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SHORT COMMUNICATION

Ouabain Binding to Sodium- and Potassium-Dependent Adenosine Triphosphatase: Inhibition by the β,γ-Methylene Analogue of Adenosine Triphosphate

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SUMMARY

TOBIN, T., AKERA, T., HOGG, R. E., AND BRODY, T. M.: Ouabain binding to sodium- and potassium-dependent adenosine triphosphatase: inhibition by the β,γ-methylene analogue of adenosine triphosphate. *Mol. Pharmacol.* **9**, 278–281 (1973).

To determine the mechanism of nucleotide-dependent, Na^+-stimulated binding of [^{3}H]-ouabain to ($Na^+ + K^+$)-ATPase (EC 3.6.1.3), we tested the ability of β,γ-methylene ATP (adenylylmethylenediphosphonate) to support [^{3}H]ouabain binding. β,γ-Methylene ATP is an analogue of ATP in which the β- and γ-phosphates are linked by a methylene group. It is not hydrolyzed by the ($Na^+ + K^+$)-ATPase. In the presence of Na^+ and Mg^{++}, β,γ-methylene ATP did not support [^{3}H]ouabain binding to rat brain ($Na^+ + K^+$)-ATPase and it inhibited ATP-dependent binding. When [^{3}H]ouabain binding to guinea pig kidney ($Na^+ + K^+$)-ATPase was determined in the presence of Na^+, Mg^{++}, and P_i, the addition of β,γ-methylene ATP was inhibitory, in contrast to the stimulation produced by ATP. These results show that β,γ-methylene ATP binds to the ($Na^+ + K^+$)-ATPase and that this interaction does not support [^{3}H]ouabain binding.

Many interpretations of the mechanism of the sodium pump [($Na^+ + K^+$)-ATPase, ATP phosphohydrolase, EC 3.6.1.3] and its partial reaction sequences are based on the assumption that the binding of nucleotide substrates produces substantial conformational changes of the enzyme which account for nucleotide-dependent, Na^+-stimulated [^{3}H]ouabain binding (1–5). An alternative hypothesis holds that these nucleotides act by phosphorylating the enzyme and that the conformational changes required for [^{3}H]-ouabain binding depend on the prior formation of phospho-enzyme (6–9). To distinguish between these mechanisms we tested the ability of β,γ-methylene ATP to support [^{3}H]ouabain binding in the presence of Na^+. β,γ-Methylene ATP is a phosphonic acid analogue of ATP in which the β- and γ-phosphates are linked by a methylene group (10). This bond, though stereochemically similar to the β,γ-pyrophosphate bond of ATP (10), is resistant to hydrolysis by ($Na^+ + K^+$)-ATPase (11) and other enzymes (10). The results show that β,γ-methylene

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ATP is unable to stimulate [^{3}H]ouabain binding in the presence of Na^+ and Mg^{++}. Furthermore, it inhibits ATP- and P_i-dependent [^{3}H]ouabain binding under similar experimental conditions. These findings support the hypothesis that the Na^+-stimulated, ATP-dependent pathway of [^{3}H]ouabain binding requires the Na^+-stimulated phosphorylation of this enzyme rather than binding of the nucleotide substrate per se.

Rat brain and guinea pig kidney ($Na^+ + K^+$)-ATPase were prepared and assayed as described previously (7, 12). [^{3}H]-Ouabain binding was assayed by the method of Tobin *et al.* (13). To allow comparison of experiments performed on different enzymes, a particular value in each experiment was arbitrarily taken as 100% and other values were expressed as a percentage of this. All values are the means of at least four separate determinations plus or minus the standard errors of the mean. Sodium ATP was obtained from the Sigma Chemical Company, and β,γ-methylene ATP was a product of Miles Laboratories.

Table 1 shows the initial rates of [^{3}H]-ouabain binding to rat brain ($Na^+ + K^+$)-ATPase in the presence of 200 mM Na^+, 3 mM Mg^{++}, 50 nM [^{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, as found previously (13). However, if β,γ-methylene ATP was substituted for ATP no stimulation of [^{3}H]ouabain binding was observed over the time period of this experiment. 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 it reduced the initial rate of [^{3}H]ouabain binding. Since the rat brain enzyme–ouabain complex is relatively stable in the presence of monovalent cations (14), these experiments suggest that β,γ-methylene ATP binds to this enzyme in the presence of Na^+ and Mg^{++} without causing any stimulation of [^{3}H]ouabain binding.

The recent observations of Hegyvary and Post (11), which suggest that Na^+ and ATP bind to the same conformation of the ($Na^+ + K^+$)-ATPase, prompted us to test the actions of β,γ-methylene ATP on equilibrium levels of [^{3}H]ouabain binding in the presence of Na^+ and Mg^{++}. Guinea pig kidney enzymes were used in these experiments because of the relative ease with which shifts in [^{3}H]ouabain binding equilibria can be demonstrated in this preparation (7, 15). In Fig. 1 guinea pig kidney enzymes were allowed to equilibrate with 500 nM [^{3}H]-ouabain in the presence of 4 mM Mg^{++}, 1 mM P_i, and 60 nM Na^+ (7). Under these conditions binding reached equilibrium at about 40% of the level in the presence of Na^+, Mg^{++}, and ATP, which allowed us to test for either stimulation or inhibition of [^{3}H]ouabain binding by nucleotides. Figure 1 shows that the addition of ATP produced a prompt in-

TABLE 1

Sodium-stimulated binding of [^{3}H]ouabain in the presence of ATP and β,γ-methylene ATP

Rat brain ($Na^+ + K^+$)-ATPase was incubated with 50 nM [^{3}H]ouabain at 37°. At zero time the nucleotides and cations were added, and the binding reaction was stopped at the indicated times. Binding is expressed as a percentage of that observed in the presence of 200 mM Na^+, 3 mM Mg^{++}, and 0.1 mM ATP at 2 min, which averaged 101 pmoles of [^{3}H]ouabain per milligram of protein.

Addition	Binding after	
	1.0 min	2.0 min
	%	%
200 mM Na^+, 3 mM Mg^{++}, 0.1 mM ATP	68.6 ± 1.0	100
200 mM Na^+, 3 mM Mg^{++}, 3 mM β,γ-methylene ATP		1.10
20 mM Na^+, 3 mM Mg^{++}, 3 mM β,γ-methylene ATP		1.03
200 mM Na^+, 3 mM Mg^{++}, 0.1 mM ATP, 3 mM β,γ-methylene ATP	44.3 ± 3.7	66.2 ± 3.5

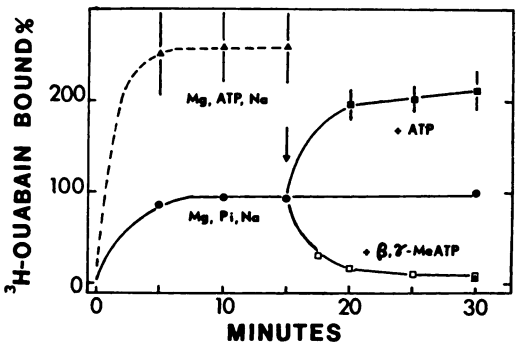

FIG. 1. *Inhibition of equilibrium level of [^{3}H]ouabain binding by β, γ-methylene ATP*

Guinea pig kidney enzymes were incubated in 50 mM Tris buffer (pH 7.4) with 1 mM P_i, 500 nM [^{3}H]ouabain, and 60 mM Na^+. The binding reaction was started by the addition of 4 mM Mg^{++} at zero time and was stopped at the indicated times. ●——●, binding in the presence of 4 mM Mg^{++}, 1 mM P_i, and 60 mM Na^+. Binding in the presence of Na^+ at 30 min was arbitrarily taken as 100%. Maximal binding (4 mM Mg^{++}, 1 mM P_i, no Na^+) averaged 94.2 pmoles of [^{3}H]ouabain per milligram of protein. After 15 min binding in the presence of Na^+, Mg^{++}, and P_i was challenged by the addition of 3 mM ATP, (■——■) or 3 mM β,γ-methylene ATP (□——□). ▲- - -▲, [^{3}H]ouabain binding in the presence of 60 mM Na^+, 3 mM Mg^{++}, and 5 mM ATP; △, binding in the presence of 4 mM Mg^{++}, 1 mM P_i, and 250 μM unlabeled ouabain. The vertical bars show those standard errors of the means greater than ±3%.

crease 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. The experiment shows that an "unhydrolyzed" nucleotide molecule bound to the enzyme is inhibitory to [^{3}H]ouabain binding, even in the presence of Na^+ and Mg^{++}.

The significance of these results depends entirely on the degree to which β,γ-methylene ATP substitutes for ATP in the formation of the ($Na \cdot E \cdot ATP \cdot Mg$) complex. It seems unlikely that these results are due to the inability of ($Na \cdot E \cdot$ β,γ-methylene ATP) to bind Mg^{++}, since the affinity constant of β,γ-methylene ATP for Mg^{++} is about twice that of Mg^{++} for ATP (16) and very low levels of Mg^{++} are sufficient to catalyze the [^{3}H]ouabain binding reaction (7). Of more importance is the observation that the apparent affinity of ($Na^+ + K^+$)-ATPase for β,γ-methylene ATP is lower than that of the enzyme for ATP (11). Although this observation might explain the inability of β,γ-methylene ATP to stimulate [^{3}H]ouabain binding in Table 1, it would not explain the observations of Table 1 and Fig. 1 showing that β,γ-methylene ATP binds tightly enough to inhibit [^{3}H]ouabain binding. Furthermore, β,γ-methylene ATP has a higher apparent affinity for this enzyme (11) than any of the other nucleoside triphosphates which have been postulated (1–5) to act simply by binding to this enzyme. Therefore it seems unlikely that these observations are atypical and due to the small alterations in bond angles, pK_a values, and bond distances (16) caused by the substitution of the methylene group into the β,γ bond of the ATP molecule, although this possibility cannot be ruled out. Comparisons of the actions of ATP and β,γ-methylene ATP on the dissociation of Mg^{++}-actomyosin at high ionic strength have shown that β,γ-methylene ATP will not dissociate this complex, in contrast to the actions of ATP (17). Therefore these experiments leave open the possibility that ATP affects this enzyme differently from β,γ-methylene ATP and that these differences may account for the inability of β,γ-methylene ATP to support [^{3}H]ouabain binding.

The possibility that the 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 the ($Na^+ + K^+$)-ATPase. Nucleoside triphosphates other than ATP have recently been shown to phosphorylate this enzyme (13, 18), weakening interpretations which were based on the assumption that only ATP can give rise to significant levels of phospho-enzyme (1, 3, 4). Recent work by Hegyvary and Post (11), which suggests that Na^+ and ATP bind to the same conformation of the enzyme, also supports these observations, for Na^+ has been demonstrated to stabilize cooperatively a non-ouabain-binding conformation of this enzyme (7). Similarly, experiments by Hansen *et al.* (9) and Tobin *et al.* (13) have shown that ADP binds to this enzyme in the

presence of Na^+ (9) or Na^+ and Mg^{++} (13) 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 (9).

If phosphorylation of this enzyme is required for the Na^+-stimulated pathway of [^{3}H]ouabain binding, transphosphorylation alone is presumably not sufficient. The phosphorylation must activate the transport machinery and start the enzyme into its transport cycle. This requirement gives these observations their mechanistic importance and may explain the minor differences between the conditions for phosphorylation and [^{3}H]ouabain binding. Siegel and Josephson (5) have recently drawn attention to the fact that the $(Na)_{0.5}$ for phosphorylation of electroplax ($Na^+ + K^+$)-ATPase is considerably less than the $(Na)_{0.5}$ for [^{3}H]ouabain binding. The explanation for this discrepancy probably lies in the actions of Na^+ on the $E_1 \sim P$ to E_2—P equilibrium, since high concentrations of Na^+ favor the $E_1 \sim P$ configuration (19). Thus, although [^{3}H]ouabain does not appear to interact with the $E_1 \sim P$ conformation (19, 20), there is no reason a priori why E_2—P should be the optimal binding conformation. The discrepancy between the Na^+ requirements for these reactions may indicate that the optimal [^{3}H]ouabain binding conformation of this enzyme is actually intermediate between the $E_1 \sim P$ and E_2—P configurations.

When Tobin and Sen (7) first proposed that Na^+ stabilized a non-ouabain-binding conformation of this enzyme and directed the system toward phosphorylation, the actions of MgATP were unclear. At that time it seemed possible that MgATP might, like Mg^{++}, give rise to ouabain binding in the absence of phosphorylation (7). This concept also received some support from the kinetic observations of Robinson (21), who showed heterotropic interactions between Na^+ and ATP in the presence of Mg^{++}. However, the data presented here, other experiments in this laboratory, and the data of Hegyvary and Post (11) all suggest that Na^+ and ATP interact with the same non-ouabain-binding conformation of this enzyme, increasing the likelihood that Na^+-, Mg^{++}-, and ATP-dependent [^{3}H]ouabain binding occurs after phosphorylation of the enzyme.

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