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Rapid inhibition of the K^+-sensitive phosphoenzyme of Na^+/K^+-ATPase by (*Z*)-5-methyl-2-[2-(1-naphthyl)ethenyl]-4-piperidinopyridine

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Membrane-bound Na^+,K^+-ATPase (0.1 mg/ml) was incubated with the K^+-site-directed probe (*Z*)-5-methyl-2-[2-(1-naphthyl)ethenyl]-4-piperidinopyridine (AU-1421) (Takada, J. et al. (1990) Biochim. Biophys. Acta 1037, 373–379) at 37°C for 30 min in the absence of ligands, then the Na^+-dependent phosphorylation level was examined in the presence of 10 μM [^{32}P]ATP at 0°C. The level was decreased to 50% and 0% by about 50 μM and 100 μM AU-1421, respectively. Addition of 1 mM K^+ during the treatment with AU-1421 resulted in complete maintenance of the phosphorylation level. When the preincubation was performed at 0°C for 10 s, even 100 μM AU-1421 did not impair the phosphorylation. In contrast to the non-phospho form of the enzyme, the K^+-sensitive phosphoenzyme formed from ATP was immediately inhibited by the addition of AU-1421 at 0°C. The reactivity of the inhibited phosphoenzyme was restored by the addition of K^+. About 1 mM K^+ gave the same maximal reactivity in the presence of various fixed concentrations (8–41 μM) of AU-1421, but the apparent affinity for K^+ decreased simply with the increase of AU-1421 concentration. From this simple competitive relationship, the apparent K_i value of AU-1421 for the phosphoenzyme was calculated to be 7.2 μM. Compared to the non-phospho form of the enzyme, the phospho form appears to be rather susceptible to AU-1421, probably because the K^+-site of the phosphoenzyme is exposed to the extracellular aqueous phase.

Introduction

The plasma membrane-spanning enzyme Na^+,K^+-ATPase transports Na^+ and K^+ in opposite directions across the membrane in animal cells. The enzyme selectively binds cations at one face, occludes them into its interior domain, and dissociates them at the opposite face. These processes are repeated consecutively for Na^+- and K^+-transport through a mechanism involving Asp-369 residue phosphorylation by ATP and its dephosphorylation [1–4]. In order to understand the mechanism of cation transport by this enzyme, information is required about amino acid residues that form the cation-binding sites and about the pathway of cation movement [4]. As an approach to this problem, we [5] tried to find suitable probes and obtained the new fluorescent compound (*Z*)-5-methyl-2-[2-(1-naphthyl)-ethenyl]-4-piperidinopyridine, AU-1421 (see Fig. 1 of Ref. 5 for the chemical structure). This hydrophobic amine appeared to interact with the K^+ occlusion center of the non-phosphorylated enzyme during a relatively prolonged incubation period at 37°C, but not at 0°C. In order to characterize further the K^+-site-directed nature of AU-1421, the effects of AU-1421 on phosphorylation and dephosphorylation of the Na^+,K^+-ATPase were investigated in the present experiments. The K^+-sensitive phosphoenzyme, E_2P (the enzyme state during turnover is expressed according to the notation of the Post-Albers scheme [1–3,7,11] throughout the present paper) was found to be inhibited rapidly by AU-1421 even at 0°C. The reactivity of E_2P was competitively restored by K^+.

Abbreviations: AU-1421, (*Z*)-5-methyl-2-[2-(1-naphthyl)ethenyl]-4-piperidinopyridine; CDTA, 1,2-cyclohexylene dinitrilotetraacetic acid.

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Materials and Methods

Materials

The HCl salt of AU-1421 was synthesized in the Chemical Laboratories of Banyu Pharmaceutical Co., Ltd. It was dissolved in distilled water and the solution was stored in a refrigirator under shielding from light. It was used within a month after dissolution. Membrane-bound Na^+,K^+-ATPase was prepared from sheep kidney outer medulla as described previously [5]. The specific activity of ATP hydrolysis was in the range of 6–11 μmol of P_i/min per mg at 37°C in the presence of 25 mM histidine (free base, pH 7.0), 114 mM NaCl, 20 mM KCl, 3 mM Na_2ATP, 4 mM $MgCl_2$, and 0.1 mM EDTA. The protein concentration was estimated by the method of Lowry using an assay kit obtained from Pierce Inc. with bovine serum albumin as a standard.

Phosphorylation and dephosphorylation

Phosphorylation of the enzyme (0.1 mg/ml) by 10 μM [^{32}P]ATP and measurements of the kinetics of dephosphorylation were carried out at 0°C in the presence of 25 mM histidine as described previously [6,7]. When the pretreatment of the enzyme with AU-1421 was required, it was performed at 37°C for 30 min unless otherwise mentioned. When protection of the enzyme from the action of AU-1421 by the presence of another substance during the preincubation was to be tested, the mixture was centrifuged and the supernatant was removed by aspiration. The recovered membranes were resuspended (0.1 mg/ml) in the reaction mixture for phosphorylation.

Results

Phosphorylation experiments

Slow action of AU-1421 on the phosphorylation from ATP

In the previous experiments, the enzyme (0.08 mg/ml) was preincubated with 60 μM AU-1421 at 37°C (25 mM histidine, pH 7.0) in the absence of ligands, then the Na^+,K^+-ATPase activity was measured by transferring aliquot of the preincubation mixture into the complete reaction mixture for the enzyme [5]. The activity was decreased to 50% after preincubation for about 4 min, and was decreased to less than 5% by preincubation for 60 min (see Fig. 2 of Ref. 5). The inactivation by AU-1421 thus proceeded on a time scale of the order of minutes at relatively high temperature. However, there was no effect on the activity after preincubation at 0°C.

AU-1421 was thought to bind tightly to the K^+-site to stabilize the enzyme [5]. If this is so, the enzyme phosphorylation from ATP should be depressed by

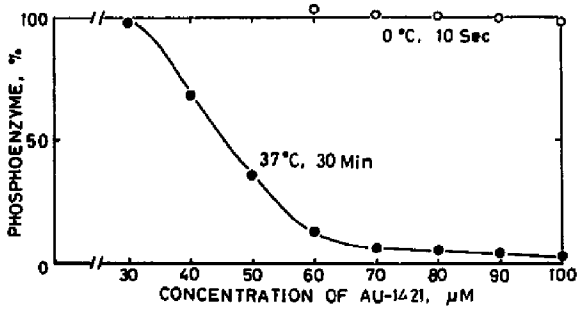


Fig. 1. Inhibition of phosphorylation of Na^+,K^+-ATPase from ATP by treatment with AU-1421 for 30 min at 37°C. The membrane protein (0.05 mg) was incubated with 30–100 μM AU-1421 in the presence of 25 mM histidine (0.5 ml) for 30 min at 37°C (●). After 30 min, the preincubation mixture was cooled in an ice-bath. Subsequently 0.5 ml of a solution containing 20 μM [^{32}P]ATP, 2 M NaCl, 2 mM $MgCl_2$, and 25 mM histidine was added to the preincubation mixture (final volume was 1 ml) to initiate the radio-labelling of the enzyme at ice-bath temperature. At 40 s after the start of the phosphorylation, it was quenched by adding 10 ml of perchloric acid containing non-radioactive 1 mM P_i and ATP. In another system (○), 60–100 μM AU-1421 was added to the enzyme standing in the ice-bath at 10 s before the initiation of the phosphorylation. Other conditions were the same as in the above experiment.

treatment with AU-1421 under certain conditions. To examine this, the enzyme (0.1 mg/ml) was preincubated with 30–100 μM AU-1421 at 37°C. After 30 min, the solution was cooled to 0°C, then the phosphorylation was started by the addition of 10 μM radioactive ATP, 1 M NaCl, and 1 mM $MgCl_2$ in an ice-bath. In this experiment, we used a high concentration of NaCl in order to observe only the phosphorylation step by stabilizing E_1P, so that we could exclude possible effects of AU-1421 on steps occurring after the formation of E_1P (for example, $E_1P \rightarrow E_2P$, or $E_2P \rightarrow E_2 + P_i$). Under these conditions, phosphorylation proceeded slowly [7], and hence the level of phosphoenzyme was estimated at 40 s after initiation of the phosphorylation (Fig. 1). The level of phosphoenzyme thus obtained was decreased by preincubation with AU-1421. The half-maximal-effect concentration of AU-1421 was 45–50 μM. Almost identical results were obtained when the preincubation mixture was centrifuged to remove the supernatant and the recovered enzyme was used for the phosphorylation reaction (not shown). Therefore, tight binding of AU-1421 to the enzyme is the cause of the decrease of the phosphoenzyme. Free AU-1421 did not interfere the phosphorylation because there was little effect on the level of phosphoenzyme, when the phosphorylation was started (without centrifugation) at 10 s after the addition of AU-1421 at 0°C (Fig. 1).

The contrasting results obtained under these two temperatures (37°C and 0°C) suggest the existence of some barrier against access of AU-1421 to its binding site. On the other hand, when the enzyme was in the

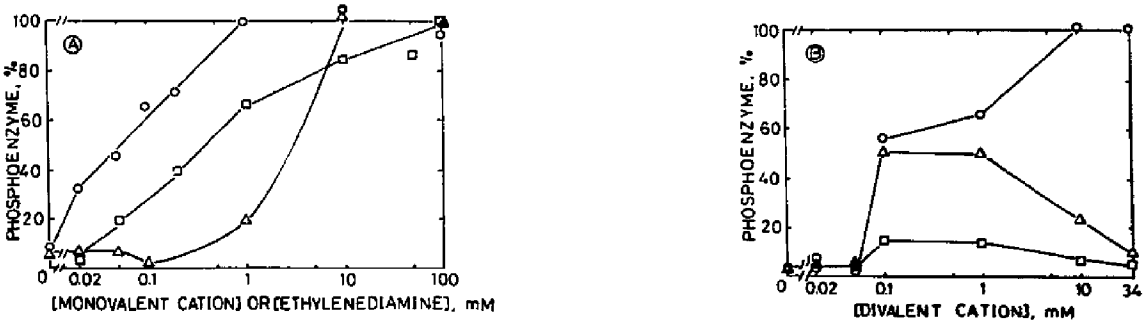


Fig. 2. (A) Protection of Na^+,K^+-ATPase from AU-1421 by K^+, ethylenediamine, or Na^+. In a volume of 1 ml of 25 mM histidine, the membrane protein (0.1 mg) was incubated with 100 μM AU-1421 at 37°C in the presence of the indicated concentrations of KCl (○), ethylenediamine (□), or NaCl (△). After 30 min, the incubation mixtures were centrifuged and the proteins recovered in the pellet were resuspended in 0.5 ml of 25 mM histidine in an ice-bath. The phosphorylation was performed as described in the legend to Fig. 1 by adding 0.5 ml of a solution containing radioactive ATP, NaCl, and $MgCl_2$ to these membrane suspensions. (B) Protection of the enzyme from AU-1421 by divalent cations. The preincubation of the enzyme with AU-1421 in the presence of the indicated concentrations of $MnCl_2$ (○), $CaCl_2$ (△), or $MgCl_2$ (□) was performed as described in the legend to Fig. 2A. Resuspension and the phosphorylation were also carried out in the same way as in the above experiment.

form of E_2P, it was inhibited immediately after the addition of AU-1421 at 0°C (see below: 'Rapid inhibition of E_2P by AU-1421 at 0°C').

Protection of the enzyme from AU-1421 by K^+-site-directed substances

Suppression of the phosphorylation by 100 μM AU-1421 was completely prevented by the presence of 1 mM K^+ during the preincubation period. About 60 μM K^+ protected 50% of the enzyme from AU-1421, while millimolar order concentration was required for protection by Na^+ (Fig. 2A). This is consistent with our previous idea that AU-1421 interacts with the K^+ occlusion center of Na^+,K^+-ATPase, and that a high concentration of Na^+ probably acts as a weak substitute for K^+ [5]. Ethylenediamine, which has been suggested to be a K^+-site-directed reagent [8,9], also prevented the action of AU-1421 when it was added during the preincubation period (Fig. 2A). Its half-maximal-effect concentration was about 0.5 mM, and 100% protection was obtained at about 100 mM. These results are consistent with the previous observations, where the overall Na^+,K^+-ATPase activity was measured after the preincubation [10].

Another reported probe for the K^+ occlusion center is Ca^{2+} [10–12]. When 0.1–1 mM Ca^{2+} was added during the preincubation of the enzyme with 100 μM AU-1421, about 50% of the phosphorylation activity remained. Ca^{2+} ions at higher concentration were less effective for the protection of the enzyme (Fig. 2B). Mg^{2+} also showed a slight preventive effect on AU-1421 action, with a similar concentration dependency to that of Ca^{2+}. Mn^{2+}, however, only had protecting action against 100 μM AU-1421; its 50% protective concentration was about 90 μM, and its 100% protective concentration was about 10 mM (Fig. 2B). When a high concentration of $CaCl_2$ was used, residual Ca^{2+} derived from the preincubation mixture might inhibit phosphorylation. Ca^{2+} is more inhibitory than Mn^{2+} to the phosphorylation [13]. Thus, as far as competition with AU-1421 is concerned, Ca^{2+} is not a specific divalent cation.

Dephosphorylation experiments

Rapid inhibition of E_2P by AU-1421 at 0°C

The ease of access of ligands to their sites is changed by phosphorylation of the enzyme [1–4,23]. E_2P interacts with K^+ easily at the external cell surface. If AU-1421 is specific to the K^+-site, it may interact with E_2P from the aqueous phase more easily than with non-phosphorylated E_2 (dominant conformation in the presence of histidine [14]), in which the K^+-site forms an occlusion center in an interior domain of the enzyme.

In order to test this possibility, K^+-sensitive E_2P was prepared by the use of 10 μM ATP in the presence of 100 mM NaCl and 0.5 mM $MgCl_2$ at 0°C. After stopping the phosphorylation by the addition of an excess amount of CDTA·Na_3 to chelate Mg^{2+}, various concentrations of AU-1421 was simply added to the phosphoenzyme (Fig. 3). Immediately after the addition, the hydrolyzing activity of E_2P was inhibited. Unlike the non-phospho form, E_2P did not require either a high temperature or a long period for the interaction with AU-1421. The half-maximal concentration of AU-1421 for the inhibition was estimated to be about 10 μM from the dose dependency (inset in Fig. 3).

Competition between AU-1421 and K^+ for binding to E_2P

When 3.8 mM K^+ was added to the AU-1421 inhibited phosphoenzyme, the latter was split immediately

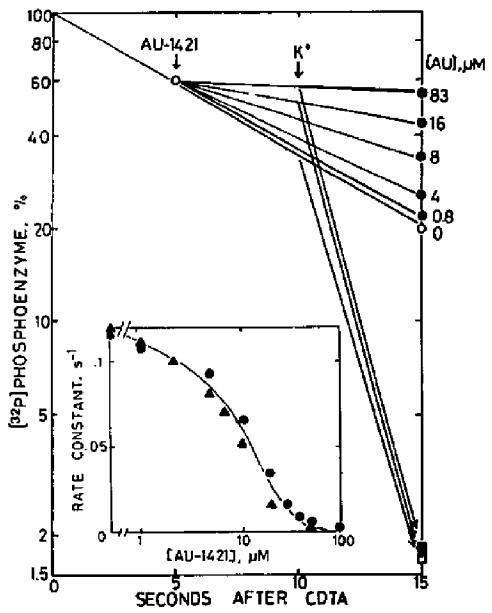


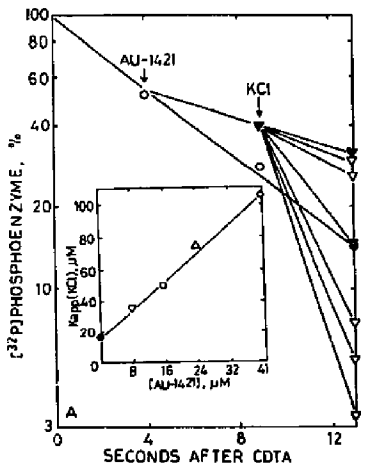
Fig. 3. Rapid inhibition by AU-1421 and its prevention by K^+ of the K^+-sensitive phosphoenzyme of Na^+,K^+-ATPase. Membrane protein (0.1 mg) was phosphorylated by adding 10 μM [^{32}P]ATP in the presence of 100 mM NaCl, 0.5 mM $MgCl_2$, and 25 mM histidine at 0 °C. The volume of the complete reaction mixture was 1 ml. At 15 s after the start of the phosphorylation, 0.1 ml of 100 mM CDTA (pH 7.2 with NaOH) was added to stop the phosphorylation. This time is indicated as zero time in the figure. At 5 s after CDTA, 0.1 ml of 0 (○, □), 1, 5, 10, 19, or 100 (●) μM AU-1421 was added to the phosphoenzyme. A further 5 s later, 0.1 ml of 5 mM KCl was added (□, ■). The rate constant of dephosphorylation after the addition of AU-1421 was estimated in the presence of 100 (●) or 10 mM (▲) NaCl (inset).

in the same way as occurs in the non-inhibited state (Fig. 3). AU-1421 appeared to be released from E_2P by the binding of K^+.

In order to investigate the relationship between AU-1421 and K^+ binding to E_2P, the concentration of added K^+ was varied in the presence of 8 μM AU-1421 (Fig. 4A). The rate of E_2P splitting became faster with increasing K^+ concentration. This was the case when the concentration of AU-1421 was 0, 16, 24, or 41 μM (not shown). With increase of the AU-1421 concentration, the apparent affinity (50% protective concentration) for K^+ simply decreased, while the maximum rate of dephosphorylation seemed to remain ultimately the same (Fig. 4B). Similar results were obtained when AU-1421 and K^+ were added simultaneously (not shown). Therefore, AU-1421 appeared to bind to E_2P reversibly and to compete with K^+. The apparent K_i value for AU-1421 was estimated to be 7.2 μM (inset in Fig. 4A). Thus, as in the non-phospho form of the enzyme (cf. Fig. 1), AU-1421 seems to interact with the K^+-site on E_2P also, but much more easily.

Interaction of AU-1421 inhibited E_2P with Ca^{2+} or ouabain

Do K^+-site-directed substances for the non-phospho form of the enzyme (see above: 'Protection of the enzyme from AU-1421 by K^+-site-directed substances') prevent the AU-1421 inhibition of E_2P too ? To examine the effect of Ca^{2+} on the AU-1421 inhibited E_2P, the enzyme was labeled with 10 μM radioactive ATP,



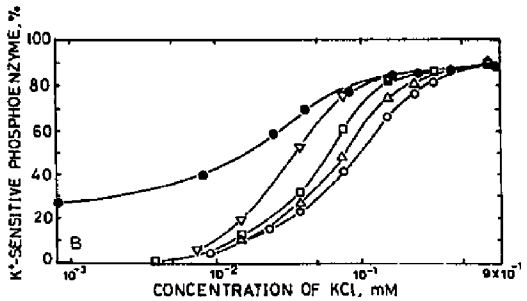


Fig. 4. Competition between AU-1421 and K^+ on the K^+-sensitive phosphoenzyme. (A) Phosphorylation of the enzyme was initiated as described in the legend to Fig. 3 in the presence of 10 mM NaCl, and chased at 15 s by the addition of CDTA (○). At 4 s after CDTA, 0.1 ml of 10 μM AU-1421 was added (▼). A further 5 s later, 0.1 ml of 0.1, 0.2, 0.5, 1, 2, or 10 mM KCl was added (▽) to obtain the amount of K^+-stimulated splitting of the phosphoenzyme. Dephosphorylation was stopped by the addition of acid at 4 s after KCl. The K^+-sensitivity at various concentrations of KCl was taken as the difference between the phosphoenzyme level found after no addition of KCl and that after addition of each concentration of KCl. (B) Percentage of the K^+-sensitive phosphoenzyme as a function of KCl concentration in the presence of various concentrations of AU-1421. The dephosphorylation experiments as described in (A) were performed in the presence of 0 (●), 8 (▽), 16 (□), 24 (△), or 41 μM (○) AU-1421. The K^+ concentration giving 50% K^+-sensitivity was plotted as a function of AU-1421 concentration (inset in (A)). The apparent K_i value for AU-1421 was estimated to be about 7.2 μM from the intercept on the abscissa.

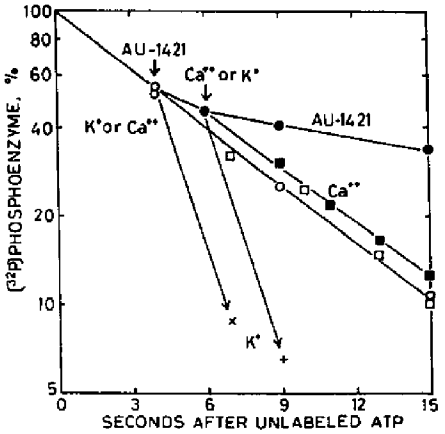


Fig. 5. Protection of the K^+-sensitive phosphoenzyme from AU-1421 by Ca^{2+}. Phosphoenzyme was formed as described in the legend to Fig. 3 in the presence of 10 mM NaCl. At zero time, 0.1 ml of 7 mM unlabeled ATP·Na_2 was added (○). At 4 s, 0.1 ml of 50 mM $CaCl_2$ (□) or 2 mM KCl (×) was added, as a control for the non-inhibited phosphoenzyme. To prepare the inhibited phosphoenzyme, 0.1 ml of 0.5 mM AU-1421 was added to the phosphoenzyme at 4 s (●). To this inhibited phosphoenzyme, 0.1 ml of 50 mM $CaCl_2$ (■) or 2 mM KCl (+) was added at 6 s.

and was chased with a 70-fold excess of unlabeled ATP (Fig. 5). Then E_2P was inhibited by adding 41 μM AU-1421. To this inhibited state of the phosphoenzyme, 42 mM Ca^{2+} was added. Because the chase was performed with unlabeled ATP (but not CDTA), this addition of divalent cation did not reactivate the phosphorylation. The high concentration of Ca^{2+} restored the dephosphorylation rate to a value equal to that of the non-inhibited E_2P, but did not stimulate it further, as K^+ did. This amount of Ca^{2+} had no effect on the dephosphorylation of the non-inhibited E_2P. The half-maximal-effect concentration of Ca^{2+} for the restoration of the splitting of E_2P under these conditions was about 3 mM, and the saturating concentration for this effect was about 20 mM (not shown). Mg^{2+} or Mn^{2+} released AU-1421 inhibition only a little (not shown). Ethylenediamine did not prevent AU-1421 inhibition of E_2P, but rather inhibited the splitting by itself at a concentration of more than 1 mM (not shown). (cf. above 'Protection of the enzyme from AU-1421 by K^+-site-directed substances'.)

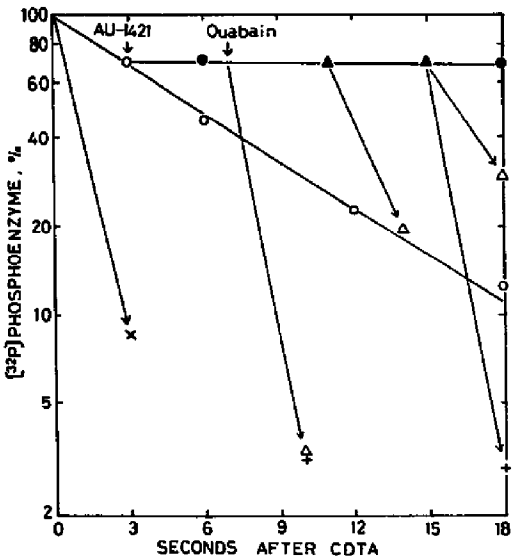


Fig. 6. Binding of ouabain to the AU-1421 inhibited phosphoenzyme. Phosphorylation of the enzyme and the chase were performed as described in the legend to Fig. 3 in the presence of 10 mM NaCl (○). At 3 s after the chase, 0.1 ml of 0.5 mM AU-1421 was added (●). At 7 s, 0.1 ml of 5 mM ouabain was further added to the AU-1421 inhibited phosphoenzyme (▲). A final concentration of 4 mM KCl was added to the phosphoenzyme treated with both AU-1421 and ouabain (△) or with AU-1421 only (+). Initially, the phosphoenzyme was sensitive to K^+ (×).

The Na^+, K^+-ATPase-specific inhibitor ouabain preferentially binds to the E_2P state of the enzyme. In order to examine whether ouabain binds to the AU-1421 inhibited E_2P, we made use of the fact that E_2P with bound ouabain loses the K^+-sensitivity [15], while E_2P with bound AU-1421 retains it (Fig. 3). When sensitivity to K^+ was tested after the addition of 0.38 mM ouabain to the AU-1421 inhibited E_2P, the enzyme gradually became insensitive, in contrast to the case without addition of ouabain (Fig. 6). This means that ouabain can bind to the AU-1421 inhibited E_2P. Together with the fact that the overall Na^+,K^+-ATPase activity was inhibited independently by ouabain and AU-1421 [16], it may be concluded that AU-1421 binds to the enzyme at a site different from that of ouabain binding.

Discussion

Our previous experiments [5] showed that AU-1421, a hydrophobic pyridine derivative, interacts with the K^+ occlusion center of the non-phospho form of Na^+,K^+-ATPase. Because AU-1421 is a fluorescent compound, it should be a useful probe for labeling the peptide region around the K^+-site or for monitoring the microenvironment around this region. The peak wavelenghs of excitation and emission of AU-1421 were 330 nm and 415 nm, respectively, in the presence of 25 mM histidine and 0.1 mM EDTA (pH 7.4 with Tris) (Nagamune, H., Takada, J. and Fukushima, Y., unpublished data).

In order to confirm the characteristics of AU-1421 as a probe for the K^+-site, or rather for the K^+-pathway of Na^+,K^+-ATPase, we investigated in the present experiments the interaction of this compound with E_2P, wich has high reactivity to K^+ among the various states in the reaction cycle of this enzyme [1–4,23]. K^+ from the external surface of the cell binds rapidly to E_2P and stimulates its splitting.

Simple competition between AU-1421 and K^+ at the mouth of the K^+-pathway

AU-1421, like K^+, interacted with E_2P immediately after the addition, but unlike K^+, it inhibited the splitting of E_2P (Fig. 3). However, the dephosphorylation was restored by K^+ following simple competitive kinetics (Fig. 4B). Therefore, it can be considered that AU-1421 binds to E_2P easily at the mouth of the K^+-pathway opening towards the outside face of the cell. Stimulation of E_2P splitting may require an interaction with K^+ at a deep part of the K^+-pocket, and AU-1421 may be able to interact only with the surface region of the K^+-pathway, but may not be able to pass easily into the deeper part of the site under these conditions. The molecular size of AU-1421 is much larger than the size of K^+.

Compared to the phosphoenzyme, the inhibition of the non-phosphorylated enzyme by AU-1421 required more energy (cf. Fig. 1). This is probably because the K^+-site is nearly isolated from the aqueous phase in the E_2 state to form the occlusion center, or because the site is only accessible through the K^+-releasing route in the E_1 state, so that AU-1421 can not readily reach the K^+-site from the aqueous phase. The results of our previous experiments [5] were interpreted as indicating that AU-1421 can reach the site through E_1. The conditions required for the inhibition of phosphorylation in the present experiments (Fig. 1) were very similar to those of the previous work. Thus, AU-1421 probably binds to the K^+-site through the K^+-releasing route to inhibit the phosphorylation.

Prevention of the AU-1421 inhibition by Ca^{2+}

That Ca^{2+} can reach the K^+ occlusion center under certain conditions is one of the recent conclusions of researchers on the characteristics of the K^+-site [10–12]. Therefore, Ca^{2+} was expected to protect the enzyme from the inhibition by AU-1421, and indeed this was the case for E_2P (Fig. 5). However, Ca^{2+} seemed not to occupy a deep position of the K^+-pocket of the E_2P from the extracellular solution, but only to replace the AU-1421 bound at the mouth of the K^+-pathway. This is presumably the reason why Ca^{2+} removed the inhibition by AU-1421, but did not act as a substitute for K^+ in the dephosphorylation of E_2P (Fig. 5). There seems to be a selective barrier against Ca^{2+} between the K^+-pocket and the aqueous phase. Vasallo and Post [11] postulated that the barrier might be a positive charge located inside or close to the access pathway between the extracellular solution and the K^+-pocket, since 'Ca^{2+} has twice the positive charge of K^+ and a slightly smaller atomic size'.

Compared to the phosphoenzyme, protection by divalent cations of the non-phosphorylated enzyme from AU-1421 was not as specific to Ca^{2+} (Fig. 2B). If these divalent cations also have access the K^+-site through the K^+-releasing pathway, as postulated for the access of AU-1421 [5], the selectivity for cations at the releasing mouth of the pathway may be lower than that at the uptake mouth of E_2P. The transport cation must be specifically selected at the binding step. Although there is another possibility, i.e., that divalent cations protect the enzyme from AU-1421 allosterically, the specificity of divalent cation passage through the K^+-pathway from the extracellular and the intracellular solutions should be investigated in more detail.

General inhibitory action by amine compounds

In general, amine compounds are inhibitors of Na^+,K^+-ATPase [17–19]. Even a buffer component such as Tris or imidazole inhibited E_2P when added at a concentration of several tens of millimolar [6,20,21]. The inhibition was, like that by AU-1421, removed completely by K^+ or Ca^{2+} [6,20]. With respect to the non-phospho form of the enzyme, the inhibition by these basic buffer substances required preincubation in the presence of another destabilizer of protein structure, such as dilute sodium dodecyl sulfate [20,22]. In this case also, monovalent cation prevented the inhibition, but ATP did not. Hydrophilic amines presumably require some loosening of the protein structure to access an interior domain of the enzyme. Thus, the contrast between the phosphorylated enzyme and the non-phosphorylated enzyme in the inhibition by Tris, etc. and AU-1421 reflects, in both cases, a degree of isolation of the interaction site of amine from the aqueous phase [22]. Hydrophobic amines work much more effectively, so that AU-1421 is utilizable as a probe.

Acknowledgements

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