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# LIGAND-DEPENDENT REACTIVITY OF ($Na^+ + K^+$)-ATPase WITH SHOWDOMYCIN

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**Showdomycin inhibited pig brain ($Na^+ + K^+$)-ATPase with pseudo first-order kinetics. The rate of inhibition by showdomycin was examined in the presence of 16 combinations of four ligands, i.e., $Na^+$, $K^+$, $Mg^{2+}$ and ATP, and was found to depend on the ligands added. Combinations of ligands were divided into five groups in terms of the magnitude of the rate constant; in the order of decreasing rate constants these were: (1) $Na^+ + Mg^{2+}$ + ATP, (2) $Mg^{2+}$, $Mg^{2+} + K^+$, $K^+$ and none, (3) $Na^+ + Mg^{2+}$, $Na^+$, $K^+ + Na^+$ and $Na^+ + K^+ + Mg^{2+}$, (4) $Mg^{2+} + K^+$ + ATP, $K^+$ + ATP and $Mg^{2+}$ + ATP, (5) $K^+ + Na^+$ + ATP, $Na^+$ + ATP, $Na^+ + K^+ + Mg^{2+}$ + ATP and ATP. The highest rate was obtained in the presence of $Na^+$, $Mg^{2+}$ and ATP. The apparent concentrations of $Na^+$, $Mg^{2+}$ and ATP for half-maximum stimulation of inhibition ($K^s_{0.5}$) were 3 mM, 0.13 mM and 4 μM, respectively. The rate was unchanged upon further increase in $Na^+$ concentration from 140 to 1000 mM. The rates of inhibition could be explained on the basis of the enzyme forms present, including $E_1$, $E_2$, ES, $E_1$-P and $E_2$-P, i.e., $E_2$ has higher reactivity with showdomycin than $E_1$, while $E_2$-P has almost the same reactivity as $E_1$-P. We conclude that the reaction of ($Na^+ + K^+$)-ATPase proceeds via at least four kinds of enzyme form ($E_1$, $E_2$, $E_1$ · nucleotide and EP), which all have different conformations.**

## Introduction

($Na^+ + K^+$)-ATPase plays an important role in directing metabolic energy to the active transport of $Na^+$ and $K^+$ across the cell membrane [1,2]. It has been proposed that the enzyme exists in different conformations during the hydrolysis of ATP and that these conformational changes are essential to the mechanism of ion transport [3,4]. In order to explore the changes in conformation, the ligand dependency of tryptic digestion [5], intrinsic tryptophan fluorescence of the enzyme [6–8], formycin triphosphate fluorescence [9], *S*-mercuric-*N*-dansylcysteine fluorescence [10], BIPM fluorescence [11], the extent of inhibition by *N*-ethylmaleimide [12–14] and the amount of *N*-ethylmaleimide bound to the enzyme [15,16] have been examined. However, the situation is not yet well understood. The difficulties in measuring the conformational changes are partly due to the small extent of the changes. One of the easiest ways to measure this change is to compare the effects of ligands on the group-specific modification of ($Na^+ + K^+$)-ATPase.

The reactivity of SH groups essential to the enzyme activity has been examined by many investigators [10–21]. Banerjee et al. [12] and Skou [13] reported some effects of ligands on the extent of *N*-ethylmaleimide inhibition.

However, a quantitative estimation of reactivity

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Abbreviations: BIPM, *N*-(*p*-(2-benzimidazolyl)phenyl)maleimide; Ado*PP*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate; Ado*PP*[$CH_2$]*P*, adenosine 5′-[β,γ-methylene]triphosphate; Ado*P*[$CH_2$]*P*, adenosine 5′-[α,β-methylene]diphosphate.

requires knowledge of the rate constant of inhibition, when the reaction is irreversible. For this purpose, showdomycin (2-(β-D-ribofuranosyl)maleimide) is preferable as an inhibitor, because showdomycin inhibited rat brain ($Na^+ + K^+$)-ATPase [22] irreversibly by a bimolecular reaction and the inhibition by showdomycin followed better pseudo first-order kinetics than that by *N*-ethylmaleimide.

The present experiments using showdomycin show that four different enzyme forms can be distinguished in terms of the magnitude of the inhibition rate.

## Methods

*Enzyme preparation.* The enzyme was prepared from pig brain essentially by using the method of Nakao et al. [23] with some modifications. Pig brain cerebrums weighing 80 g were chopped with scissors and homogenized in an Ultra-Turrax with 4 vol. of homogenizing solution consisting of 0.25 M sucrose, 10 mM Tris-HCl (pH 7.1), 1 mM EDTA and 30 mM KCl. The homogenate was mixed gently with an equal volume of ice-cold homogenizing solution containing 0.25% sodium deoxycholate (Difco) and the whole was allowed to stand for 30 min on ice. The mixture was then centrifuged at 27 000 × *g* for 30 min (Sorvall SS 34 rotor). The upper two-thirds of the supernatant was recovered by aspiration and centrifuged again at 100 000 × *g* for 60 min (Beckman 45 Ti rotor). The pellet was resuspended in 50 ml of 1 mM EDTA, 3 mM Tris-HCl (pH 7.3) and the suspension was centrifuged at 180 000 × *g* for 30 min (Beckman 60 Ti rotor). The resultant pellet was suspended in redistilled water and stored at −70°C. These microsomal preparations were treated with NaI as described by Nakao et al. [23] and washed five times with redistilled water. All procedures were carried out at 0–4°C. The NaI-treated enzyme was used throughout. The specific activity of the preparation was 230–250 μmol $P_i$/mg per h and ouabain-insensitive activity was less than 0.5% of the total activity. Protein was estimated according to the method of Lowry et al. [24] with bovine serum albumin as a standard.

*Measurement of the inhibition rate.* About 100 μg of the enzyme were incubated at 37°C in 0.5 ml of 50 mM Tris-HCl (pH 7.4), 0.01 mM EDTA with various ligands, as described elsewhere for each experiment. Inhibition was started by adding 2 mM inhibitor to the mixture. At appropriate intervals, 20-μl aliquots of the mixture were each transferred to 500 μl of the enzyme assay mixture containing 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$, 3 mM ATP, 0.5 mM EDTA, 50 mM Tris-HCl (pH 7.4) and 1 mM β-mercaptoethanol. The solution was incubated for 16 min at 37°C, and released $P_i$ was measured according to the method of Fiske and SubbaRow [25]. Further inhibition by the inhibitor during enzyme assay was negligible (Fig. 1). We calculated the rate constants for pseudo first-order inhibition by a standard linear regression analysis method. The coefficient of determination was less than −0.97 in all cases.

*Phosphorylation of the enzyme by ATP.* The reaction mixture contained 0.25 mg of NaI-treated enzyme, 1.1 mM $MgCl_2$, 0.22 mM EDTA, 4.4 mM imidazole hydrochloride (pH 7.5) and various concentrations of NaCl in a total volume of 0.9 ml. Phosphorylation was started by adding 0.1 ml of 100 μM [γ-$^{32}$P]ATP to the medium. After 20 s at 25°C, the reaction was stopped by adding 8 ml of a solution containing 0.25 M trichloroacetic acid, 10 mM $H_3PO_4$ and 5 mM $Na_2ATP$. The precipitated protein was collected on a Millipore filter (3 μm) and washed six times with 10 ml of 0.25 M trichloroacetic acid containing 10 mM $H_3PO_4$. The filter was dissolved in 10 ml of acetone and the Cherenkov radiation from $^{32}$P was measured in a scintillation counter. The nonspecific adsorption of [γ-$^{32}$P]ATP on the filter with precipitated protein was measured when 10 mM KCl was added instead of NaCl. It was about 4% of maximal $^{32}$P incorporated. [γ-$^{32}$P]ATP was synthesized by using the method of Glynn and Chappell [26].

*Materials.* ATP was purchased from Kyowa Hakko Kogyo Co., Ltd, and other nucleotides from Sigma Chemical Co. The sodium salts of the nucleotides were converted to Tris salts by ion-exchange chromatography. ATP contaminating the ADP preparation was reduced to less than 0.5% by chromatography on Dowex-1. The purity of the ADP was checked by HPLC (MicroPak-$NH_2$ column, with 0.4 M $KH_2PO_4$ in 30% methanol as an eluent).

## Results

*Inhibition of ($Na^+ + K^+$)-ATPase activity by showdomycin or N-ethylmaleimide*

Pig brain ($Na^+ + K^+$)-ATPase was inhibited by pre-

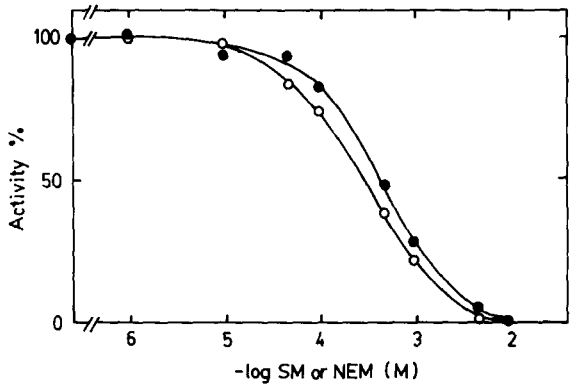



Fig. 1. Inhibition of $(Na^+ + K^+)$-ATPase activity by showdomycin or *N*-ethylmaleimide. The enzyme (7 μg/ml) was preincubated with showdomycin (SM) (•) or *N*-ethylmaleimide (NEM) (○) at the indicated concentrations in the presence of 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA and 50 mM Tris-HCl (pH 7.4) at 37°C. After 30 min, 3 mM ATP was added to the mixture and the enzyme activity was assayed for 16 min.

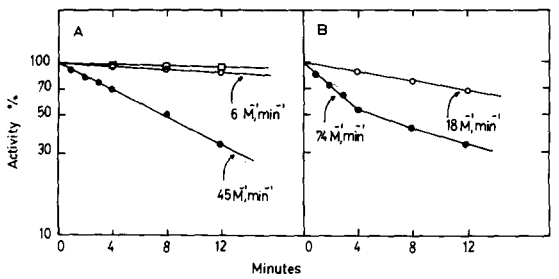



Fig. 2. Inactivation of $(Na^+ + K^+)$-ATPase by showdomycin or *N*-ethylmaleimide. About 100 μg of the enzyme were incubated with 2 mM showdomycin (A) or 2 mM *N*-ethylmaleimide (B) in 0.5 ml of 50 mM Tris-HCl (pH 7.4), 0.01 mM EDTA, with and without 0.1 mM ATP at 37°C. At the indicated times after addition of inhibitor, an aliquot of the enzyme was removed and assayed for $(Na^+ + K^+)$-ATPase activity as described in Methods. (•) Time course of inhibition in the absence of added ATP; (○) enzyme inhibition in the presence of 0.1 mM ATP; (□) enzyme activity in the absence of both inhibitor and ATP. Enzyme activities are expressed as a percentage of control activity preincubated with inhibitor for 0 min.

incubation with showdomycin in much the same way as with *N*-ethylmaleimide. The shapes of the inhibition curves were similar with showdomycin and *N*-ethylmaleimide (Fig. 1) and the concentrations required for half-maximum inhibition were $4.4 \cdot 10^{-4}$ and $3.1 \cdot 10^{-4}$ M for showdomycin and *N*-ethylmaleimide, respectively. Fig. 2 shows the differences in the rates of inhibition of $(Na^+ + K^+)$-ATPase by *N*-ethylmaleimide and showdomycin and in the protective effect of ATP against the inhibition. The inhibition by showdomycin followed pseudo first-order kinetics during the first 12 min, while that by *N*-ethylmaleimide did not. The presence of 0.1 mM ATP decreased the rates of inhibition by showdomycin and *N*-ethylmaleimide to 1/7 and 1/4, respectively. In the absence of inhibitors, inactivation of the enzyme activity during the preincubation was negligible. These results show that the use of showdomycin is preferable for detecting any change induced by ligands, including ATP. These data are in agreement with those of Tobin and Akera [22]. As the apparent rates of inhibition by showdomycin were directly proportional to the concentration of the reagent in the range of 0.5–10 mM, the inhibition of $(Na^+ + K^+)$-ATPase occurred as a bimolecular reaction. The rate constant ($k$) (in $M^{-1} \cdot min^{-1}$) may be calculated as follows:

$$k = \frac{\ln \frac{100}{b}}{t[I]}$$

where $I$ is the molar concentration of inhibitor, $b$ is the percentage of residual activity and $t$ is the time in min. The rate constant for showdomycin inhibition of $(Na^+ + K^+)$-ATPase in the absence of ligand was 45 $M^{-1} \cdot min^{-1}$ (Fig. 2).

*Effects of various ligands on the inhibition rate by showdomycin*

The $k$ values (inhibition rate constants) were measured in the presence of 16 combinations of 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$ and/or 0.1 mM ATP (Table I). Inhibition in the presence of any combination of ligands except $Na^+ + Mg^{2+}$ + ATP followed pseudo first-order kinetics for at least the initial 9 min (data not shown). In the case of $Na^+ + Mg^{2+}$ + ATP, inhibition followed the same kinetics for the initial 5 min and then the rate decreased (Fig. 4). The $k$ values varied according to the combinations

TABLE I

EFFECTS OF VARIOUS LIGANDS ON THE INHIBITION RATE BY SHOWDOMYCIN

The enzyme was preincubated with 2 mM showdomycin in 50 mM Tris-HCl (pH 7.4), 0.01 mM EDTA and various combinations of ligands, i.e., combinations of 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$ and 0.1 mM ATP. Inhibition rates were measured as described in Fig. 2A.

| Group No. | Conditions | Rate constant ($M^{-1} \cdot min^{-1}$) | $n$ |
|---|---|---|---|
| 1 | $Na^+$, $Mg^{2+}$, ATP | 108 ± 7 | 10 |
| 2 | $Mg^{2+}$ | 59 ± 5 | 5 |
| | $Mg^{2+}$, $K^+$ | 55 ± 5 | 5 |
| | $K^+$ | 50 ± 5 | 5 |
| | No ligand | 50 ± 5 | 5 |
| 3 | $Na^+$, $Mg^{2+}$ | 37 ± 5 | 5 |
| | $Na^+$ | 36 ± 7 | 5 |
| | $K^+$, $Na^+$ | 35 ± 6 | 5 |
| | $Na^+$, $K^+$, $Mg^{2+}$ | 35 ± 2 | 5 |
| 4 | $Mg^{2+}$, $K^+$, ATP | 39 ± 1 | 3 |
| | $K^+$, ATP | 38 ± 3 | 3 |
| | $Mg^{2+}$, ATP | 34 ± 2 | 5 |
| 5 | $K^+$, $Na^+$, ATP | 21 ± 7 | 3 |
| | $Na^+$, ATP | 20 ± 5 | 5 |
| | $Na^+$, $K^+$, $Mg^{2+}$, ATP | 14 ± 2 | 3 |
| | ATP | 12 ± 3 | 7 |

of ligands added. As the $k$ value obtained in the presence of $Na^+ + Mg^{2+}$, $Na^+$, $K^+ + Na^+$ or $Na^+ + K^+ + Mg^{2+}$ was smaller than that in the presence of $Mg^{2+}$, no ligand, $K^+$ or $K^+ + Mg^{2+}$, respectively, $Na^+$ seems to decrease $k$ values. On the other hand, the presence of $Mg^{2+}$ with no ligand, $K^+$ or $Na^+$ gave higher $k$ values than the absence of $Mg^{2+}$. Flashner et al. [27] and Forgac [28] have also observed antagonism between the bindings of $Na^+$ and $Mg^{2+}$ to the enzyme. Addition of $K^+$ had little effect on the $k$ values. The effects of cations shown here are not due to the change of ionic strength, because a similar effect of cations was observed when the ionic strength was kept constant by the addition of choline chloride (data not shown). ATP markedly decreased $k$ in every combination except $Na^+ + Mg^{2+}$, but increased it 3-fold in the presence of $Na^+ + Mg^{2+}$ (from 37 to 108 $M^{-1} \cdot min^{-1}$). Under the same conditions, ATP also accelerated the inhibition by *N*-ethylmaleimide, but the resulting increment in the rate of inhibition by

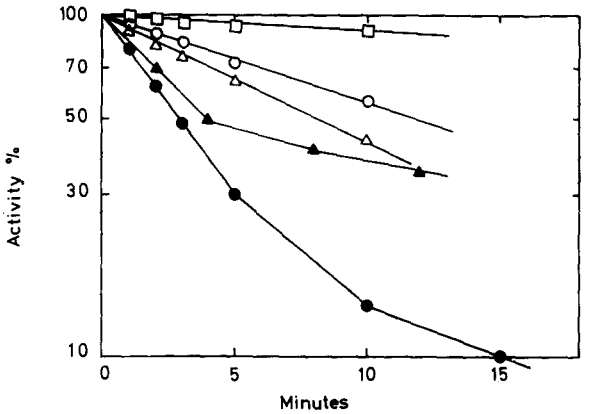



Fig. 3. Inactivation of $(Na^+ + K^+)$-ATPase by showdomycin or *N*-ethylmaleimide in the presence of $Ma^+ + Mg^{2+}$. The enzyme was preincubated with the following solutions under conditions otherwise the same as for Fig. 2. 140 mM NaCl and 3 mM $MgCl_2$ (□), 140 mM NaCl and 3 mM $MgCl_2$ plus either 2 mM showdomycin (○, ●) or 2 mM *N*-ethylmaleimide (△, ▲). With 0.1 mM ATP (●, ▲) and without ATP (○, △).

*N*-ethylmaleimide (54 to 92 $M^{-1} \cdot min^{-1}$) was smaller than that in the case of showdomycin (Fig. 3).

Combination of ligands could be divided into five groups as shown in Table I, according to the magnitude of the rate constant. Group 1 consisted of $Na^+ + Mg^{2+}$ + ATP. The $k$ value of this group was highest: 108 $M^{-1} \cdot min^{-1}$. Group 2 consisted of no ligand, $K^+$, $Mg^{2+}$ and $Mg^{2+} + K^+$. The $k$ values were 50–60 $M^{-1} \cdot min^{-1}$. Group 3 consisted of $Na^+$, $Na^+ + Mg^{2+}$, $K^+ + Na^+$ and $Na^+ + K^+ + Mg^{2+}$. The $k$ values were 35–40 $M^{-1} \cdot min^{-1}$. Group 4 consisted of $K^+$ + ATP, $Mg^{2+}$ + ATP and $Mg^{2+} + K^+$ + ATP. The $k$ values were almost the same as those of group 3. Group 5 consisted of ATP, $Na^+$ + ATP, $K^+ + Na^+$ + ATP and $Na^+ + K^+ + Mg^{2+}$ + ATP. The $k$ values were 10–20 $M^{-1} \cdot min^{-1}$. It is clear from this classification that ATP has two kinds of effect on the inhibition rate, deceleration and acceleration. First, addition of 0.1 mM ATP decreased $k$ values by about 20 $M^{-1} \cdot min^{-1}$ (group 2 to group 4, group 3 to group 5). The greatest protection against the inhibition was observed in the absence of any ligands (50 to 12 $M^{-1} \cdot min^{-1}$). Second, in contrast, addition of ATP to the enzyme in the presence of $Na^+ + Mg^{2+}$ greatly increased $k$ (group 1). When 1 mM ADP was used instead of 0.1 mM ATP, the $k$ values were divided into the same five groups.

*The stimulation of inhibition by $Na^+ + Mg^{2+} + ATP$*

$Na^+ + Mg^{2+} + ATP$ (group 1) is a combination of ligands required for EP formation. In order to clarify the mechanism of the acceleration of inhibition, the apparent concentrations of $Na^+$, $Mg^{2+}$ and ATP required for half-maximum acceleration of inhibition ($K^s_{0.5}$) were examined as shown in Fig. 4. $K^s_{0.5}$ for $Na^+$ with 1 mM ATP plus 3 mM $MgCl_2$ and $K^s_{0.5}$ for $Mg^{2+}$ with 140 mM NaCl plus 1 mM ATP were 3.1 and 0.13 mM, respectively. An accurate value of $K^s_{0.5}$ for ATP could not be determined because of enzymatic hydrolysis of ATP during the inhibition by showdomycin. In order to decrease ($Mg^{2+} + Na^+$)-ATPase activity during preincubation with showdomycin, the concentrations of NaCl and $MgCl_2$ were reduced to 14 and 1 mM, respectively, and the inhibition period was reduced as much as possible. As shown in Fig. 4, $K^s_{0.5}$ for ATP was 4 $\mu$M under these conditions.

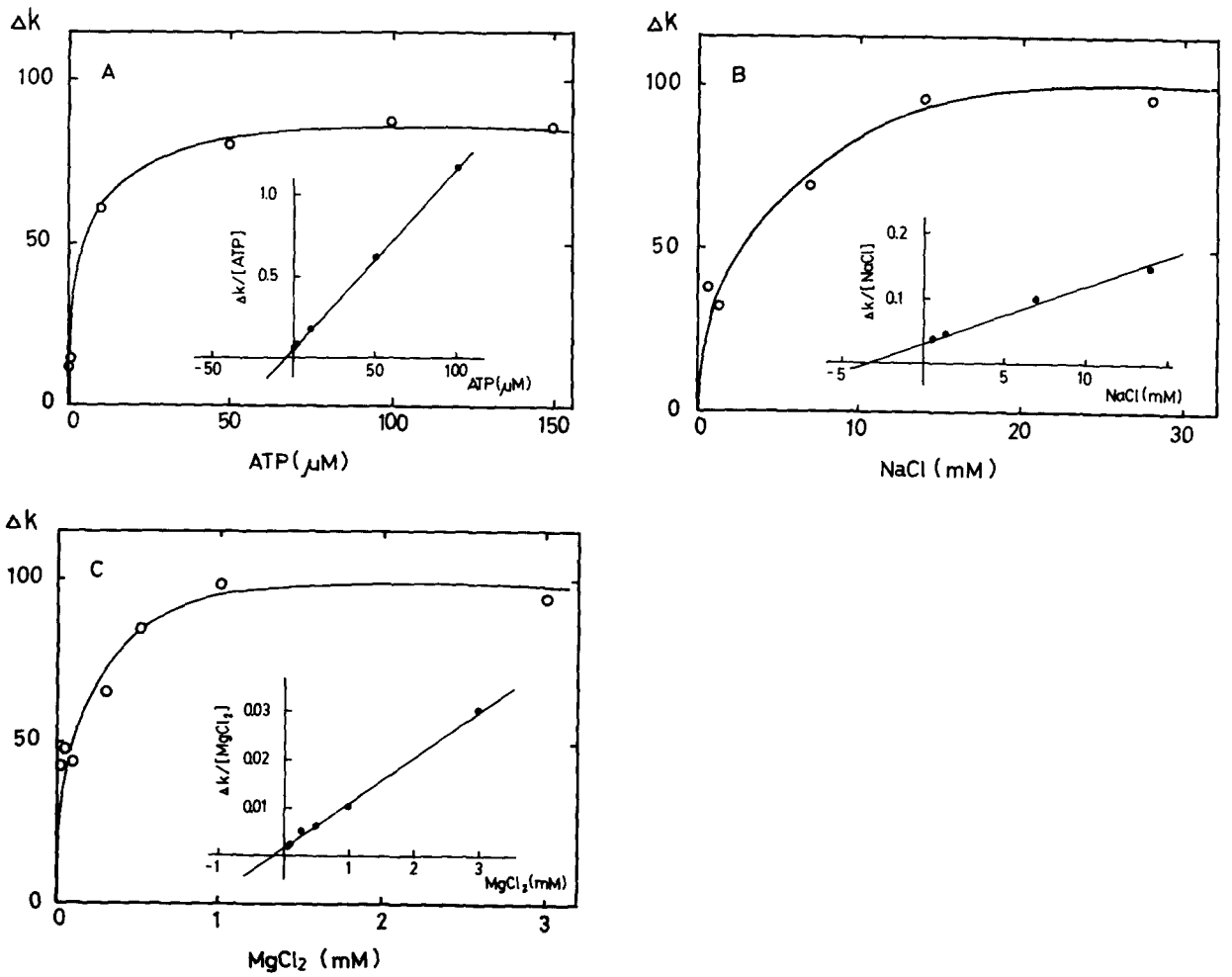



Fig. 4. Apparent concentration of $Na^+$, $Mg^{2+}$ or ATP for the half-maximum stimulation of inhibition ($K^s_{0.5}$). The enzyme was preincubated with showdomycin under the following conditions: 50 mM Tris-HCl (pH 7.4), 0.01 mM EDTA plus 14 mM NaCl, 1 mM $MgCl_2$ and various concentrations of ATP (A), 3 mM $MgCl_2$, 1 mM ATP and various concentration of NaCl (B), 140 mM NaCl, 1 mM ATP and various concentrations of $MgCl_2$ (C). The ordinate shows the increment of the inhibition rate ($\Delta k$) caused by the addition of ligand, and the abscissa shows the concentration of ligand. In the inset, the ordinate shows $\Delta k$/[ligand], and the abscissa shows the concentration of ligand.

TABLE II

EFFECTS OF VARIOUS NUCLEOTIDES OR PHOSPHATE COMPOUNDS ON THE RATE OF INHIBITION

The inhibition rates ($k$) were measured in the presence of 1 mM nucleotide or 1 mM phosphate compound, with and without both 140 mM NaCl and 3 mM $MgCl_2$ as described in Fig. 2A.

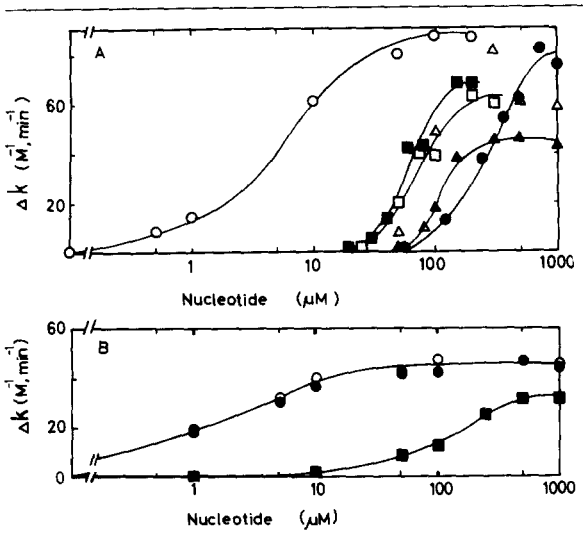
| Ligand | Rate constant ($M^{-1} \cdot min^{-1}$) | |
|---|---|---|
| | Without $Na^+ + Mg^{2+}$ | With $Na^+ + Mg^{2+}$ |
| None | 62 | 33 |
| ATP | 14 | 112 |
| ADP | 19 | 82 |
| Deoxy ATP | 14 | 98 |
| Deoxy ADP | 19 | 67 |
| CTP | 36 | 104 |
| ITP | 51 | 108 |
| GTP | 37 | 96 |
| UTP | 46 | 72 |
| *p*-Nitrophenylphosphate | 45 | 79 |
| Ado*PP*[NH]*P* | 16 | 23 |
| Ado*PP*[$CH_2$]*P* | 16 | 16 |
| Ado*P*[$CH_2$]*P* | 20 | 23 |
| AMP | 62 | 27 |
| *p*-Nitrophenol | 61 | 36 |
| $P_i$ | 68 | 30 |

*Effects of nucleotides on the stimulation of showdomycin inhibition*

Nucleoside di- and triphosphates could replace ATP with respect to the stimulative effect on the inhibition. 1 mM ADP, deoxy ATP, deoxy ADP, CTP, ITP, GTP or UTP accelerated the inhibition in the presence of both 140 mM NaCl and 3 mM $MgCl_2$, as shown in Table II. *p*-Nitrophenylphosphate also had this effect. Substrate analogs, Ado*PP*[NH]*P*, Ado*PP*[$CH_2$]*P* and Ado*P*[$CH_2$]*P* could not replace ATP. Of the above nucleotides, ATP produced the largest rate constant of inhibition and had the highest affinity for the enzyme (Fig. 5A). Though $K^s_{0.5}$ for ADP was much higher than that for ATP, the maximal increment of the rate with ADP was about the same as that with ATP, as shown in Fig. 5A. The effect of ADP was not due to contaminating ATP in the ADP preparation used, because the amount of contaminating ATP was less than 0.5%.

*Protective effects of nucleotides against showdomycin inhibition*

All the nucleotides used in our experiments protected the enzyme against inhibition by showdomycin in the absence of cations. 1 mM deoxy ATP,



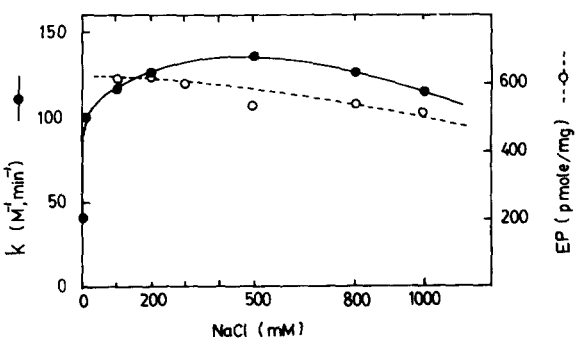




Fig. 5. Effects of nucleotides on the rate of inhibition by showdomycin. A, the enzyme was preincubated in the presence of 140 mM NaCl, 3 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), 0.01 mM EDTA and various concentrations of nucleotides except for ATP. In the case of ATP, the enzyme was preincubated in the presence of 14 mM NaCl, 1 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), 0.01 M EDTA and various concentrations of ATP. Inhibition rates were measured as described in Fig. 2 and Methods. B, the enzyme was preincubated in the presence of 50 mM Tris-HCl, 0.01 mM EDTA and various concentrations of nucleotides: ATP (○), ADP (●), ITP (□), CTP (■), deoxy ATP (△), *p*-nitrophenylphosphate (▲).

Fig. 6. Effect of NaCl on the steady-state EP level and the inhibition rate. In the presence of 1 mM ATP, 3 mM $MgCl_2$ and various concentrations of NaCl (0–1000 mM), the EP level (●) and the inhibition rate (○) were examined as described in Methods.

Ado*PP*[NH]*P* or Ado*PP*[$CH_2$]*P* protected the enzyme to the same extent as 1 mM ATP (Table II). The concentrations of ATP and ADP required for half-maximum protection ($K^p_{0.5}$) were similar and amounted to 1.5 $\mu$M (Fig. 5B). AMP, *p*-nitrophenol or $P_i$ had neither a protective effect nor an accelerative effect on the inhibition.

*The rate constant of inhibition of phosphorylated intermediates*

It is now generally accepted that the hydrolysis of ATP by ($Na^+ + K^+$)-ATPase proceeds by a multiple-step reaction sequence and that two forms of phosphoenzyme ($E_1$-P, $E_2$-P) occur as intermediates in the reaction pathway [3,29,30]. In the presence of 140 mM NaCl, 0.1 mM ATP and 5 mM $MgCl_2$, 80% of the phosphoenzyme exists in the $E_2$-P form and the remainder in the $E_1$-P form (data not shown). As it is known that high concentrations of $Na^+$ inhibit the net transformation of $E_1$-P to $E_2$-P, accompanied by the accumulation of the $E_1$-P form [31], the effect of high concentrations of NaCl on the rate of inhibition was examined in the presence of $Mg^{2+}$ + ATP (Fig. 6). The rate decreased slightly with increase in $Na^+$ concentration from 140 to 1000 mM. The level of the phosphorylated enzyme in the presence of $Mg^{2+}$ + ATP also decreased slightly with increase in $Na^+$ concentration. Thus, the apparent inhibition rate of the phosphoenzyme (rate/EP level) was unchanged by the increase in $Na^+$ concentration. As the $k$ value obtained with 3 mM $Mg^{2+}$ plus 3 mM $P_i$ was 90 $M^{-1} \cdot min^{-1}$, it appears that $E_2$-P formed from $P_i$ also had high reactivity with showdomycin.

## Discussion

The rates of inhibition by showdomycin varied according to the combination of ligands added. Assuming that the ligands added bind to all enzyme molecules uniformly, we can classify the enzyme forms induced by ligands into five groups in terms of the magnitude of the inhibition rate. Group 1 contains both phosphorylated forms and a kind of E-S complex, because ADP and *p*-nitrophenylphosphate could substitute for ATP in terms of the acceleration of inhibition by showdomycin. These forms have the highest reactivity with showdomycin as shown in Table I. Group 2 consists of E, $EK^+$, $EMg^{2+}$ and $EK^+Mg^{2+}$, which could be considered to be $E_2$ forms of the enzyme [4,32,33]. Group 3 consists of $ENa^+$, $ENa^+K^+$, $ENa^+Mg^{2+}$ and $ENa^+K^+Mg^{2+}$, which were considered to be $E_1$ forms of the enzyme, because the $Na^+$ concentration was sufficiently high [13,34]. Group 4 consists of combinations of $E_2$ forms with nucleotide ($E_2 \cdot$ nucleotide). However, the possibility remains that this group contains mixtures of $E_1 \cdot$ nucleotide and $E_2$. Group 5 consists of combinations of $E_1$ forms with nucleotide ($E_1 \cdot$ nucleotide). From these classifications, we can reach the following conclusions. (1) There are at least four kinds of enzyme form having different $k$ values, i.e., $E_1$, $E_2$, $E_1 \cdot$ nucleotide and EP. (2) $E_1$ forms have lower reactivity with showdomycin than $E_2$ forms. Some differences in chemical properties between $E_1$ and $E_2$ were also reported [5,7–9,35]. (3) The combined forms of $E_1$ with nucleotide ($E_1 \cdot$ nucleotide) have lower reactivity with showdomycin than those of $E_2$ ($E_2 \cdot$ nucleotide). (4) Addition of ATP to the $E_1$ or $E_2$ form decreased the rate constant except in the presence of $Na^+ + Mg^{2+}$. (5) In the presence of $Na^+ + Mg^{2+}$, ATP markedly increased the $k$ value.

Several studies have shown that the reactivity of the enzyme to SH-blocking reagents, such as *N*-ethylmaleimide [16], fluorescein mercuric acetate [21] and *S*-mercuric-*N*-dansylcysteine [10], increased markedly in the presence of $Na^+ + Mg^{2+}$ + ATP or $Mg^{2+} + P_i$. For the following reasons, we conclude that the enzyme forms having the highest reactivity with showdomycin in the presence of $Na^+ + Mg^{2+}$ + ATP are phosphoenzymes. First, the values of $K^s_{0.5}$ for $Na^+$ and ATP in the inhibition reaction were similar in magnitudes to those for the acceleration of phosphorylation; $K^s_{0.5}$ values for ATP and $Na^+$ were 4 $\mu$M and 3.3 mM while $K_m$ values for ATP and $Na^+$ in phosphorylation were reported to be 3.6 $\mu$M [36] and 5.4 mM [37], respectively. Second, nucleotides incapable of phosphorylating the enzyme (Ado*PP*-[$CH_2$]*P* and Ado*PP*[NH]*P*) did not increase the inhibition rate.

We can also conclude that the two phosphoenzymes ($E_1$-P and $E_2$-P) have nearly the same reactivity with showdomycin, because the rate of inhibition by showdomycin in the presence of $Na^+ + Mg^{2+}$ + ATP was unchanged on further increase in $Na^+$ concentration to 1000 mM, at which the conversion of $E_1$-P to $E_2$-P would be blocked [31]. Previous studies on

tryptophan fluorescence [7,9], *S*-mercuric-*N*-dansylcysteine fluorescence [10] and *N*-ethylmaleimide binding [16] showed that the chemical reactivities of the two forms differ from each other. The difference from our observation may be due to the difference in methodology, because we measured only the reactivity of SH groups essential for the enzyme activity.

ADP and *p*-nitrophenylphosphate have a stimulative effect on inhibition in the presence of $Na^+$ + $Mg^{2+}$, like ATP, though the two compounds are unable to phosphorylate the enzyme. Thus, we suggest that the E-S complex, produced exclusively in the presence of $Na^+$ + $Mg^{2+}$ + substrate, has as high a reactivity with showdomycin as phosphoenzymes have, and may be in a pre-EP state. $K^s_{0.5}$ for *p*-nitrophenylphosphate was 90 $\mu$M (Fig. 5A). Since this value is much lower than the $K_m$ value for $K^+$-dependent *p*-nitrophenylphosphatase activity (1.2 mM) [38], the stimulative action of *p*-nitrophenylphosphate appears to differ in nature from its activity as a substrate for $K^+$-dependent *p*-nitrophenylphosphatase. $K^s_{0.5}$ for ADP was 270 $\mu$M (Fig. 5A). This value is far larger than that for ADP binding [39,40], but is similar to that for ADP inhibition of overall ATP hydrolysis [41,42]. Thus, the site for ADP observed in our experiments may be the low-affinity site described by Robinson [42].

As shown in Table II, nucleotide specificity with regard to the stimulation of inhibition was strict, while that for protection against inhibition was broad. Since $K^s_{0.5}$ for ATP was about 30–70-times smaller than that for any other nucleoside triphosphate or ADP, it appears that ATP is specific for the stimulation of inhibition by showdomycin (Fig. 5A). On the other hand, protection occurred with a variety of nucleotides. Though the two effects differed in nucleotide specificity, the $K_{0.5}$ values for ATP in the protection and in the acceleration were similar. As these values were in agreement with the $K_d$ values of ATP binding to the enzyme (0.1–2.0 $\mu$M) [39,40] and the $K_m$ value of the high-affinity ATP site (3.3 $\mu$M) [36], ATP would produce either stimulation or protection through its binding to the high-affinity ATP binding site [42–44]. The nucleotide specificity of the ATP binding site might be modified, probably accompanied by conformational changes induced by ligands.

Showdomycin was a more sensitive probe than *N*-ethylmaleimide for monitoring conformational changes induced by ATP, because ATP had greater effects on both the rate and the degree of inhibition by showdomycin than on those by *N*-ethylmaleimide. The inhibition of pig brain ($Na^+$ + $K^+$)-ATPase by showdomycin followed better pseudo first-order kinetics than that by *N*-ethylmaleimide, probably owing to the greater water solubility of showdomycin. Schoot et al. [45] reported that the inhibition of kidney ($Na^+$ + $K^+$)-ATPase by *N*-ethylmaleimide obeyed pseudo first-order kinetics. The difference from our result may be due to organ specificity [46].

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