Na^+,K^+-ATPase: (Ca^{2+} + OUABAIN)-DEPENDENT PHOSPHORYLATION BY P_i

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1. Introduction

The active transports of Na^+ and K^+ are done by Na^+,K^+-ATPase (EC 3.6.1.3) of the plasma membrane [1]. The enzyme, which requires Mg^{2+} and is inhibited by ouabain and related cardiac glycosides, is also considered to be the receptor for some, if not all, of the cardiac effects of these drugs [2]. Studies on the interaction of Ca^{2+} with this enzyme are of special interest because of the long-established interrelation between the effects of Ca^{2+} and cardiac glycosides on the heart [3]. Inhibition of enzyme activity by Ca^{2+}, and the effects of Ca^{2+} on enzyme phosphorylation by ATP have been studied [2,4,5]. Na^+,K^+-ATPase is also phosphorylated by P_i in the presence of either Mg^{2+} or Mg^{2+} and ouabain [6–9]. Here we report that the enzyme may also be phosphorylated by P_i in the presence of Ca^{2+}, but that this process has an obligatory requirement for ouabain. The maximal level of phosphoenzyme formed with P_i + Ca^{2+} + ouabain is half of that obtained with P_i + Mg^{2+} + ouabain. The maximal level of bound ouabain is the same under both conditions. The findings provide support for Ca^{2+} + ouabain-induced half-of-the-sites reactivity in the oligomeric enzyme.

2. Methods

The highly purified enzyme of the dog kidney outer medulla was prepared and assayed as in [10]. The specific activities of the various preparations used here were 900–1500 μmol ATP converted to P_i . mg^{-1} . h^{-1}. All phosphorylation experiments were done at 37°C with 0.1 mg enzyme protein suspended in 0.2 ml solution containing 20 mM Tris–HCl (pH 7.2), and the indicated concentrations of ligands. Measurement of acid-stable phosphoenzyme, and SDS gel electrophoresis of the labeled enzyme, were done as in [10]. Values for ^{32}P-incorporation were corrected for ^{32}P-incorporation into the denatured enzyme. The latter was <5% of the total as noted [9]. Ouabain binding to the enzyme was measured by conventional filtration techniques [2]. [^3H]Ouabain and $^{32}P_i$ were obtained from New England Nuclear (Boston MA). $^{32}P_i$ was purified [11] immediately prior to use.

3. Results

Fig.1 shows the time-course of phosphorylation of the enzyme by P_i in the presence of Ca^{2+} and Mg^{2+}. In agreement with [8,9], when Mg^{2+} is used a steady

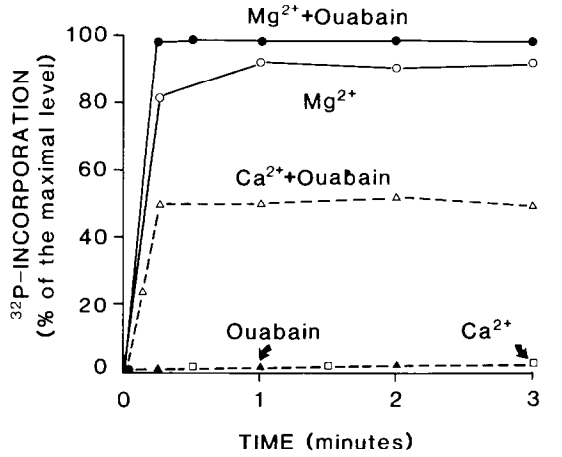

Fig.1. Time-course of enzyme phosphorylation by P_i in the presence of Mg^{2+}, Ca^{2+} and ouabain. All reaction mixtures contained 0.2 mM $^{32}P_i$ and 0.5 mM EDTA. The ligands were added as indicated: 2 mM Mg^{2+}; 2 mM Ca^{2+}; 0.1 mM ouabain. Other reaction conditions were as in section 2. The maximal level of ^{32}P-incorporation was 3.1 nmol P_i/mg.

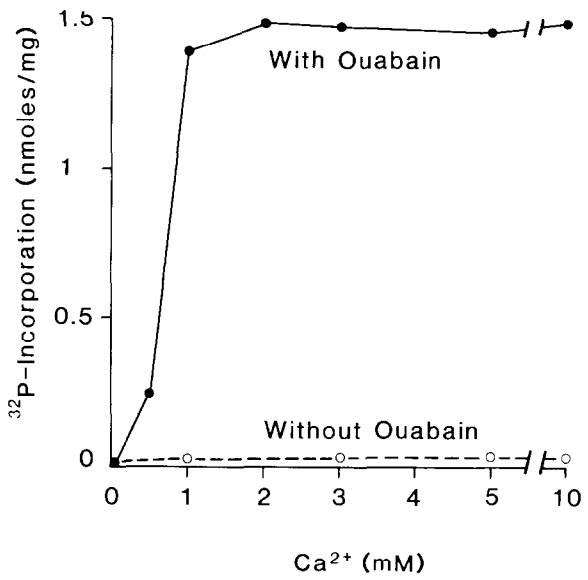

Fig.2. Effects of varying concentrations of Ca^{2+} on enzyme phosphorylation by P_i in the presence or absorbence of ouabain. Each incubation was done for 3 min under the conditions described in fig.1. All reactions mixtures contained 0.5 mM EDTA. The enzyme preparation used here was that used in fig.1.

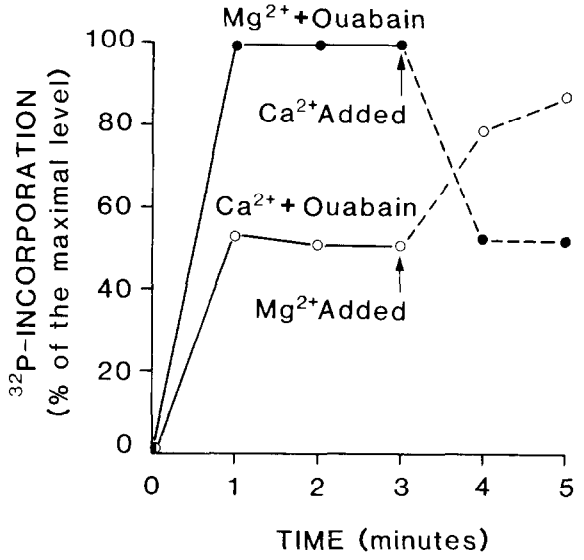

Fig.3. Relationship between Ca^{2+}-induced and Mg^{2+}-induced phosphorylation: (•) phosphorylation was initiated in the presence of 0.6 mM Mg^{2+} and ouabain; after 3 min 5 mM Ca^{2+} was added; (○) phosphorylation was initiated with 1 mM Ca^{2+} and ouabain; after 3 min 5 mM Mg^{2+} was added. Other conditions were as in fig.1. The maximal level of ^{32}P-incorporation in this enzyme preparation differing from fig.1,2, was 2.6 nmol/mg.

state level of ^{32}P-incorporation is attained in <1 min regardless of the presence or absence of ouabain. With Ca^{2+}, however, phosphorylation does not occur unless ouabain is also present. Ouabain without Ca^{2+} does not induce phosphorylation. Fig.2 shows that the [Ca^{2+}] used in fig.1 is optimal for Ca^{2+} + ouabain-induced phosphorylation. In separate experiments it was established that [ouabain], [P_i] and [Mg^{2+}] used in fig.1 and 2 are also optimal (not shown). It is evident, therefore, that the maximal level of phosphoenzyme obtained in the presence of Ca^{2+} + ouabain is half of that obtained in the presence of Mg^{2+} + ouabain (fig.3, table 1). The data of fig.3 show that Ca^{2+} + ouabain-induced phosphorylation and Mg^{2+} + ouabain-induced phosphorylation are not independent processes. When the phosphoenzyme is formed in the presence of ouabain and a low, but still optimal, [Mg^{2+}]; and is then exposed to a high [Ca^{2+}], the phosphoenzyme level is reduced to that obtained with Ca^{2+} + ouabain. Conversely, when the phosphoenzyme is first formed in the presence of Ca^{2+} and ouabain, and then exposed to Mg^{2+}, the level rises and approaches that obtained in the presence of Mg^{2+} + ouabain. With numerous combinations of [Ca^{2+}] and [Mg^{2+}] other than those shown in fig.3, it was not possible to obtain a level of phosphoenzyme greater than that obtained with Mg^{2+} + ouabain.

Similarities of the Ca^{2+}-induced and the Mg^{2+}-induced phosphoenzyme were also revealed by the following experiments for which data are not pre-

Table 1
Maximal levels of ouabain binding and ^{32}P-incorporation in the presence of Ca^{2+} or Mg^{2+}

Reaction condition	Phosphoenzyme (nmol ^{32}P/mg protein)	Bound ouabain (nmol/mg protein)
Mg^{2+} + P_i + ouabain	2.19 ± 0.02	2.07 ± 0.13
Ca^{2+} + P_i + ouabain	1.13 ± 0.02	2.01 ± 0.12

Reaction conditions and ligand concentrations were as described in legend to fig.1. In one set of reactions, $^{32}P_i$ and unlabeled ouabain were used; and in the other unlabeled P_i and [^{3}H]ouabain were used. Reaction time was 30 min. Values of bound ouabain were corrected for non-specific binding to heat-denatured enzyme. Each indicated value is the average and range of 6 separate experiments. The same enzyme preparation was used in all experiments. Note that this preparation was different from the enzyme preparation used in experiments in fig.1–3

sented: When the enzyme was reacted with $^{32}P_i$ + Ca^{2+} + ouabain, then subjected to SDS–polyacrylamide gel electrophoresis at pH 2.4, ^{32}P-label was detected only in the catalytic subunit (the α-subunit) of the enzyme. Exclusive labeling of this subunit upon reaction with Mg^{2+} + P_i has been shown [9].

In [1,2] the maximal level of ouabain binding to the purified enzyme occurred in the presence of P_i + Mg^{2+}, and the no. ouabain molecules bound/unit of enzyme was the same as the no. P_i molecules incorp./unit of enzyme. The data of table 1 confirm this. Interestingly, however, the data show that when Mg^{2+} is replaced with Ca^{2+}, 2 molecules of ouabain are bound for each P_i incorporated.

4. Discussion

The first points to be considered are the implications of these findings in the context of the pharmacologic effects of cardiac glycosides. There is now ample evidence to suggest that the cardiac effects of these drugs may be due to their interactions with Na^+,K^+-ATPase [2]. It has also been known for decades that there is a synergism between the effects of Ca^{2+} and cardiac glycosides on the heart [3]. Studies on the effects of Ca^{2+} and ouabain on the reactions of ATP with Na^+,K^+-ATPase, have provided no clues to suggest that direct interactions of the 2 agents with Na^+,K^+-ATPase may be involved in the above synergism. Hence, the central theme of most current hypotheses on the mechanism of pharmacologic effects of cardiac glycosides is that these drugs inhibit the enzyme directly, but that alterations in membrane fluxes of Ca^{2+} are indicrect consequences of the functional inhibition of the enzyme [12]. These data show that in vitro there is a functional state of the enzyme with obligatory requirement for Ca^{2+} and ouabain. Although the physiological significance of enzyme phosphorylation by P_i is not clear, the mere demonstration of such state of the enzyme indicates that serious attention should be paid to the possibility of direct involvement of Na^+,K^+-ATPase in the mediation of the interrelated effects of Ca^{2+} and ouabain in the intact tissue.

The above considerations aside, these data are of help in the clarification of some uncertainties concerning the quaternary structure of Na^+,K^+-ATPase. A mainstay of the arguments for the existence of $\geqslant 2$ α-subunits in the functional unit of the enzyme has been the observation that only half of the α-subunit content of the enzyme is phosphorylated in the presence of either Mg^{2+} + P_i or Mg^{2+} + Na^+ + ATP (e.g., [13,14]). Due to inherent difficulties in the determination of this stoichiometry, however, the possibility that in a rigorously purified enzyme all α-subunits may be phosphorylated under the above conditions has not been ruled out (e.g., [15]). We now show that the replacement of Mg^{2+} with Ca^{2+} halves the maximal level of the phosphoenzyme. This points to the existence of an α-oligomer, regardless of how the above-mentioned uncertainties are resolved. If with Mg^{2+} all of the α-subunits are phosphorylated, then with Ca^{2+} half must be phosphorylated; suggesting a minimum of two associated α-subunits exhibiting ligand-induced half-of-the-sites reactivity with respect to phosphorylation, but not to ouabain binding. However, if half of the α-subunits are phosphorylated in the presence of Mg^{2+}, one must conclude that the minimum structure is an α-tetramer, and that in the presence of Ca^{2+} only one subunit is phosphorylated, and two bind ouabain. This pattern of ligand interaction with the enzyme would be consistent with the results of crosslinking experiments on Na^+,K^+-ATPase which also suggest that the existence of an α-tetramer exhibiting ligand-induced negative cooperativity across 2 intersubunit domains [10,14].

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