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K^+-site-directed pyridine derivative, AU-1421, activates hydrolysis of the K^+-sensitive phosphoenzyme of sarcoplasmic reticulum Ca^{2+}-ATPase and inactivates that of K^+-transporting ATPases

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(*Z*)-5-Methyl-2-[2-(1-naphthyl)ethenyl]-4-piperidinopyridine, AU-1421, interacted at 0°C with the K^+-sensitve phosphoenzymes of three transport ATPases, Ca^{2+}-, H^+/K^+- and Na^+/K^+-ATPase. In the case of Ca^{2+}-ATPase, AU-1421 at about 80 μM stimulated 6-fold the rate of splitting of the phosphoenzyme, on which K^+ simply functions as an accelerator from one side of the membrane. Probably AU-1421 also simply interacts with the K^+-binding site of the phosphoenzyme that is easily accessible from the aqueous phase. In the cases of H^+/K^+- and Na^+/K^+-ATPases, AU-1421 stabilized the phosphoenzymes which accept K^+ as the translocating ion. The rate constants of dephosphorylation for H^+/K^+-ATPase and Na^+/K^+-ATPase were decreased to half by AU-1421 at about 5 and 10 μM, respectively. Presumably after binding of AU-1421 to a K^+-recognition site of the phosphoenzyme, local motion of the peptide region near the binding site that serves to move the bound ion into the ion-transport pathway (occlusion center) might be inhibited. Thus AU-1421 may be able to distinguish two modes of K^+ action on the K^+-sensitive phosphoenzymes.

Introduction

Cation transport ATPases, i.e. Ca^{2+}-ATPase of sarcoplasmic reticulum, H^+/K^+-ATPase of gastric mucosa and Na^+/K^+-ATPase of animal cell membrane, are stimulated by K^+. The stimulation is due mainly to acceleration of the splitting of one form of the aspartyl-phosphoenzyme formed from ATP by transfer of the terminal phosphate group during the reaction sequence for coupling between ATP hydrolysis and cation movement. The K^+-sensitive phosphoenzyme is usually designated E_2P in the general reaction scheme for these pumping systems [1–3]. K^+-acceleration of the E_2P splitting is an initial step for the translocation of K^+ in H^+/K^+- and Na^+/K^+-ATPase, whereas in Ca^{2+}-ATPase of SR, K^+ is not translocated across the membrane, but simply interacts with E_2P at the cytoplasmic surface of the SR to accelerate the hydrolysis of the phosphoester bond [4].

The hydrophobic amine AU-1421 is thought to occupy the K^+-site of the K^+-translocating ATPases, thereby inhibiting them [5–9]. E_2P of Na^+/K^+-ATPase is rapidly inhibited by AU-1421, because the drug binds easily to the mouth of the K^+-pathway opening towards the aqueous phase, but, unlike K^+, it does not undergo subsequent translocation into the interior domain of the pathway [9]. In contrast to the K^+-translocating ATPases, stimulation of the hydrolysis of E_2P in the Ca^{2+}-ATPase of SR may occur simply through binding of AU-1421 to an exterior site. This paper describes the different actions of AU-1421 on the E_2P of Ca^{2+}-ATPase and those of the K^+-translocating ATPases.

Abbreviations: AU-1421, (*Z*)-5-methyl-2-[2-(1-naphthyl)ethenyl]-4-piperidinopyridine; SR, sarcoplasmic reticulum; CDTA, 1,2-cyclohexylene dinitrilotetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); SCH 28080, 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy) imidazo[1,2-a]pyridine.

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Materials and Methods

Materials

Ca^{2+}-ATPase of SR was prepared from rabbit skeletal muscle following the method of Weber et al. with some modifications [10]. The membranes were suspended in 5 mM Tris maleate (pH 6.5) containing 100 mM KCl, and stored at 0°C. The specific activity of ATP hydrolysis was 4.0 μmol P_i/min per mg at 37°C in the presence of 50 μM $CaCl_2$, 4 mM $MgCl_2$, 100 mM KCl, 3 mM ATP · Na_2, 0.01% Triton X-100 and 20 mM Hepes (pH 7.2 with imidazole). H^+/K^+-ATPase-rich membranes were obtained from microsomes of pig gastric mucosa by Ficoll density gradient centrifugation in the presence of 250 mM sucrose. The interface fraction between 0 and 7% Ficoll was colleted, lyophilized, and stored at −80°C [11]. The specific activity was 1.1 and 1.2 μmol P_i/min per mg at 37°C in 40 mM Tris-HCl (pH 7.4) containing 15 mM KCl, 3 mM ATP · Na_2 and 3 mM $MgCl_2$. These activities were not inhibited by addition of 1 mM ouabain. When 120 mM NaCl were further added, ouabain-inhibitable activity amounted to 0.037 to 0.078 μmol P_i/min per mg. Membrane-bound Na^+/K^+-ATPase was prepared from crude microsomes of the outer medulla of sheep kidney by the use of sodium dodecyl sulfate, as described previously [7], and stored at −80°C in 250 mM sucrose, 20 mM histidine and 0.7 mM EDTA (pH 7.2). The specific activity was 8.7 μmol P_i/min per mg at 37°C in the presence of 25 mM histidine (free base, pH 7.1), 114 mM NaCl, 20 mM KCl, 3 mM ATP · Na_2, 4 mM $MgCl_2$ and 0.1 mM EDTA. Protein was determined according to the method of Lowry et al. [12, 13] using an assay kit obtained from Pierce Inc. with bovine serum albumin as a standard. ATPase activity was determined following the modified Fiske-SubbaRow method [13].

AU-1421 (for the chemical structure, see Ref. 5 or 7) was synthesized as the HCl salt at the Chemical Laboratories of Banyu Pharmaceutical Co., Ltd. It was dissolved (2 mM) in distilled water and the solution was stored shielded from light in a refrigerator.

Phosphorylation and dephosphorylation

As the enzymes were stored with inorganic cation for stabilization, they were washed once (H^+/K^+- and Na^+/K^+-ATPases) or three times (Ca^{2+}-ATPase) with inorganic cation-free 25 mM histidine (free base, pH 7.0–7.2 with Tris) before every experiment to minimize modification of AU-1421 action on the enzyme (e.g., see Refs. 5 and 7 for details of the weakening of AU-1421 action on H^+/K^+- or Na^+/K^+-ATPase by K^+ or other cations). Labelling of the enzyme (0.1 mg/ml) was performed in the presence of 25 mM histidine (pH 7.0–7.2), 10 μM [γ-^{32}P]ATP, 0.5 mM $MgCl_2$ and other inorganic cations required for the transphosphorylation of each ATPase (indicated in each experiment), with or without AU-1421, at 0°C. In the experiment on SR ATPase, 0.01% Triton X-100 was employed to prevent saturation with Ca^{2+} inside the vesicles as a result of the uptake cycling of Ca^{2+}-ATPase. Splitting of the [^{32}P]phosphoenzyme was exposed by the addition of an excess amount of CDTA · Tris to remove free Mg^{2+}, which is a common requirement for the phosphorylation of these ATPases. To observe its effect on the dephosphorylation, AU-1421 was added during the exposure of the dephosphorylation. The amounts of the [^{32}P]phosphoenzyme were determined as described previously [9].

Tryptic digestion of H^+/K^+-ATPase and electrophoresis

Tryptic digestion of H^+/K^+-ATPase was done by a modification of the method of Helmich-de Jong et al. [14]. Gastric vesicles (400 μg of protein) were incubated with 8 μg of TPCK-trypsin (Sigma) at 37°C for 5 min in 10 mM Pipes-Tris (pH 7.4) containing 1 M glycerol, 1 mM EDTA and 250 mM sucrose. After 5 min, the digestion was terminated by addition of 50 μg of soybean trypsin inhibitor (Sigma) and the samples were frozen in liquid N_2. From the digested samples, aliquots were taken and treated with 2% SDS and 5% 2-mercaptoethanol at 37°C for 5 min, then applied to SDS-polyacrylamide (10%) gel.

Results and Discussion

Activation by AU-1421 in the hydrolysis of E_2P of Ca^{2+}-ATPase

Washed SR (0.1 mg/ml) was phosphorylated by addition of 10 μM [^{32}P]ATP · Tris in the presence of 0.5 mM $MgCl_2$, 7 μM $CaCl_2$, 0.01% Triton and 25 mM histidine at 0°C. Since the concentration of Ca^{2+} was low, all the phosphoenzyme became E_2P within 30 s; it was insensitive to ADP, but was split rapidly by 100 mM K^+ with rate constant of 0.51 s^{-1}, after stopping the phosphorylation by addition of CDTA. Under these conditions, AU-1421 stimulated the splitting of E_2P, as did K^+ (Fig. 1). The rate constant (0.03 s^{-1}) of the E_2P splitting without any addition increased to 0.18 s^{-1} after addition of 83 μM AU-1421, where the stimulatory effect was almost saturated (inset, Fig. 1). Though the stimulation by AU-1421 was apparently partial in comparison with the K^+-stimulation, the affinity of AU-1421 for E_2P was much higher than that of K^+. The half-maximal concentration of KCl for the stimulation of E_2P splitting was about 10 mM (not shown, but see Ref. 15). The limited effect of AU-1421 may have been a result of poor solubility. The maximum solubility was reduced to about 0.1 mM at these salt concentrations, which was substantially less than that in plain water (see also Materials).

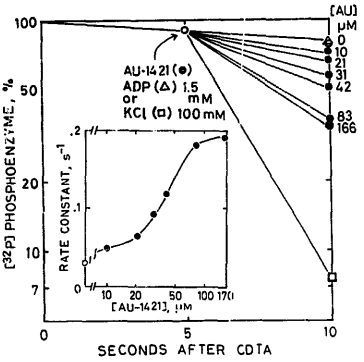

Fig. 1. Accerelation by AU-1421 in the hydrolysis of the K^+-sensitive phosphoenzyme of Ca^{2+}-ATPase. SR membranes were washed, just before use, with 25 mM histidine (free base, pH 7.1) three times to decrease the amount of KCl (which had been presented in the storage solution). The membrane protein (0.1 mg) suspended in 0.9 ml of 28 mM histidine containing 0.56 mM $MgCl_2$, 7.8 μM $CaCl_2$ and 0.011% Triton X-100 was phosphorylated with 0.1 ml of 0.1 mM [^{32}P]ATP at 0°C. At 30 s after the addition of ATP, 0.1 ml of 100 mM CDTA·Tris was added to expose the dephosphorylation (time zero in the figure) (○). A further 5 s later, 0.1 ml of AU-1421 (●), KCl (□) or ADP·Tris (△) was added to give the indicated final concentrations. The rate constant of the dephosphorylation (inset) was obtained by stopping the reaction with 10 ml of perchloric acid containing 10 mM inorganic phosphate and 5 mM unlabeled ATP at 5 s after the above additions.

From this activation of E_2P splitting, we expected that a low concentration of AU-1421 might replace K^+ in the overall Ca^{2+}-ATPase reaction. However, no activation was found upon addition of 0.8 to 100 μM AU-1421 in the absence of K^+ and in the presence of 50 μM $CaCl_2$, 4 mM $MgCl_2$, 3 mM ATP·Tris, 0.008% Triton and 20 mM Hepes (pH 7.2 with imidazole) at 37°C (not shown). On the contrary, AU-1421 reduced the Ca^{2+}-ATPase activity in the presence of 100 mM KCl (not shown). At a concentration of 60 μM, AU-1421 reduced the ATPase activity to 50%. Probably the inhibitory action of AU-1421 was prominent before the formation of E_2P.

In fact, AU-1421 was inhibitory to the phosphorylation when added at 10 s before the simultaneous addition of 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 μM [^{32}P]ATP in the presence of 0.01% Triton and 25 mM histidine (Fig. 2). As a large amount of $CaCl_2$ (1 mM) was added in this experiment, the formed phosphoenzyme was mainly K^+-insensitive (so-called E_1P), and therefore the decrease in the phosphoenzyme level was not due to stimulation of the dephosphorylation by AU-1421 (cf. Fig. 1), but must have been due to another interfering effect.

Inactivation by AU-1421 in the hydrolysis of E_2P of K^+-transporting ATPases

In order to compare the action of AU-1421 on the splitting of E_2P in Ca^{2+}-ATPase, which does not transport K^+, and in K^+-transporting ATPase, H^+/K^+-ATPase (0.1 mg/ml) was phosphorylated with 10 μM [^{32}P]ATP·Tris in the presence of 0.5 mM $MgCl_2$ and 25 mM histidine at 0°C. At 20 s after the initiation of phosphorylation, the reaction was disturbed by 10 mM CDTA, and a further 5 s later, 1–50 μM AU-1421 or 50 mM KCl was added (Fig. 3). The phosphoenzyme was K^+-sensitive (thus being E_2P) and was stabilized soon after the addition of AU-1421. AU-1421 at several micromolar seemed to reduce the rate of splitting to half, and at several tens of micromolar AU-1421 completely stabilized E_2P of H^+/K^+-ATPase. This result is similar to that observed with another K^+-transporting enzyme, Na^+/K^+-ATPase (see Fig. 3 of Ref. 9, where the half-maximal inhibition concentration of AU-1421 was about 10 μM), and is opposite to that obtained with Ca^{2+}-ATPase (cf. Fig. 1). A more

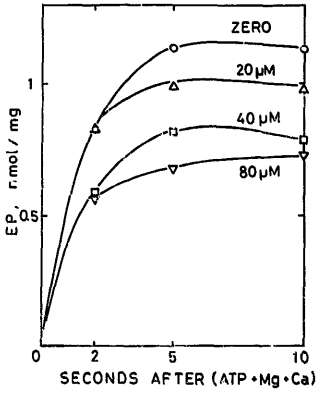

Fig. 2. Inhibition by AU-1421 of the phosphorylation of Ca^{2+}-ATPase. The washed membranes as described in the legend for Fig. 1 were suspended (0.1 mg protein) in 0.8 ml of 32 mM histidine (pH 7.1) containing 0.0125% Triton X-100 at 0°C. To this, 0.1 ml of 0 (○), 0.2 (△), 0.4 (□) or 0.8 (▽) mM AU-1421 was added. Then, 10 s later, the phosphorylation was started by adding 0.1 ml of 0.1 mM [^{32}P]ATP containing 10 mM $MgCl_2$ and 10 mM $CaCl_2$.

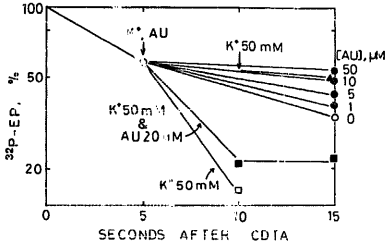

Fig. 3. Inactivation by AU- 421 in the hydrolysis of K^+-sensitive phosphoenzyme of H^+/K^+-ATPase. Washed H^+/K^+-ATPase preparation (0.1 mg) suspended in 0.8 ml of 32 mM histidine containing 0.625 mM $MgCl_2$ was phosphorylated with 0.1 ml of 0.1 mM [^{32}P]ATP at 0°C. After 20 s, 0.1 ml of 100 mM CDTA-Tris was added (this is zero time in the figure) to follow the splitting of the phosphoenzyme (○). A further 5 s later, 0.1 ml of AU-1421 (●), KCl (□) or both (■) was added to give the final concentrations indicated. After adding 10 μM AU-1421, 0.1 ml of 600 mM KCl was further added (▲) to see if K^+ could release the AU-1421 inhibition.

specific and potent inhibitor for H^+/K^+-ATPase, SCH 28080, has also been shown to inhibit the E_2P state of the enzyme [2,16].

The E_2P state of Ca^{2+}-ATPase and that of K^+-transporting ATPase have a K^+ binding site near the surface of the enzyme molecule. Particularly in the latter case, interaction of the binding site with the ligand should induce some motion of the binding site, resulting in translocation of the bound ligand into an ion pathway (or occlusion center) located in an interior domain of the enzyme. Since AU-1421 is larger than K^+, it may interfere with this motion.

A similar contrast between Ca^{2+}-ATPase and Na^+/K^+-ATPase has been observed previously with respect to hydrophilic amines, such as Tris [17], although they are much weaker effectors than AU-1421. E_2P splitting of the Ca^{2+}-ATPase was slightly accelerated by Tris at certain concentrations, whereas that of Na^+/K^+-ATPase was inhibited. The concentration range of Tris used in those experiments were of the order of millimolar to hundreds of millimolar.

When 20 μM AU-1421 was added to E_2P of the H^+/K^+-ATPase together with 50 mM KCl, acceleration of the hydrolysis was scarcely inhibited (Fig. 3). K^+ appeared to prevent AU-1421 inhibition. This is consistent with the results obtained for Na^+/K^+-ATPase, in which AU-1421 simply competed with K^+ [8,9]. K^+ and AU-1421 also competed in the overall ATP-hydrolysis (at 37°C) by both Na^+/K^+- and H^+/K^+-ATPase [5–7].

When K^+ was added afterwards to the E_2P of H^+/K^+-ATPase inhibited by AU-1421, K^+ did not restore the reactivity of E_2P (Fig. 3). Dissociation of AU-1421 from E_2P seemed to be slow under these conditions. This is similar to the mode of inhibition of E_2P by SCH 28080 [16].

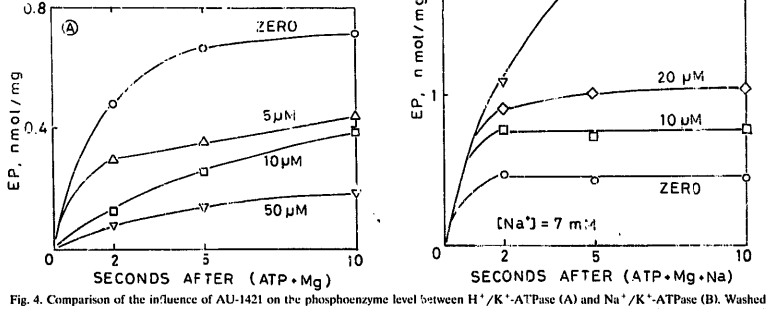

Fig. 4. Comparison of the influence of AU-1421 on the phosphoenzyme level between H^+/K^+-ATPase (A) and Na^+/K^+-ATPase (B). Washed membrane preparations (0.1 mg) were suspended in 0.8 ml of 32 mM histidine at 0°C, then 0.1 ml of 0 (○), 50 (△), 100 (□), 200 (◇) or 500 (▽) μM AU-1421 was added. The phosphorylation was initiated at 10 s later by adding 0.1 ml of 0.1 mM [^{32}P]ATP containing 5 mM $MgCl_2$ (A) or containing 5 mM $MgCl_2$ and 50 mM NaCl (B).

Difference between H^+/K^+-ATPase and Na^+/K^+-ATPase

In comparison with Na^+/K^+-ATPase, H^+/K^+-ATPase was easily attacked by AU-1421, in the non-phosphorylated form.

When the phosphorylation of H^+/K^+-ATPase was performed after preincubation with AU-1421 for 10 s at 0°C in the absence of inorganic cation, the level of the phosphoenzyme was decreased to half by the presence of 10 μM AU-1421 (Fig. 4A). Probably AU-1421 interacted with the K^+-site of the non-phosphorylated H^+/K^+-ATPase [5] to stabilize the enzyme in the K^+-induced conformation, E_2, which has low affinity for ATP.

In fact, when H^+/K^+-ATPase was digested with trypsin in the presence of AU-1421, and the peptide fragments produced were observed by gel electrophoresis [14], the pattern of the fragments on the gel was similar to that obtained after digestion of the enzyme in the presence of KCl (E_2 form), and different from that obtained in the presence of ATP (E_1 form) (Fig. 5).

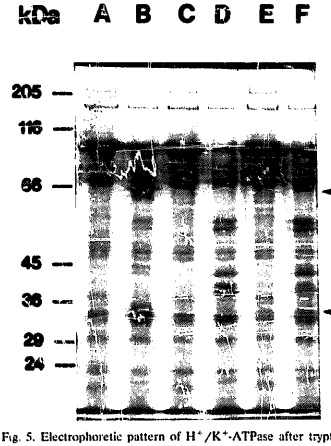

Fig. 5. Electrophoretic pattern of H^+/K^+-ATPase after tryptic digestion. Gastric vesicles (2 mg/ml) were incubated with 40 μg/ml TPCK-trypsin at 37°C for 0 (lanes A, C, E) or 5 min (lanes B, D, F) in 200 μl of 10 mM Pipes-Tris (pM 7.4) containing 1 M glycerol, 1 mM EDTA and 250 mM sucrose in the presence of 1 mM ATP (lanes A, B), 1 mM KCl (lanes C, D), or 10 μM AU-1421 (lanes E, F). Each sample was incubated with 2% SDS and 2-mercaptoethanol at 37°C for 5 min, and aliquots (45 μg protein) were subjected to SDS-polyacrylamide (10%) gel. K^+-specific fragments of 56 and 42 kDa and ATP-specific fragments of 67 and 35 kDa are indicated by arrows.

On the other hand, AU-1421 did not occupy the K^+-site of the non-phosphorylated Na^+/K^+-ATPase during a brief preincubation for 10 s at 0°C. Little influence was observed on the rate of phosphorylation of Na^+/K^+-ATPase. So, in the presence of 7 mM NaCl, accumulation of E_2P occurred owing to the stabilization induced by AU-1421 (Fig. 4B). At a high concentration of NaCl, 1 M, K^+-insensitive E_1P is formed slowly (75% of the total phosphoenzyme was E_1P at the steady state) [18], and the effect of AU-1421 was much less (not shown, but see Fig. 1 of Ref. 9). Inhibition of the phosphorylation of Na^+/K^+-ATPase by AU-1421 required a longer incubation period (more than several min) at 37°C with the drug [9].

This is consistent with the previous results [19] showing a difference between H^+/K^+- and Na^+/K^+-ATPase with respect to the acceptance of AU-1421 in the non-phosphorylated form (Figs. 4A and B). Compared with the K^+-site of H^+/K^+-ATPase, the K^+-site of Na^+/K^+-ATPase seems to present a greater barrier against access to the K^+-occlusion center from the aqueous phase [19,20]. Identification of the K^+-occluded form of H^+/K^+-ATPase is less well documented than that of Na^+/K^+-ATPase [2].

Conclusion

K^+ is the only ion shared by the three transport ATPases studied in the present experiments, and binding to their E_2P is the main common action of K^+. As AU-1421 possessed higher affinity than K^+ for all of these E_2Ps, we thought that AU-1421 interacted with the K^+-sites of the phosphoenzyme, and we did not positively conclude that AU-1421 exerted an allosteric action on E_2P at a site different from that for K^+. The K^+-site of these ATPases can accept a variety of ligands [2,7,15], and they appear to open toward the aqueous phase outside the enzyme molecule in the E_2P state, making it apparently easy for AU-1421 to bind [7,9]. Subsequent motion of the E_2Ps differs depending upon the movement of K^+; when K^+ is a simple activator for E_2P splitting from one side of the membrane, AU-1421 is an activator (Fig. 1), but when K^+ is translocated through a pathway in the enzyme, AU-1421 restricts the motion of the enzyme effecting the inhibition of E_2P splitting (Fig. 3 and Fig. 3 of Ref. 9).

AU-1421 inhibited the phosphorylation by ATP of these ATPases (Figs. 2 and 4A, and Ref. 9). This is consistent with the idea that AU-1421 binds to the K^+-site to stabilize the E_2 state (Fig. 5). Occupation of the K^+-occlusion center of Na^+/K^+-ATPase by AU-1421 particularly required incubation for several minutes or longer at 37°C [7,9]. Otherwise, only stabiliza-

tion of the formed E_2P was observed (Fig. 4B). With respect to the K^+-binding site of Ca^{2+}- or H^+/K^+-ATPases, occupation by AU-1421 was accomplished during incubation for several seconds at 0°C. This difference (Figs. 4A and B) is though to reflect the degree of isolation of the K^+-site of the non-phosphorylated enzyme from the aqueous phase.

Acknowledgements

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