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### **ACYLPHOSPHATASE: A POTENTIAL MODULATOR OF HEART SARCOLEMMMA NA<sup>+</sup>,K<sup>+</sup> PUMP**

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## Acylphosphatase: A Potential Modulator of Heart Sarcolemma $\text{Na}^+$ , $\text{K}^+$ Pump<sup>†</sup>

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**ABSTRACT:** Acylphosphatase, purified from cardiac muscle, catalyzes the hydrolysis of the phosphorylated intermediate of heart sarcolemmal  $\text{Na}^+$ , $\text{K}^+$ -ATPase. This effect was remarkable even using acylphosphatase amounts (100–300 units/mg of membrane protein) near the lower limit of the physiological range; besides the low value of the apparent  $K_m$ , on the order of  $10^{-7}$  M, indicates that the enzyme has a high affinity for this special substrate. The results of a dot-immunobinding assay suggest the possibility of an interaction between acylphosphatase and native, undenatured  $\text{Na}^+$ , $\text{K}^+$ -ATPase. Moreover, when added to sarcolemmal vesicles, acylphosphatase was found to affect the functional properties of the  $\text{Na}^+$ , $\text{K}^+$  pump with regard to the rate of both ATP hydrolysis and cation transport. However, while ATPase activity and  $\text{Na}^+$  uptake were stimulated, the last at a greater extent, the active  $\text{K}^+$  transport was inhibited, so that the  $\text{Na}^+/\text{K}^+$  ratio, which was calculated as 1.50 without acylphosphatase, rose to 6.68 in the presence of 300 units/mg of vesicle protein of this enzyme. Taken together, the reported results indicate that acylphosphatase, because of its hydrolytic activity on the phosphoenzyme intermediate, induces a sort of “uncoupling” effect on the heart sarcolemmal membrane  $\text{Na}^+$ , $\text{K}^+$  pump. Possible mechanisms for such an effect, which suggests a potential role of acylphosphatase in the control of this active transport system, are discussed.

The  $\text{Na}^+$ , $\text{K}^+$  pump, otherwise named  $\text{Na}^+$ , $\text{K}^+$ -ATPase<sup>1</sup> because of its enzymatic properties consisting of the catalysis of  $\text{Na}^+$ , $\text{K}^+$ -dependent ATP hydrolysis, is, in fact, a ubiquitous active transport system which is present in the plasma membrane of most mammalian cells. Here it acts as an energy transducer converting chemical energy from ATP hydrolysis to an electrochemical gradient of the  $\text{Na}^+$  and  $\text{K}^+$  ions since in normal conditions, for each ATP hydrolyzed three  $\text{Na}^+$  ions are transported out and two  $\text{K}^+$  ions into the cell (Skou, 1988). This electrochemical gradient represents an energy source for many cellular processes. In heart muscle,  $\text{Na}^+$ , $\text{K}^+$ -ATPase, located in the sarcolemma, appears to have a special function in the excitation and in the contraction–relaxation cycle of this tissue. Indeed, the electrochemical gradient generated by the  $\text{Na}^+$ , $\text{K}^+$  pump is essential to establish membrane potential and considerably contributes to regulate the intracellular  $\text{Ca}^{2+}$  concentration through the activity of the  $\text{Na}^+$ – $\text{Ca}^{2+}$  exchange system and the potential dependent permeability of  $\text{Ca}^{2+}$  channels (Caroni et al., 1980).

In spite of a lot of studies, the molecular mechanisms for the action of the  $\text{Na}^+$ , $\text{K}^+$ -ATPase are still controversial, and various models have been proposed to describe the functioning of this active transport system. There is no doubt, however, that the transient formation of an acylphosphorylated phosphoenzyme (EP) intermediate, due to a  $\text{Na}^+$ -dependent phosphorylation and a  $\text{K}^+$ -dependent dephosphorylation of a specific aspartyl residue in the  $\alpha$ -subunit of  $\text{Na}^+$ , $\text{K}^+$ -ATPase, is a crucial event in the production of the conformational changes needed to modify the affinity toward the two cations and to couple their transport to ATP hydrolysis (Skou, 1988).

Acylphosphatase (EC 3.6.1.7) is a well-represented cytosolic enzyme that catalyzes the hydrolysis of the carboxyl phosphate bond present in compounds such as 3-phosphoglyceroyl phosphate (Ramponi et al., 1967), carbamoyl phosphate (Ramponi et al., 1961), and succinoyl phosphate (Berti et al., 1977). Studies from our laboratory about the structural and functional properties of acylphosphatase isolated from different sources indicate that in mammalian tissues this enzyme is present in two isoforms; one is prevalent in skeletal and in cardiac muscle, the other in erythrocyte. The two forms appear to be true isoenzyme consisting of the same number of amino acids but rather different in their primary structure and also in kinetic properties as regards catalytic potency, affinity toward substrates, and sensitivity to inhibitors (Liguri et al., 1986).

We have already reported that both acylphosphatase isoenzymes, in addition to the above-mentioned soluble low molecular weight substrates, can also act on the phosphorylated intermediates of various transport ATPases (Stefani et al., 1981; Nassi et al., 1991, 1993; Nediani et al., 1992), notably that heart muscle acylphosphatase can hydrolyze the

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<sup>1</sup> Abbreviations:  $\text{Na}^+$ , $\text{K}^+$ -ATPase, sodium–potassium ion-dependent adenosinetriphosphatase; EP, phosphoenzyme; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PEP, phosphoenolpyruvate monosodium salt.

phosphoenzyme intermediate of cardiac sarcolemma Na<sup>+</sup>,K<sup>+</sup>-ATPase (Nassi et al., 1994).

In the present paper, we report the results of studies aimed to examine more closely this effect in order to evaluate the plausibility of its occurrence in physiological conditions and the possible consequences on the functional properties of the Na<sup>+</sup>,K<sup>+</sup> pump.

## MATERIALS AND METHODS

All reagents, substrates, and coenzymes were from Sigma except lactate dehydrogenase and pyruvate kinase which were obtained from Boehringer, Mannheim. Adenosine-5'-triphosphatase (sodium- and potassium-activated), acetylcholinesterase, and  $\gamma$ -glutamyltranspeptidase were from Sigma. Nitrocellulose sheets were from Sartorius (Firenze, Italy). Fetal calf serum was from GIBCO Co. (Canada). [ $\gamma$ -<sup>32</sup>P]-ATP (6000 Ci/mmol) was purchased from New England Nuclear. Acetylene was from SIO (Società Italiana Ossigeno). ECL (enhanced chemiluminescence method) reagents were from Amersham. Mouse monoclonal antibodies to the muscular form of acylphosphatase were produced and purified according to Degl' Innocenti et al. (1990). Goat anti-mouse IgG antibody-HRP conjugate was from AF-FINITI (Nottingham, U.K.).

Acylphosphatase (muscular form) was purified from bovine heart according to Ramponi et al. (1969) for the extraction step and according to Stefani et al. (1985) for the other steps. The enzyme, isolated as a pure product, had a specific activity of 3650 units/mg of protein using benzoyl phosphate as substrate (Ramponi et al., 1966). Benzoyl phosphate was synthesized as per Camici et al. (1976). Sarcolemmal vesicle's protein content was assayed by the biuret method of Beisenherz et al. (1953).

Sarcolemmal vesicles were isolated from trimmed calf ventricles by the method of Jones (1988). The final pellet was resuspended in 30 mM Tris-HCl, pH 7.4, to a final concentration of 6–10 mg of protein/mL and stored frozen at –20 °C. A yield of 6–8 mg of vesicle protein per 100 g of wet tissue was routinely obtained.

Na<sup>+</sup>,K<sup>+</sup>-ATPase (see below), cytochrome *c* oxidase (Remacle et al., 1980), and glucose 6-phosphatase activities (Baginsky et al., 1974) were measured to determine the extent of contamination of the sarcolemmal fraction vesicles by mitochondria and sarcoplasmic reticulum. Vesicle sidedness and leakiness were estimated by the method of Caroni and Carafoli (1983).

To prepare Na<sup>+</sup>,K<sup>+</sup>-ATPase, [<sup>32</sup>P]phosphoenzyme sarcolemmal vesicles were first treated with SDS (0.3 mg/mL) as described in previous studies (Nassi et al., 1994) and after were phosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP according to Nassi et al. (1993). Briefly, SDS-treated sarcolemmal vesicles (1 mg/mL) were phosphorylated for 5 s in ice in a medium containing 30 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EGTA, 3 mM MgCl<sub>2</sub>, and 10  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (150–300 cpm/pmol). The reaction was stopped by adding 4 volumes of ice-cold 6% trichloroacetic acid containing 1 mM ATP and 5 mM NaH<sub>2</sub>PO<sub>4</sub>. The precipitated membranes were pelleted at 4 °C at 30000g for 10 min and washed 4 times by repelleting once with the above stop solution and 3 additional times in ice-cold 30 mM Tris-HCl, pH 7.4. The final pellet was resuspended in 30 mM Tris-HCl, pH 7.4, and the radioactivity was determined by counting an aliquot of the pellet, dissolved in 3% SDS, in a liquid scintillation

mixture. The phosphoenzyme level was calculated by subtracting nonspecific <sup>32</sup>P bound to vesicles phosphorylated in the same conditions 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. The reaction was terminated in ice, and the suspensions were centrifuged at 13000g for 10 min. Aliquots of the supernatants were used for measuring free <sup>32</sup>P radioactivity. In another series of experiments, differing amounts of phosphorylated membranes were incubated with a fixed amount of acylphosphatase (100 units). Controls for spontaneous hydrolysis were incubated under the same conditions, except that acylphosphatase was omitted.

For cation transport and Na<sup>+</sup>,K<sup>+</sup>-ATPase activity measurements, sarcolemmal vesicles were first equilibrated overnight at 4 °C and then preincubated for 30 min at 37 °C with an equal volume of a mixture to attain a final concentration of 30 mM Tris-HCl, pH 7.4, 3 mM MgCl<sub>2</sub>, 90 mM choline chloride, 1 mM EGTA, and 10 mM KCl, in order to obtain K<sup>+</sup>-preloaded vesicles.

Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was determined by a coupled optical test at 340 nm and at 30 °C using a Perkin-Elmer 4B spectrophotometer. For the optimized assay, the medium contained 30 mM Tris-HCl, pH 7.4, 3 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM EGTA, 1 mM PEP, 3 mM Tris-ATP, 1 mM ouabain (see below), 0.4 mM NADH, 6 units of pyruvate kinase, 5.5 units of lactate dehydrogenase, and 50  $\mu$ g (100  $\mu$ L of a 0.5 mg/mL K<sup>+</sup>-preloaded vesicles) of vesicle protein in a final volume of 1 mL. Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was calculated as the difference in ATP hydrolysis in the presence and in the absence of 0.1 mM strophanthidine and was expressed as nanomoles of ATP split per minute per milligram of vesicle protein.

Cation transport measurements were carried out at 30 °C in a medium containing 30 mM Tris-HCl, pH 7.4, 3 mM MgCl<sub>2</sub>, 100 mM NaCl, and 1 mM EGTA in the presence and in the absence of 3 mM ATP. One volume of K<sup>+</sup>-preloaded vesicles was added to 9 volumes of the buffer described above to start the reaction. After 1 min, the reaction was stopped by adding 10 mL of ice-cold stop solution containing 0.16 M KCl in 30 mM Tris-HCl, pH 7.4, for Na<sup>+</sup> influx, or 0.16 M NaCl in 30 mM Tris-HCl, pH 7.4, for K<sup>+</sup> efflux. Diluted vesicles were filtered on 0.45  $\mu$ m pore Sartorius filters and washed twice with the above ice-cold stop solutions. The filters were immersed in 2 mL of warm 50 mM KOH, for Na<sup>+</sup> uptake, or 2 mL of warm 50 mM NaOH, for K<sup>+</sup> transport, to dissolve the vesicle membranes and to free the cations. After 2 h, the filters were removed, and an aliquot of these media was used to measure Na<sup>+</sup> or K<sup>+</sup> concentration by a Perkin-Elmer 3100 atomic absorption spectrometer using single-element hollow cathode lamps (0.7 slit) at 589 and 766 nm, respectively, for Na<sup>+</sup> and K<sup>+</sup> determinations. The used oxidant fuel was air-acetylene. The calibration curve was carried out with 12.5, 25, 50, and 100  $\mu$ M NaCl in 50 mM KOH or 2.5, 5, and 10  $\mu$ M KCl in 50 mM NaOH against a blank. Na<sup>+</sup> influx was determined as the difference in Na<sup>+</sup> taken up by vesicles in the presence and in the absence of 3 mM ATP and was expressed as nanomoles per minute per milligram of vesicle protein. K<sup>+</sup> efflux was measured as the difference of K<sup>+</sup> retained into K<sup>+</sup>-preloaded vesicles in the presence and in the absence of 3 mM ATP; it was also expressed as nanomoles per minute per milligram of vesicle protein.

In all experiments of ATP hydrolysis and cation transport, acylphosphatase, when present, was added at a concentration of 50, 150, 300 units/mg of vesicle protein.

Dot-immunobinding assay was performed according to Hawkes et al. (1982) with slight modifications.  $\text{Na}^+, \text{K}^+$ -ATPase and acylphosphatase were applied in 1  $\mu\text{L}$  aliquots onto nitrocellulose strips which were then saturated by immersing them in TBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 2.7 mM KCl) containing 1% bovine serum albumin (BSA) and 10% fetal calf serum (FCS) at room temperature on an orbital shaker. After 1.5 h, the strips were washed for 30 min in several changes of TBS containing 0.1% Tween 20 (T-TBS). One nitrocellulose strip on which we dotted 200 ng of  $\text{Na}^+, \text{K}^+$ -ATPase and 0.7 ng of acylphosphatase was incubated at 30 °C in 1 mL of 30 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM KCl, 3 mM  $\text{MgCl}_2$ , and 3 mM ATP. Two aliquots of 200 ng of  $\text{Na}^+, \text{K}^+$ -ATPase were dotted on another strip and incubated in the same medium containing 1  $\mu\text{g}$  of acylphosphatase. After 3 min of incubation, the strips were washed several times in T-TBS for 30 min and then incubated overnight at 4 °C with primary anti-acylphosphatase antibody diluted 1:500 with T-TBS, 1% BSA, and 10% FCS. The primary antibody was removed, and the strips were washed for 30 min as before. After being washed, the strips were incubated with goat anti-mouse-HRP conjugate second antibody diluted 1:3000 with T-TBS, 1% BSA, and 10% FCS. Incubation was carried out for 2 h at room temperature, and then the strips were washed in 0.5% T-TBS for 30 min with several changes. The strips were incubated for 1 min with the ECL detection reagents in a dark room, drained off from the excess of the detection solutions, and exposed to the autoradiography film for 2 min. Control experiments to assess the specificity of acylphosphatase interaction with  $\text{Na}^+, \text{K}^+$ -ATPase were performed in the same conditions using other membrane-bound enzymes such as acetylcholinesterase and  $\gamma$ -glutamyltranspeptidase.

## RESULTS

Sarcolemmal vesicles used in these studies were estimated to consist of 28% leaky, 20% inside-out, and 52% right-side-out vesicles. The mitochondrial and sarcoplasmic reticulum contaminations in the sarcolemmal fraction vesicles were negligible, since cytochrome *c* oxidase and glucose 6-phosphatase activities were absent.

Table 1 shows the release of phosphate from sarcolemmal  $\text{Na}^+, \text{K}^+$ -ATPase phosphoenzyme in the absence and in the presence of acylphosphatase from 50 to 300 units/mg of vesicle protein. Such concentrations were used because they are near the lower limit of the physiological range which was estimated to be from 100 to 800 units/mg of sarcolemmal protein, on account of the acylphosphatase activity in heart muscle and the protein content of cardiac sarcolemmal membrane (Berti et al., 1987). When acylphosphatase was present, the release of phosphate was constantly higher than spontaneous hydrolysis and rose significantly with the increase in acylphosphatase/membrane protein ratio; with 300 units/mg of sarcolemmal protein, the phosphate release was about 2-fold over spontaneous hydrolysis. No significant enhancement of phosphate release was observed using heat-denatured acylphosphatase.

This indicates that acylphosphatase, even when used at low concentrations, efficiently hydrolyzes the phosphorylated intermediate of sarcolemmal  $\text{Na}^+, \text{K}^+$  pump, a result which

Table 1: Effect of Different Acylphosphatase Concentrations on the  $\text{Na}^+, \text{K}^+$ -ATPase Phosphorylated Intermediate from Sarcolemmal Vesicles<sup>a</sup>

acylphosphatase addition (units/mg of vesicle protein)	phosphate release [pmol (mg of protein) <sup>-1</sup> min <sup>-1</sup> ]
none	89.88 ± 4.45
50	120.03 ± 6.20
150	140.62 ± 4.71
300	157.50 ± 6.36
heat-inactivated	91.16 ± 4.56

<sup>a</sup> Labeled vesicles (1 mg of protein/mL of assay medium) were incubated as described under Materials and Methods with varying amounts of acylphosphatase. The phosphoenzyme level was calculated as 215 pmol of phosphate bound/mg of vesicle protein. Each point is the mean ± SE of five experiments performed on differing vesicle preparations. All the changes in phosphate release induced by active acylphosphatase were statistically significant ( $p < 0.01$  by the one-way analysis of variance). Heat-inactivated acylphosphatase (2 h at 100 °C) was added at a concentration corresponding to 300 units of active enzyme/mg of membrane protein.

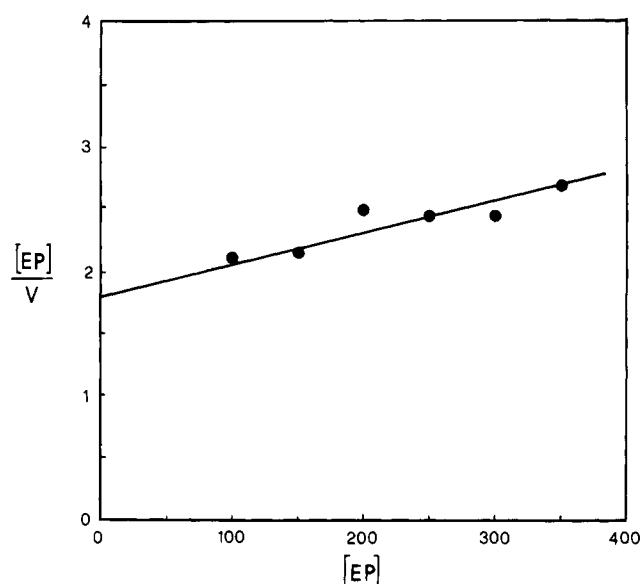


FIGURE 1: Hanes-Woolf plot of the dependence of the initial rate of acylphosphatase-induced dephosphorylation of  $\text{Na}^+, \text{K}^+$ -ATPase phosphoenzyme on EP concentration. 100 units of acylphosphatase was incubated in 30 mM Tris-HCl (pH 7.4 at 37 °C) with differing amounts of labeled membranes. EP concentration in the medium was expressed as picomoles of <sup>32</sup>P bound per milliliter. The initial rate of acylphosphatase-induced dephosphorylation (*V*), net for spontaneous hydrolysis, was expressed as picomoles of <sup>32</sup>P released per minute per milliliter. When  $[\text{EP}]/V = 0$ , the intercept on the  $[\text{EP}]$  axis gives  $-K_m$ . Each point represents the mean value of six determinations, and the line was fitted by linear regression.

is to be added to the high affinity of our enzyme toward this particular substrate that we found when we incubated a fixed amount of acylphosphatase with variable amounts of phosphorylated vesicles and measured the rate of acylphosphatase-induced hydrolysis as a function of EP concentration. In these conditions, the rate of phosphate release rose with increasing phosphoenzyme concentration, and from a Hanes-Woolf plot of the data obtained, a low apparent  $K_m$  ( $6.9 \times 10^{-7}$  M) was calculated (Figure 1).

Since the above results were obtained with an acid-denatured phosphoenzyme, in order to verify the possibility of a physical interaction between acylphosphatase and native, undenatured  $\text{Na}^+, \text{K}^+$ -ATPase, we dotted these two proteins onto nitrocellulose sheets and subjected them to the immunobinding assay described under Materials and Methods. As shown in Figure 2, the immunoreaction was positive, but at

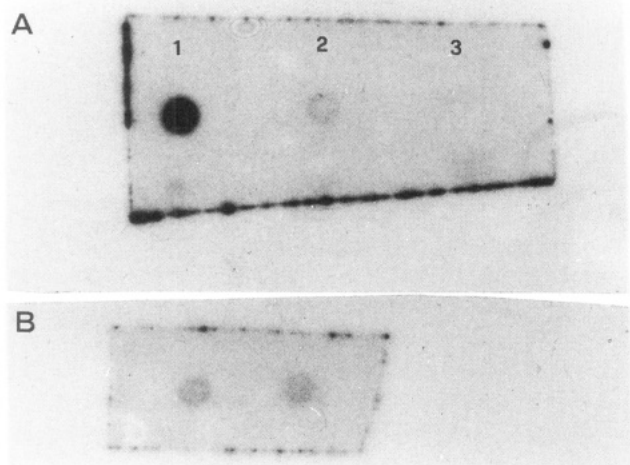


FIGURE 2: Dot-immunobinding assay revealing an interaction between acylphosphatase and Na<sup>+</sup>,K<sup>+</sup>-ATPase. (A) Positive reaction with 7 ng (1) and 0.7 ng (2) of dotted acylphosphatase while no visible spot can be seen with the application of 200 ng of Na<sup>+</sup>,K<sup>+</sup>-ATPase (3). (B) Positive reaction with the same amount of Na<sup>+</sup>,K<sup>+</sup>-ATPase after this protein was incubated with a medium containing 1  $\mu$ g of acylphosphatase. For details, see the text.

a different intensity level, corresponding to the two amounts used (7 and 0.7 ng) of dotted acylphosphatase. No visible spot was evident corresponding to Na<sup>+</sup>,K<sup>+</sup>-ATPase, although it was applied in a much larger amount (200 ng). However, a positive reaction also was detected for this protein when it was incubated with acylphosphatase. The composition of the medium containing acylphosphatase (1  $\mu$ g of enzyme in 1 mL of medium) was very similar to those used for ATPase and cation transport assays (see below), so as to induce EP formation which is supposed to be essential for the acylphosphatase interaction. Taken together, these findings lead, therefore, to exclude a false positiveness, due to a cross-reaction of our primary antibody with Na<sup>+</sup>,K<sup>+</sup>-ATPase, and suggest the establishment of binding between this protein and acylphosphatase. Dot immunobinding analysis were also performed, under the same experimental conditions, applying onto nitrocellulose strips other membrane-bound enzymes such as acetylcholinesterase and  $\gamma$ -glutamyltranspeptidase. No positive reactions were observed when they were incubated in medium containing acylphosphatase (data not shown).

To see whether the action of acylphosphatase on EP resulted in modified functional properties of Na<sup>+</sup>,K<sup>+</sup>-ATPase, we studied the effect of our enzyme on the rate of ATP hydrolysis and of cation (Na<sup>+</sup>,K<sup>+</sup>) transport in sarcolemmal vesicles used as a source of the Na<sup>+</sup>,K<sup>+</sup> pump as it exists *in situ*. The ATPase activity due to the Na<sup>+</sup>,K<sup>+</sup> pump was calculated, according to Harvey and Blostein (1986), as the

difference in the rate of ATP hydrolysis without and with 0.1 mM strophantidine, which, being membrane permeant, was added to inhibit total Na<sup>+</sup>,K<sup>+</sup>-ATPase-sensitive activity: furthermore, ouabain was present in all assays to inhibit the activity of right-side, permeable, or broken vesicles. Under these conditions, the strophantidine-sensitive activity was considered as due to the Na<sup>+</sup>,K<sup>+</sup>-ATPase activity of unleaky inside-out vesicles, the only ones that, in our experimental conditions, should be considered competent for cation transport, at the same time having the phosphorylation site accessible to our enzyme. The effect of acylphosphatase on Na<sup>+</sup>,K<sup>+</sup>-ATPase activity is illustrated in Table 2, where a significant enhancement in the maximal rate of ATP hydrolysis in the presence of increasing amounts of our enzyme is evident. No significant effect was evident with heat-denatured acylphosphatase or the active enzyme exhibited *per se* ATPase activity. The rate of ATP hydrolysis was also determined as a function of ATP and Na<sup>+</sup> concentrations. For the ATP dependence, sarcolemmal vesicles, preloaded with K<sup>+</sup> as described under Materials and Methods, were incubated with ATP concentrations ranging from 0.1 to 3 mM while the Na<sup>+</sup> concentration was kept constant at 100 mM; the Na<sup>+</sup> dependence was explored by incubating the same vesicles with Na<sup>+</sup> concentrations ranging from 1 to 200 mM at a constant ATP concentration of 3 mM. Acylphosphatase, when present, was added at 300 units/mg of vesicle protein, the amount that in the present study proved to give the maximal effect. From the results shown in Figures 3 and 4, it is evident that acylphosphatase enhanced the rate of ATP hydrolysis with all the used ATP and Na<sup>+</sup> concentrations, what suggests an increased affinity toward both these ligands. In fact, double-reciprocal plots of the data reported in the above figures (see insets) indicate that the concentrations giving half-maximal velocity of ATP hydrolysis (apparent  $K_m$  values) are  $0.55 \pm 0.06$  mM ATP and  $8.01 \pm 1.14$  Na<sup>+</sup> without acylphosphatase, while these values in the presence of the enzyme were significantly lowered to  $0.27 \pm 0.04$  mM ATP and to  $5.61 \pm 0.24$  mM Na<sup>+</sup> (mean values  $\pm$  SE;  $p < 0.05$ ). Acylphosphatase effects on cation transport were studied by measuring, in the absence and in the presence of our enzyme, the ATP-dependent influx into sarcolemmal vesicles, equivalent to the normal efflux from the cell, and the ATP-dependent K<sup>+</sup> efflux from loaded vesicles, equivalent to the normal influx into the cell. In these experiments, the temperature was set at 30  $^{\circ}$ C to prevent a too rapid depletion of K<sup>+</sup> from loaded vesicles. ATP and Na<sup>+</sup> concentrations were 3 mM and 100 mM, respectively; these are the highest used for ATPase assays, which were also performed at 30  $^{\circ}$ C. An assay period of 1 min was chosen for cation transport experiments, after we

Table 2: Effect of Acylphosphatase on Strophantidine-Sensitive Na<sup>+</sup>,K<sup>+</sup>-ATPase Activity of Sarcolemmal Vesicles<sup>a</sup>

acylphosphatase (units/mg of vesicle protein)	strophantidine-sensitive Na <sup>+</sup> ,K <sup>+</sup> -ATPase activity [nmol min <sup>-1</sup> (mg of vesicle protein) <sup>-1</sup> ]	increase (n-fold)
none	10.52 $\pm$ 0.15	
50	11.42 $\pm$ 0.23	1.08
150	12.68 $\pm$ 0.31	1.20
300	14.05 $\pm$ 0.25	1.33
heat-inactivated	10.22 $\pm$ 0.16	

<sup>a</sup> Strophantidine-sensitive Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was assayed as described under Materials and Methods. Each value is the difference in the mean  $\pm$  SE of five determinations performed with and without 0.1 mM strophantidine. Changes observed with increasing amounts of acylphosphatase were statistically significant ( $p < 0.01$  by the one-way analysis of variance). Heat-inactivated acylphosphatase (2 h at 100  $^{\circ}$ C) was added at a concentration corresponding to 300 units of active enzyme/mg of membrane protein.

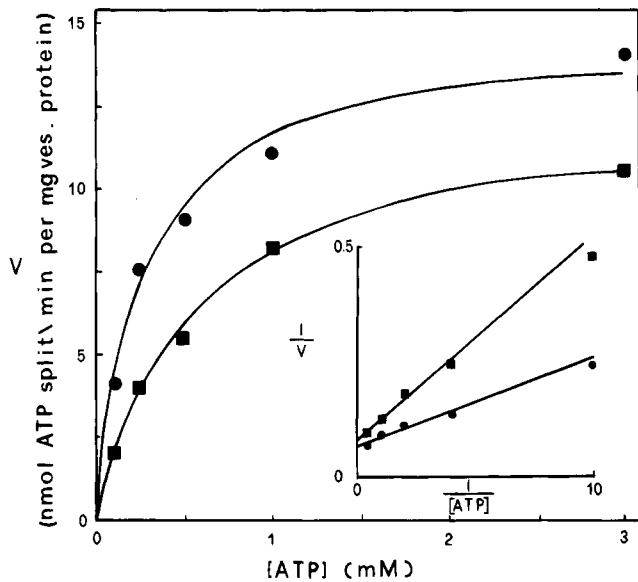


FIGURE 3: Sarcolemmal vesicle  $\text{Na}^+, \text{K}^+$ -ATPase activity as a function of ATP concentration. Except for varying ATP concentrations,  $\text{Na}^+, \text{K}^+$ -ATPase activity was measured as described under Materials and Methods in the presence (●) and in the absence (■) of acylphosphatase (300 units/mg of vesicle protein). Each point represents the mean value of five determinations. The inset shows the same data as Lineweaver-Burk plot with lines fitted by linear regression.

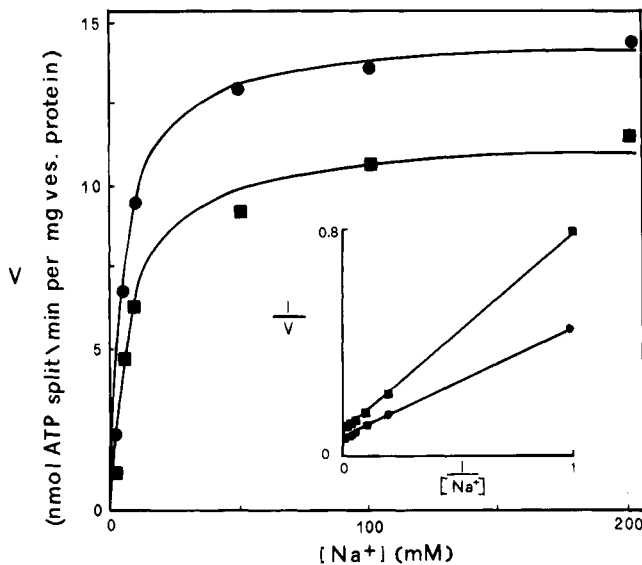


FIGURE 4: Sarcolemmal vesicle  $\text{Na}^+, \text{K}^+$ -ATPase activity as a function of  $\text{Na}^+$  concentration. Except for varying  $\text{Na}^+$  concentrations,  $\text{Na}^+, \text{K}^+$ -ATPase activity was measured as described under Materials and Methods in the presence (●) and in the absence (■) of acylphosphatase (300 units/mg of vesicle protein). Each point represents the mean value of five determinations. The inset shows the same data as a Lineweaver-Burk plot with lines fitted by linear regression.

found that  $\text{Na}^+$  uptake, measured at 30 s intervals, proceeded linearly over a 2 min period. After 1 min, the vesicles retained more than 90% of the  $\text{K}^+$  amount initially present.  $\text{Na}^+$  and  $\text{K}^+$  transport rates were measured as described under Materials and Methods using an absorption spectrometry procedure. For both cations, the values observed without ATP were subtracted in order to exclude the contribution of nonactive transport mechanisms. As shown in Figure 5, acylphosphatase addition resulted in a significant increase in the ATP-dependent  $\text{Na}^+$  uptake, and more markedly so with higher enzyme amounts; with 300 units/mg of vesicle

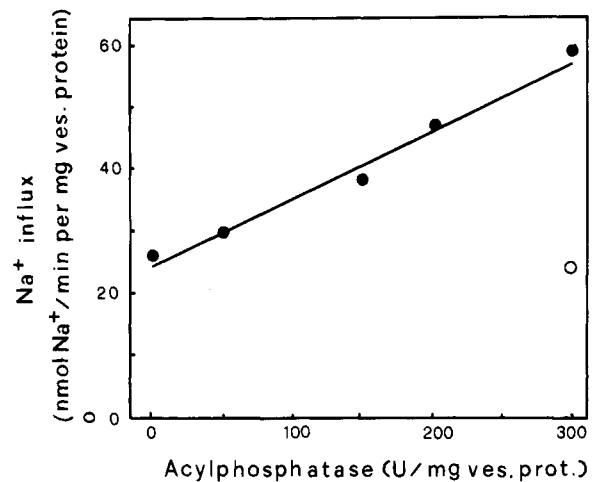


FIGURE 5: Effect of acylphosphatase on  $\text{Na}^+$  transport into sarcolemmal vesicles.  $\text{Na}^+$  influx was assayed as described under Materials and Methods with differing amounts of acylphosphatase. Each point represents the mean of five determinations. (○) indicates the value obtained with an amount of heat-inactivated acylphosphatase corresponding to 300 units of the active enzyme.

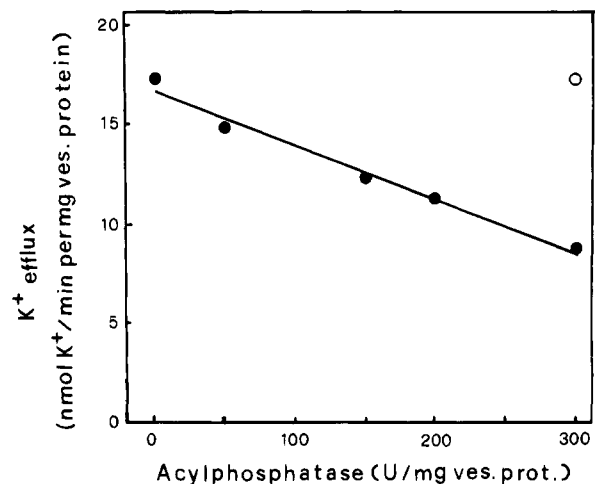


FIGURE 6: Effect of acylphosphatase on  $\text{K}^+$  transport in sarcolemmal vesicles.  $\text{K}^+$  efflux was assayed as described under Materials and Methods with differing amounts of acylphosphatase. Each point represents the mean of five determinations. (○) indicates the value obtained with an amount of heat-inactivated acylphosphatase corresponding to 300 units of the active enzyme.

protein,  $\text{Na}^+$  uptake was 2.3-fold the basal value. Conversely, in the presence of acylphosphatase, the efflux of  $\text{K}^+$  from loaded vesicles (Figure 6) was strongly and significantly decreased, even at the lowest enzyme concentration; with 300 units/mg of vesicle protein, a 50% decrease in the rate of  $\text{K}^+$  transport was observed. Heat-denatured acylphosphatase had no significant effect on the rate of cation transport, or the active enzyme modified the ATP-independent  $\text{Na}^+$  and  $\text{K}^+$  fluxes.

Due to all these effects, acylphosphatase addition resulted in a modified stoichiometry of ATP-driven cation transport by the  $\text{Na}^+, \text{K}^+$  pump, as indicated by the  $\text{Na}^+/\text{ATP}$  and  $\text{Na}^+/\text{K}^+$  ratios that we calculated from the rate of ATP hydrolysis and of cation transport observed in the same experimental conditions without and with a definite amount of acylphosphatase (Table 3).

## DISCUSSION

In the present study, we found that the phosphorylated intermediate of heart sarcolemma  $\text{Na}^+, \text{K}^+$ -pump is actively

Table 3: Effect of Acylphosphatase on Na<sup>+</sup>/ATP and Na<sup>+</sup>/K<sup>+</sup> Stoichiometry<sup>a</sup>

acylphosphatase addition (units/mg of vesicle protein)	strophantidine-sensitive		ATP-dependent Na <sup>+</sup> influx (nmol mg <sup>-1</sup> min <sup>-1</sup> )	ATP-dependent K <sup>+</sup> efflux (nmol mg <sup>-1</sup> min <sup>-1</sup> )	Na <sup>+</sup> /ATP ratio	Na <sup>+</sup> /K <sup>+</sup> ratio
	Na <sup>+</sup> /K <sup>+</sup> -ATPase activity (nmol mg <sup>-1</sup> min <sup>-1</sup> )	ATP-dependent Na <sup>+</sup> influx (nmol mg <sup>-1</sup> min <sup>-1</sup> )				
none	10.52 ± 0.15	26.46 ± 0.62	17.17 ± 0.33	2.51	1.50	
50	11.42 ± 0.23	29.66 ± 0.58	14.66 ± 0.20	2.59	2.02	
150	12.68 ± 0.31	37.66 ± 0.14	12.20 ± 0.16	2.97	3.08	
300	14.05 ± 0.25	59.01 ± 0.48	8.67 ± 0.07	4.20	6.68	

<sup>a</sup> Strophantidine-sensitive Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, ATP-dependent Na<sup>+</sup> influx (normal efflux), and K<sup>+</sup> efflux (normal influx) were assayed as described under Materials and Methods. Each value shown is the mean ±SE of five determinations, and all changes induced by active acylphosphatase were statistically significant ( $p < 0.01$  by the one-way analysis of variance).

hydrolyzed by acylphosphatase, even when this enzyme is added at concentrations near the lower limit of the physiological range. Furthermore, the measurement of the initial rate of acylphosphatase-induced hydrolysis as a function of EP concentration gave an apparent  $K_m$  value on the order of  $10^{-7}$  M, which suggests a high affinity toward this special substrate. In fact, the  $K_m$  values still now observed for other potentially competing acylphosphatase substrates, that is, various low molecular weight cytosolic components (see the introduction), were always higher than  $10^{-4}$  M. All the above experiments were conducted using heart sarcolemma membranes which did not undergo disruption, proteolysis, or other drastic treatment; however, it was necessarily the acid-denatured EP which was subjected to the hydrolytic activity of our enzyme. Thus, to exclude that the observed effects were to be considered simple artifacts due to the denaturing procedure, we thought it was important to verify if acylphosphatase could interact with untreated purified Na<sup>+</sup>,K<sup>+</sup>-ATPase, at the same time affecting the functional properties of this active transport system.

As for the first question, the chosen approach was a dot-immunobinding assay, an extremely versatile method which has the advantage of being quick and simpler than other comparable procedures. Moreover, for our purposes, the method proved to be sensitive enough, since less than 1 ng of acylphosphatase was clearly detectable. Purified Na<sup>+</sup>,K<sup>+</sup>-ATPase used for these experiments was a commercial form from dog kidney, hence not from bovine heart muscle as acylphosphatase, but it is well-known that the Na<sup>+</sup>,K<sup>+</sup> pump exhibits, especially in its  $\alpha$ -subunits, a high structural homology through all mammalian species and tissues (Peterson & Hokin, 1988; Baxter-Lowe & Hokin, 1989). In any case, we think that the observed results cannot find another explanation than admitting that, at least in the assay conditions (which were very similar to those used for ATPase activity and cation transport measurements), binding was established between acylphosphatase and Na<sup>+</sup>,K<sup>+</sup>-ATPase. Obviously, these data are merely qualitative, and no conclusion may be inferred about the quantitative aspects of this interaction. As for the specificity of these results, the absence of a positive reaction between acylphosphatase and acetylcholinesterase or  $\gamma$ -glutamyltranspeptidase confirms that the observed binding of our enzyme with Na<sup>+</sup>,K<sup>+</sup>-ATPase was not an artifact but reflected a rather selective interaction.

We also found that acylphosphatase induced significant modifications in the functional properties of the Na<sup>+</sup>,K<sup>+</sup> pump, notably in the rate of ATP hydrolysis and of cation transport. In order to compare these effects and to obtain a reliable estimation of the coupling between ATP hydrolysis and pumping activity, all the experiments were conducted under the same experimental conditions as regards the temperature and the concentrations of intravesicular K<sup>+</sup>,

extravesicular Na<sup>+</sup>, and ATP. In such conditions, that ensured good efficiency of the pump at the same time preventing a too rapid loss of K<sup>+</sup> from vesicles, the system without added acylphosphatase exhibited a Na<sup>+</sup>/ATP ratio near the generally accepted value of 3/1, and also the ratio for the ATP-dependent Na<sup>+</sup>/K<sup>+</sup> exchange corresponded to the normal value of 3/2. In the presence of added acylphosphatase, and more markedly so with increasing enzyme amounts, we observed changes in the rate both of ATP hydrolysis and of cation transport. However, while ATPase activity and Na<sup>+</sup> uptake were stimulated, the latter at an even greater extent, the ATP-dependent K<sup>+</sup> efflux from loaded sarcolemmal vesicles was reduced, as seen in the observed alterations in the Na<sup>+</sup>/ATP and, especially, Na<sup>+</sup>/K<sup>+</sup> ratios. In this connection, it is noteworthy that acylphosphatase does not exhibit ATPase activity *per se*; moreover, the heat-inactivated enzyme (which had no effect on phosphate release from EP) did not produce appreciable modifications in sarcolemmal vesicle ATPase and pumping activity, nor did the active enzyme have significant effects on the ATP-independent Na<sup>+</sup> and K<sup>+</sup> fluxes in and from vesicles. This lead us to exclude either that acylphosphatase acts simply like a protein, independently from its catalytic properties, or that the modifications induced by this enzyme on Na<sup>+</sup> and K<sup>+</sup> transport are due to changes in the passive permeability of the sarcolemmal membrane to these cations.

A possible interpretation of acylphosphatase effects may be proposed on the basis of the most widely used models that describe the functioning of the Na<sup>+</sup>,K<sup>+</sup> pump. On the assumption that the interactions with Na<sup>+</sup>, K<sup>+</sup>, and ATP are consecutive, the sequence of chemical reactions and conformational transitions associated with cation transport may be described as in the scheme shown in Figure 7. In this scheme, it is assumed that Na<sup>+</sup>,K<sup>+</sup>-ATPase exists in two main conformations, denoted E<sub>1</sub>, with high Na<sup>+</sup>-low K<sup>+</sup> affinity, and E<sub>2</sub>, with high K<sup>+</sup>-low Na<sup>+</sup> affinity; intermediate conformational states are postulated to justify the release of bound cations. Briefly, interaction with ATP facilitates the transition from E<sub>2</sub> to E<sub>1</sub>, allowing the binding of Na<sup>+</sup>. Phosphorylation by ATP stimulates the conversion of E<sub>1</sub> into E<sub>2</sub> and the outward transport of Na<sup>+</sup>. K<sup>+</sup> binds to the E<sub>2</sub>P phosphoenzyme, inducing its dephosphorylation, which, together with the subsequent interaction with ATP, is responsible for the inward release of K<sup>+</sup> and the recovery of the initial state. In this context, it seems reasonable to suppose that the observed acylphosphatase effects are the result of an accelerated turnover of EP. In fact, although the modifications induced by acylphosphatase on cation transport and ATP hydrolysis do not exactly parallel those of EP hydrolysis, these effects are of the same order of magnitude and, in our opinion, sufficiently correlated, all the more so that phosphate release was tested on the acid-

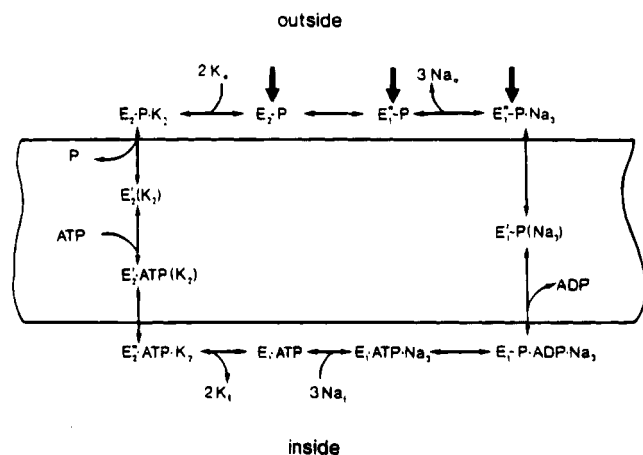


FIGURE 7: Scheme for  $\text{Na}^+\text{-K}^+$  exchange by  $\text{Na}^+\text{,K}^+\text{-ATPase}$ .  $\text{E}_1$  represents the form with high  $\text{Na}^+\text{-low K}^+$  affinity and  $\text{E}_2$  the form with high  $\text{K}^+\text{-low Na}^+$  affinity.  $\text{E}$ ,  $\text{E}'$ , and  $\text{E}''$  represent transient conformational states and  $\text{EP}$  the phosphorylated intermediate. Parentheses indicate that the cations are occluded, i.e., not available to be released toward the cytoplasm or the extracellular medium. Boldface arrows indicate the supposed sites of acylphosphatase action.

denatured phosphoenzyme while cation transport and ATP hydrolysis were assayed using intact sarcolemmal vesicles. On the basis of the previously reported  $\text{Na}^+/\text{ATP}$  and  $\text{Na}^+/\text{K}^+$  ratios, the proposed mechanism is that the acylphosphatase-induced hydrolysis of the phosphorylated intermediate, in competition with its own hydrolytic catalysis, would occur after the conformational changes needed for  $\text{Na}^+$  transport but before the binding of  $\text{K}^+$ . This would result in a short-circuit of the system which could account either for the increase in the rate of ATP hydrolysis and  $\text{Na}^+$  transport or for the inhibition of  $\text{K}^+$  transport, since this cation binds only to the phosphorylated form of  $\text{Na}^+\text{,K}^+$  pump. As regards the lowering of the apparent  $K_m$  values for  $\text{Na}^+$  and ATP that we observed when studying the effect of acylphosphatase on the kinetics of ATP hydrolysis, this probably represents the result of a change in the contribution of various elementary steps to the rate limitation of the entire reaction cycle. More in particular, the increase in the apparent affinity for ATP may be consistent with less dependence of the overall reaction on  $\text{K}^+$  deocclusion. The basis for the apparent increased affinity for  $\text{Na}^+$  is more difficult to explain. However, if acylphosphatase hydrolyzes the phosphoenzyme at the stages that we postulated (see the arrows in the scheme), the requirement for  $\text{K}^+$  stimulation of EP hydrolysis and hence coupling of the reaction to  $\text{K}^+$  translocation is likely to be reduced. Another consequence of this acylphosphatase effect might be that the rate of reversal of the reactions by which  $\text{Na}^+$  is bound, translocated, and released is reduced, as seen in an increase in the apparent affinity for this cation.

In conclusion, the results of the present study indicate that acylphosphatase, owing to its catalytic activity on the phosphorylated intermediate, can alter the functioning of  $\text{Na}^+\text{,K}^+\text{-ATPase}$ , inducing a sort of "uncoupling" effect on this active transport system. This represents, at least to our knowledge, the first evidence concerning modifications of the functional properties of the heart sarcolemma  $\text{Na}^+\text{,K}^+$  pump that are induced by an enzyme normally present in the same tissue. Some aspects of acylphosphatase action— notably its high affinity toward the phosphorylated intermediate and its ability of acting significantly even at low

concentration and of binding to  $\text{Na}^+\text{,K}^+\text{-ATPase}$ —lead us to consider as not merely speculative the hypothesis that our enzyme may contribute to the physiological regulation of heart sarcolemma  $\text{Na}^+\text{,K}^+$  pump. Further investigations, aimed to probe more deeply the action mechanism of acylphosphatase, to identify the molecular basis for its interaction with the  $\text{Na}^+\text{,K}^+$  pump and eventual factors inducing its recruitment toward the sarcolemmal membrane would be therefore of interest.

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