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Mechanism of action of AZD0865, a K^+-competitive inhibitor of gastric H^+,K^+-ATPase

Karin Gedda *, *Carin Briving, Karin Svensson, Ingela Maxvall, Kjell Andersson*

AstraZeneca R&D, Mölndal S-431 83, Sweden

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ABSTRACT

AZD0865 is a member of a drug class that inhibits gastric H^+,K^+-ATPase by K^+-competitive binding. The objective of these experiments was to characterize the mechanism of action, selectivity and inhibitory potency of AZD0865 in vitro. In porcine ion-leaky vesicles at pH 7.4, AZD0865 concentration-dependently inhibited K^+-stimulated H^+,K^+-ATPase activity (IC_{50} 1.0 ± 0.2 μM) but was more potent at pH 6.4 (IC_{50} 0.13 ± 0.01 μM). The IC_{50} values for a permanent cation analogue, AR-H070091, were 11 ± 1.2 μM at pH 7.4 and 16 ± 1.8 μM at pH 6.4. These results suggest that the protonated form of AZD0865 inhibits H^+,K^+-ATPase. In ion-tight vesicles, AZD0865 inhibited H^+,K^+-ATPase more potently (IC_{50} 6.9 ± 0.4 nM) than in ion-leaky vesicles, suggesting a luminal site of action. AZD0865 inhibited acid formation in histamine- or dibutyryl-cAMP-stimulated rabbit gastric glands (IC_{50} 0.28 ± 0.01 and 0.26 ± 0.003 μM, respectively). In ion-leaky vesicles at pH 7.4, AZD0865 (3 μM) immediately inhibited H^+,K^+-ATPase activity by $88 \pm 1\%$. Immediately after a 10-fold dilution H^+,K^+-ATPase inhibition was 41%, indicating reversible binding of AZD0865 to gastric H^+,K^+-ATPase. In contrast to omeprazole, AZD0865 inhibited H^+,K^+-ATPase activity in a K^+-competitive manner (K_i 46 ± 3 nM). AZD0865 inhibited the process of cation occlusion concentration-dependently (IC_{50} 1.7 ± 0.06 μM). At 100 μM, AZD0865 reduced porcine renal Na^+,K^+-ATPase activity by $9 \pm 2\%$, demonstrating a high selectivity for H^+,K^+-ATPase. Thus, AZD0865 potently, K^+-competitively, and selectively inhibits gastric H^+,K^+-ATPase activity and acid formation in vitro, with a fast onset of effect.

1. Introduction

The gastric H^+,K^+-ATPase, which is responsible for the production of gastric acid, belongs to a class of ion-translocating ATPases that are characterized by the formation of a covalently phosphorylated enzyme intermediate as part of their catalytic cycle [1]. Other enzymes in this class include Na^+,K^+-ATPase and Ca^+-ATPases. The catalytic cycle of gastric H^+,K^+-ATPase involves moving between the conformational states E_1, in which the cation-binding site faces the parietal cell cytoplasm with low affinity for K^+, and E_2, with the cation-binding site facing the canaliculus with high affinity for K^+ [2]. K^+ plays a vital part in this catalytic cycle as it is required for the dephosphorylation of the H^+,K^+-ATPase and the subsequent conformational changes.

The final step of gastric acid secretion can be inhibited by agents that are competitive with respect to K^+ binding to the parietal cell gastric H^+,K^+-ATPase [3–6]. The identity of the binding site (or sites) for these agents has not been ascertained fully, but recent mutational studies have demonstrated that the binding site is distinct from the K^+ binding site and that amino acid residues at or near the K^+ binding site are involved

* *Corresponding author*. Tel.: +46 31 776 1036; fax: +46 31 776 3761.
E-mail address: karin.gedda@astrazeneca.com (K. Gedda).

Abbreviations: PPI, proton pump inhibitor; DMSO, dimethylsulphoxide; MESG, 2-amino-6-mercapto-7-methyl-purine riboside; PNP, purine nucleoside phosphorylase

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in the binding. It is known that these agents have a luminal site of action [5–7], binding preferentially to the E_2 conformation. Recent evidence suggests the presence of a binding site in the cavity formed by the M1, −4, −5, −6, and −8 transmembrane segments and the extracellular loops formed by M5/M6, M7/M8 and M9/M10, present only in the E_2 conformation of the enzyme [8–13]. By reversibly binding to this cavity in the gastric H^+,K^+-ATPase and preventing K^+ from occupying its binding site, these agents may prevent the conformational changes necessary for ion transport, thereby inhibiting the H^+,K^+-ATPase and blocking acid secretion.

AZD0865 (8-[(2,6-dimethylbenzyl)amino]-N-[2-hydroxyethyl]-2,3-dimethylimidazo[1,2-*a*]pyridine-6-carboxamide) is a novel member of the class of compounds that inhibit gastric H^+,K^+-ATPase by K^+-competitive binding (Fig. 1A) [14]. It is a lipophilic weak base, with a pK_a value of 6.1 and a log K_d value of 4.2. Here, we report on the mechanism of action, inhibitory potency, and selectivity of AZD0865 in vitro. H^+,K^+-ATPase containing gastric membrane vesicles from pig and glands from rabbit gastric mucosa have been used as test systems to evaluate the inhibitory mechanism of the compound. The inhibitory potency of AZD0865 was evaluated at different pH values and the effect was compared to the effect of its permanent cation analogue AR-H070091 (Fig. 1B). A method to continuously measure ATPase activity was used to study the onset of inhibitory activity and the restoration of enzyme activity following washout. To study the effect of AZD0865 on the cation occlusion step, thallium was used as a K^+ cognate [15,16]. The selectivity of AZD0865 was studied using pig renal Na^+,K^+-ATPase. Finally, the effect of an active metabolite (Fig. 1C), generated at a low level in vivo, was determined in ion-leaky vesicles (pH 7.4) and in the gastric gland model.

2. Materials and methods

2.1. Materials

2.1.1. Preparation of H^+,K^+-ATPase

Ion-tight and ion-leaky membrane vesicles enriched in gastric H^+,K^+-ATPase were derived from pig stomach according to the method of Saccomani [17] with some modifications. Briefly, tissue was homogenized and a microsomal fraction was obtained by differential centrifugation. The pelleted material was separated on a discontinuous density gradient and the fraction at the interface between the 0.25 M sucrose and 0.25 M sucrose plus 7.5% Ficoll layers (i.e., the ion-tight membrane vesicle fraction) was collected, mixed with an equal volume of 60% sucrose and stored in aliquots at −70 °C. To obtain the ion-leaky membrane vesicles, the ion-tight fraction was diluted with 1 mM Pipes/Tris (pH 7.4) to give a 1% sucrose concentration, homogenized and centrifuged at 100,000 × *g* for 2 h. The

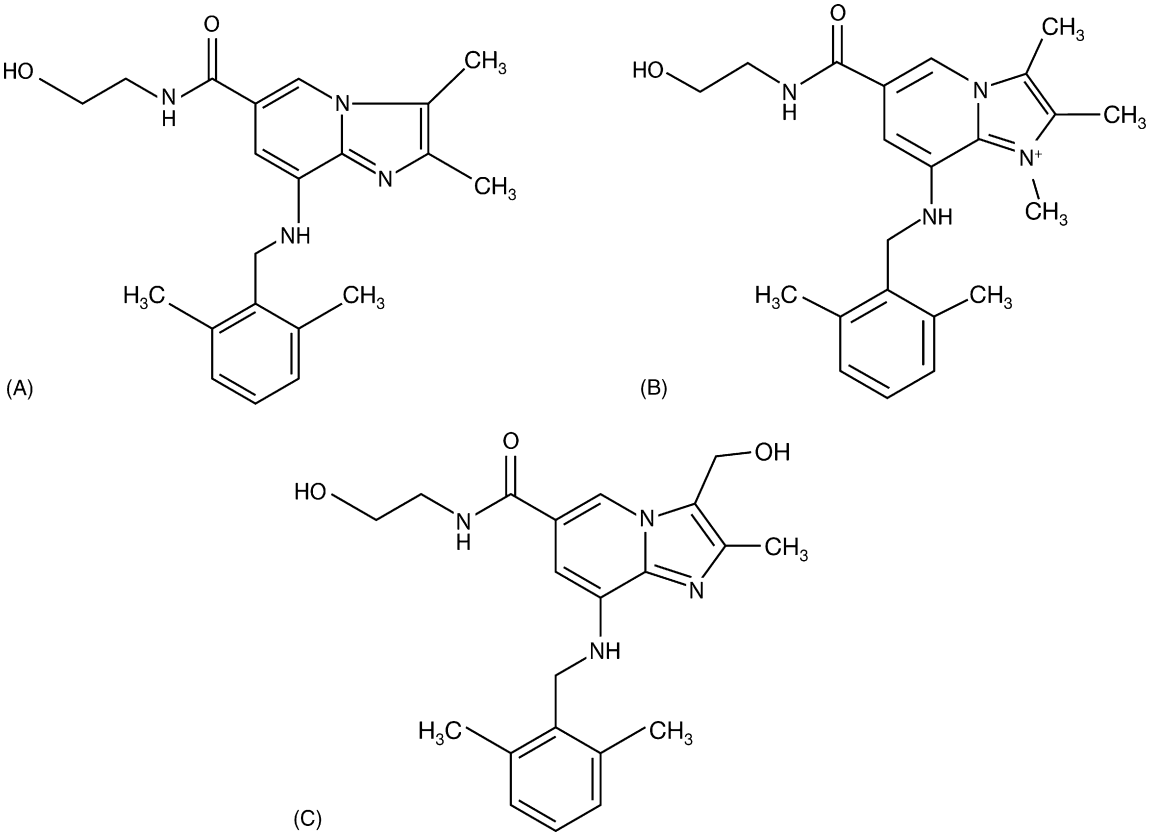

Fig. 1 – Chemical structure of AZD0865 ((8-[(2,6-dimethylbenzyl)amino]-N-(2-hydroxyethyl)-2,3-dimethylimidazo[1,2-*a*]pyridine-6-carboxamide (A), the AZD0865 permanent cation analogue AR-H070091 (8-[(2,6-dimethylbenzyl)amino]-6-{[(2-hydroxyethyl)amino]carbonyl}-1,2,3-trimethylimidazo[1,2-*a*]pyridin-1-ium) (B), and the AZD0865 metabolite AR-H044881 (8-[(2,6-dimethylbenzyl)amino]-N-(2-hydroxyethyl)-3-(hydroxymethyl)-2-methylimidazo[1,2-*a*]pyridine-6-carboxamide (C).

resulting pellet was suspended in water, lyophilised twice and stored at −70 °C prior to use.

2.1.2. Preparation of gastric glands

Gastric glands were isolated from the oxyntic mucosa of freely fed male New Zealand White rabbits, according to the method of Berglindh and Obrink [18]. Briefly, the minced gastric oxyntic mucosa was digested with collagenase at 37 °C for approximately 60 min. After digestion, the glands were rinsed three times and resuspended in assay medium containing 132.5 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1 mM $CaCl_2$, 11 mM D-glucose, 10 μM indomethacin, and 2 mg/mL albumin. The gastric glands were used immediately after the isolation procedure.

2.1.3. Preparation of Na^+,K^+-ATPase

Na^+,K^+-ATPase was prepared from fresh hog kidney red outer medulla, as described by Jorgensen [19].

2.1.4. Additional chemicals and materials

The test compounds were diluted in DMSO, unless stated otherwise, at a stock concentration of 10 mM, and serially diluted in the same solvent. The final concentration of solvent never exceeded 1% in the assay medium.

Adenosine 5′-triphosphate (ATP) disodium salt, dibutyryl cyclic adenosine monophosphate (db-cAMP) sodium salt, collagenase (EC 3.4.24.3) type IA, indomethacin, *p*-methylaminophenol sulphate (ELON), rabbit serum albumin (fraction V), valinomycin (minimum 90% HPLC) and Dowex 50X8-100 were obtained from Sigma, USA. Nα-Tosyl-L-Lysine chloro-methyl ketone (trypsin inhibitor) was obtained from Calbiochem, USA. Histamine dihydrochloride, ammonium heptamolybdate tetrahydrate, sodium fluoride and thallium(I) nitrate were from Merck, Germany. Bio-Rad protein assay and bovine γ-globulin were purchased from Bio-Rad, USA. [Dimethylamine-^{14}C]-aminopyrine was obtained from Amersham, Sweden. The $^{204}TlNO_3$ was from Isotoplaboratoriet Risø, Denmark. Scintillation cocktail, Ready Safe®, was purchased from Beckman Instruments, USA. EnzCheck™ Phosphate Assay Kit was purchased from Molecular Probes, USA. Eppendorf UVette 50–2000 μL, was from Eppendorf, Germany. All other chemicals used were commercially available and of analytical grade or of the highest purity available.

2.2. Experimental procedures

2.2.1. H^+,K^+-ATPase activity in ion-leaky gastric vesicles

Membrane vesicles (5 μg membrane protein) were incubated for 15 min at 37 °C in 1000 μL 18 mM PIPES/Tris buffer pH 7.4 or 6.4 containing 2 mM $MgCl_2$, 10 mM KCl, 2 mM ATP and increasing concentrations of AZD0865 (0.01–100 μM at pH 7.4; 0.01–10 μM at pH 6.4), AR-H070091 (0.1–100 μM), or AR-H044881 (0.01–100 μM, pH 7.4). H^+,K^+-ATPase activity was estimated by the release of inorganic phosphate, P_i, according to the method of LeBel [20]. The Mg^{2+}-ATPase activity was determined in the absence of K^+ and inhibitor and subtracted from the total ATPase activity in the presence of Mg^{2+} and K^+.

2.2.2. H^+,K^+-ATPase activity in ion-tight gastric vesicles

15 μg of protein was pre-incubated at 37 °C for 5 min in 2 mM HEPES-NaOH buffer, pH 7.4, containing 175 mM KCl, 2 mM $MgCl_2$ and increasing concentrations of AZD0865 (0.0001–1 μM) in the presence and absence of valinomycin, 1 μg/μg protein. ATPase activity was initiated by the addition of 2 mM ATP (final volume 1 mL) and inorganic phosphate (P_i) released after 20 min was determined according to the method of LeBel [20]. The difference in ATPase activity in the presence and absence of valinomycin (Δ valinomycin) was used to determine the activity from ion-tight vesicles only.

2.2.3. Inhibitory effect on acid formation in gastric glands

Acid formation in isolated gastric glands was measured by the distribution of ^{14}C-aminopyrine between the glands and the incubation medium as previously described [21]. The glands were incubated in medium with increasing concentrations of AZD0865 or AR-H044881 (0.01–100 μM) in a total volume of 1.5 mL. The medium consisted of 132.5 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1 mM $CaCl_2$, 11 mM D-glucose, 10 μM indomethacin, 2 mg/mL albumin and 0.05 μCi ^{14}C-AP (approximately 0.3 μM). The glands were stimulated with 0.1 mM histamine or 1 mM dibutyryl-cAMP and incubated for 1 h at 37 °C. Following centrifugation, the radioactivity in the gland pellet and in the supernatant was counted in a liquid scintillation counter.

2.2.4. Continuous measurements of H^+,K^+-ATPase activity

Washout experiments were performed in order to characterize the binding properties of AZD0865. Inhibition of K^+-stimulated H^+,K^+-ATPase activity was followed using the EnzChek™ Phosphate Assay Kit that continuously measures P_i released from the hydrolysis of ATP. The assay was performed according to the manufacturer's guidelines. Ion-leaky gastric vesicles (2 μg) were preincubated at 37 °C for 5 min in reaction buffer (20 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM NaN_3, 0.2 mM MESG, 1 U/mL PNP and 10 mM KCl). Enzyme activity was initiated by addition of ATP plus $MgCl_2$ to give a final concentration of 2 mM of each agent. The absorbance was recorded until a stable increase was obtained. AZD0865, dissolved in methanol, was added to give a final concentration of 3 μM, and the absorbance was recorded for 1.5 min. Finally, the sample was diluted 10 times with reaction buffer including Mg^{2+}-ATP but without gastric vesicles and the absorbance was recorded for 1.5 min. Spontaneous hydrolysis of ATP and basal Mg^{2+}-ATPase activity during the time-course of the experiment were estimated in separate experiments, as well as the inhibition of K^+-stimulated activity at 0.3 μM of AZD0865 and an enzyme concentration of 0.2 μg. The results were compared with those obtained above with an initial concentration of 3 μM AZD0865 after 10 times dilution with reaction buffer including Mg^{2+}-ATP. In separate experiments, the activated form of omeprazole (i.e., the sulphenamide) was used in the inhibitory step at a final concentration of 10 μM.

2.2.5. Enzyme kinetics and inhibition constant

The inhibition kinetics were determined in relation to the activation of H^+,K^+-ATPase activity by K^+. The ATPase activity was estimated as above for ion-leaky membrane vesicles at pH 7.4. Assays were done in duplicate and at three concentrations

(30, 100 and 300 nM) of AZD0865 for each K^+ concentration. Inhibition was studied at five different concentrations of K^+ (0.3, 0.7, 1.0, 2.0 and 3.0 mM). The inhibitory effect of AZD0865 on basal Mg^{2+}-ATPase activity at each inhibitor concentration was determined and subtracted from the corresponding activity in the presence of K^+ and Mg^{2+}. One series of experiments were performed using the activated form of omeprazole at 100, 300 and 1000 nM.

2.2.6. *Thallium occlusion in ion-leaky gastric vesicles*

The occlusion of Tl^+ by H^+,K^+-ATPase was studied in ion-leaky membrane vesicles using the radioactive isotope $^{204}Tl^+$ and using AlF_4^- to stabilise the E_2 confirmation of the H^+,K^+-ATPase [15,16,22]. 40 μg of protein was incubated in a total volume of 200 μL with 40 mM PIPES/Tris pH 6.7, 2 mM $MgCl_2$, 1 mM NaF, 0.1 mM $AlCl_3$, 1.5 μM $^{204}TlNO_3$, 24 μM $TlNO_3$ and increasing concentrations of AZD0865 (0.01–100 μM). Samples were incubated for 10 min at room temperature and then chilled on ice for 20 min. Free Tl^+ was separated from bound Tl^+ using Dowex-Tris columns at 0 °C as described by Shani et al. [23]. Elution from the columns was accomplished by addition of 250 mM sucrose. The eluates were mixed with acidified Ready Safe® and radioactivity estimated in a liquid scintillation counter.

2.2.7. *Na^+,K^+-ATPase activity*

2 μg of protein was incubated for 15 min at 37 °C in 1000 μL 18 mM PIPES/Tris buffer pH 7.4 containing 2 mM $MgCl_2$, 10 mM KCl, 100 mM NaCl, 2 mM ATP and 1–100 μM AZD0865. The ATPase activity was estimated as release of inorganic phosphate from ATP, as described by LeBel et al. [20]. The Mg^{2+}-ATPase activity was determined in the absence of K^+ and inhibitor. At each AZD0865 concentration, Mg^{2+}-ATPase activity was subtracted from the overall ATPase activity.

2.2.8. *Protein determination*

The protein contents of the membrane vesicle preparations were determined according to Bradford [24], using the Bio-Rad protein kit with bovine γ-globulin as the standard.

2.3. *Data analysis*

The data presented correspond to the mean ± S.E.M. from three experiments and preparations. The concentration response curves in the membrane vesicle and gland assays were constructed in Microsoft Excel Software using the add-in XlFit V2.0. The inhibition constant, K_i, and the Michaelis–Menten constant, K_M, for K^+ binding were determined by nonlinear regression using the program GraFit V4.0.13 (Erithacus Software).

3. Results

3.1. *Inhibition of H^+,K^+-ATPase activity*

In ion-leaky membrane vesicles at pH 7.4 and with a K^+ concentration of 10 mM, AZD0865 was found to concentration-dependently inhibit the H^+,K^+-ATPase activity with an IC_{50} value of 1.0 ± 0.2 μM (Fig. 2A). At pH 6.4, the IC_{50} value was found to be 0.13 ± 0.01 μM (Fig. 2A). When the effect of the permanent cation analogue AR-H070091 was evaluated, the corresponding IC_{50} values were 11 ± 1.2 μM at pH 7.4 and 16 ± 1.8 μM at pH 6.4. The metabolite AR-H044881 was found to inhibit the H^+,K^+-ATPase activity with an IC_{50} value of 2.7 ± 0.1 μM in the ion-leaky vesicles at pH 7.4. In the ion-tight membrane vesicles AZD0865 was found to inhibit the K^+ stimulated H^+,K^+-ATPase activity, in the presence of valinomycin, concentration-dependently with an IC_{50} value of 6.9 ± 0.4 nM (Fig. 2B).

3.2. *Inhibitory effect of stimulated acid formation in gastric glands*

The acid formation in isolated gastric glands was stimulated either at the receptor level using histamine or intracellularly by the use of db-cAMP. AZD0865 concentration-dependently inhibited the stimulated acid formation, as estimated from the aminopyrine accumulation in the glands. The IC_{50} value was 0.28 ± 0.01 μM when histamine was used as the agonist and

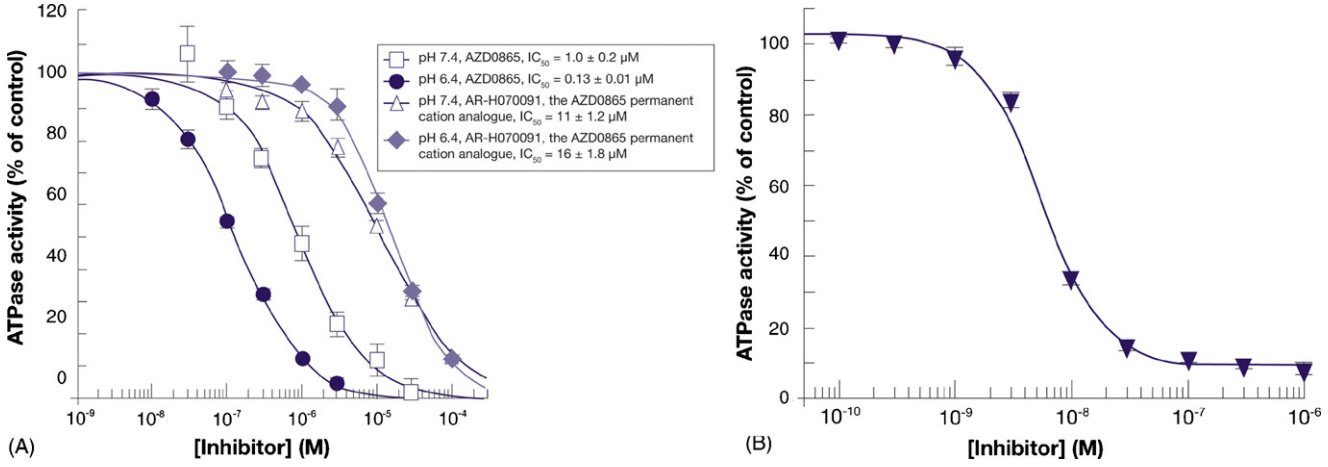

Fig. 2 – Concentration dependent inhibition of H^+,K^+-ATPase activity (% of control, mean ± S.E.M. from three experiments) in ion-leaky membrane vesicles by AZD0865 and AR-H070091 (the AZD0865 permanent cation analogue) at pH 6.4 and 7.4 (A) and in ion-tight membrane vesicles by AZD0865 (B).

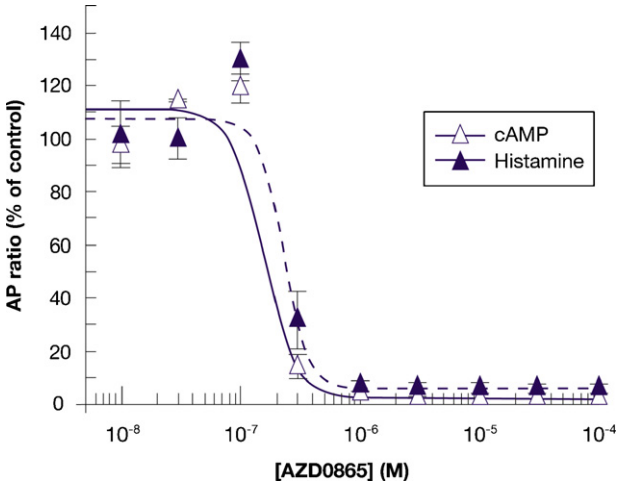

Fig. 3 – Concentration dependent inhibition by AZD0865 on acid formation (AP ratio) in gastric glands stimulated using either db-cAMP or histamine (% of control, mean ± S.E.M. from three experiments).

0.26 ± 0.003 μM when acid formation was stimulated by db-cAMP (Fig. 3). The metabolite AR-H044881 also inhibited the acid formation concentration-dependently with an IC_{50} value of 2.3 ± 0.3 μM in gastric glands stimulated with db-cAMP.

3.3. *Time-course of inhibition and restoration of H^+,K^+-ATPase activity*

In the ion-leaky membrane vesicles at pH 7.4, AZD0865 rapidly inhibited H^+,K^+-ATPase activity such that within seconds of administration of a 3 μM concentration, the activity of H^+,K^+-ATPase decreased to 12 ± 1% of control (Fig. 4A). Immediately following the washout (within seconds), H^+,K^+-ATPase activity was restored to 59% of control (Fig. 4B). The remaining inhibition of AZD0865 corresponds to the effect of the concentration of compound following the dilution (i.e., 0.3 μM, when measured in separate experiments; data not shown). When the effect of 10 μM of omeprazole was evaluated, the H^+,K^+-ATPase activity was decreased to 41% of the control, but ATPase activity was not restored following the washout step (Fig. 4C).

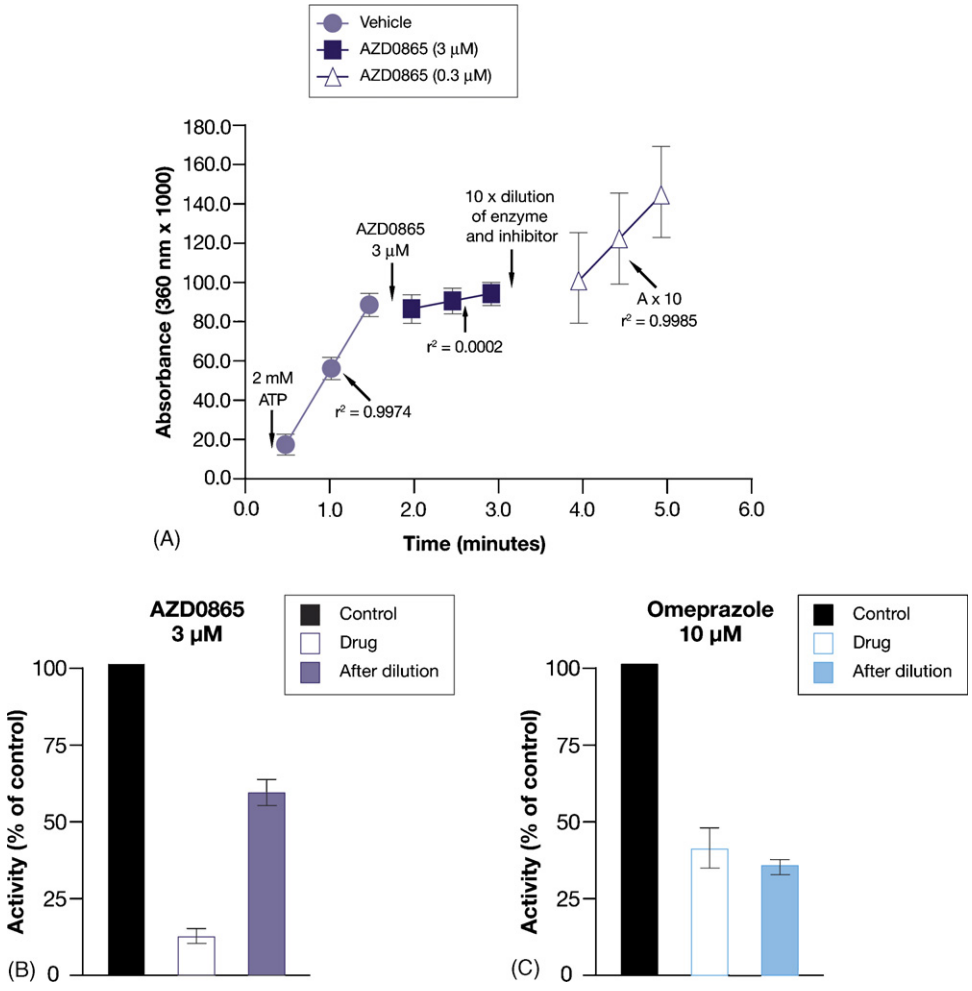

Fig. 4 – The effect of 3 μM AZD0865 on the rate of H^+, K^+-ATPase activity and the rate of recovery immediately following dilution (mean ± S.E.M. from three experiments) in ion-leaky vesicles (A). The effect on the ATPase activity expressed as the percentage of pre-treatment ATPase activity (in the absence of test compound) of AZD0865 (B) or the sulphenamide of omeprazole (C).

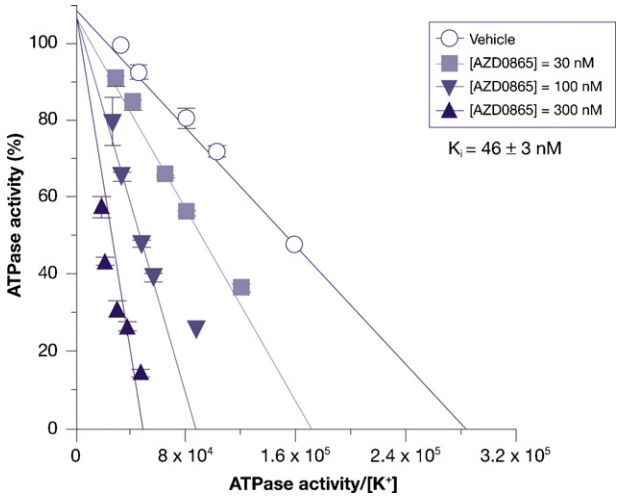

Fig. 5 – Enzyme kinetic plot showing H^+,K^+-ATPase activity (% of control) vs. the ratio of H^+,K^+-ATPase activity to K^+ concentration, for various doses of AZD0865.

3.4. *Enzyme kinetics and inhibition constant*

In ion-leaky pig gastric vesicles at pH 7.4, steady-state kinetic analysis showed that AZD0865 inhibited H^+,K^+-ATPase activity in a K^+-competitive manner, with a K_i of 46 ± 3 nM (Fig. 5). The K_M for K^+ was determined to be 0.41 ± 0.02 mM. The Eadie–Hofstee plot shows H^+,K^+-ATPase activity versus the ratio of H^+,K^+-ATPase activity to K^+ concentration, for various concentrations of AZD0865, and demonstrates a common intercept with the Y-axis typical of competitive inhibition.

3.5. *Effect on cation occlusion*

At pH 6.7 and a thallium concentration of 25.5 μM, AZD0865 was found to inhibit thallium occlusion in a concentration-dependent manner, with an IC_{50} value of 1.7 ± 0.06 μM (Fig. 6).

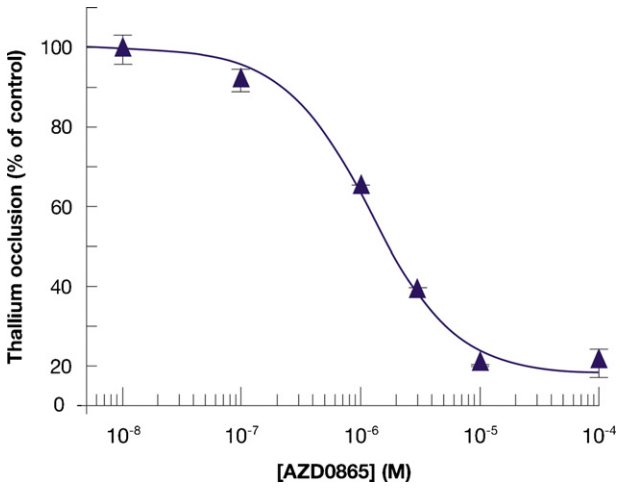

Fig. 6 – Concentration dependent inhibition of thallium occlusion in ion-leaky membrane vesicles at pH 7.4 by AZD0865 (mean ± S.E.M. from three experiments).

3.6. *Na^+,K^+-ATPase activity*

AZD0865 demonstrated high selectivity for H^+,K^+-ATPase over Na^+,K^+-ATPase. At a concentration of 100 μM (i.e., a concentration 100 times higher than the IC_{50} value for inhibition of H^+,K^+-ATPase activity at pH 7.4), AZD0865 inhibited the Na^+,K^+-ATPase activity by 9 ± 2%.

4. Discussion

AZD0865 is a lipophilic weak base that will concentrate in acidic environments. Theoretically, the concentration will be 100,000 times higher in the parietal cell canaliculi than in the plasma, and the compound will immediately be protonated in this acidic space. The inhibitory effect was monitored at different pH values to assess what impact the degree of protonation had on H^+,K^+-ATPase activity. As AZD0865 has a pK_a value of 6.1, only a fraction (~5%) of the compound is protonated at pH 7.4. At pH 6.4, approximately 33% of the compound is protonated. The increased potency of AZD0865 at pH 6.4 versus pH 7.4 (IC_{50}: 0.13 μM versus 1.00 μM) corresponds to the theoretical increase in protonated compound. The inhibitory effect of the permanent cation analogue, AR-H070091, was not affected by the decrease in pH. Taken together, these findings indicate that, like other agents in this class [4–6,25], AZD0865 is active in its protonated form and that its potency increases with decreasing pH.

The IC_{50} value for inhibition of K^+-stimulated ATPase activity in ion-tight vesicles, under conditions allowing an acidic interior to be formed, was found to be approximately 150 times lower than the corresponding value determined in ion-leaky vesicles incubated at the same pH. H^+,K^+-ATPase in ion-tight vesicles is orientated so that the luminal K^+-binding site faces the interior of the vesicle [17], and thus the interior of the vesicle is analogous to the parietal cell canaliculus. Since the Δ valinomycin ATPase activity is derived only from ion-tight vesicles, and a high K^+ concentration (175 mM) is used in the assay, inhibition of the enzyme in the ion-tight vesicles is consistent with a luminal site of action. Previous studies have reported that the binding site of similar agents is also on the luminal face of the enzyme [9,10,26]. The binding site of AZD0865 is not known; however, in common with similar agents (e.g., SCH28080), it is likely that the binding site is at or near the K^+ binding site [11–13,27]. The lower IC_{50} value in ion-tight vesicles, compared with in ion-leaky vesicles most likely reflects the concentration of the protonated form of AZD0865 in the highly acidic interiors of the ion-tight vesicles. However, since neither the intravesicular pH or K^+ concentration is known, it is hard to directly compare the IC_{50} values obtained in the different models. Moreover, the ability of AZD0865 to inhibit the enzyme in ion-tight vesicles indicates that it is relatively membrane permeable.

Inhibition of the acid formation in vitro by AZD0865 was demonstrated in the rabbit gastric gland, a more complex model than the isolated membrane vesicles. The luminal part of the H^+,K^+-ATPase is within the gland tissue and surrounded by undefined concentrations of inhibitor and K^+. Also, the gland experiments are run in the presence of serum albumin, which affects the amount of unbound compound. In this more

complex model, AZD0865 inhibited the H^+,K^+-ATPase activity with high potency. This inhibitory effect was similar following stimulation either at the receptor level using histamine or intracellularly using db-cAMP (IC_{50} values 0.28 and 0.26 μM, respectively), which is in accordance with an accumulation of protonated AZD0865 in the canaliculi of the parietal cells and inhibition of the final step in acid secretion.

Continuous measurements of the H^+,K^+-ATPase activity in the ion-leaky vesicles was used to study the onset of inhibitory effect in vitro. The onset of inhibition was immediate, and following washout of AZD0865 the H^+,K^+-ATPase activity was restored quickly, in keeping with a noncovalent, ionic binding to the enzyme. Restoration of enzyme function on dilution is also a feature of other agents in this class [28–31]. In contrast, the effect of omeprazole was not reversed upon dilution of the sample, which reflects the covalent nature of PPI binding to the gastric H^+,K^+-ATPase.

The mechanism of inhibition by AZD0865 was investigated by measuring H^+,K^+-ATPase activity at defined K^+ concentrations. The resulting enzyme-kinetic pattern is characteristic of competitive binding. This K^+-competitive binding property of AZD0865 is shared with other agents in this class [3,5,6,25,29,32]. The inhibitory constant K_i of AZD0865 and the K_M value for K^+ obtained in the kinetic study at pH 7.4 can be used to derive an IC_{50} value for inhibition by AZD0865 at 10 mM KCl. This value (1.2 μM) is similar to that obtained in the dose-response experiment at pH 7.4 (1.0 ± 0.2 μM).

Several different cations can be used to activate the ATPase in vitro. The stimulatory effect, expressed as the Michaelis–Menten constant, K_M, for these cations is in the order $Tl^+ < K^+ < Rb^+ < NH_4^+$ [33]. During the cycling of the enzyme between the E_1 and E_2 forms, the enzyme becomes phosphorylated (i.e., it assumes the E_2P form) and K^+ becomes temporarily occluded. When the effect of AZD0865 on cation occlusion was studied, thallium was used as a K^+ congener rather than rubidium used previously [15,16], resulting in a decreased nonspecific cation binding (data not shown). AZD0865 concentration-dependently prevented the occlusion of Tl^+. As AZD0865 prevents cation occlusion, it appears to be binding to and stabilizing gastric H^+,K^+-ATPase in the E_2P form. This is again consistent with AZD0865 competing with K^+ to block the enzyme. In support of this contention, it has been reported that SCH28080 exhibits high affinity binding to the phosphorylated form of the gastric H^+,K^+-ATPase [5,25,34].

The amino acid sequences of H^+,K^+-ATPase and Na^+,K^+-ATPase are highly homologous; for example, the human H^+,K^+-ATPase is approximately 60% homologous with human Na^+,K^+-ATPase [35]. Despite such a high degree of homology, AZD0865 was more than 100 times more selective for H^+,K^+-ATPase over Na^+,K^+-ATPase. Since only a minute effect of AZD0865 on Na^+,K^+-ATPase was detected at 100 μM (a concentration much higher than the C_{max} values achieved in healthy volunteers at expected therapeutic doses [36]), the effect on Na^+,K^+-ATPase is very likely to be without biological significance. Varying degrees of selectivity for H^+,K^+-ATPase over Na^+,K^+-ATPase have been reported for previously investigated K^+-competitive inhibitors [3,6,29,37].

A low level of the active metabolite AR-H044881 is generated in vivo (B. Holstein, manuscript in preparation). In the in vitro systems tested in this study (ion-leaky vesicles and db-cAMP stimulated gastric glands) the inhibitory potency was three to nine times less than that of AZD0865. Hence, the contribution of the active metabolite to the acid inhibitory effect in the in vivo situation is likely to be of minor importance.

In summary, these results demonstrate that AZD0865 is a potent and K^+-competitive inhibitor of acid secretion, with high selectivity for the gastric H^+,K^+-ATPase. AZD0865 produces a concentration-dependent inhibition of the gastric H^+,K^+-ATPase, thus blocking acid secretion. In contrast to the covalent binding and irreversible mechanism of action of the PPIs, AZD0865 binds reversibly to H^+,K^+-ATPase. The increased potency of AZD0865 at pH 6.4 versus pH 7.4, and its increased potency in ion-tight versus ion-leaky vesicles, together with the high potency in gastric glands, suggests that AZD0865 concentrates in the acidic compartment and has a luminal site of action.

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