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STUDIES ON THE MECHANISM OF ACTION OF A80915A, A SEMI-NAPHTHOQUINONE NATURAL PRODUCT, AS AN INHIBITOR OF GASTRIC (H^+-K^+)-ATPase*

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Abstract—A semi-naphthoquinone natural product, A80915A, produced by *Streptomyces aculeolatus* was found to be a potent inhibitor of gastric (H^+-K^+)-ATPase, the enzyme responsible for acid secretion in the stomach. Enzyme activity was measured by potassium-stimulated hydrolysis of ATP or *p*-nitrophenolphosphate with enzyme prepared from the stomach fundic mucosa of pigs. Concentration-dependent inhibition was observed with an IC_{50} of about 2–3 μM for both ATPase and *p*-nitrophenylphosphatase. A Hill plot indicated that the enzyme has two binding sites for A80915A. Inhibition was not affected by the presence of the reducing agent dithiothreitol, indicating a lack of involvement of enzyme sulfhydryl groups. A 30-min incubation of enzyme with increasing drug concentrations followed by a 10-fold dilution did not alter the IC_{50}, indicating that A80915A does not covalently modify the enzyme. Coincubation of enzyme with 3.8 μM A80915A resulted in time-dependent inhibition. The rate of inhibition was slowed significantly by the presence of 20 mM potassium, rubidium and ammonium but not by 20 mM sodium, lithium and choline, or by 40 mM sucrose. The level of inhibition was influenced by the order of addition of potassium and drug to the enzyme. Taken together, these studies indicate that inhibition by A80915A is dependent on the conformation of gastric (H^+-K^+)-ATPase and that potassium slows the rate of inhibition by converting the enzyme to a conformation where the drug binding site is not as accessible. The mode of action of A80915A is distinct from that of two well characterized proton pump inhibitors, omeprazole and SCH 28080.

Acid secretion is mediated by gastric (H^+-K^+)-ATPase, a membrane-bound protein located on the luminal side of the parietal cell of the stomach fundic mucosa [1–4]. The gastric (H^+-K^+)-ATPase hydrolyzes ATP to exchange intracellular protons for luminal potassium ions in equal stoichiometry. The enzyme maintains a large proton gradient (10^6-fold) across the stomach mucosa and has been referred to as the "proton pump". Due to the critical role of the enzyme in acid secretion, the development of compounds that block the function of the enzyme has been pursued as an effective method for controlling acid secretion [2, 5, 6].

Three classes of proton pump inhibitors have been identified. The first class is substituted benzimidazole sulfoxides for which omeprazole‡ (Fig. 1) is the prototype and the first clinically useful drug [7–13]. Omeprazole is a prodrug that is transformed at low pH conditions to a more reactive species, a sulfenamide, that reacts with thiol groups on gastric (H^+-K^+)-ATPase [9, 14–16]. Consequently, omeprazole is an irreversible inhibitor that covalently modifies the enzyme by formation of a disulfide bond with cysteine residues. A second class of inhibitors is a group of compounds that contain a protonatable amine and includes SCH 28080 (Fig. 1), which is the best studied, and substituted 2-guanidinothiazoles and certain calcium channel antagonists such as verapamil and diltiazem [17–20]. SCH 28080 inhibits gastric (H^+-K^+)-ATPase by a reversible mechanism, and competes for a high affinity potassium site located on the luminal side of the enzyme [21–25]. Recently, a third class of inhibitors has been described which are polypeptides, such as melittin, a 26-amino acid helical polypeptide isolated from bee venom [26, 27], and the pumilacidins, cyclic acylheptapeptide antibiotics, produced by *Bacillus pumilus* [28]. Inhibition of gastric (H^+-K^+)-ATPase by melittin is non-covalent. The binding site is distinct from that of SCH 28080 and is located on the cytosolic portion of the enzyme. The melittin binding site has been proposed as a regulatory site for peptides to modulate enzymatic activity [27]. The mechanism of action of pumilacidins has not been reported.

During the pharmacological evaluation of a new

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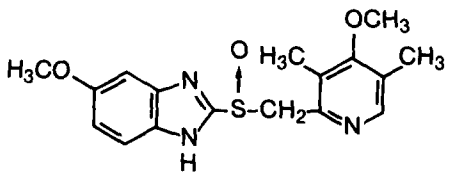
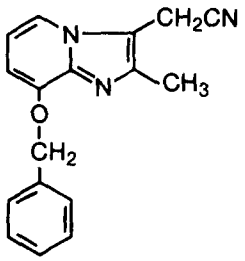
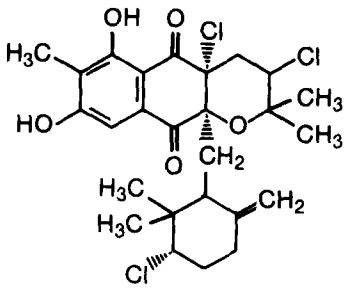
‡ Abbreviations: omeprazole, 5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl]-1*H*-benzimidazole; SCH 28080, (3-cyanomethyl-2-8-phenylmethoxy)imidazo[1,2-*a*]pyridine; A80915A, 3,4a-dichloro-3,4,4a,10a-tetrahydro-6,8-dihydroxy-2,2,7-trimethyl-10a-[(2,2-dimethyl-3-chloro-6-methylenecyclohexyl)methyl]-2*H*-naphtho[2,3,*b*]pryan-5,10-dione; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid]; MES, 2-[*N*-morpholino]ethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; (H^+-K^+)-ATPase, magnesium-dependent, hydrogen-ion transporting, and potassium-stimulated adenosine triphosphatase (EC 3.6.1.36); p-NPP, *p*-nitrophenylphosphate; p-NPPase, *p*-nitrophenylphosphatase; p-NP, *p*-nitrophenol; and FITC, fluorescein isothiocyanate.



Omeprazole



SCH 28080



A80915A

Fig. 1. Structures of omeprazole, SCH 28080, and A80915A.

semi-naphthoquinone antibiotic, A80915A (Fig. 1) was determined to be a potent inhibitor of pig gastric (H^+-K^+)-ATPase. This natural product is a low molecular weight metabolite produced in the fermentation broth of *Streptomyces aculeolatus* [29]. The structure of A80915A is chemically distinct from that of benzimidazoles and polypeptide agents and contains no protonatable amine. The present studies examined the mechanism of action of A80915A as an inhibitor of gastric (H^+-K^+)-ATPase.

MATERIALS AND METHODS

Reagents. SCH 28080 was provided by the Schering Corp. (Kenilworth, NJ); omeprazole was a gift of A. B. Hässle Laboratories (Mölndal, Sweden). A80915A (molecular weight of 529.9) was isolated as previously described [29]. Nigericin was obtained from Eli Lilly and Co. (Indianapolis, IN). *p*-Nitrophenol (p-NP) was purchased from the Eastman Kodak Co. (Rochester, NY). Porcine kidney Type IV γ-glutamyltransferase (EC 2.3.2.2) and porcine intestinal mucosal Type IV alkaline phosphatase (EC 3.1.3.1), the indicated diagnostic kits, and other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO).

Preparation of gastric membrane vesicles. The stomachs of freshly slaughtered pigs were obtained from a slaughterhouse in Anderson, IN. Stomachs were transported on ice, immersed in ice-cold buffer containing 250 mM sucrose and 50 mM HEPES–TRIS, pH 7.2. Membrane vesicles were prepared according to the procedure of Skrabanja *et al.* [30]. The pellet obtained after centrifugation at 39,000 rpm was resuspended with a 25 gauge needle syringe and frozen overnight at −70°. The following day the suspension was thawed and layered on top of a 40% sucrose solution (w/v) and centrifuged for 2 hr at 40,000 rpm. The opalescent band at the interface was removed with a Wintrobe tube syringe cannula, frozen in 0.3-mL aliquots, and stored at −70°. The resulting preparation was assayed for K^+-stimulated ATPase or K^+-stimulated *p*-nitrophenylphosphatase (p-NPPase) activity as described below. The enzyme preparation contained no detectable ouabain-sensitive (Na^+-K^+)-ATPase or mitochondrial oligomycin- or azide-sensitive (H^+)-ATPase. (H^+-K^+)-ATPase activity was enhanced about 2-fold by the presence of potassium and nigericin (10 μg/mL) in the assay buffer. p-NPPase activity was stimulated 14.5-fold by the presence of potassium in the assay buffer.

(H^+-K^+)-*ATPase activity.* Enzyme activity was measured at 30° in a 96-well microtiter dish in a total assay volume of 260 μL. Hydrolysis of ATP was followed by measuring the production of inorganic phosphate. The assay mixture contained about 1 μg protein of enzyme preparation, 2 mM ATP, 32 mM HEPES, pH 7.4, 2 mM $MgSO_4$, 5.3 mM NaN_3, 10 μg/mL of nigericin in the absence and presence of 20 mM KCl. After a 30-min incubation, the assay was terminated with the addition of a phosphate detection solution [31]. Samples were read at 690 nm in a Titertek Multiskan MCC/340 microtiter dish reader. The instrument was blanked on wells containing the assay mixture without enzyme. The rate of formation of inorganic phosphate was linear over the assay time period. The amount of inorganic phosphate was determined from the linear portion of a standard curve of inorganic phosphate.

K^+-*stimulated p-NPPase activity.* Enzyme activity was measured at 37° in a 96-well microtiter dish in a total assay volume of 250 μL following the hydrolysis of the artificial substrate, *p*-nitrophenylphosphate (p-NPP). The assay mixture contained about 1 μg protein of enzyme preparation, 5 mM p-NPP, 5 mM $MgSO_4$, 5 mM NaN_3, and 30 mM HEPES, pH 6.9, in the presence and absence of 20 mM KCl. The assay was terminated after 60 min by the addition of 50 μL of 5 N sodium hydroxide. The samples were read at 405 nm on a Titertek Multiskan MCC/340 microtiter dish reader. The reader was blanked on wells containing assay mixture lacking enzyme to correct for any absorption due to p-NPP or non-enzymatic hydrolysis of p-NPP. The amount of p-NP formed was determined from the linear portion of a standard curve of p-NP. K^+-stimulated p-NPPase activity is the difference between the activity measured in the presence of 20 mM KCl and in the absence of KCl. Initial studies indicated that the rate of p-NP formation was linear over the 60-min assay time period. In studies where the effect of

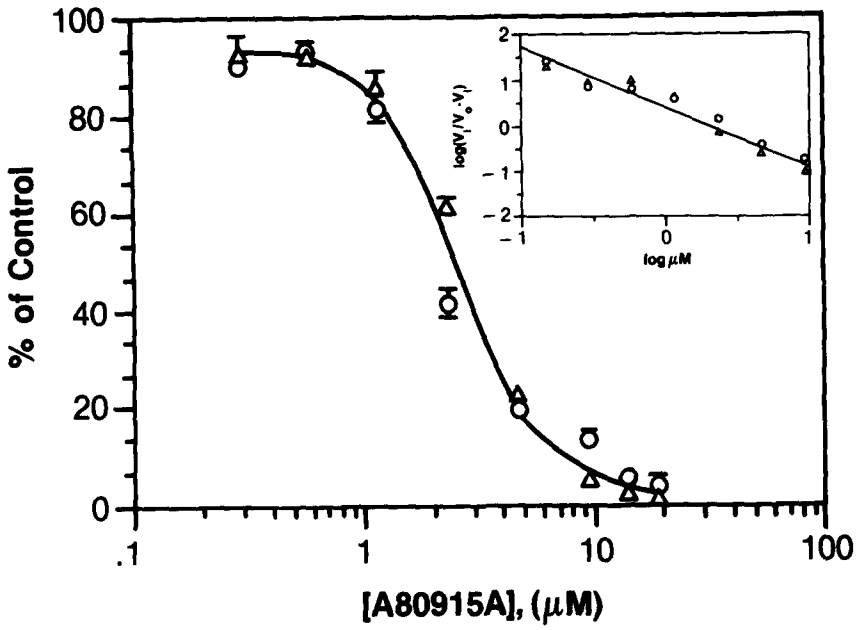


Fig. 2. Effect of A80915A on enzyme activity. Enzyme was incubated for 30 min at 37° with 1.5 to 95 μM A80915A, diluted 5-fold, and assayed for either (H^+-K^+)-ATPase activity in the presence of 10 μg/mL nigericin (△) or K^+-stimulated p-NPPase (○) activity as described in Materials and Methods. The control activity for (H^+-K^+)-ATPase was 0.48 μmol P_i formed/min/mg protein and for K^+-stimulated p-NPPase 0.57 μmol p-NP formed/min/mg protein. Values are means ± SD (N = 4). The final drug concentration present in the assay mixture is given on the abscissa. Inset: a Hill plot of these data. The line was drawn by the method of least squares (correlation coefficient = 0.95). The slope was 1.2 to 1.3 for (H^+-K^+)-ATPase and K^+-stimulated p-NPPase.

cations or sucrose on activity was examined, a final concentration of 19.2 mM was present for the indicated cation or 38.4 mM sucrose.

Coincubation of enzyme with drug. Drugs were dissolved in dimethyl sulfoxide (DMSO) and fresh solutions were prepared daily. In most experiments gastric (H^+-K^+)-ATPase was coincubated with SCH 28080 and A80915A for 20–30 min with enzyme in 30 mM HEPES, pH 6.9, containing 5 mM $MgSO_4$ and 5 mM NaN_3 and in the absence of potassium or other cations unless indicated otherwise. Since omeprazole is a prodrug and must be activated under acidic conditions, the enzyme was coincubated for 30 min at 37° with omeprazole in 5 mM MES, pH 5.8, containing 5 mM $MgSO_4$ and 5 mM NaN_3. Afterwards, the pH was adjusted to a final pH of 6.9 with HEPES. Unless otherwise noted, the enzyme was added last to the incubation mixture. Enzyme activity was measured either by addition of concentrated stocks of ATP, p-NPP, or KCl to yield the final concentration indicated above resulting in 1.04-fold dilution of the preincubation enzyme or by dilution of the preincubated enzyme 5- to 10-fold into the assay mixture containing the substrate and potassium.

γ-Glutamyltransferase. Porcine kidney γ-glutamyltransferase was preincubated in the absence and presence of 2–37 μM A80915A for 30 min at 37°. Enzyme activity was measured for 20 min at 37° with a γ-glutamyltransferase Sigma diagnostic kit (Procedure No. 545). The assay was conducted using one-half of all recommended volumes. Each assay tube contained 0.0165 enzyme units. After color development, 200-μL aliquots were transferred to wells in a microtiter dish and read at 540 nm with a Titertek Multiskan MCC/340 microtiter dish reader. Samples were assayed in quadruplicate.

Alkaline phosphatase. Porcine intestinal alkaline phosphatase was incubated in the absence and presence of 6–37 μM A80915A for 30 min at 37° in a 96-well microtiter dish. Alkaline phosphatase activity was measured using the Sigma alkaline phosphatase diagnostic kit (Procedure No. DG 1245). A 5-min time course was run with each sample. Each sample was run in triplicate and product formation was determined at 2-, 3-, 4- and 5-min time points. Samples were read at 405 nm on a Titertek Multiskan MCC/340 microtiter dish reader. The amount of p-NP formed was determined from the linear portion of a standard curve of p-NP. The rate of p-NP formation was 4.35 μmol/min/mg.

Protein determination. Protein was determined by the method of Lowry *et al.* [32].

RESULTS

Effect of A80915A on enzyme activity. In the initial evaluation of A80915A as a potential enzyme inhibitor, the activities of three membrane-associated enzymes were examined. The presence of 37 μM A80915A had no effect on the activities of alkaline phosphatase or γ-glutamyltransferase whereas lower

Table 1. Effect of dithiothreitol on inhibition

Compound*	% Inhibition†	
	−DTT	+DTT
Omeprazole (5.8 μM)	94 ± 3	1 ± 8
SCH 28080 (18 μM)	66 ± 3	72 ± 1
A80915A (3.8 μM)	96 ± 2	99 ± 3

* Enzyme was incubated for 30 min with either SCH 28080 or A80915A in 30 mM HEPES, pH 6.9 (or 5 mM MES, pH 5.8, for omeprazole) in the presence or absence of 5 mM DTT, diluted 5-fold, and K^+-stimulated p-NPPase activity assayed for 60 min. The indicated drug concentration was the final concentration in the assay mixture.

† The control enzyme activity was 0.83 μmol p-NP formed/min/mg protein after incubation at pH 6.9 in the absence of DTT, A80915A and SCH 28080. Enzyme activity was 0.32 μmol p-NP formed/min/mg protein after incubation at pH 5.8 in the absence of omeprazole and DTT. Values are means ± SD (N = 4).

concentrations inhibited the activity of pig gastric (H^+-K^+)-ATPase (data not shown). To further examine the effect of A80915A on gastric (H^+-K^+)-ATPase, the enzyme was coincubated with increasing concentrations of inhibitor prior to measuring enzyme activity by K^+-stimulated hydrolysis of the natural substrate ATP or of the artifical substrate, p-NPP. For measuring (H^+-K^+)-ATPase activity, the ionophore nigericin (which exchanges H^+ and K^+) was added to the assay buffer to provide potassium to the luminal potassium-site of the enzyme [33]. In contrast, K^+-stimulated p-NPPase is not dependent on potassium on the luminal side of the enzyme and is not stimulated by the presence of the ionophore [34] and nigericin was not present during the assay. Figure 2 illustrates that a concentration-dependent inhibition of enzyme activity was measured with both substrates; the apparent IC_{50} values of the K^+-stimulated ATPase and K^+-stimulated p-NPPase activities were similar, about 2 μM. A Hill plot of these data (inset, Fig. 2) indicated that the Hill coefficient was 1.2 to 1.3, suggesting the presence of two binding sites. The experiments described below were conducted to further examine A80915A as a proton pump inhibitor. Enzymatic activity was monitored by measuring K^+-stimulated p-NPPase activity.

Involvement of sulfhydryl groups. Next, the effect of the presence of a thiol-reducing agent, dithiothreitol (DTT), on the inhibition of (H^+-K^+)-ATPase by A80915A, omeprazole, and SCH 28080 was examined. Table 1 shows that all three drugs inhibited K^+-stimulated p-NPPase activity from 66 to 96% in the absence of reducing agent. However, omeprazole in the presence of DTT in the incubation mixture had little effect on enzyme activity, consistent with it binding to thiol-groups on the enzyme [14]. In contrast, inhibition by A80915A or SCH 28080 was unaltered (72–99% inhibition) by the presence of DTT in the incubation mixture.

Effect of dilution on enzyme inhibition. To further elucidate the mode of action of A80915A, studies were conducted to ascertain whether the compound binds irreversibly to the enzyme. The IC_{50} was determined by two procedures. (a) The enzyme was incubated for 30 min with increasing drug concentrations from 0.2 to 30 μM and then K^+-stimulated p-NPPase activity was measured. (b) Alternatively, the enzyme was incubated for 30 min with a 10-fold higher drug concentration ranging from 2 to 300 μM and was diluted subsequently 10-fold prior to assaying K^+-stimulated p-NPPase activity. The two protocols should give the same IC_{50} if the compound reversibly inhibits the enzyme since the final drug concentration range in the assay mixture is identical. However, if the drug binds irreversibly to the enzyme, then the enzyme would be exposed to much higher concentrations during the 30-min preincubation, so the IC_{50} after dilution would appear to be shifted 10-fold. When enzyme inhibition by omeprazole was examined, the IC_{50} shifted approximately 11-fold from 4 to 0.3 μM, consistent with its irreversible mode of action (Fig. 3A) [14]. By contrast the IC_{50} values of SCH 28080 and A80915A were about 1.8 μM (Fig. 3B) and 1.5 to 2.0 μM (Fig. 3C), respectively, under both incubation conditions. Thus, A80915A, unlike omeprazole, does not covalently modify the enzyme and does reversibly inhibit the enzyme similar to SCH 28080 [35].

Time-dependence of enzyme inhibition by A80915A. Next, pig gastric (H^+-K^+)-ATPase was incubated with a 3.8 μM concentration of the natural product in the presence and absence of potassium for increasing time intervals prior to being assayed for activity. The top curves of Fig. 4 illustrate that there was little to no reduction in enzyme activity when enzyme was incubated at 37° without inhibitor in the presence and absence of 20 mM KCl. By contrast, the bottom curves of Fig. 4 show that the addition of 3.8 μM A80915A resulted in a time-dependent inhibition of K^+-stimulated p-NPPase activity. In the absence of potassium, enzyme inhibition was nearly complete (about 93%) by 20 min. The rate of inhibition by A80915A, however, was slowed by the presence of potassium. Enzyme activity was inhibited about 90% within 10 min in the absence of potassium and within 20–40 min in the presence of potassium.

Effect of potassium and other cations on enzyme inhibiton. To determine if the effect of potassium observed in Fig. 4 may be due to an osmotic effect on the gastric vesicles rather than an effect on gastric (H^+-K^+)-ATPase itself, the effect of the presence of several cations and sucrose on drug inhibition was examined. Enzyme activity was determined after a fixed time exposure (20 min) of the enzyme to drug during which time potassium protects the enzyme from inhibition, as demonstrated previously (Fig. 4). As illustrated in Fig. 5, a 20-min coincubation of the enzyme with 3.8 μM A80915A in the presence of 20 mM potassium, rubidium, and ammonium partially protected the enzyme from inhibition (99% inhibition of the control versus 73, 87 and 90%, respectively, in the presence of potassium, rubidium, and ammonium), whereas coincubation with 20 mM sodium, lithium, choline, or 40 mM sucrose had no effect (96–98%). Inhibition by A80915A during a

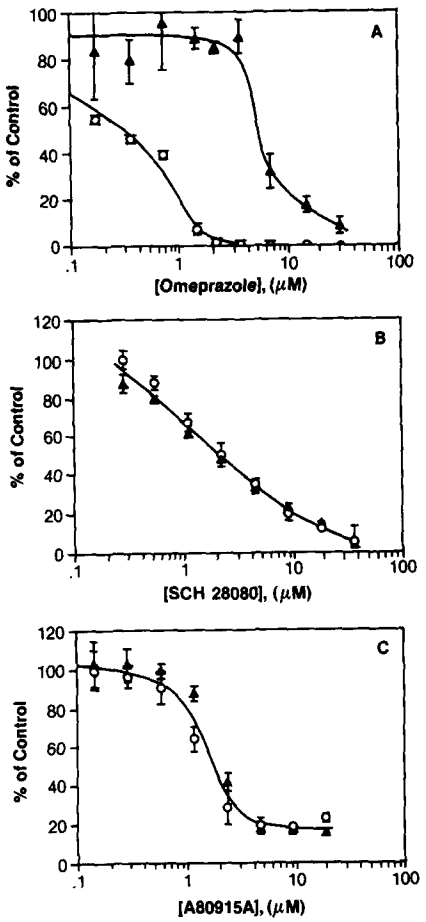


Fig. 3. Effect of dilution on enzyme inhibition by A80915A. The effect of a 10-fold dilution on inhibition of gastric (H^+-K^+)-ATPase by omeprazole, SCH 28080 or A80915A was determined after a 30-min incubation of enzyme with drug in the absence of potassium. Two procedures were used. (a) Enzyme (0.9 μg protein) was incubated for 30 min at 37° with the indicated drug concentrations ranging from 0.2 to 30 μM (▲), prior to measuring K^+-stimulated p-NPPase activity without dilution. (b) Alternatively, enzyme (9.0 μg protein) was incubated for 30 min at 37° with 10-fold higher drug concentrations (2–300 μM), followed by a 10-fold dilution of this incubation mixture (○) before assaying K^+-stimulated p-NPPase activity. For omeprazole, at the end of the 30-min incubation the pH was adjusted from 5.8 to 6.9 with a concentrated stock of HEPES to give a final concentration of 30 mM. The abscissa indicates the final drug concentration present during the assay. Values for inhibition by omeprazole (panel A) are means ± range (N = 2), for inhibition by SCH 28080 (panel B) means ± range (N = 2), and for inhibition by A80915A (panel C) means ± SD (N = 3–4). The control K^+-stimulated p-NPPase activity was 0.70, 0.75, and 0.43 μmol p-NP formed/min/mg protein for panels A, B, and C, respectively.

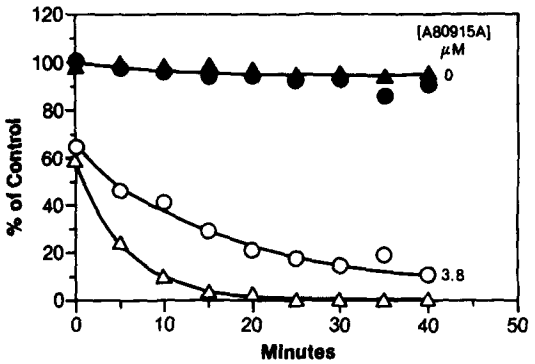


Fig. 4. Time-dependence of enzyme inhibition by A80915A and the effect of potassium. In the lower two curves, enzyme was coincubated with 3.8 μM A80915A for 0–40 min at 37° in the presence (○) and absence (△) of 20 mM KCl. In the upper two curves, enzyme was incubated without inhibitor for 0–40 min in the presence (●) and absence (▲) of 20 mM KCl. Afterwards, the final concentration of KCl was adjusted to 20 mM and K^+-stimulated p-NPPase activity was measured for 60 min. Values are means ± range (N = 2). Without incubation and in the absence of the inhibitor, p-NPPase activity was 0.51 μmol p-NP formed/min/mg protein. In a separate experiment, K^+-independent p-NPPase activity (which was 0.044 μmol p-NP formed/min/mg protein representing 8.4% of the total p-NPPase activity of the uninhibited control) remained constant throughout a 40-min pre-incubation time course (data not shown). In the presence of inhibitor (without preincubation) the rate of p-NP formation was nearly linear over the 60-min assay period ($r^2 = 0.92$; data not shown). The curves shown are representative of three independent experiments.

10-min preincubation was also reduced by potassium, rubidium and ammonium (84% in the absence of cation versus 64, 77 and 78%, respectively, in the presence of potassium, rubidium, and ammonium) (data not shown). Thus, the diminished inhibition by A80915A in the presence of potassium observed in Figs. 4 and 5 is not due to a change in osmolarity.

Sequence of addition of potassium and the inhibitor. Next, the order of addition of inhibitor and potassium to the incubation mixture was examined. Table 2 summarizes a study in which gastric (H^+-K^+)-ATPase was incubated 20 min with either 3.8 μM A80915A or 20 mM potassium, followed by an additional 20-min incubation with 20 mM potassium or inhibitor prior to measuring K^+-stimulated p-NPPase activity. In the control, a 20-min incubation of enzyme with A80915A resulted in almost complete (86%) inhibition of enzyme activity (Incubation 1, Table 2). As previously illustrated in Figs. 4 and 5, incubation of the enzyme with K^+ reduced drug inhibition from the control value of 86% to 42.8 to 56.4% whether K^+ was added simultaneously with A80915A for either a 20-min or a 40-min incubation (Incubations 2 and 3 of Table 2) or whether the enzyme was preexposed to potassium for 20 min prior to A80915A addition (42.8%, Incubation 4 of Table 2). In contrast, if the pump was initially exposed for 20 min to drug, and 20 mM K^+ was added for an additional 20-min incubation, potassium had no effect on inhibition of gastric (H^+-K^+)-ATPase by A80915A (83.4%, Incubation 5 versus the control 85.9%, Incubation 1). Thus, the effect of potassium is to slow the rate of inhibition.

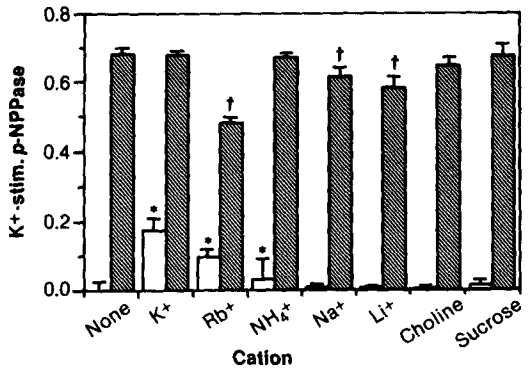


Fig. 5. Effect of the presence of cations or sucrose on inhibition by A80915A. Enzyme was incubated for 20 min at 37° with 3.8 μM A80915A in the presence of a 20 mM concentration of the chloride salts of potassium, rubidium, ammonium, sodium, lithium, choline, or 40 mM sucrose. Subsequently, enzyme activity was assayed by the addition of potassium and p-NPP to all assay conditions (open bars). Alternatively, enzyme was incubated for 20 min in the absence of A80915A with each cation or sucrose and then assayed for K^+-stimulated p-NPPase activity (stripped bars). Values are means ± SD (N = 3–4) and were corrected for p-NPPase activity measured in the absence of potassium and the indicated cation or sucrose. The units of K^+-stimulated p-NPPase activity indicated on the ordinate are μmol p-NP formed/min/mg protein. Data were analyzed by a Student's *t*-test. An asterisk (*) indicates that inhibition by A80915A in the presence of the indicated cation during the coincubation was significantly different ($P < 0.05$) from inhibition obtained in the absence of the cation. A dagger (†) indicates that enzyme activity measured in the presence of the indicated cation without drug was significantly different ($P < 0.05$) from activity obtained in the absence of the cation and drug. The control enzyme activity measured without the addition of cations or drug was 0.68 μmol p-NP formed/min/mg protein (N = 4).

Table 2. Order of addition of potassium and A80915A to (H^+-K^+)-ATPase

Incubation*	Addition†	% Inhibition‡
(1) E	I	85.9 ± 1.0
(2) E	K^+, I	45.0 ± 7.4§
(3) E, K^+, I	None	56.4 ± 5.9§
(4) E, K^+	I	42.8 ± 3.2§
(5) E, I	K^+	83.4 ± 1.6‖

* Enzyme (E) was incubated for 20 min at 37° with and without 20 mM K^+ and/or 3.8 μM A80915A (I) as indicated.

† K^+ (20 mM) and/or A80915A (3.8 μM) was added to the incubation mixture containing the enzyme. After incubation for an additional 20 min, K^+-stimulated p-NPPase activity was measured in the presence of 20 mM K^+ (final concentration) as described in Materials and Methods.

‡ Percent inhibition was calculated based on enzyme incubated for 40 min at 37° in the absence of A80915A and K^+ and assayed by the addition of 20 mM K^+ and p-NPP. The control K^+-stimulated p-NPPase activity was 0.76 μmol/min/mg protein. Values are means ± SD (N = 4).

§ Significantly ($P < 0.001$) different from Incubation 1 (Student's *t*-test).

‖ Not significantly ($P > 0.5$) different from Incubation 1 (Student's *t*-test).

DISCUSSION

The present report is the first demonstration of a semi-naphthoquinone compound as an inhibitor of gastric (H^+-K^+)-ATPase. The compound A80915A had an IC_{50} of 2–3 μM that is comparable to that of omeprazole (4 μM, Fig. 3A; 2.2 μM [35]) and SCH 28080 (1.3 to 2.5 μM, Fig. 3B) [17, 21]. The mode of action of A80915A, however, was different from that of omeprazole and SCH 28080. Unlike omeprazole, inhibition by A80915A did not involve sulfhydryl groups and the enzyme was not covalently modified. In these respects the properties of A80915A are similar to those of SCH 28080. Unlike SCH 28080 [22] (data not shown), however, enzyme inhibition by A80915A was time dependent. The latency observed in inhibition may be due, in part, to the time required for A80915A to equilibrate across the membrane of the gastric vesicles. Nevertheless, the length of time required to achieve the same level of enzyme inhibition increased in the presence of potassium. Two other cations, rubidium and ammonium, that are known to substitute for potassium at the K^+-site on the enzyme [2, 36, 37] also reduced the rate of inhibition. Thus, the cation effect on inhibition by A80915A must be due to an interaction of potassium with the enzyme. Non-specific effects of A80915A on the gastric membrane vesicles, such as an osmotic effect or perturbation of the membrane phospholipid, would not be expected to result in the observed cation selectivity. Membrane perturbation would not be easily reversed by dilution and might be expected to effect the activities of the other two membrane-associated proteins examined.

Potassium is the physiologically relevant cation and plays a key role in the kinetic mechanism of the enzyme [1, 38]. Gastric (H^+-K^+)-ATPase is a member of the P-type class of plasma membrane ion-pumping ATPases that includes (Na^+-K^+)-ATPase, Ca^{2+}-ATPase, and *Neurospora crassa* (H^+)-ATPase [1–3]. During the catalytic cycle of this class of ATPases, the enzyme has two conformational states, E_1 and E_2. During ATP hydrolysis, an intermediate aspartyl-phosphoenzyme (E_1~P) is formed and the enzyme is dephosphorylated subsequently by K^+ binding to a high affinity potassium-site located on the luminal side of the proton pump resulting in the formation of the E_2 conformer [1]. Even without ATP hydrolysis or the presence of ATP, the proton pump can be converted from the E_1 to the E_2 conformation by binding K^+ [27, 36, 39]. Thus in the present studies, when the enzyme was coincubated with potassium, a conformational change in the enzyme would be expected.

In the present studies, the effect of potassium on the coincubation of enzyme with drug was to slow the rate of inhibition by A80915A since the same level of inhibition could be achieved after a longer

incubation period in the presence of potassium. One possible explanation is that A80915A binds to one conformation of the enzyme and less well—or not at all—to the other conformation that has potassium bound, perhaps due to lack of accessibility of the A80915A-binding site(s). Consequently, the presence of potassium, rubidium, or ammonium converts the uninhibited form (E_1) of the enzyme to the cation-form thereby reducing the amount of enzyme available for binding of A80915A. Thus, a change in the conformation results in the apparent reduction in the rate of inhibition in the presence of potassium. If binding of the drug is freely reversible as demonstrated by the effect of dilution (Fig. 3), the addition of potassium to the inhibited enzyme might be expected to reduce enzyme inhibition by the drug. This was not observed (Table 2, Incubation 5). This finding suggests that the drug and potassium do not bind to the same enzyme conformation. Consequently, the uninhibited enzyme exists in two conformations which interconvert in the absence of potassium and ATP. One conformation (corresponding to E_1) binds potassium; the other conformation (E_D) binds drug. The interconversion of E_D to E_1 is slow relative to the rate of drug binding, resulting in little to no effect of the presence of potassium. The binding of SCH 28080 to gastric (H^+-K^+)-ATPase in the absence of ATP is also proposed to be dependent on a conformational change of the enzyme [23].

Unlike SCH 28080, the proton pump possesses two binding sites for A80915A that exhibit cooperativity. Gastric (H^+-K^+)-ATPase is also reported to possess two binding sites for the substrates, ATP, p-NPP, and K^+ [38, 40, 41]. Whether the two binding sites for A80915A are identical in their binding properties is not known. The locations of the binding sites on the enzyme are also not known.

The mode of action of A80915A is reminiscent of that recently reported for melittin [26, 27, 42]. Melittin inhibits (H^+-K^+)-ATPase and K^+-stimulated p-NPPase activities by binding to the enzyme at a site that is distinct from the luminal potassium (SCH 28080)-binding site [27]. Studies conducted with melittin and (Na^+-K^+)-ATPase indicate that enzyme inhibition is slowly reversible and that the enzyme may be protected from inhibition by the addition of KCl or NaCl. Binding of Na^+, K^+, or melittin to fluorescein isothiocyanate (FITC)-labeled (Na^+-K^+)-ATPase results in the formation of the E_2 form as measured by fluorescence quenching [42]. Whether melittin and A80915A share a common binding site and whether the binding of A80915A to the enzyme results in a conformational change remains to be determined.

More studies are required to determine the kinetic mechanism by which A80915A inhibits the proton pump. Further elucidation of the mode of action of inhibitors such as A80915A and melittin should give greater insight into the mechanism and conformational forms of the enzyme. Such studies should aid in further defining the functional domains of gastric (H^+-K^+)-ATPase. Ultimately, new sites may be revealed for the development of novel antisecretory agents.

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