological Membranes, 2nd Edn., Vol. 3 (Martonosi, A.N., ed.), pp. 35–114. Plenum Press, New York.
2 Robinson, J.D. and Flashner, M.S. (1979) Biochim. Biophys. Acta 549, 145–176.
3 Post, R.L., Toda, G. and Rogers, F.N. (1975) J. Biol. Chem. 250, 691–701.
4 Askari, A., Huang, W.H. and McCornick, P.W. (1983) J. Biol. Chem. 258, 3453–3460.
5 Schuurmans, S.F. and Bonting, S.L. (1981) Physiol. Rev. 61, 1–76.
6 Campos, M., Berberián, G. and Beaugé, L. (1988) Biochim. Biophys. Acta 940, 43–50.
7 Campos, M., Berberián, G. and Beaugé, L. (1988) Biochim. Biophys. Acta 938, 7–16.
8 Beaugé, L., Berberián, G., Campos, M. and Pedemonte, C. (1985) in The Sodium Pump (Glynn, I. and Ellory, C., eds.), pp. 321–333. The Company of Biologists, Cambridge, U.K.
9 Inturrisi, C.E. and Titus, E. (1970) Mol. Pharmacol. 6, 99–107.
10 Karlish, S.J.D. (1980) J. Bioenerg. Biomembr. 12, 111–136.
11 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 256, 36–52.
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
13 Markwell, M.A.K., Hass, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210.
14 Karlish, S.J.D. and Pick, U. (1981) J. Physiol. 312, 505–529.
15 Karlish, S.J.D. and Stein, W.D. (1982) J. Physiol. 328, 295–316.
16 Schuurmans Stekhoven, F.M.A.H., Van Heeswijk, M.P.E., De Pont, J.J.H.H.M. and Bonting, S.L. (1976) Biochim. Biophys. Acta 442, 210–224.
17 Cantley, L.C., Resh, M.D. and Guidotti, G. (1978) Nature 272, 552–554.
18 Beaugé, L. and Berberián, G. (1984) Biochim. Biophys. Acta 772, 411–414.
19 Beaugé, L., Berberián, G. and Campos, M. (1984) Biochim. Biophys. Acta 773, 157–164.
20 Stadtman, E.R. (1957) Methods Enzymol. 3, 228–231.
21 De Meis, L., Martins, O.B. and Alves, E.W. (1980) Biochemistry 19, 4252–4261.
22 Glynn, I.M. and Chappell, J.B. (1964) Biochem. J. 90, 147–149.
23 De Meis, L. (1974) Biochemistry 11, 2460–2465.

24 Fiske, C.H. and SubbaRow, H.Y. (1925) J. Biol. Chem. 66, 375–400.
25 Karlish, S.J.D., Beaugé, L. and Glynn, I.M. (1979) Nature 282, 333–335.
26 Skou, J.C. (1974) Biochim. Biophys. Acta 39, 258–273.
27 Kapakos, J.G. and Steinberg, M. (1986) J. Biol. Chem. 261, 2090–2095.
28 Steinberg, M. and Karlish, S.J.D. (1989) J. Biol. Chem. 264, 2726–2734.
29 Taniguchi, K., Tosa, H., Suzuki, K. and Kamo, Y. (1988) J. Biol. Chem. 263, 12943–12947.
30 Rephaeli, A., Richards, D.E. and Karlish, S.J.D. (1986) J. Biol. Chem. 261, 12437–12440.
31 Amler, E., Teiseinger, J. and Svoboda, P. (1987) Biochim. Biophys. Acta 905, 376–382.
32 Berberián, G. and Beaugé, L. (1985) Biochim. Biophys. Acta 821, 17–29.
33 Hegyvary, C. and Jørgensen, P.L. (1981) J. Biol. Chem. 256, 6296–6303.
34 Glynn, I.M., Hara, Y., Richards, D.E. and Steinberg, M. (1987) J. Physiol. 383, 477–485.
35 Rephaeli, A., Richards, D.E. and Karlish, S.J.D. (1986) J. Biol. Chem. 261, 6248–6254.
36 Pedemonte, C. and Beaugé, L. (1986) Arch. Biochim. Biophys. 244, 596–606.
37 Van der Hijden, H.T.W.M. and De Pont, J.J.H.H.M. (1989) Biochim. Biophys. Acta 983, 142–152.
38 Yoda, A. and Yoda, S. (1988) J. Biol. Chem. 263, 10320–10325.
39 Dudding, W.F. and Winter, C.G. (1971) Biochim. Biophys. Acta 241, 57–61.
40 Drapeau, P. and Blostein, R. (1980) J. Biol. Chem. 258, 7827–7834.
41 Beaugé, L. and Berberián, G. (1983) Biochim. Biophys. Acta 727, 336–350.
42 Senear, D.F., Betts, G. and Bernhard, S.A. (1985) Biochemistry 24, 6789–6798.
43 Post, R.L., Sen, A.K. and Rosenthal, A.S. (1965) J. Biol. Chem. 240, 1437–1449.
44 Beaugé, L. and Campos, M. (1983) Biochim. Biophys. Acta 729, 137–149.
45 Mardh, S. (1975) Biochim. Biophys. Acta 391, 448–463.
46 Kaplan, J.H., Kenney, L.J. and Webb, M.R. (1985) in The Sodium Pump, (Glynn, I. and Ellory, E., eds.), pp. 415–421, The Company of Biologists, Cambridge, U.K.