MOLECULAR PHARMACOLOGY, 8, 256–263

SHORT COMMUNICATION

Nucleotide Specificity of the Na^+-Stimulated Phosphorylation and [^{3}H]Ouabain-Binding Reactions of (Na^+ + K^+)-Dependent Adenosine Triphosphatase

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(Received December 9, 1971)

SUMMARY

ATP, CTP, and ITP supported a rapid initial rate of [^{3}H]ouabain binding to rat brain (Na^+ + K^+)-ATPase, up to 4 times that supported by ADP. These nucleotides also prevented the rapid initial incorporation of the [γ-^{32}P]phosphate of [γ-^{32}P]ATP into the enzyme. [γ-^{32}P]Cytidine triphosphate labeled the enzyme to the same extent as [γ-^{32}P]ATP, suggesting that nucleotides other than ATP phosphorylate this enzyme. In support of this, the phosphorylation from [γ-^{32}P]CTP required Mg^{2+}, was stimulated by Na^+, and was not observed in the presence of K^+, and the label incorporated into the enzyme from [γ-^{32}P]CTP turned over at the same rate as that from [γ-^{32}P]ATP. In other experiments the initial rates of hydrolysis of ATP, ITP, UTP, and ADP in the absence of added K^+ matched the initial rates of [^{3}H]ouabain binding supported by these substrates. The results suggest that these substrates give rise to sufficient phosphoenzyme to account for the initial rates of [^{3}H]ouabain binding supported by them. Concentrations of ADP sufficient to inhibit the phosphorylation of this enzyme by ATP also inhibited the initial rate of the ATP-supported binding of [^{3}H]ouabain, suggesting that ADP can bind to this enzyme without stimulating [^{3}H]ouabain binding.

Many of the arguments which have been developed concerning the effects of nucleotides on the (Na^+ + K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) and the relationships among the partial reaction sequences of this enzyme system hinge on the assumption that only ATP can give rise to significant amounts of phosphoenzyme (1).

This work was supported by a grant from the Michigan Heart Association, Grant M. H. 12783-05 from the National Institute of Mental Health, and General Research Support Grant NIH RR 05623-04 to the College of Veterinary Medicine, Michigan State University, from the National Institutes of Health.

In particular, the role of the phosphorylated intermediate of the (Na^+ + K^+)-ATPase in the Na^+-stimulated binding of [^{3}H]ouabain to this enzyme is unclear. Some authors have suggested that the sodium-stimulated binding of ouabain occurs after the sodium-stimulated phosphorylation of the enzyme (2–4). Other workers have proposed that binding of a nucleotide to this enzyme is sufficient to stimulate [^{2}H]ouabain binding (5–7). Such proposals are based on the apparently low nucleotide specificity of the [^{3}H]ouabain-binding reaction (5–7) as compared with the high specificity of the over-all (Na^+ + K^+)-ATPase reaction for ATP (8, 9), the assump-

tion being that the nucleotide specificity of the phosphorylation step is the same as that of the over-all reaction sequence. However, [^{3}H]ouabain binding experiments are usually performed in the absence of K^+, i.e., conditions under which the rate of hydrolysis of the phosphoenzyme is minimal (3). Thus, because of the slow rate of dephosphorylation under these conditions, relatively poor substrates of this enzyme may give rise to considerable amounts of phosphoenzyme and thus stimulate [^{3}H]ouabain binding. In this communication we present direct evidence that [γ-^{32}P]CTP is able to phosphorylate this enzyme fully, as well as indirect evidence which suggests that other nucleotide triphosphates can also form variable amounts of phosphoenzyme. The release of P_i from these nucleotides in the absence of K^+ was also measured, and compared well with the initial rates of ouabain binding supported by these substrates. The results support suggestions that nucleotides other than ATP can give rise to considerable amounts of phosphoenzyme under appropriate conditions and that the sodium-stimulated mechanism of [^{3}H]ouabain binding is directly related to the formation of phosphoenzyme.

Rat brain ($Na^+ + K^+$)-ATPase (specific activity, 100–250 μmoles of P_i per milligram of protein per hour) was prepared and assayed by the method of Akera *et al.* (10). The hydrolysis of nucleotide substrates in the absence of K^+ was measured by modifying the assay method as follows. The enzyme concentration was increased 4–6-fold to 60–90 μg of protein per milliliter, and the assay period was extended to 30 min. Both these measures increased the yield of P_i in the absence of K^+. The concentration of Na^+ was reduced to 10 mM, since this concentration produces full phosphorylation of the enzyme (11) and allows maximal turnover in the absence of added K^+ (12). Hydrolysis in the absence of Na^+ and the presence of ouabain was deducted as background Mg^{2+}-dependent ATPase (10). [^{3}H] Oubain binding studies were performed by the method of Tobin and Sen (4), with the following modifications. Since only the initial rates of binding were being measured, the ouabain concentration was lowered to 20 nM to give an easily measurable initial rate of binding. Rat brain enzyme was used because the stability of the ouabain-enzyme complex in this species (13) ensures that the measured initial rates of [^{3}H]ouabain binding will be close to the true initial rates. The binding reaction was terminated by adding 3 ml of 2.5 mM unlabeled ouabain in ice-cold 25 mM Tris buffer, pH 7.4. This addition immediately stopped the binding of labeled ouabain and cooled the reaction mixture to about 10°, at which temperature the enzyme-ouabain bond is relatively stable (4). The tubes were then cooled to 0° in an ice bath and centrifuged, and the precipitates were solubilized and counted as previously described (4).

Labeling of the enzyme with [γ-^{32}P]nucleotides was performed by a modification of the method of Post *et al.* (11). [γ-^{32}P]ATP was obtained from New England Nuclear Corporation and diluted with carrier ATP as required. [γ-^{32}P]CTP was obtained from Amersham/Searle and diluted with carrier CTP as required. All labeling experiments were performed at 0° in 15-ml Corex centrifuge tubes. Each tube contained 0.2–0.3 mg of enzyme protein, 50 mM Tris buffer (pH 7.4), 100 mM Na^+, 1 mM $MgCl_2$, and other additions as indicated in a final volume of 1 ml. The labeling reaction was started by the addition of 0.05 μmole of the [γ-^{32}P]nucleotide and stopped at the indicated times by the addition of 5.0 ml of ice-cold 5% trichloracetic acid containing 1 mM unlabeled ATP and P_i. This solution was then filtered through a Millipore filter of 25-mm over-all diameter, pore size 0.8 μ. Each filter was washed four times with 5 ml of the trichloracetic acid–ATP–P_i solution, transferred to a counting vial, dissolved in Bray's scintillation medium, and counted in a Beckman LSE 100 liquid scintillation spectrophotometer. Each experiment was performed at least four times, and values shown are means ± standard errors of at least four experiments. Because different enzyme preparations were used in each experiment, the highest individual group of values in each experiment was designated as 100%, and other values were expressed as a percentage of those values.

Previous workers have shown that various

nucleotide triphosphates and diphosphates will support [^{3}H]ouabain binding by this enzyme (5–7). However, because of the relative stability of the ouabain-enzyme complex (6), the initial rates of ouabain binding might be expected to show the greatest nucleotide-dependent differences, rather than estimations of binding after arbitrary periods of up to 30 min (7). Figure 1 shows that in rat brain enzyme preparations, ATP and ITP produced essentially the same initial rates of [^{3}H]ouabain binding. UTP was somewhat less effective, and ADP produced only about 25% of the initial rate observed

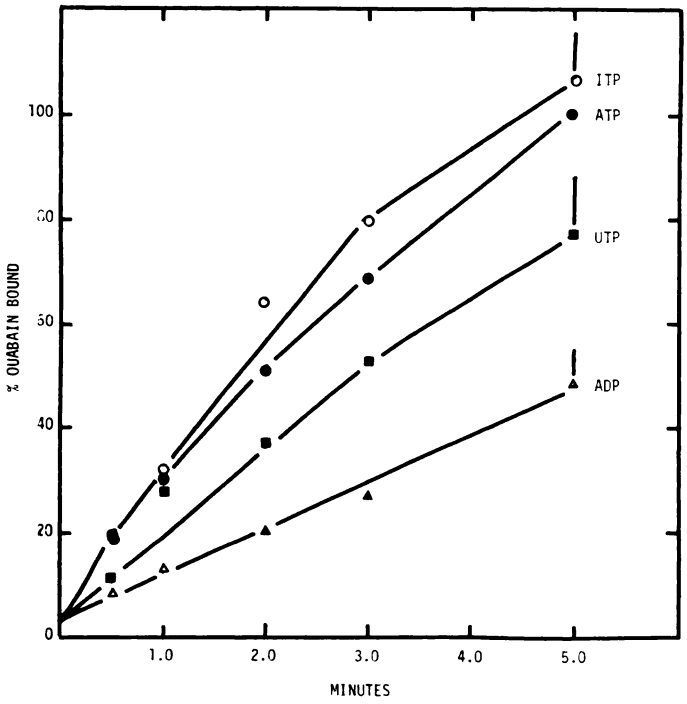

FIG. 1. *Initial rates of [^{3}H]ouabain binding supported by nucleotide substrates*

Rat brain enzyme was incubated with 20 nM [^{3}H]ouabain in the presence of 100 mM Na^+, 5 mM Mg^{2+}, and 50 mM Tris buffer, pH 7.4. At zero time the binding reaction was started by the addition of the indicated nucleotide (2 mM) and stopped at the time points indicated. Binding at each time point is expressed as a percentage of that occurring in the presence of ATP at 5 min (53.9 ± 5.3 pmoles/mg of protein). Each point is the mean ± standard error of four experimental determinations.

TABLE 1

Initial rates of [^{3}H]ouabain binding in the presence of ATP and CTP, as percentages of maximal binding with ATP

Rat brain ($Na^+ + K^+$)-ATPase was incubated with the specified nucleotides (5 mM), and the reaction was stopped as described in the text. Other conditions were as described in Fig. 1, except that the [^{3}H]ouabain concentration was 60 nM. Binding at 5 min in the presence of ATP averaged 75.1 pmoles of [^{3}H]ouabain per milligram of protein. All values are expressed as a percentage of this ± the standard error of the mean of four determinations.

Nucleotide	0 min	1 min	2 min	3 min	4 min	5 min
	%	%	%	%	%	%
ATP	4.4 ± 0.8	29.7 ± 9.1	61.0 ± 2.7	73.2 ± 5.1	91.8 ± 5.3	100
CTP	4.3 ± 0.6	39.5 ± 6.1	59.3 ± 8.0	74.9 ± 13.1	91.7 ± 7.1	105.1 ± 11.4

TABLE 2

Effect of Na^+ on [^3H]ouabain binding supported by different nucleotides, expressed as percentage of binding in the presence of Na^+-ATP

Rat brain ($Na^+ + K^+$)-ATPase was incubated with 20 nM [^{3}H]ouabain and the indicated nucleotides (2 mM nucleotide and 2 mM Mg^{2+}) in the presence and absence of 100 mM Na^+ for 5 min. Binding in the presence of Na^+, Mg^{2+}, and ATP (64.5 ± 9.5 pmoles/mg of protein) was taken as 100%, and other values are expressed as a percentage of this ± the standard error of the mean of four determinations.

Na^+	ATP	ADP	ITP	UTP
	%	%	%	%
+	100	47.0 ± 6.3	118 ± 4.0	75.0 ± 8.0
−	45 ± 4.25	25.0 ± 5.3	57 ± 5.0	40 ± 3.6

TABLE 3

Nucleotide inhibition of phosphorylation by [γ-^{32}P]ATP

Rat brain enzyme was incubated with the indicated nucleotide (0.1 mM) for 40 sec and 0° and then labeled with 0.04 mM [γ-^{32}P]ATP. The labeling reaction was stopped after 2 sec, as indicated in the text. The values indicate the K^+-sensitive incorporation of ^{32}P into the membranes, expressed as a percentage of that occurring with ATP alone (about 250 pmoles of ^{32}P per milligram of protein). Values are the means ± standard errors of four determinations.

Nucleotide	[^{32}P]Intermediate
	%
None	100
CTP	16.0 ± 12
ITP	17.5 ± 7
UTP	36.2 ± 13
ADP	18.8 ± 7.0

with ATP. In other experiments, CTP supported binding at the same initial rate as ATP (Table 1), and the binding supported by these nucleotides was stimulated in each case by Na^+ (Table 2).

These different initial rates could conceivably reflect differing abilities of the nucleotides to phosphorylate the enzyme. However, since most of these nucleotides are not available with labeled terminal phosphate groups, the over-all experimental approach was necessarily indirect. One test for phosphorylation by these nucleotides would be to add the test nucleotide and then challenge with [γ-^{32}P]ATP. A decrement in the initial incoraportion of ^{32}P might be due to the prior formation of phosphoenzyme by the test nucleotide. Table 3 shows that in four such experiments all the nucleotides tested produced substantial inhibition of ^{32}P incorporation from [γ-^{32}P]ATP at 2 sec. However, an equally plausible interpretation of these data might be that simple binding of the unlabeled nucleotides prevented the phosphorylation from [γ-^{32}P]ATP.

The recent availability of [γ^{32}P]CTP allowed us to test this phosphorylation hypothesis directly with two terminally labeled nucleotides, as shown in Table 4. If rat brain enzyme was exposed to [γ-^{32}P]ATP or [γ-^{32}P]CTP under indentical conditions for 5 sec, [γ-^{32}P]CTP gave rise to 82% of the amount of phosphoenzyme that [γ-^{32}P]ATP did, and at 30 sec the amounts of phosphoenzyme were similar. However, if unlabeled ATP was added 5 sec prior to the [γ-^{32}P]CTP, no labeling was observed, nor was any observed at 30 sec if unlabeled ATP was added immediately after the [γ-^{32}P]CTP. These results suggest that while CTP by itself is able to phosphorylate the enzyme, it cannot do so in the presence of ATP. Other experiments (Table 5) showed that this labeling from [γ-^{32}P]CTP has many of the properties of the labeling from [γ-^{32}P]ATP. Formation of the phosphoenzyme required Mg^{2+}, was stimulated by Na^+, and was not observed in the presence of K^+ or of Na^+ plus K^+. If ouabain was allowed to bind to the enzyme prior to the addition of Na^+ and CTP, the Na^+-stimulated labeling was not observed. Once formed, the phosphate incorporated from CTP turned over at the same rate as that from ATP (Fig. 2), suggesting that the same phosphoenzyme was formed in each

TABLE 4

Labeling from [γ-^{32}P]CTP and effects of unlabeled ATP

The nucleotides were added to the rat brain ($Na^+ + K^+$)-ATPase at the times indicated by the substrates, and the reaction was stopped at the times indicated by the figures. Incorporation of ^{32}P is expressed as a percentage of that occurring from [γ-^{32}P]ATP at 5 sec (336 pmoles of ^{32}P per milligram of protein). Prior addition of unlabeled ATP reduced the labeling from [γ-^{32}P]CTP to less than that observed with [γ-^{32}P]CTP plus 16 mM K^+, and is indicated as 0% labeling. All nucleotides were added to give a final concentraion of 0.05 mM, except for unlabeled ATP added after CTP, which had a final concentration of 0.2 mM. Each value is the mean ± standard error of four experiments.

0 sec	5 sec	10 sec	35 sec
—[a]	[γ-^{32}P]ATP	100%	—
—	[γ-^{32}P]CTP	82.0 ± 6.3%	108 ± 11.3%
ATP	[γ-^{32}P]CTP	0%	2.6 ± 1.2%
—	[γ-^{32}P]CTP	ATP	6.2 ± 1.4%

[a] Dashes signify that no addition or measurement was made.

TABLE 5

Characteristics of cation-dependent labeling from [γ-^{32}P]CTP

Rat brain enzyme in 50 mM Tris buffer, pH 7.4, was incubated for 5 sec with 0.05 mM [γ-^{32}P]CTP in the presence of the indicated ligands. The ligand concentrations were as follow: $MgCl_2$, 5 mM; Tris-EDTA, 5 mM; NaCl, 16 mM; KCl, 16 mM; P_i, 1 mM; ouabain, 0.25 mM. For the experiment with ouabain the enzyme was first incubated with the Mg^{2+}, P_i, and ouabain for 15 min. NaCl was then added, and the labeling reaction was started within 20 sec. The values represent the amount of ^{32}P trapped in the Millipore filter, as a percentage of that trapped after labeling in the presence of Mg^{2+} and Na^+ (370 pmoles of ^{32}P per milligram of protein). Each point represents the mean ± standard error of four experiments.

Additions	^{32}P incorporation
	%
Tris-EDTA	6.7 ± 1.1
Tris-EDTA + Na^+	7.7 ± 2.3
Mg^{2+}	28.9 ± 2.5
Mg^{2+} + K^+	9.2 ± 2.2
Mg^{2+} + Na^+ + K^+	11.2 ± 1.4
Mg^{2+} + Na^+ + ouabain + P_i	16.4 ± 0.7
Mg^{2+} + Na^+	100.0 ± 0

case. It seems reasonable to assume that CTP phosphorylates this enzymes in the same manner and to the same extent as ATP does, but not in the presence of ATP (see ref. 14).

If all the nucleotides tested phosphorylate this enzyme, then the phosphoenzymes formed from each substrate should be chemically similar and thus the incorporated phosphates should turn over at the same rate (Fig. 2). Thus the amount of P_i released from each substrate in the absence of K^+ should be directly proportional to the steady state levels of *E*-P. Table 6 shows that the rate of release of P_i from the nucleotide substrates used in Fig. 1 correlates well with the initial rates of [^{3}H]ouabain binding supported by them. These data agree with other evidence that ITP and UTP can phosphorylate this enzyme (15–17), and suggest that the different levels of phosphorylation of the enzyme by these substrates account for the observed differences in the initial rates of [^{3}H]ouabain binding.

Among the nucleotides tested, ADP is atypical in that it is a much more effective inhibitor of phosphorylation by [γ-^{32}P]ATP (Table 3) than a substrate for this enzyme (Table 6). This discrepancy is presumably due to ADP competitively displacing ATP without phosphorylating the enzyme (11, 14). If this is so, it should be possible to inhibit the ATP-dependent binding of [^{3}H] ouabain with concentrations of ADP which prevent the phosphorylation of this enzyme by ATP. Figure 3 shows that concentrations of ADP which almost completely inhibited the phosphorylation of this enzyme by ATP (11) markedly inhibited ATP-dependent [^{3}H]ouabain binding. The data show that ADP at millimolar concentrations bound to the enzyme (14) tightly enough to block the phosphorylation from ATP, but did not sup-

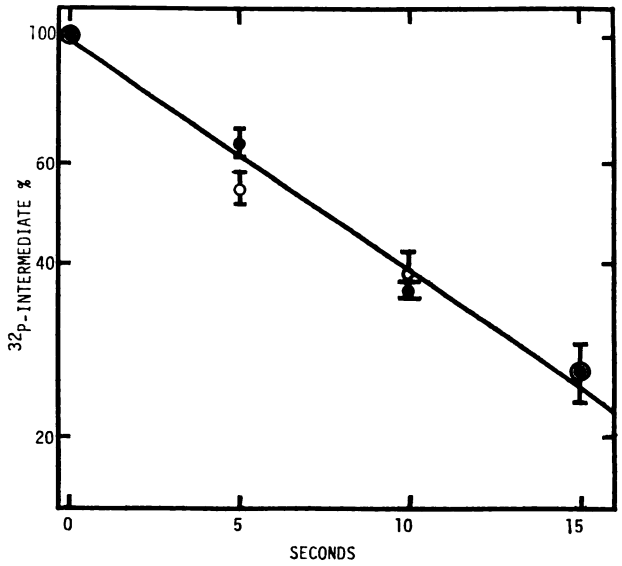

FIG. 2. *Rate of turnover of phosphoenzyme formed from* [γ-^{32}P]*ATP and* [γ-^{32}P]*CTP*

Rat brain enzyme was labeled with [γ-^{32}P]ATP or [γ-^{32}P]CTP as described in the text. Five seconds after starting the labeling reaction (indicated as zero time), 2 mM unlabeled ATP or CTP was added, and the reaction was stopped at the indicated times. The amount of labeling found at each time point was plotted as a percentage of that observed at zero time with ATP (●) or CTP (○). Each point is the mean ± standard error of four experiments.

port more ouabain binding than could be accounted for by the release of P_i. The data suggest that the Na^+-Mg^{2+}-ADP-enzyme complex per se is not the binding species.[1]

These results show that CTP can phosphorylate the (Na^+ + K^+)-ATPase, and strongly suggest that the other nucleotide triphosphates can also. This hypothesis is borne out by other data which indicate that ITP and UTP can also phosphorylate the (Na^+ + K^+)-ATPase (15–17). The observation that [γ-^{32}P] CTP-dependent labeling was displaced by ATP after 30 sec explains the report by Fahn *et al.* (18) that only ADP reduced ^{32}P incorporation from [γ-^{32}P]ATP at 1 min after labeling. However, their observation that [γ-^{32}P]ITP did not phosphorylate electric eel (Na^+ + K^+)-ATPase is more difficult to explain, and may not apply to enzymes from mammalian species (15, 16; but see ref. 17).

Many authors, assuming that the nucleotide-dependent formation of phosphoenzyme is highly specific for ATP, have attributed the effects of CTP, ADP, and other nucleotides (5–7, 19, 20) to simple binding of these

TABLE 6

Sodium-stimulated nucleotide hydrolysis

Rat brain enzyme, 10 mM Na^+, 5 mM (Mg^{2+}-nucleotide), and 50 mM Tris buffer (pH 7.4) were incubated for 30 min at 37°. Ouabain-inhibitable release of P_i is expressed as a percentage of that occurring from ATP (8.7 ± 1.6 μmoles of P_i per milligram of protein per hour. Results are the means ± standard errors of the number of determinations shown in the last column.

Nucleotide	Hydrolysis	n
	%	
ATP	100	6
ITP	96.9 ± 14.0	4
UTP	62.3 ± 10.6	4
ADP	24.8 ± 5.3	6

[1]When this paper was submitted for publication, Hansen, Jensen, and Norby had reported [*Nature New Biol.* 236, 122 (1971)] that NaADP does not support ouabain binding and that ADP inhibits NaATP-dependent [^{3}H]ouabain binding. The data presented here support these observations and indicate that in the presence of Mg^{2+}, ADP also inhibits [^{3}H]ouabain binding due to ATP.

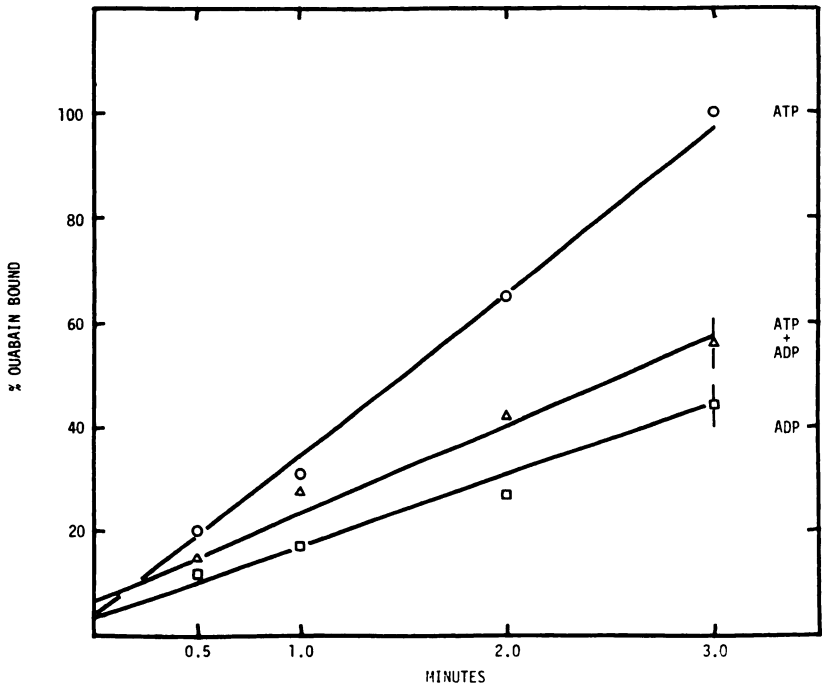

FIG. 3. *Inhibition of ATP-supported [³H]ouabain binding by ADP*

The curves show the initial rates of [³H]ouabain binding supported by 0.1 mM ATP (○), 4 mM ADP (□), and these substrates combined (△). Other conditions were the same as described in Fig. 1. Values are expressed as a percentage of the binding observed with 0.1 mM ATP at 3 min (25 ± 5.5 pmoles of [³H]oubain per milligram of protein). Each value is the mean ± standard error of four determinations.

nucleotides by the enzyme. They have suggested that binding of nucleotides by (Na^+ + K^+)-ATPase induces conformational changes in the enzyme which are the basis of the nucleotide-dependent modifications of its partial reaction sequences [[³H]ouabain binding (5) and *p*-nitrophenyl phosphatase activity (20)]. These interpretations must be reassessed in the light of the data presented here, which support interpretations attributing a primary role in these events to the formation of phosphoenzyme. In particular these observations support the suggestions of Robinson (21), Sen and co-workers (3, 4, 22), and Matsui and Schwartz (2) concerning the role of the phosphoenzyme in the nucleotide-stimulated phosphatase and [³H]ouabain-binding reactions.

The mechanism by which the ouabain-inhibitable release of P_i from ADP occurs is not clear. "ADPase" values of 1.5% (9), 10% (23), and even 68% (24) of the rates of hydrolysis of ATP have been reported by various authors. This variation from preparation to preparation supports suggestions that the release may occur via contaminating adenylate kinase (4, 23, 24). Furthermore, other experiments in this laboratory have shown that the addition of glucose and hexokinase can significantly reduce the initial rate of [³H]ouabain binding due to ADP. However, under some circumstances dADP, reportedly a poor substrate for adenylate kinase (24), can support the same initial rate of [³H]ouabain binding as ATP. This phenomenon is being investigated.

ACKNOWLEDGMENTS

The authors wish to thank Mrs. Annie Han and Mr. Roxy So for excellent technical assistance.

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