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OUABAIN BINDING TO $(Na^+ + K^+)$-ATPase

EFFECTS OF NUCLEOTIDE ANALOGUES AND ETHACRYNIC ACID

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SUMMARY

To determine the mechanism of Na^+, Mg^{2+} and ATP stimulated [^{3}H]ouabain binding to $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) the effects on this reaction of ethacrynic acid and analogues of ATP were investigated. β,γ-Methylene-ATP inhibited [^{3}H]ouabain binding to this enzyme under all conditions tested. This inhibition was synergistic with that of Na^+, and Na^+ acted to increase the apparent affinity of the enzyme for β,γ-methylene-ATP. β,γ-Methylene ADP also inhibited [^{3}H]ouabain binding to this enzyme. Similarly, the overall effect of β,γ-imido-ATP on [^{3}H]ouabain binding to guinea pig kidney $(Na^+ + K^+)$-ATPase was inhibitory. Ethacrynic acid prolonged transient pulses of [^{3}H]ouabain binding supported by ATP or ADP. Ethacrynic acid produced this effect by stabilizing the form of the enzyme which interacted with ouabain rather than by affecting the rates at which ouabain was bound to or dissociated from the enzyme. The data suggest that nucleotides with a stable terminal phosphate bond cannot support [^{3}H]ouabain binding. These observations are consistent with the hypothesis that Na^+-stimulated phosphorylation of the $(Na^+ + K^+)$-ATPase is a primary event in the Na^+-stimulated binding of [^{3}H]-ouabain to this enzyme.

INTRODUCTION

The $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) or "sodium pump" enzyme appears to be the biochemical basis for the active transport of Na^+ and K^+ across the plasma membrane of mammalian cells [1]. This enzyme system is very selectively inhibited by cardiac glycosides and investigation of the mechanism of this inhibition has yielded much information concerning the mechanism of action of this pump [2–4]. One of the major problems in determining the mechanism by which cardiac glycosides interact with $(Na^+ + K^+)$-ATPase in the presence of ATP is to distinguish between ligand actions of ATP and those due to phosphorylation of the enzyme by ATP [1]. The initial experimental approach to this problem was to test the ability of nucleotides other than ATP to support [^{3}H]ouabain binding [2, 3, 5] since nucleotides other than ATP are generally relatively poor substrates for the $(Na^+ + K^+)$-ATPase. Such nucleotides are CTP, ITP, ADP, and UTP, none of which are

hydrolyzed by the $(Na^+ + K^+)$-ATPase at more than 10% of the rate at which ATP is hydrolyzed [6–8]. However, these agents are just as effective as ATP in supporting [^{3}H]ouabain binding in the presence of Na^+ [3, 5, 8, 9] suggesting that the mechanism by which they stimulate [^{3}H]ouabain binding is distinct from the mechanism by which they are hydrolyzed by this enzyme [3, 5, 9]. These observations have led to suggestions that nucleotide-dependent, Na^+-stimulated [^{3}H]ouabain-binding is dependent simply on the binding of substrates to the $(Na^+ + K^+)$-ATPase and does not necessarily require the formation of phosphoenzyme [3, 5, 9].

This interpretation is weakened by the observation that though nucleotide substrates other than ATP are hydrolyzed relatively slowly by the $(Na^+ + K^+)$-ATPase they are quite effective in phosphorylating the enzyme under the conditions of a [^{3}H]-ouabain binding experiment. UTP, which in the presence of Na^+ and K^+ is hydrolyzed at about 5% of the rate at which ATP is hydrolyzed, is just as affective as ATP in phosphorylating this enzyme [10]. Similarly, CTP, another poor substrate, also phosphorylates this enzyme to the same extent as ATP [8]. These data show that the various nucleotides substrates of this enzyme cannot be used to distinguish between ligand actions of nucleotides and those that may be due to the formation of phospho-enzyme.

This communication presents the results of a further investigation of this problem. The method used has been to stabilize the various phosphate bonds involved in the $(Na^+ + K^+)$-ATPase reaction and to investigate the effects of this stabilization on the binding of [^{3}H]ouabain to this enzyme. Thus, the enzyme–phosphate (E–P) bond of the phospho-form of the $(Na^+ + K^+)$-ATPase was stabilized by exposing the enzyme to ethacrynic acid [11]. Such exposure produced a marked prolongation of transient pulses of [^{3}H]ouabain binding, consistent with the concept that binding occurs to the phosphoenzyme.

The other aspect of this approach was to study the actions of nucleotide analogues on the binding of [^{3}H]ouabain to this enzyme. The analogues used were, β,γ-methylene-ATP, β,γ-methylene-ADP and β,γ-imido-ATP. In these analogues, the terminal bridge oxygen of the nucleotide molecule has been replaced by either a methylene or an imido group. These analogues are structurally similar to ATP [12] and at least in the case of β,γ-methylene-ATP they bind to this enzyme without being hydrolyzed [13]. When the terminal phosphate bond of the nucleotide molecule is stabilized in this way, these nucleotides lose their ability to stimulate [^{3}H]ouabain binding and become inhibitory. These observations are consistent with recent data which suggest that the ligand actions of ATP tend to return this enzyme to a non-ouabain binding configuration [13, 14] and support suggestions that transfer of the terminal phosphate of nucleotide substrates to this enzyme is required for Na^+-stimulated [^{3}H]ouabain binding to occur. A preliminary report has been communicated [16].

MATERIALS AND METHODS

Enzyme preparation and assay

Rat brain $(Na^+ + K^+)$-ATPase was prepared as described by Akera and Brody [17] and guinea pig kidney $(Na^+ + K^+)$-ATPase by the method of Post and Sen [18]. $(Na^+ + K^+)$-ATPase activity was assayed as previously described [19] and the protein

content of the enzymes was estimated by the method of Lowry et al. [20]. Total ATP-ase activity was between 150 and 300 μmoles P_i/mg protein per h for the rat brain enzymes and between 90 and 180 μmoles P_i/mg protein per h for the guinea pig kidney enzymes. In each case about 90–95% of the total activity was ouabain sensitive.

Phosphorylation and [^3H]ouabain binding

Phosphorylation and [^{3}H]ouabain binding were carried out as described previously [19] with the exception of Fig. 8B, where [^{3}H]ouabain binding was performed as described by Tobin and Sen [4]. In most experiments, to allow comparison of data obtained on enzymes of differing specific activities, the highest value in each experiment was arbitrarily set at 100% and other values expressed as a percentage of this [8].

Reagents, chemicals

[γ-^{32}P]ATP was obtained from New England Nuclear Ltd, Boston, Mass. and was diluted with carrier ATP to give about $5 \cdot 10^6$ cpm per μmole of ATP. [^{3}H]-ouabain, (New England Nuclear), was diluted with carrier ouabain to give 500 Ci/mole, the high specific activity being required by the relatively low concentration of [^{3}H]ouabain used in the initial rate of binding experiments [8]. ATP and ouabain were obtained from Sigma Chemical Co., St. Louis, Mo. β,γ-Methylene-ATP and α,β-methylene-ADP were obtained from Miles Laboratories Ltd, Kankakee, Ill. β,γ-Imido-ATP was obtained as the lithium salt from Boehringer Ltd, Mannheim, Germany and was converted to the Na^+ salt on a Dowex column as previously described [4]. In other experiments β,γ-imido-ATP as its sodium salt was obtained from I.C.N., Ltd, Irvine, Calif. Ethacrynic acid was a gift of Dr John E. Baer of the Merck Institute for Therapeutic Research, West Point, Pa. Unless otherwise indicated, all experiments were repeated at least four times and the data presented are the means of at least four experimental determinations on different enzyme preparations, plus or minus the standard errors of the means (S.E.). Lack of a vertical bar indicating the S.E. indicates that the calculated S.E. was less than 3%. Where appropriate, statistical significance was calculated by the t test, the criterion for significance being $P<0.05$.

RESULTS

ATP alone has not been observed to stimulate [^{3}H]ouabain binding to this enzyme but has always been observed to be partially effective in the presence of Mg^{2+} [2, 9]. The mechanism of this action of ATP in the presence of Mg^{2+} is unclear. It could be due to a ligand action of Mg^{2+} and ATP to change the conformation of the enzyme or it could result from an action of ATP to phosphorylate this enzyme in the presence of Mg^{2+}. In an attempt to answer this question the actions of ATP and β,γ-methylene-ATP on the equilibrium levels of [^{3}H]ouabain binding to this enzyme in the presence of Mg^{2+}, and Mg^{2+} and P_i were compared.

Fig. 1 shows the effects of ATP or β,γ-methylene-ATP on [^{3}H]ouabain binding to this enzyme in the presence of Mg^{2+} and P_i. Adenosine triphosphate reduced binding to about 60% of control levels (Fig. 1A). A similar inhibition of binding was observed if the binding reaction was started in the presence of ATP, showing

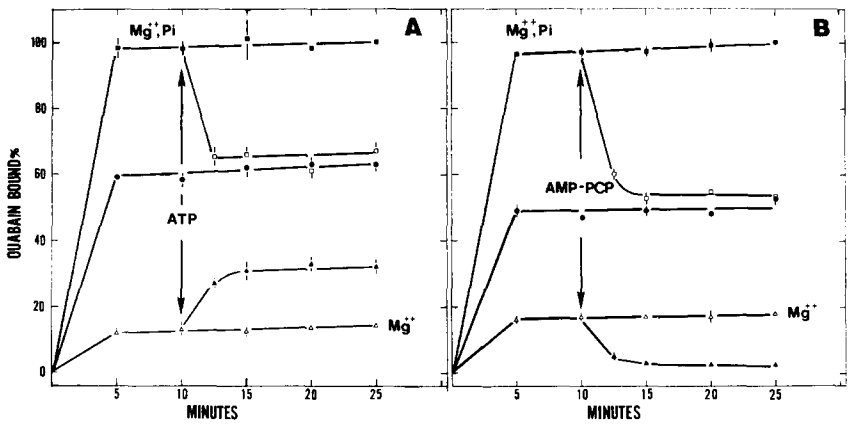

Fig. 1. Effects of ATP and β,γ-methylene-ATP (AMP-PCP) on Mg^{2+} and Mg^{2+} and P_i dependent [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase. (A) Guinea pig kidney enzymes were allowed to equilibrate with $5 \cdot 10^{-7}$ M [^{3}H]ouabain in the presence of 4 mM Mg^{2+} (△–△), 4 mM Mg^{2+} and 1 mM P_i, (■–■) or 4 mM Mg^{2+}, 1 mM P_i and 2 mM ATP (●–●) at 37 °C. In each case the binding reaction was started by the addition of Mg^{2+} at indicated zero time. At 10 min binding in the presence of Mg^{2+} and Mg^{2+} and P_i was challenged by the addition of 2 mM ATP. (□–□) shows the subsequent binding of [^{3}H]ouabain in the presence of Mg^{2+}, ATP and P_i ,while (▲–▲) shows binding in the presence of Mg^{2+} and ATP. (B) Matched experiments in which β,γ-methylene-ATP was substituted for ATP. In each experiment binding occurring in the presence of an excess of unlabeled ouabain was deducted out as background labeling. Binding is expressed as a percentage of that in the presence of 4 mM Mg^{2+} and 1 mM P_i, which averaged 113.42 ± 8.02 pmoles [^{3}H]ouabain/mg protein.

that the reaction goes to equilibrium. The inhibition develops very rapidly and is much more marked than that observed by Hansen et al. [21]. This inhibitory action of ATP is consistent with observations that [^{3}H]ouabain binding to this enzyme in the presence of Mg^{2+} and P_i is consistently greater than that observed in the presence of Na^+, Mg^{2+} and ATP [4, 22].

In contrast to the inhibitory actions of ATP on Mg^{2+} and P_i dependent binding, binding in the presence of Mg^{2+} alone was stimulated by ATP and equilibrated at about 30% of the level in the presence of Mg^{2+} and P_i (Fig. 1A). This observation precluded the hypothesis that ATP is simply an inhibitor of [^{3}H]ouabain binding and made it mandatory to test the actions of non-hydrolyzable nucleotide analogues on the binding of [^{3}H]ouabain to the enzyme under these conditions.

Fig. 1B shows the action of β,γ-methylene-ATP on equilibrium levels of [^{3}H]-ouabain binding to this enzyme under the same conditions as in Fig. 1A. In the presence of Mg^{2+} and P_i the action of β,γ-methylene-ATP is similar to that of ATP, producing about a 50% inhibition of the level of [^{3}H]ouabain binding. In other experiments increasing the concentration of β,γ-methylene-ATP increased the amount of inhibition observed. However, in the presence of Mg^{2+} alone, β,γ-methylene-ATP, in contrast to the actions of ATP, is also inhibitory to [^{3}H]ouabain binding. The data are consistent with the concept that bound ATP is inhibitory to [^{3}H]ouabain binding and that the low level of stimulation observed in the presence of Mg^{2+} and ATP may be the result of phosphorylation of the enzyme by Mg^{2+} and ATP, possibly due to the presence of residual Na^+ in these binding systems [23].

TABLE I

EFFECTS OF Na^+ AND β,γ-METHYLENE-ATP ON Mg^{2+} AND P_i STIMULATED [^{3}H]-OUABAIN BINDING

Guinea pig kidney enzymes were allowed to equilibrate with $2.5 \cdot 10^{-7}$ M [^{3}H]ouabain in the presence of 4 mM Mg^{2+} and 1 mM P_i for 15 min at 37 °C. Binding observed under these conditions was taken as 100 % binding, which averaged 232.5 ± 8.15 pmoles [^{3}H]ouabain/mg protein. The table shows the binding observed under these conditions and when Na^+ or β,γ-methylene-ATP, or these agents combined were included in the binding system.

Ligand concentration	n	[^{3}H]Ouabain binding (%)
—	4	100.0
15 mM Na^+	4	81.2 ± 2.0
1.0 mM β,γ-methylene-ATP	4	64.3 ± 3.8
Na^+ plus β,γ-methylene-ATP	4	17.0 ± 1.5

This inhibitory action of β,γ-methylene-ATP on the equilibrium level of [^{3}H]-ouabain binding is similar to that previously reported for Na^+ on Mg^{2+} and P_i supported [^{3}H]ouabain binding, where Na^+ also acts to reduce the equilibrium level [^{3}H]ouabain binding [4]. In view of this finding and the recent observations of Hegyvary and Post [13] that Na^+ and ATP appear to bind to the same conformation of the $(Na^+ + K^+)$-ATPase, it seemed possible that Na^+ and ATP might cooperate to inhibit [^{3}H]ouabain binding. Table I shows the results of an experiment where this possibility was investigated. In this study 15 mM Na^+ alone produced a 19% inhibition of [^{3}H]ouabain binding, and 1 mM β,γ-methylene-ATP produced a 36% inhibition of [^{3}H]ouabain binding. A simple additive action of these agents would be expected to produce a 55% inhibition of binding. Together they produced an 83% inhibition of [^{3}H]ouabain binding, which suggests a synergistic inhibitory action of Na^+ and β,γ-methylene-ATP on [^{3}H]ouabain binding to this enzyme.

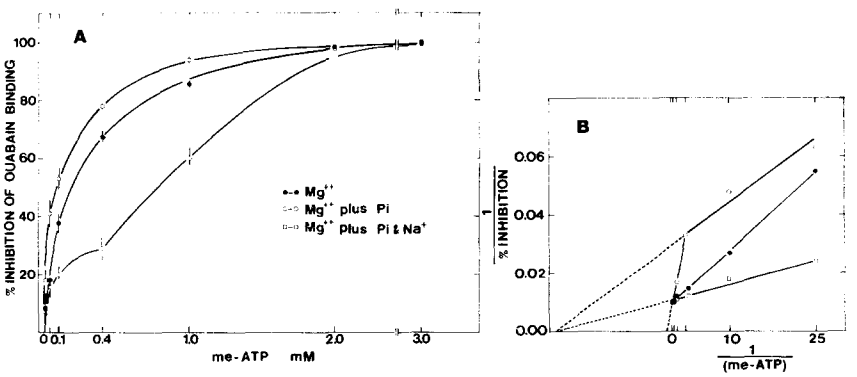

Fig. 2. Effects of β,γ-methylene-ATP on [^{3}H]ouabain binding to $(Na^+ + K^+)$-ATPase in the presence of Mg^{2+}, Mg^{2+} and P_i and Mg^{2+}, P_i and Na^+. (A) Guinea pig kidney enzymes were allowed to equilibrate with $5 \cdot 10^{-7}$ M [^{3}H]ouabain in the presence of the indicated ligands for 10 min at 37 °C. (●–●) shows inhibition by β,γ-methylene-ATP of [^{3}H]ouabain binding occurring in the presence of 2 mM Mg^{2+}. (○–○) shows inhibition by the indicated concentrations of β,γ-methylene-ATP in the presence of 2 mM Mg^{2+} and 1 mM P_i. (□–□) shows inhibition by β,γ-methylene-ATP of [^{3}H]ouabain binding occurring in the presence of 2 mM Mg^{2+}, 1 mM P_i and 15 mM Na^+. (B) shows a double reciprocal transformation of this data.

Fig. 2 shows a more detailed study of this interaction. If the enzyme system was allowed to equilibrate with [^{3}H]ouabain in the presence of Mg^{2+} alone, β,γ-methylene-ATP readily inhibited [^{3}H]ouabain binding, with an apparent K_i of about $2 \cdot 10^{-4}$ M. When P_i was added to the system the interaction became more complex. The β,γ-methylene-ATP inhibition "curve" was shifted to the left and the apparent affinity of the system for β,γ-methylene-ATP was reduced to about $8 \cdot 10^{-4}$ M. However, if 15 mM Na^+ was added to the system the action of P_i was completely antagonized and β,γ-methylene-ATP again readily displaced [^{3}H]ouabain. The data show an antagonism between P_i and β,γ-methylene-ATP on this enzyme system and they suggest that the presence of Na^+ increased the apparent affinity of the enzyme for β,γ-methylene-ATP.

The unusual shape of the β,γ-methylene-ATP inhibition curve in the presence of Mg^{2+} and P_i invited further analysis and Fig. 2B shows a double reciprocal analysis of this data. This suggests that the curve is composed of two distinct portions, one of which is hyperbolic and the other sigmoidal. The hyperbolic portion appears to have the same apparent affinity for β,γ-methylene-ATP as the system has in the presence of Na^+, i.e., a portion of the enzyme may be resistant to P_i. On the other hand, the bulk of the enzyme appears to interact with P_i and if the hyperbolic portion of the curve is deducted the remaining inhibition curve is sigmoidal. This sigmoidicity suggests that there is more than one nucleotide binding site on each functional unit of the enzyme. Thus, β,γ-methylene-ATP appears to cooperatively inhibit Mg^{2+} and P_i dependent [^{3}H]ouabain binding and though 15 mM Na^+ has minimal effects on the level of Mg^{2+} and P_i dependent [^{3}H]ouabain binding it greatly increased the apparent

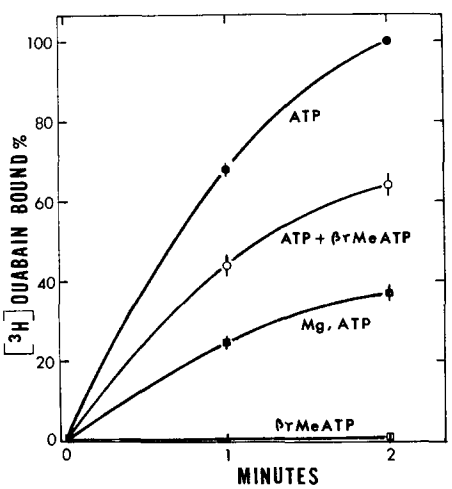

Fig. 3. Nucleotide dependent [^{3}H]ouabain binding in the presence of ATP and β,γ-methylene-ATP. Rat brain enzymes were incubated with $5 \cdot 10^{-8}$ M [^{3}H]ouabain at 37 °C. At indicated zero time the following nucleotide and cation combinations were added and the binding reaction stopped at the indicated times. (●–●) 200 mM Na^+, 3 mM Mg^{2+}, and 0.1 mM ATP; (□–□) 200 mM Na^+, 3 mM Mg^{2+} and 3 mM β,γ-methylene-ATP; (■–■) 3 mM Mg^{2+} and 3 mM ATP; (○–○) 200 mM Na^+, 3 mM Mg^{2+}, 0.1 mM ATP and 3 mM β,γ-methylene-ATP. Binding is expressed as a percentage of that in the presence of 200 mM Na^+, 3 mM Mg^{2+}, and 0.1 mM ATP at 2 min which averaged 101 pmoles [^{3}H]ouabain/mg protein.

affinity of the system for β,γ-methylene-ATP and eliminated the cooperativity of this inhibition.

The data presented above suggest that the actions of Na^+ and β,γ-methylene-ATP together are to inhibit [^{3}H]ouabain binding, even in the presence of Mg^{2+}. Since this observation contrasts with suggestions concerning the mechanism of Na^+, Mg^{2+} and ATP dependent [^{3}H]ouabain binding [3, 5, 9] a series of experiments were conducted in which the actions of ATP and different analogues of ATP on [^{3}H]-ouabain binding in the presence of Na^+ were directly compared. Fig. 3 shows a typical experiment which compares the initial rates of [^{3}H]ouabain binding occurring to rat brain ($Na^+ + K^+$)-ATPase in the presence of 200 mM Na^+, 3 mM Mg^{2+}, $5 \cdot 10^{-8}$ M [^{3}H]ouabain and either 0.1 mM ATP, 3 mM β,γ-methylene-ATP or these substrates combined. In the presence of 0.1 mM ATP a relatively rapid initial rate of [^{3}H]ouabain binding was observed [8]. However, if β,γ-methylene-ATP was substituted for the ATP no binding was observed over the time period of the experiment. In other studies, similar results were observed if the concentration of Na^+ in the system was reduced to 20 mM. However, if β,γ-methylene-ATP was added to the system in the presence of 0.1 mM ATP, the initial rate of [^{3}H]ouabain binding was reduced. The experiment shows that β,γ-methylene-ATP binds to this enzyme system in the presence of Na^+ and Mg^{2+} but that this interaction was not sufficient to stimulate [^{3}H]ouabain binding under these conditions.

Though Fig. 3 shows that β,γ-methylene-ATP is not able to stimulate [^{3}H]-ouabain binding it fails to demonstrate any possible inhibitory actions of β,γ-methylene-ATP on [^{3}H]ouabain binding to this enzyme. Fig. 4 shows a direct comparison

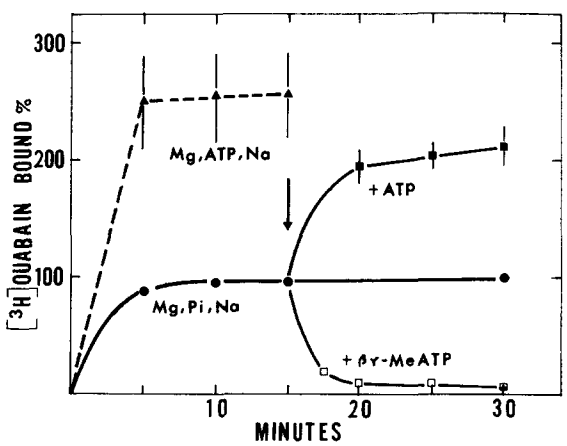

Fig. 4. Inhibition of the equilibrium level of [^{3}H]ouabain binding by β,γ-methylene-ATP. Guinea pig kidney enzymes were incubated with $5 \cdot 10^{-7}$ M [^{3}H]ouabain at 37 °C. The binding reaction was started by the addition of 4 mM Mg^{2+} at zero time and stopped at the indicated times. (●–●) shows binding in the presence of 4 mM Mg^{2+}, 1 mM P_i and 60 mM Na^+. After 15 min. binding in the presence of Na^+, Mg^{2+} and P_i was challenged by the addition of 3 mM ATP, (■–■), or 3 mM β,γ-methylene-ATP, (□–□). (▲–▲) shows [^{3}H]ouabain binding in the presence of 60 mM Na^+, 3 mM Mg^{2+} and 5 mM ATP and (△) shows binding in the presence of 4 mM Mg^{2+}, 1 mM P_i and $2.5 \cdot 10^{-4}$ M unlabeled ouabain. Binding in the presence of Na^+, Mg^{2+} and P_i at 30 min was arbitrarily taken as 100 %. Maximal binding (4 mM Mg^{2+}, 1 mM P_i without Na^+) averaged 94.2 pmoles [^{3}H]ouabain/mg protein.

of the actions of ATP and β,γ-methylene-ATP in an experiment designed to allow the demonstration of either stimulation or inhibition of [^{3}H]ouabain binding by these agents in the presence of Na^+. In Fig. 4 guinea pig kidney enzymes were allowed to equilibrate with [^{3}H]ouabain in the presence of 4 mM Mg^{2+}, 1 mM P_i and 60 mM Na^+. Under these conditions binding equilibrated at about 40% of the level in the presence of Na^+, Mg^{2+} and ATP. As shown in Fig. 4 the addition of ATP produced a prompt increase in the equilibrium level of [^{3}H]ouabain binding whereas β,γ-methylene-ATP produced equally prompt and essentially complete inhibition of [^{3}H]ouabain binding. This experiment supports the hypothesis that an unhydrolyzed nucleotide molecule on the nucleotide binding site is inhibitory to [^{3}H]ouabain binding, even in the presence of Na^+ and Mg^{2+} [21].

In rate dialysis experiments on ($Na^+ + K^+$)-ATPase, β,γ-methylene-ATP has only about one hundredth of the apparent affinity of ATP for this enzyme system [13]. Thus, it might be argued that ATP produces its stimulatory effect by binding at a high affinity site. This site would be unavailable to β,γ-methylene-ATP, whose inhibitory actions could be due to binding at other lower affinity sites [13]. Therefore, the ability of CTP and UTP to stimulate [^{3}H]ouabain binding to guinea pig kidney enzymes under the conditions of Fig. 4 was examined. Fig. 5 shows that these nucleotides are as effective as ATP in stimulating [^{3}H]ouabain binding, despite the fact that their apparent affinities for the ($Na^+ + K^+$)-ATPase are between ten and one hundred times less than that of β,γ-methylene-ATP for ($Na^+ + K^+$)-ATPase [13]. Thus, it appears unlikely that the sharp contrast between the actions of ATP and β,γ-methylene-ATP observed in Fig. 4 is related to the lower affinity of β,γ-methylene-ATP for this enzyme system.

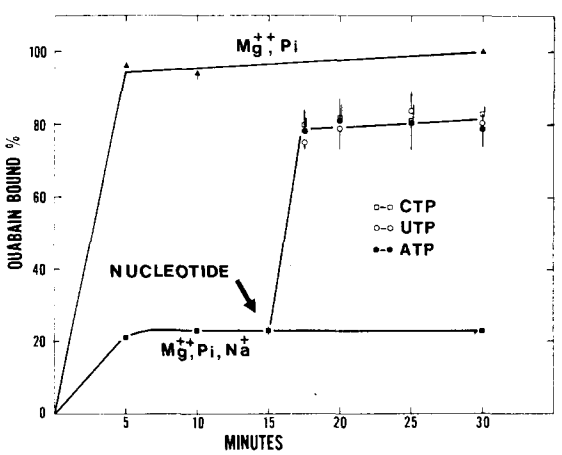

Fig. 5. Effects of CTP and UTP on Na^+, Mg^{2+} and P_i dependent [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase. (▲–▲) shows [^{3}H]ouabain binding to guinea pig kidney ($Na^+ + K^+$) ATPase in the presence of 4 mM Mg^{2+} and 1 mM P_i at 37 °C, with binding at 30 min taken as 100 %. (■–■) shows [^{3}H]ouabain binding occurring in the presence of 4 mM Mg^{2+}, 1 mM P_i and 60 mM Na^+. At 15 min binding under these conditions was challenged by the addition of 3 mM each of ATP, (●–●) CTP, (□–□) or UTP (○–○). The symbols show the amount of [^{3}H]ouabain binding observed at each time point with the indicated nucleotide. Binding is expressed as a percentage of that observed in the presence of 4 mM Mg^{2+} and 1 mM P_i at 30 min, which averaged 260.35 ± 27.74 pmoles [^{3}H]ouabain/mg protein.

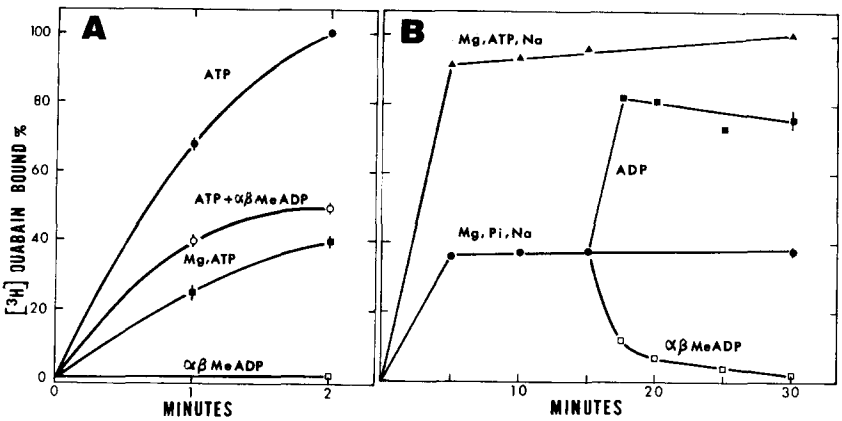

Fig. 6. Effects of α,β-methylene-ADP on [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase. (A) The experimental conditions are as in Fig. 3 except that α,β-methylene-ADP is substituted for β,γ-methylene-ATP. The open square (□) shows binding in the presence of 200 mM Na^+, 3 mM Mg^{2+} and 3 mM α,β-methylene-ADP. (■–■) shows binding in the presence of 3 mM Mg^{2+} and 3 mM ATP. (○–○) shows binding in the presence of 200 mM Na^+, 3 mM Mg^{2+}, 0.1 mM ATP and 3 mM α,β-methylene-ADP. (●–●) shows binding in the presence of 200 mM Na^+, 3 mM Mg^{2+} and 0.1 mM ATP. Binding is expressed as a percentage of that observed in the presence of Na^+, Mg^{2+} and ATP at 2 min, which averaged 158.12±6.28 pmoles [^{3}H]ouabain/mg protein. (B) The experimental conditions are as in Fig. 4. (●–●) shows ouabain binding to guinea pig kidney ($Na^+ + K^+$)-ATPase in the presence of 60 mM Na^+, 4 mM Mg^{2+} and 1 mM P_i. At 15 min [^{3}H]ouabain binding was challenged by the addition of 3 mM α,β-methylene-ADP, (□–□), or 3 mM ADP, (■–■). (▲–▲) shows [^{3}H]ouabain binding in the presence of 60 mM Na^+, 3 mM Mg^{2+} and 3 mM ATP with ouabain binding expressed as a percentage of that observed under these conditions at 30 min which averaged 312±7 pmoles [^{3}H]ouabain/mg protein. All points are single experimental points except those at 30 min, which are the means±S.E. of four separate experimental determinations.

If these experiments were repeated using other nucleotide analogues, similar results were obtained. Figs 6A and 6B show that essentially similar results were obtained with α,β-methylene-ADP. Figs 7A and 7B show the actions of β,γ-imido-ATP. While β,γ-imido-ATP produced produced a small initial rate of [^{3}H]ouabain binding to rat brain enzymes in the presence of Na^+ and Mg^{2+} this rate was only about 2.5% of the initial rate observed with ATP and its overall effect on [^{3}H]ouabain binding to guinea pig kidney enzymes was inhibitory.

These data suggest that Na^+ stimulated binding of [^{3}H]ouabain may require the formation of phosphoenzyme for binding to occur. If this is the case, then factors which tend to stabilize the phosphoenzyme should favor the binding of [^{3}H]ouabain. The next series of experiments examined this possibility. Because formation of the phosphoenzyme is usually complete or at "maximal" steady state levels in the absence of K^+ [15], it is not possible to significantly increase the steady state levels of phosphoenzyme above those which would be observed in the absence of a stabilizer. Therefore, the approach taken was to use limiting amounts of substrate, under which conditions the incorporation of ^{32}P into this enzyme appears as a rapid transient pulse of ^{32}P incorporation [24]. Under these conditions, agents which stabilize the phosphorylated form of the enzyme should act to increase the duration of a [^{3}H]ouabain binding pulse if the hypothesis that ouabain binding occurs to the phosphoenzyme is correct.

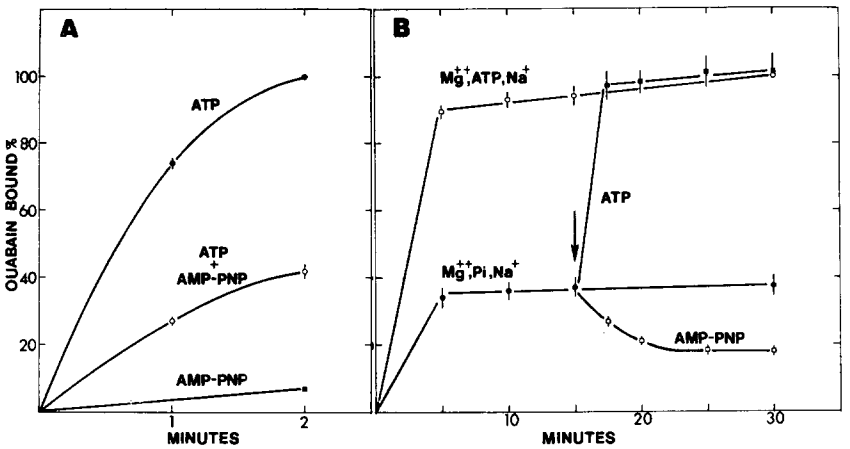

Fig. 7. Effects of β, γ-imido-ATP (AMP-PNP) on [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase. (A) The experimental conditions are as in Fig. 3 except that β,γ-imido-ATP was substituted for β,γ-methylene-ATP. (■) shows binding in the presence of 200 mM Na^+, 3 mM Mg^{2+} and 2 mM β,γ-imido-ATP. (○–○) shows binding in the presence of 200 mM Na^+, 3 mM Mg^{2+}, 0.1 mM ATP and 2 mM β,γ-imido-ATP. (●–●) shows binding in the presence of 200 mM Na^+, 3 mM Mg^{2+} and 0.1 mM ATP. Binding is expressed as a percentage of that observed in the presence of Na^+, Mg^{2+} and ATP at 2 min, which averaged 226.5 ± 7.7 pmoles [^{3}H]ouabain /mgprotein. (B) The experimental conditions are as in Fig. 4. (●–●) shows ouabain binding to guinea pig kidney ($Na^+ + K^+$)-ATPase in the presence of 60 mM Na^+, 4 mM Mg^{2+} and 1 mM P_i. At 15 min [^{3}H]ouabain binding was challenged by the addition of 2 mM β,γ-imido-ATP, (□–□), or 2 mM ATP, (■–■). (○–○) shows [^{3}H]ouabain binding in the presence of 60 mM Na^+, 3 mM Mg^{2+} and 2 mM ATP with ouabain binding expressed as a percentage of that observed under these conditions at 30 min 273.69 ± 8.27 pmoles [^{3}H]ouabain mg/ protein. All points are the means ± S.E. of four separate experimental determinations.

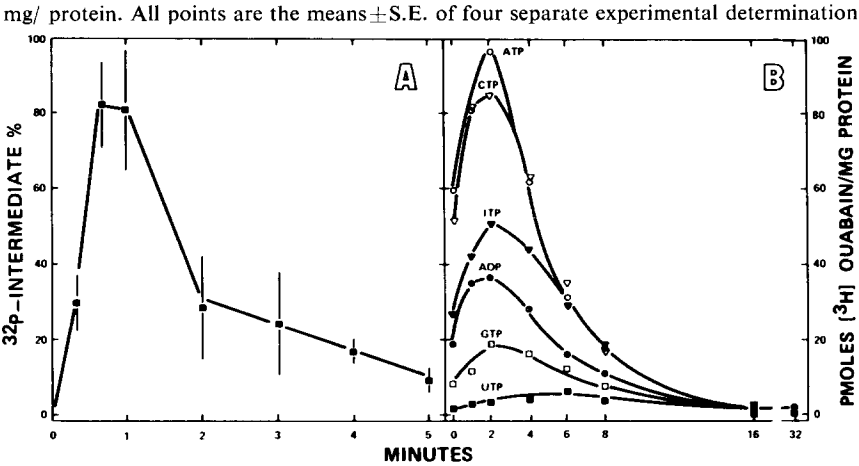

Fig. 8. Time courses of [γ-^{32}P]ATP dependent labeling and nucleotide supported [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase. (A) Guinea pig kidney enzymes were incubated with 100 mM Na^+ and 5 mM Mg^{2+} at 37 °C. At indicated zero time 25 μM (γ-^{32}P)ATP was added to start the labeling reaction. The reaction was stopped with trichloroacetic acid (■–■) shows the amount of ^{32}P-labeling of the enzyme at the indicated time points. Labeling is expressed as a percentage of peak labeling for each of four different experiments which averaged 122 pmoles ^{32}P/mg protein. (B) At indicated zero time the binding reaction was started by the addition of 50 μM of each of the indicated nucleotides and stopped by centrifugation at the indicated time points. The symbols show the binding of [^{3}H]ouabain supported by each of the indicated nucleotides with no deductions made for background labeling.

Fig. 8 shows the relationship between the time course of phosphorylation of this enzyme from [γ-^{32}P]ATP and [^{3}H]ouabain binding supported by small amounts of different nucleotides at 37 °C. The time course of labeling from [γ-^{32}P]ATP is transient, peaking at about one minute and declining rapidly thereafter. Similarly, [^{3}H]ouabain binding follows a transient time course, peaking at about two minutes and then declining at about the normal rate of dissociation of ouabain from guinea pig kidney ($Na^+ + K^+$)-ATPase [4, 25]. The parallelism between these two patterns is consistent with the hypothesis that [^{3}H]ouabain binding occurs to the E–P form of the ATPase; the slightly delayed peaking of the [^{3}H]ouabain binding curve being consistent with the [^{3}H]ouabain present in the binding system prolonging hydrolysis of the nucleotide supporting [^{3}H]ouabain binding.

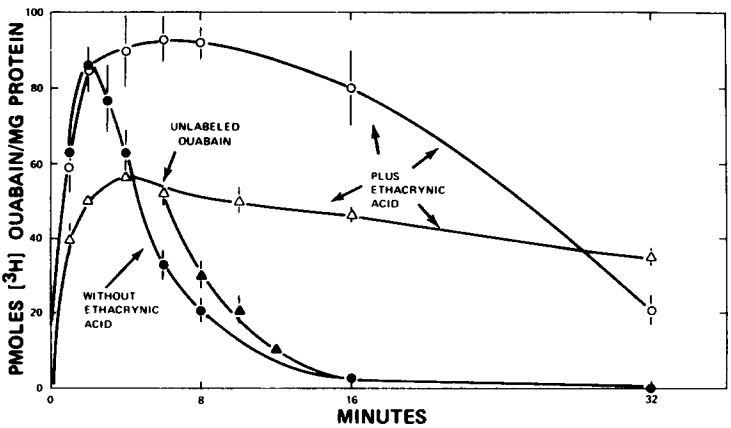

Fig. 9. Effects of ethacrynic acid on an ATP supported pulse of [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase. Guinea pig kidney ($Na^+ + K^+$)-ATPase was incubated with 100 mM Na^+, 5 mM Mg^{2+} and $2.5 \cdot 10^{-7}$ M [^{3}H]ouabain at 37 °C. At indicated zero time the [^{3}H]ouabain binding reaction was started by the addition of ATP. (●–●) shows the time course of a pulse of [^{3}H]ouabain binding supported by 30 μM ATP. (○–○) shows the time course when 1 mM ethacrynic acid was added just prior to the ATP; (△–△) shows the time course of [^{3}H]ouabain binding when the enzyme was incubated with 1 mM ethacrynic acid for 10 min before the addition of ATP; (▲–▲) show the time course of [^{3}H]ouabain binding when $2.5 \cdot 10^{-4}$ M unlabeled ouabain was added 6 min after the ATP.

Fig. 9 shows the effects of ethacrynic acid on such transient pulses of [^{3}H]-ouabain binding to the ($Na^+ + K^+$)-ATPase. The solid circles show the time course of a pulse of [^{3}H]ouabain binding supported by ATP at 37 °C. The open circles show the same experiment repeated in the presence of 1.0 mM ethacrynic acid. The presence of ethacrynic acid in the system produced a marked prolongation of the pulse of [^{3}H]ouabain binding, increasing the area under the curve more than four-fold. If the enzyme was exposed to ethacrynic acid for 10 min at 37 °C before the binding reaction was started, the binding curve was similarly prolonged though the maximum height of the binding curve was markedly reduced. The experiments show that ethacrynic acid can markedly potentiate the ability of ATP to support transient pulses of [^{3}H]-ouabain binding to guinea pig kidney ($Na^+ + K^+$)-ATPase.

To eliminate the possibility that ethacrynic acid prolonged these transient pulses of [^{3}H]ouabain binding by directly stabilizing the enzyme–ouabain complex, the spontaneous dissociation of bound [^{3}H]ouabain was exposed by adding unlabeled ouabain. The data show that [^{3}H]ouabain bound to ethacrynic acid inhibited enzymes dissociates at the about normal rate for the guinea pig kidney ($Na^+ + K^+$)-ATPase–ouabain complex [4, 25]. This observation suggests that ethacrynic acid promotes [^{3}H]ouabain binding by stabilizing the ouabain binding conformation of the enzyme rather than by interfering with the dissociation of the bound glycoside.

Because ADP also supports [^{3}H]ouabain binding to the ($Na^+ + K^+$)-ATPase by a mechanism apparently similar to that of ATP [8], the effects of ethacrynic acid on an ADP supported pulse of [^{3}H]ouabain binding were examined. Fig. 10 shows that ethacrynic acid also markedly potentiates the ability of ADP to support a tranient pulse of [^{3}H]ouabain binding to the guinea pig kidney ($Na^+ + K^+$)-ATPase. In this study the concentration of [^{3}H]ouabain in the binding system was reduced to show that ethacrynic acid could increase both the peak height and duration of a nucleotide supported pulse of [^{3}H]ouabain binding.

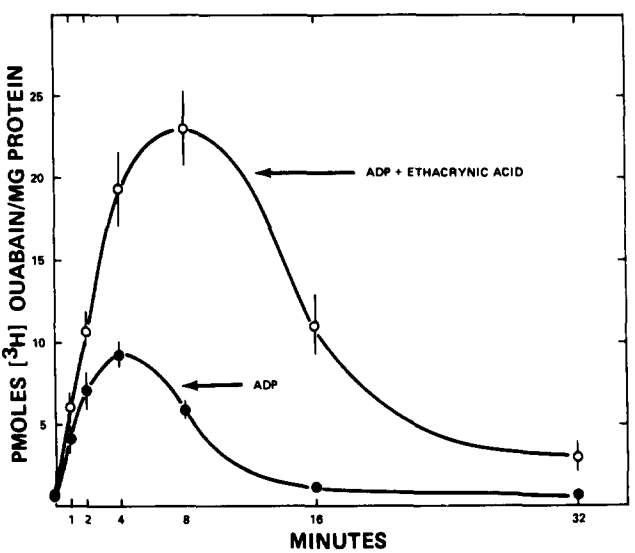

Fig. 10. Effect of ethacrynic acid on an ADP supported pulse of [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase. Guinea pig kidney enzymes were incubated with 100 mM Na^+, 5 mM Mg^{2+} and $1.2 \cdot 10^{-7}$ M [^{3}H]ouabain at 37 °C. At indicated zero time the binding reaction was started by the addition of 25 μM ADP. (●–●) shows binding in the presence of ADP; (○–○) binding when the reaction was started with 25 μM ADP and 1 mM ethacrynic acid.

DISCUSSION

The results obtained with the methylene analogues of ATP and ADP show that these analogues inhibited [^{3}H]ouabain binding to the ($Na^+ + K^+$)-ATPase under all conditions tested. These conditions include the presence of Mg^{2+}, Mg^{2+} and P_i, or in the presence of Na^+, Mg^{2+} and P_i. The inhibition of [^{3}H]ouabain binding by β,γ-methylene-ATP was antagonized by P_i but markedly potentiated by Na^+, Na^+

acting to greatly increase the apparent affinity of the system for β,γ-methylene-ATP. These results suggest that the primary action of β,γ-methylene-ATP is to stabilize a configuration of this enzyme with a relatively high affinity for Na^+ and a negligible affinity for [^{3}H]ouabain. The marked contrast between the actions of ATP and β,γ-methylene-ATP on [^{3}H]ouabain binding in the presence of Na^+, Mg^{2+} and P_i (Fig. 4) suggests a fundamental difference between the actions of ATP and β,γ-methylene-ATP on this enzyme system and it is tempting to conclude that these differences are due to the inability of the methylene analogues of ATP and ADP to give rise to the phospho-form of the ($Na^+ + K^+$)-ATPase.

The validity of this conclusion depends on a number of factors. One possibility is that β,γ-methylene-ATP does not substitute for ATP in the formation of the (Na–Mg–E–ATP) complex which some [3, 5, 9, 26] consider to be the primary complex involved in Na^+ stimulated [^{3}H]ouabain binding. There is, however, little evidence to support this view. Fig. 2, showing that Na^+ markedly alters the apparent affinity of this enzyme for β,γ-methylene-ATP in the presence of Mg^{2+}, P_i and [^{3}H]ouabain indicates that under these conditions the enzyme retains its affinity for Na^+. Similarly, Figs 3 and 4 show that the enzyme also retains its affinity for β,γ-methylene-ATP. Though the possibility remains that Mg^{2+} does not bind to the (Na–E–β,γ-methylene-ATP) complex [23], this appears unlikely since the affinity constant of Mg^{2+} for β,γ-methylene-ATP is about twice that of ATP for Mg^{2+} and extremely low levels of Mg^{2+} are sufficient to catalyze the [^{3}H]ouabain binding reaction [4, 27]. Thus, from the data presented here it appears unlikely that the inability of β,γ-methylene-ATP to support [^{3}H]ouabain binding is due to the inability of this nucleotide analogue to form the (Na–Mg–E–β,γ-methylene-ATP) complex.

Another possible problem is that the apparent affinity of these nucleotide analogues for the ($Na^+ + K^+$)-ATPase is apparently less than that of ATP for this enzyme system [13] in the absence of Mg^{2+}. Although this observation might explain the inability of these analogues to stimulate [^{3}H]ouabain binding in the rat brain enzyme preparations they would not serve to explain the inhibitory actions of these analogues on [^{3}H]ouabain binding to the guinea pig kidney preparations where nucleotides of lower apparent affinity than β,γ-methylene-ATP are stimulatory (Fig. 6). β,γ-Methylene-ATP has a higher apparent affinity for this enzyme than any of the other nucleotide substrates of this enzyme, which substrates are just as effective as ATP in stimulating [^{3}H]ouabain binding. Therefore, it seems unlikely that the observations reported here are atypical and due to the small changes in pK_a values, bond angles and distances introduced by the substitution of the methylene group into the β–γ bond of the ATP molecule, though this possibility cannot be ruled out.

To confirm the results obtained with β,γ-methylene-ATP, the actions of β,γ-imido-ATP on [^{3}H]ouabain binding to this enzyme were also studied. β,γ-Imido-ATP (adenylimido-diphosphate) is an analog of ATP in which an NH group replaces the terminal bridge oxygen of the ATP molecule [12]. β,γ-Imido-ATP appears to be a better analogue of ATP than β,γ-methylene-ATP, at least under some circumstances [28]. Because the pK_a of the terminal phosphate group of β,γ-imido-ATP is closer to that of ATP than β,γ-methylene-ATP, β,γ-imido-ATP is likely to carry a net charge more similar to ATP than β,γ-methylene-ATP at physiological pH. Also, the P–N–P bond angles is much closer to the P–O–P bond angle than the P–C–P bond angle. These differences are presumably reflected in the fact that β,γ-imido-ATP is the most

potent inhibitor known of heavy meromyosin ATPase and readily dissociates Mg^{2+} actomyosin, whereas β,γ-methylene-ATP is not effective in either of these actions. These observations suggest that β,γ-imido-ATP is a better analogue of ATP than β,γ-methylene-ATP [12, 28].

Despite these observations, Fig. 7 shows that the data obtained with β,γ-imido-ATP were not nearly as clearcut as the data obtained with β,γ-methylene-ATP. In the presence of Na^+ and Mg^{2+}, β,γ-imido-ATP produced a small initial rate of [^{3}H]-ouabain binding, about 2.5% of the initial rate observed in the presence of ATP. However, despite this small stimulation of [^{3}H]ouabain binding by β,γ-imido-ATP, it was a more potent inhibitor of Na^+, Mg^{2+} and ATP supported [^{3}H]ouabain binding than β,γ-methylene-ATP. Similarly, when [^{3}H]ouabain binding in the presence of Na^+, Mg^{2+} and P_i was challenged with β,γ-imido-ATP, inhibition of [^{3}H]ouabain was observed, though the inhibition was not as marked as that occurring in the presence of β,γ-methylene-ATP. The reason for this relative lack of inhibitory effectiveness of β,γ-imido-ATP is not clear. One possibility was that partial hydrolysis of the β,γ-imido-ATP occurred on the resin columns used to remove the lithium from lithium β,γ-imido-ATP, as suggested by Barnett (personal communication). However, when the Na^+ salt of β,γ-imido-ATP was obtained and tested, results indistinguishable from those of Fig. 7 were obtained.

The results obtained with the nucleotide analogs contrast markedly with the results obtained in the presence of ethacrynic acid. The primary action of ethacrynic acid on this enzyme is to stabilize the E_2–P form. Thus, if the E_2–P form is the ouabain binding configuration of the $(Na^+ + K^+)$-ATPase one might expect ethacrynic acid to prolong the duration of a transient pulse of nucleotide supported [^{3}H]ouabain binding. Fig. 9 shows such an experiment. In this experiment the presence of ethacrynic acid markedly prolonged an ATP supported [^{3}H]ouabain binding pulse, increasing the area under the curve at least four-fold. Ethacrynic acid produced this effect without affecting the rates at which [^{3}H]ouabain either bound to or dissociated from the enzyme and thus apparently acts by stabilizing the configuration of the enzyme to which [^{3}H]ouabain binds.

These observations, however, leave open the mechanism by which ethacrynic acid actually stabilizes the ouabain binding configuration. Though Banerjee et al. [11] have shown that the action of ethacrynic acid is to stabilize the ouabain binding, E_2–P form, of the $(Na^+ + K^+)$-ATPase, it could also be argued that ethacrynic acid acted simply by slowing the hydrolysis of the nucleotide substrates. In this way the prolonged binding pulse would be due simply to prolonged binding of the nucleotide to the enzyme. However, the results obtained with the nucleotide analogs suggest that the simple binding of nucleotides to $(Na^+ + K^+)$-ATPase is unlikely to support [^{3}H]ouabain binding to any significant extent.

The hypothesis that nucleotide-dependent, sodium-stimulated pathway of [^{3}H]ouabain binding requires phosphorylation of the enzyme is well supported by recent observations on the mechanism of $(Na^+ + K^+)$-ATPase. Nucleotide triphosphates other than ATP have recently been shown to phosphorylate this enzyme [8, 10, 24] weakening interpretations which were based on the assumption that only ATP can give rise to significant levels of phosphoenzyme. Recent work by Hegyvary and Post [13], which suggests that Na^+ and ATP bind to the same conformation of the enzyme, also supports these observations, for Na^+ has been demonstrated to

cooperatively stabilize a non-ouabain-binding form of this enzyme [4]. Similarly, experiments by Hansen et al. [21] and Tobin et al. [8] have shown that ADP binds to this enzyme in the presence of Na^+ [21] or Na^+ and Mg^{2+} [8] and inhibits binding due to ATP, supporting suggestions that a nucleotide molecule on the substrate-binding site renders the enzyme inaccessible to [^{3}H]ouabain [21]. In support of the conclusion that Na^+ stimulated binding of [^{3}H]ouabain requires formation of phosphoenzyme it may be appropriate at this time to review the parallelisms between the conditions for Na^+ stimulated [^{3}H]ouabain binding and the Na^+ stimulated formation of phosphoenzyme.

1. Both [γ-^{32}P]ATP dependent labeling and [^{3}H]ouabain binding are transient, being lost when the ATP is consumed (Fig. 8).
2. Na^+ is the only alkali metal cation which stimulates both events [29, 30].
3. Both events require Mg^{2+} and are inhibited by high concentrations of EDTA [4, 27].
4. The nucleotide specificity of both events is low [8].
5. Ethacrynic acid both stablizes the phosphoenzyme [11] and potentiates the ability of nucleotides to support [^{3}H]ouabain binding (Figs 9 and 10).
6. Nucleotide analogues apparently unable to phosphorylate this enzyme are essentially unable to stimulate [^{3}H]ouabain binding (Figs 1–7).
7. ADP inhibits [γ-^{32}P]ATP dependent phosphorylation [31] and [^{3}H]ouabain binding to this enzyme [8, 21].
8. K^+ ion reduces the amount of both E–^{32}P [30] and [^{3}H]ouabain binding [4].
9. Ca^{2+} protects both E–P and [^{3}H]ouabain binding against the actions of K^+ [32].

If phosphorylation of this enzyme from ATP is required for the Na^+ stimulated pathway of [^{3}H]ouabain binding then transphosphorylation alone is presumably not sufficient to stimulate [^{3}H]ouabain binding. Phosphorylation of the enzyme must activate the transport machinery and start the enzyme into its transport cycle. This requirement for a phosphorylation dependent configurational change in the enzyme system gives these observations their mechanistic significance. Together Na^+ and ATP act to direct the enzyme to the ATP dependent, Na^+ stimulated pathway of [^{3}H]ouabain binding. In vitro, therefore, [^{3}H]ouabain binding in the presence of high concentrations of Na^+ and ATP is an indication that the chemical events of transphosphorylation and at least a partial configuration change towards E_2–P have occurred. The identification of these steps in the Na^+ stimulated pattern of [^{3}H]-ouabain binding and their probable relationship to the transport cycle of this enzyme should aid considerably in the design and interpretation of future experiments.

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