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FEBS Letters 337 (1994) 109–113

FEBS 13501

FEBS LETTERS

Modifications induced by acylphosphatase in the functional properties of heart sarcolemma Na^+,K^+ pump

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Received 14 October 1993; revised version received 25 November 1993

Abstract

Acylphosphatase purified from cardiac muscle actively hydrolyzes the phosphoenzyme intermediate of heart sarcolemma Na^+,K^+-ATPase. This effect occurred with acylphosphatase amounts (up to 800 units/mg membrane protein) that fall within the physiological range and the low value of the apparent K_m (0.69×10^{-7} M) indicates a considerable affinity of the enzyme towards this specific substrate. Acylphosphatase addition to purified sarcolemmal vesicles significantly increased the rate of Na^+,K^+-dependent ATP hydrolysis. Maximal stimulation, observed with 800 units/mg protein, resulted in an ATPase activity which was about 2-fold over basal value. The same acylphosphatase amounts significantly stimulated, in a similar and to an even greater extent, the rate of ATP driven Na^+ transport into sarcolemmal vesicles. These findings lead to suppose that an accelerated hydrolysis of the phosphoenzyme may result in an enhanced activity of heart sarcolemmal Na^+,K^+ pump, therefore suggesting a potential role of acylphosphatase in the control of this active transport system.

Key words: Heart sarcolemma; Na^+,K^+-ATPase; Sodium pump; Acylphosphatase

1. Introduction

The Na^+,K^+ pump, identified as a Na^+,K^+-dependent membrane-bound ATP hydrolyzing enzyme, hence the name of Na^+,K^+-ATPase, is present in the membrane of most mammalian cells where it acts as an energy transducer converting chemical energy from ATP hydrolysis to a gradient of Na^+ and K^+ ions.

The system is electrogenic since, under normal conditions, for each ATP hydrolyzed, three Na^+ ions are transported out and two K^+ ions into the cell [1], and this electrochemical gradient, in turn, represents a free energy source for many important cellular processes. In this connection, heart sarcolemma Na^+,K^+-ATPase appears to have a special function in the excitation and in the contraction–relaxation cycle of cardiac muscle. In fact, the electrochemical gradient produced by the Na^+,K^+ pump is essential to establish the membrane potential and to regulate the intracellular Ca^{2+} concentration through the Na^+-Ca^{2+} exchange system and the effect on the permeability of potential dependent calcium channels [2]. Furthermore, cardiac sarcolemma Na^+, K^+-ATPase activity is tightly correlated with that of a Na^+–H^+ exchange system, which appears to be the principal mechanism for pH regulation in heart muscle [3].

Despite numerous studies, the molecular mechanism for the action of Na^+,K^+ pump is still debatable. In any case, the transient formation of an acylphosphorylated phosphoenzyme (EP) intermediate through a Na^+-dependent phosphorylation and a K^+-dependent dephosphorylation of a specific aspartyl residue in the α-subunit of Na^+,K^+-ATPase is a crucial event for the production of the conformational modifications needed to couple the cation transport to ATP hydrolysis [1].

Acylphosphatase (EC 3.6.1.7) is a widespread cytosolic enzyme that catalyzes the hydrolysis of the carboxylphosphate bond of acylphosphates such as 3-phosphoglyceroyl phosphate [4], carbamoyl phosphate [5], succinoyl phosphate [6]. Studies conducted in our laboratory for several years about the structural and functional properties of acylphosphatase isolated from different sources lead us to conclude that in mammalian tissues this enzyme is present in two isoenzymatic forms: one is prevalent in skeletal and cardiac muscle, the other in red blood cells. Although the two isoenzymes have a similar molecular weight, they exhibit remarkable differences in catalytic potency, affinity towards substrates and sensibility to inhibitors [7]. We have previously reported that muscle acylphosphatase, in addition to the

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Abbreviations: Na^+,K^+-ATPase, sodium-potassium ion-dependent adenosine triphosphatase; EP, the phosphorylated form of Na^+,K^+-ATPase.

above soluble, low molecular weight substrates can actively act on the acylphosphorylated intermediates of Ca^{2+}-ATPase from both skeletal muscle sarcoplasmic reticulum [8] and heart sarcolemma [9].

In this paper we describe the results of studies aimed to evaluate whether similar effect of acylphosphatase on the phosphoenzyme intermediate of heart sarcolemma Na^+,K^+-ATPase resulted in modified functional properties of this active transport system.

2. Materials and methods

All reagents were of the highest purity available. Tris-ATP, monensin, strophantidine, ouabain, paranitrophenylphosphate were from Sigma; [γ-^{32}P]ATP (6000 Ci/mmol), ^{22}NaCl 730 mCi/mg were from New England Nuclear.

Acylphosphatase was purified from bovine heart according to Ramponi et al. [10] for the extraction and to Stefani et al. [11] for the other steps. The enzyme, isolated as a pure product, had a specific activity of 3650 units/mg protein using benzoyl phosphate as substrate [12]. Benzoyl phosphate was synthesized as per Camici et al. [13]. Sarcolemmal vesicles protein content was assayed by the biuret method of Beisenherz et al. [14].

2.1. Preparation of calf heart sarcolemmal vesicles

Cardiac sarcolemmal vesicles were prepared from calf heart as reported by Jones [15]. The final pellet was resuspended in 30 mM Tris-HCl, pH 7.4, to a final concentration of 6–10 mg protein/ml and stored frozen at −20°C. Yields of 6–8 mg vesicle protein per 100 g of wet tissue were routinely obtained.

Na^+,K^+-ATPase activity (see below) and K^+-*p*-nitrophenylphosphate activity [16] were measured to follow the enrichment of the sarcolemmal preparation from homogenate, cytochrome *c* oxidase [17] and glucose 6-phosphatase activities [18] to determine the extent of contamination by mitochondria and sarcoplasmic reticulum. Vesicle sidedness and leakiness were estimated as in Caroni and Carafoli [19].

2.2. Preparation of Na^+,K^+-ATPase [^{32}P]phosphoenzyme and its incubation with acylphosphatase

Sarcolemmal vesicles were diluted 1:10 in a solution containing 30 mM Tris-HCl, pH 7.4, and 0.3 mg SDS/ml. After incubation for 30 min at room temperature, the suspension was centrifuged at $105{,}700 \times g_{max}$ for 30 min. The pellet, washed in ice-cold H_2O, was recentrifuged and then resuspended in 30 mM Tris-HCl, pH 7.4 [20]. After this treatment vesicles were phosphorylated with [γ-^{32}P]ATP according to Nassi et al. [21]. The phosphoenzyme level was calculated by subtracting non-specific ^{32}P bound to vesicles phosphorylated by the same procedure except that 100 mM KCl was used in place of NaCl.

Labeled vesicles (1 mg/ml) were incubated in 30 mM Tris-HCl, pH 7.4, at 30°C with differing amounts of acylphosphatase for 1 min. In another series of experiments differing amounts of phosphorylated vesicles were incubated with a fixed amount of acylphosphatase. Controls for spontaneous hydrolysis were incubated under the same conditions, except that acylphosphatase was omitted.

2.3. SDS-polyacrylamide gel electrophoresis and autoradiography

^{32}P labeled vesicles (about 330 μg protein) were incubated in 30 mM Tris-HCl, pH 7.4, at 37°C for 30 min with and without acylphosphatase at a concentration of 300 units/mg vesicle protein. The reaction was stopped in ice and the suspension was centrifuged at $12000 \times g_{max}$ for 3 min. The pellet, resuspended in a buffer containing 10% glycerol, 2% SDS, 5% 2-β-mercaptoethanol, 0.0012% Bromophenol blue, 62 mM Tris-HCl, pH 6.8, was applied to the polyacrylamide gel. Electrophoresis was performed at 4°C with a mini-PROTEAN II slab cell (Bio-Rad) on gels of 0.75 mm thickness according to Fairbanks et al. [22]. The run time was approximately 2.5 h with a constant 16 mA current. The acrylamide concentration was 4% (stacking gel) and 5.6% (separating gel). At the end of the run gels were stained in 0.1% Coomassie blue R 250, dissolved in 40% methanol and 10% acetic acid, and dried under vacuum. For autoradiography, the gel slabs were exposed to Kodak X-Omat AR film with an Agfa-Gevaert (Curix MR 800) intensifier screen at −80°C for three days and the film was then developed.

2.4. Na^+,K^+-ATPase activity assay

Na^+,K^+-ATPase was measured as the rate of strophantidine-sensitive ATP hydrolysis. Vesicles (50 μg protein) were assayed at 30°C in 1 ml of a medium containing 3 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 5 mM NaN_3, 0.05 mM ouabain, 1 mM EGTA, 20 μM monensin, 3 mM Tris-ATP, 30 mM Tris-HCl, pH 7.4, and variable amounts of acylphosphatase. When present strophantidine was 0.1 mM. Ouabain, being not membrane permeant, was added to inhibit the activities of right side and leaky vesicles (see below). Reaction was started with Tris-ATP and terminated after 10 min with 1 ml of 2% ascorbic acid in 10% trichloroacetic acid. The inorganic phosphate released was determined by the method of Baginsky [23].

2.5. Na^+ influx into sarcolemmal vesicles

Prior to use, vesicles were equilibrated overnight at 4°C and then preincubated for 30 min at 30°C with 3 mM $MgCl_2$, 180 mM choline chloride, 10 mM KCl, 5 mM NaN_3, 1 mM EGTA, 30 mM Tris-HCl, pH 7.4. Na^+ transport was measured as the difference in ^{22}Na influx into vesicles in the presence and in the absence of 3 mM ATP. Assays were carried out at 30°C in 200 μl of medium containing 3 mM $MgCl_2$, 100 mM NaCl, 5 mM NaN_3, 1 mM EGTA, 1 μCi ^{22}Na, 100 μg vesicle protein, 30 mM Tris-HCl, pH 7.4, and variable amounts of acylphosphatase. After 1 min the reaction was terminated by the addition of 10 ml of cold 0.16 M KCl, 30 mM Tris-HCl, pH 7.4, and the suspension was immediately filtered through 0.45 μm pore Sartorius filters and washed twice with 10 ml of the same cold stopping solution. The filters were immersed in 10 ml of scintillation fluid and then counted.

3. Results and discussion

Sarcolemmal vesicles used in the present study evaluated for sidedness and leakiness as indicated above, were estimated to consist of 30% leaky, 30% inside-out and 33% right side-out vesicles. Mitochondrial and sarcoplasmic reticulum contaminations were negligible, since cytochrome *c* oxidase and glucose 6-phosphatase were virtually absent.

The Na^+,K^+-ATPase phosphoenzyme (EP) was prepared by phosphorylating sarcolemmal vesicles with [γ-^{32}P]ATP as described in section 2. Under these conditions the phosphate associated to the Na^+,K^+ pump was, on average, 215 pmol/mg membrane protein, a value that agrees with those reported by other authors [20,24].

Fig. 1 shows the autoradiography of [^{32}P]phosphorylated sarcolemmal vesicles subjected to electrophoresis after incubation with and without acylphosphatase. Phosphorylation performed in the presence of Na^+ resulted in a band of about 100 kDa, corresponding to the α-subunit of Na^+,K^+-ATPase, which was not evident in the vesicles phosphorylated with K^+ instead of Na^+. The 100 kDa band disappeared when the vesicles were incubated with acylphosphatase, what clearly indicates the ability of the enzyme to remove the labeled phosphate from the phosphorylated intermediate of Na^+,K^+ pump.

To determine whether the rate of EP dephosphorylation was dependent on acylphosphatase concentration, we measured the phosphate released by labeled vesicles

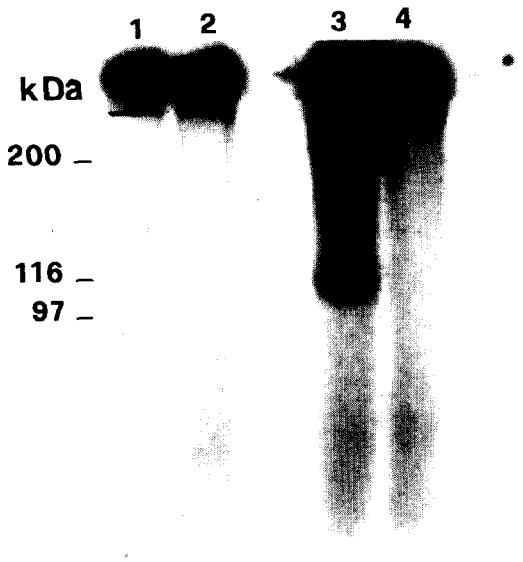

Fig. 1. Autoradiography of [^{32}P]phosphorylated sarcolemmal vesicles after electrophoresis. Phosphorylation of sarcolemmal vesicles was performed as described in section 2. Lanes 1 and 2, vesicles phosphorylated in the presence of 100 mM KCl; lanes 3 and 4, vesicles phosphorylated in the presence of 100 mM NaCl; lanes 2 and 4, phosphorylated vesicles incubated with acylphosphatase (300 units/mg vesicle protein).

incubated with varying amounts of this enzyme. Concentrations from 100 to 800 units per mg of sarcolemmal protein were used because they represent the lower and upper limits of physiological range as it may be estimated on the account of acylphosphatase activity in heart muscle and on protein content of cardiac sarcolemmal membrane [25].

As shown in Fig. 2A, in the presence of acylphosphatase, the release of phosphate was constantly higher than spontaneous hydrolysis, and rose significantly with the increase in acylphosphatase/membrane protein ratio. Furthermore, heat inactivated acylphosphatase did not produce any significant modification in phosphate release with respect to the spontaneous dephosphorylation of phosphoenzyme.

To evaluate the affinity of acylphosphatase towards EP we incubated a fixed amount of enzyme with variable amounts of ^{32}P-labeled vesicles and measured the initial rates of acylphosphatase-induced dephosphorylation, as a function of EP concentrations. A double reciprocal plot (Fig. 2B) of the initial rates of enzymatic dephosphorylation and of EP concentration gave an apparent K_m of 6.9×10^{-7} M. This low value, suggesting a high affinity in our enzyme for EP, may be consistent with the relatively low number of Na^+,K^+ pump units in sarcolemmal membrane, all the more so since the K_m values observed for muscle acylphosphatase towards potentially competing substrates, i.e. several low molecular weight cytosolic components, were always higher than 10^{-4} M. To determine whether the above action of acylphosphatase on the phosphoenzyme modified the functional properties of Na^+,K^+ pump we studied the effect of the enzyme on Na^+,K^+-dependent ATP hydrolysis and ATP-dependent Na^+ transport. For these studies we used

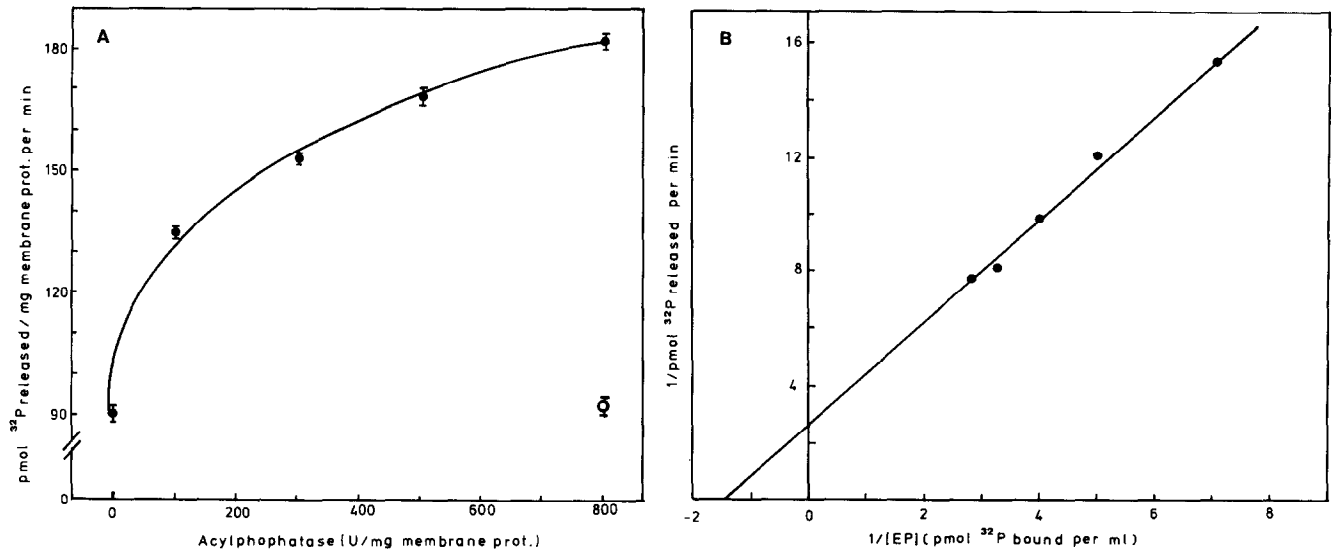

Fig. 2. Effect of acylphosphatase on the phosphate release from sarcolemmal Na^+,K^+-ATPase phosphorylated intermediate (EP). (A) Labeled vesicles were incubated in 30 mM Tris-HCl, pH 7.4, at 30°C for 1 min with varying amounts of acylphosphatase. The phosphoenzyme level was 215 pmol phosphate bound/mg membrane protein. Each point is the mean ± S.E.M. of five experiments. All the changes in phosphate release induced by active acylphosphatase were statistically significant ($P < 0.01$ by the one-way analysis of variance). ● indicates the value obtained with inactivated acylphosphatase (2 h at 100°C) at a concentration corresponding to 800 units of the active enzyme per mg membrane protein. (B) 800 units of acylphosphatase were incubated with different amounts of labeled membranes in the conditions above described. EP concentration in the medium was expressed as pmol ^{32}P bound/ml. Values are net for spontaneous hydrolysis and presented as a double reciprocal plot. Each point represents the mean value, corrected per 10^3, of five determinations.

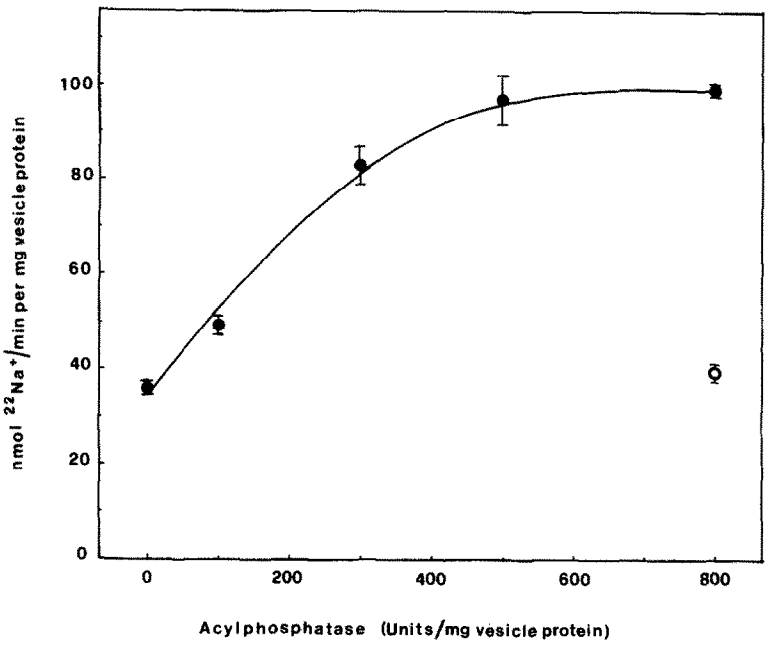

Fig. 3. Effect of acylphosphatase on ^{22}Na transport into sarcolemmal inside-out vesicles. Vesicles were equilibrated and assayed as described in section 2. Na^+ transport, measured as the difference in $^{22}Na^+$ influx into vesicles in presence and in absence of 3 mM ATP, is expressed as nmol $^{22}Na^+$/min per mg vesicle protein. Each point represents the mean ± S.E.M. of five determinations. ● indicates the value obtained with inactivated acylphosphatase (an amount corresponding to 800 units of the active enzyme). Changes observed with varying

sarcolemmal vesicles with the above mentioned characteristics. In order to compare the effects on ATP hydrolysis and Na^+ transport all the experiments were conducted in the same conditions, as regards temperature, Na^+,K^+ and ATP concentrations. Temperature was set at 30°C to prevent, in Na^+ transport assays, a too rapid depletion of K^+ from loaded vesicles. Since our preparation consisted of inside-out, right side-out, and leaky vesicles, the ATPase activity due to the Na^+,K^+ pump was calculated as the difference in the rate of ATP hydrolysis without and with strophantidine. This compound, being membrane permeant, inhibited total glycoside-sensitive activity; however, ouabain was present in all assays to inhibit right side-out and leaky vesicle activities. Under these conditions, the strophantidine-sensitive Na^+,K^+-ATPase activity was considered as that due to unleaky inside-out vesicles, the only competent for active Na^+ transport.

Table 1 illustrates the effect of acylphosphatase on strophantidine-sensitive Na^+,K^+-ATPase activity. The addition of acylphosphatase, which does not exhibit per se ATPase activity, significantly increased the rate of strophantidine-sensitive ATP hydrolysis and this effect was more marked with increasing amounts of the enzyme. Maximal stimulation, obtained with 800 U/mg vesicle protein, resulted in a 20-fold increase with respect to basal value (24.2 ± 2 against 12.2 ± 2 nmol/min/mg vesicle protein, mean values ± S.E.M.).

The effect on cation transport was studied by measuring the ATP-dependent Na^+ influx into sarcolemmal vesicles, equivalent to the physiological efflux from the cell. Obviously only inside-out vesicles were responsible for this process. Reported values are the difference of those obtained in presence and in absence of 3 mM ATP, in order to exclude the contribution by other non-active transport systems such as ion channels.

As shown in Fig. 3, increasing amounts of acylphosphatase significantly stimulated the rate of Na^+ influx. Maximal stimulation, obtained in the presence of

Table 1

Effect of acylphosphatase on strophantidine-sensitive Na^+,K^+-ATPase activity of sarcolemmal inside-out vesicles

Acylphosphatase (units/mg vesicle prot.)	Strophantidine-sensitive Na^+,K^+-ATPase activity (nmol/min per mg vesicle prot.)	Increase *n*-fold
None	12.2 ± 2.1	1.00
100	14.6 ± 4.2	1.19
300	17.4 ± 2.5	1.42
500	21.4 ± 1.9	1.75
800	24.2 ± 2.7	1.98
Heat-inactivated	12.9 ± 2.2	1.05

Strophantidine-sensitive Na^+,K^+-ATPase activity was assayed as under section 2. Each value is the mean ± S.E.M. of 5 determinations. Changes observed with differing amounts of active acylphosphatase were statistically significant ($P < 0.05$ by the one-way analysis of variance). Heat-inactivated acylphosphatase (2 h at 100°C) was added at a concentration corresponding to 800 units of the active enzyme per mg vesicle protein.

800 U/mg vesicle protein, resulted in 99.21 ± 1.3 against 36.39 ± 2.1 nmol/min per mg vesicle protein. However the increase in Na^+ influx with this enzyme concentration was not significantly different from that obtained using 500 U/mg vesicle protein (96.89 ± 5.17 nmol/min/mg vesicle protein).

It is noteworthy that the effects of acylphosphatase on EP hydrolysis, Na^+,K^+-ATPase activity and Na^+ transport were quantitatively similar since all these processes were enhanced and maximal stimulation resulted in levels which were about 2-fold over basal values. Furthermore, as for EP hydrolysis, heat-inactivated acylphosphatase did not produce appreciable modifications in ATPase activity and Na^+ transport, nor did the active enzyme have significant effect (data not shown) on the ATP-independent Na^+ influx. This led to conclude either that acylphosphatase acts rather like a protein than an enzyme or that the effect on Na^+ transport is simply due to alterations induced in the passive permeability of sarcolemmal membrane to this cation.

Thus, the present study provides evidence that heart muscle acylphosphatase owing to its catalytic activity, efficiently hydrolyzes the phosphorylated intermediate of sarcolemmal membrane Na^+,K^+ pump, at the same time affecting the functional properties of this active transport system. A reasonable interpretation of the results here reported is that an accelerated EP turnover induced by acylphosphatase, may result in an enhanced activity of Na^+,K^+ pump, whence an increase in the rate of both ATP hydrolysis and of ATP-driven Na^+ transport. Moreover, the coupling between these two processes does not appear to be impaired by acylphosphatase since this enzyme stimulated Na^+ transport at a similar (or even greater) extent with respect to ATP hydrolysis. It may be therefore supposed that acylphosphatase-induced EP hydrolysis takes place without altering the normal ordered reaction sequence underlying the function of Na^+,K^+ pump [1], in any case without preventing the conformational changes needed for Na^+ transport.

In conclusion, the present paper represents the first report, at least to our knowledge, about a modification in the activity of heart muscle sarcolemma Na^+,K^+ pump by an enzyme present in the same tissue and used at concentrations within the normal range. Further studies would be of interest to probe more deeply the mechanism of acylphosphatase action and the possibility of a physiological significance of these findings.

Acknowledgements: This work was supported by grants from Ministero dell' Università e della Ricerca Scientifica e Tecnologica, Fondi 60% e 40% (Progetto Nazionale Insufficienza Cardiaca) and from Consiglio Nazionale delle Ricerche (Target Project Invecchiamento).

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