Gastric ($H^+ + K^+$)-ATPase: modulation of the inhibitory properties of the novel potent antisecretagogue Ro 18-5364 by sulfhydryl reagents and nucleotides

Kristine Sigrist-Nelson*+, Rita K.M. Müller and Albert E. Fischli+

*Department of Biochemistry, University of Bern, CH-3012 Bern and Pharmaceutical Research Department, F. Hoffmann-La Roche & Co. Ltd, CH-4002 Basle, Switzerland

Received 21 December 1985

The sulfoxide Ro 18-5364, a potential metabolite of the IND Ro 18-5362, is a powerful inhibitor of gastric mucosal ($H^+ + K^+$)-ATPase, decreasing enzymatic activity with an apparent K_i of 0.1 μM. Exposure of Ro 18-5364-treated gastric membranes to dithiothreitol fully restored ($H^+ + K^+$)-ATPase activity. ATP protected the enzyme against Ro 18-5364-induced inactivation of enzymatic activity. In addition, Ro 18-5364 inhibited vesicular proton uptake. In proton translocation experiments reduced lipoic acid methyl ester partially restored transport properties. Dithiothreitol and mercaptoethanol were without effect. The results are discussed with respect to the possible location of essential sulfhydryl groups for enzyme activity and proton transport.

($H^+ + K^+$)-ATPase *Enzyme inhibition* *Reversible inhibition* *Sulfhydryl reagent* *Nucleotide* *Ro 18-5364*

1. INTRODUCTION

Gastric acid secretion has been shown to be mediated by the ($H^+ + K^+$)-ATPase localized within the microvillus membrane of the parietal cell secretory canaliculus [1–4]. Therefore, inhibition of ($H^+ + K^+$)-ATPase assumes marked importance with regard to possible treatment in ulcer therapy. Recently the sulfoxide Ro 18-5364 (5,7-dihydro-2-{[(4-methoxy-3-methyl-2-pyridyl)-methyl]sulfinyl}-5,5,7,7-tetramethylindeno-[5,6-d] imidazol-6(1H)-one, a potential metabolite of the IND Ro 18-5362, was shown to be an extremely potent inhibitor of the gastric ($H^+ + K^+$)-ATPase [5]. Data from reversibility studies are presented here.

+ To whom correspondence should be addressed

2. MATERIALS AND METHODS

2.1. *Preparation of porcine gastric membrane vesicles*

Membrane vesicles were prepared basically according to [6]. Stomachs from freshly slaughtered pigs were placed immediately in ice-cold 150 mM NaCl, 5 mM Tris (pH 7.3) solution. All of the following procedures were carried out at 4°C. The mucosal scrapings from the fundic region of the stomach were homogenized in 250 mM sucrose, 2 mM $MgCl_2$, 1 mM EGTA and 2 mM Hepes-Tris (pH 7.3); 10 g tissue/100 ml buffer. The homogenate was centrifuged at 14500 × *g* for 15 min and the resulting supernatant recentrifuged at 78000 × *g* for 60 min. The crude microsomal pellet was suspended in the sucrose buffer and layered over a sucrose gradient consisting of equal volumes of 30 and 40% (w/w) sucrose. Following centrifugation for 60 min at 100000 × *g* the membrane banding above the 30% sucrose was col-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

lected and stored at −70°C. The $(H^+ + K^+)$-ATPase activity varied from 60 to 80 μmol phosphate/h per mg protein.

2.2. *$(H^+ + K^+)$-ATPase assay*

The assay medium (1 ml) consisted of 1 mM $MgCl_2$, 2 mM ATP-Tris (pH 7.3), 50 mM Hepes-Tris (pH 7.3) with or without 10 mM KCl and nigericin (10 μg/ml). Membrane vesicles (10–15 μg protein) were incubated in the assay medium for 8–10 min at 37°C. The reaction was terminated by addition of 1 ml of cold trichloroacetic acid (12%) to which 0.15 g acid-washed granular charcoal had been added. The charcoal assay medium suspension was centrifuged for 10 min at 2500 × *g*. P_i was determined according to [7] and protein as in [8] with serum albumin serving as standard. For experiments involving the effect of sulfhydryl reagents and nucleotides on the Ro 18-5364-induced ATPase inhibition, two separate successive preincubations (10 min each, 37°C) were carried out before the ATPase activity was assayed. Conditions during the preincubations are given in table 1 for experiments involving the effect of dithiothreitol. Dithiothreitol was used at 1 mM, the solutions being freshly prepared for each experiment. The inhibitor Ro 18-5364 was present at 20 μM. Conditions involving the effect of nucleotides on Ro 18-5364-induced $(H^+ + K^+)$-ATPase inhibition are noted in table 2. The nucleotides ATP, GTP and ITP were present at 0.5 mM in the preincubation medium and Ro 18-5364 at 20 μM. In all cases appropriate amounts of alcohol were added to control membranes and had no effect on either the enzymatic activity or vesicular proton transport.

2.3. *Vesicular proton transport experiments*

The transport of protons from the extravesicular medium was monitored fluorimetrically. Measurements were made at 22°C with a water-jacketed cuvette in 2 ml of a medium containing 1 mM $MgCl_2$, 150 mM KCl, 5 mM Pipes-Tris (pH 6.5), 0.1 mM EGTA, 2 μM valinomycin and 4 μM acridine orange. Wavelengths used were 493 nm (excitation) and 530 nm (emission). Membranes (5–15 μg protein) were added and proton transport was initiated by the addition of 1 mM ATP-Tris (pH 6.5). Measurement of initial proton transport is expressed as *K*, a rate constant for the fluorescence quenching assuming a first-order process. Preincubation experiments concerning the effects of sulfhydryl reagents and nucleotides on the inhibitory properties of Ro 18-5364 were carried out as described in section 2.2, with the exception that 5 mM Mes-Tris buffer (pH 6) was utilized.

2.4. *Materials*

Ro 18-5364 and the reduced lipoic acid methyl ester were synthesized at F. Hoffmann-La Roche & Co. Ltd, Basle. Dithothreitol, mercaptoethanol and the ionophores valinomycin and nigericin were purchased from Sigma and acridine orange from Eastman. All other reagents used were of the highest purity commercially available.

3. RESULTS

The potency of Ro 18-5364 (fig.1) in inhibiting gastric $(H^+ + K^+)$-ATPase is demonstrated in fig.2. An apparent K_i of 0.1 μM was determined.

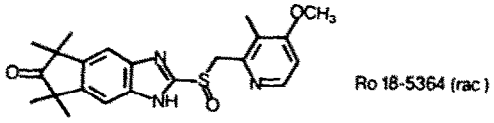

Fig.1. Structural formula of Ro 18-5364. 5,7-Dihydro-2-{[(4-methoxy-3-methyl-2-pyridyl)methyl]sulfinyl}-5,5,7,7-tetramethylindeno[5,6-d]imidazol-6(1H)-one.

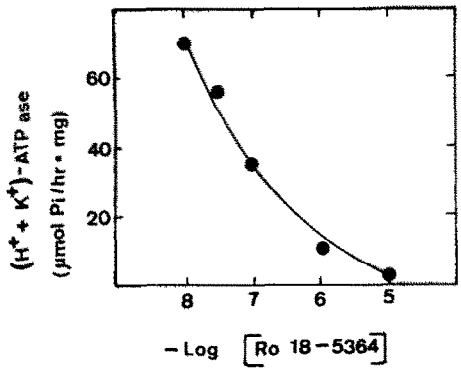

Fig.2. Concentration-dependent inhibition of gastric $(H^+ + K^+)$-ATPase activity by Ro 18-5364. Gastric membranes (15 μg protein) were incubated with varying concentrations of Ro 18-5364 in 50 mM Mes-Tris (pH 6) for 10 min at 37°C. $(H^+ + K^+)$-ATPase activity was assayed as described in section 2. Prior to the ATPase assay the pH was adjusted to 7.3. Enzyme activity in the absence of inhibitor was 73 μmol phosphate/h per mg.

The effect of dithiothreitol in reversing Ro 18-5364-induced inhibition of ATPase is shown in table 1. Preincubation of gastric membranes with Ro 18-5364 at pH 6 resulted in complete inhibition of $(H^+ + K^+)$-ATPase activity. Gastric membranes identically treated with the inhibitor, but exposed shortly to dithiothreitol prior to the ATPase assay, showed completely restored enzymatic activity. Incubation of control membranes with dithiothreitol had no significant effect on the enzymatic activity. Mercaptoethanol and dihydrolipoic acid methyl ester were also able to reverse the inhibitory effects of Ro 18-5364 (not shown).

Various nucleotides were examined for their ability to protect the $(H^+ + K^+)$-ATPase from inhibition by Ro 18-5364. The results are summarized in table 2. Of the nucleotides tested only ATP produced complete protection. Preincubation of gastric membranes with ITP or GTP did not reduce the inhibitory effect of Ro 18-5364.

The effect of sulfhydryl reagents on the restoration of $(H^+ + K^+)$-ATPase-associated proton translocation in gastric vesicles is presented in table 3. Exposure of gastric membrane vesicles to Ro 18-5364 produced complete inhibition of proton translocation. In contrast to the experiments where enzymatic activity was measured, subsequent incubation of the Ro 18-5364-treated membranes with either dithiothreitol or mercaptoethanol failed to reverse the inhibition of proton transport. Reduced lipoic acid methyl ester, however, partially restored proton translocation.

Table 1

Effect of dithiothreitol on the Ro 18-5364-induced inhibition of gastric $(H^+ + K^+)$-ATPase activity

Incubation conditions	$(H^+ + K^+)$-ATPase activity[a] (μmol phosphate/h per mg)
(A) Control, no additions	72 (0)
(B) 1 mM dithiothreitol	74 (+3)
(C) 20 μM Ro 18-5364 (10 min)	0 (−100)
(D) 20 μM Ro 18-5364 for 10 min followed by addition of 1 mM dithiothreitol for 10 min	75 (+4)

[a] In parentheses: decrease (−) and increase (+) in percent

Gastric microsomal membranes (15 μg protein) were incubated at 37°C for two sequential 10 min periods in 50 mM Mes-Tris (pH 6) with the listed additions. Prior to the ATPase assay the pH was adjusted to 7.3. Enzymatic activity was assayed as detailed in section 2. Similar values were obtained for control membranes (A), after 10 and 20 min incubation with 1 mM dithiothreitol (B) and in experiment D

Table 2

Effect of nucleotides on Ro 18-5364-induced inhibition of gastric $(H^+ + K^+)$-ATPase activity

Incubation conditions	$(H^+ + K^+)$-ATPase activity[a] (μmol phosphate/h per mg)
(A) Control, no additions	64 (0)
(B) 0.5 mM ATP	70 (+9)
(C) 0.5 mM ITP	67 (+5)
(D) 0.5 mM GTP	68 (+6)
(E) 20 μM Ro 18-5364	0 (−100)
(F) 0.5 mM ATP for 10 min followed by 20 μM Ro 18-5364 for 10 min	65 (+2)
(G) 0.5 mM ITP for 10 min followed by 20 μM Ro 18-5364 for 10 min	0 (−100)
(H) 0.5 mM GTP for 10 min followed by 20 μM Ro 18-5364 for 10 min	0 (−100)

[a] In parentheses: decrease (−) and increase (+) in percent

Incubation and assay procedures were performed as described in table 1. The incubation conditions utilized for the membrane samples are specified above

4. DISCUSSION

The complete restoration by dithiothreitol, mercaptoethanol and dihydrolipoic acid methyl ester of the ATPase of gastric membranes inhibited by Ro 18-5364 strongly suggests the involvement of sulfhydryl groups in the inhibitory mechanism. The importance of thiol groups for gastric $(H^+ + K^+)$-ATPase has been shown by Lee et al. [9], Forte et al. [10] and Schrijen et al. [11]. Distribution of reactive sulfhydryl groups in

Table 3

Effect of various thiol reagents on the inhibition by Ro 18-5364 of proton translocation in $(H^+ + K^+)$-ATPase-containing membrane vesicles

Incubation conditions	Specific reaction velocity constant K^a (10^{-3} s^{-1})
(A) Control, no additions	3.21 (0)
(B) 1 mM dithiothreitol	3.31 (+3)
(C) 50 mM mercaptoethanol	3.18 (−1)
(D) 1 mM reduced lipoic acid methyl ester	3.25 (+1)
(E) 20 μM Ro 18-5364	0.14 (−96)
(F) 20 μM Ro 18-5364 for 10 min followed by addition of 1 mM dithiothreitol for 10 min	0.19 (−94)
(G) 20 μM Ro 18-5364 for 10 min followed by addition of 50 mM mercaptoethanol for 10 min	0.10 (−97)
(H) 20 μM Ro 18-5364 for 10 min followed by addition of 1 mM reduced lipoic acid methyl ester for 10 min	1.28 (−60)

[a] In parentheses: decrease (−) and increase (+) in percent

Gastric membrane vesicles (10 μg protein) were incubated for two subsequent 10 min periods in 5 mM Mes-Tris (pH 6.5), 1 mM $MgCl_2$, 150 mM KCl, 0.1 mM EDTA, 2 μM valinomycin as described below. Following the incubations acridine orange (4 μM) was added. Initial proton transport (22°C), measured at 40–60 s after addition of 1 mM ATP, is expressed as the rate constant K, a specific reaction velocity constant for description of fluorescence quenching velocity assuming a first-order process

gastric microsomal vesicles highly enriched in $(H^+ + K^+)$-ATPase was studied by Nandi et al. [12]. Recent investigations utilizing a similar substituted benzimidazole suggested that the inhibitor may oxidize essential sulfhydryl groups in the $(H^+ + K^+)$-ATPase [6]. In another study, inhibition of the enzyme by a disulfide linkage between the enzyme and inhibitor was postulated [13]. ATP has been shown to protect $(H^+ + K^+)$-ATPase from inactivation by the sulfhydryl reagents 5,5'-dithiobis(2-nitrobenzoic acid) [11], bis(5-carboxy-2-pyridyl) disulfide and *N*-(1-naphthyl)maleimide [12].

Dithiothreitol completely reversed Ro 18-5364 -induced inhibition of enzymatic activity but was ineffective in reactivating proton translocation. Similarly, mercaptoethanol completely restored $(H^+ + K^+)$-ATPase activity but failed to restore proton uptake in gastric membrane vesicles. However, reduced lipoic acid methyl ester was successful in partially reactivating the $(H^+ + K^+)$-ATPase associated vesicular proton uptake. The difference between restoration of enzymatic activity and proton translocation may be due to the involvement of sulfhydryl groups located at different structural domains of the enzyme. That enzymatic activity was restored by dithiothreitol ($p = 0.12$; *Note:* p, calculated partition coefficients (*n*-octanol/aqueous phase, pH 7.4), calculated with the software CLOGP, Med. Chem. Project, Pomona College, Clairmont, CA) suggests that the sites involved in this activity are probably exposed to the aqueous phase. Mercaptoethanol ($p = 0.47$) would be expected to accumulate weakly in the membrane and to a higher extent in the intravesicular aqueous phase. It should therefore weakly react with disulfides in the membrane showing at the same time strong reduction of disulfides accessible from both the exterior and interior aqueous phase. That reduced lipoic acid methyl ester ($p = 220$), highly accumulated in membranes, was effective in partially restoring transport properties may indicate enhanced hydrophobicity in the proximity of the sulfhydryl groups required for proton transport.

REFERENCES

[1] Forte, J.G., Forte, T.M. and Saltman, P. (1967) J. Cell. Physiol. 69, 293–304.
[2] Sachs, G., Chang, H.H., Rabon, E., Schackmann, R., Lewin, M.J.M. and Saccomani, G. (1967) J. Biol. Chem. 251, 7690–7698.
[3] Forte, J.G., Machen, T. and Obrink, K.J. (1980) Annu. Rev. Physiol. 42, 111–126.
[4] Wolosin, J.M. (1985) Am. J. Physiol. 248, G595–G607.
[5] Sigrist-Nelson, K., Krasso, A., Müller, R.K.M. and Fischli, A.E., J. Biol. Chem., in preparation; cf. also European Patent Application no.85.104041.0, filed April 3, 1985.

[6] Im, W.B., Sih, J.C., Blakeman, D.P. and McGrath, J.P. (1985) J. Biol. Chem. 260, 4591–4597.
[7] Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–380.
[8] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[9] Lee, H.C., Breitbart, H., Berman, M. and Forte, J.G. (1979) Biochim. Biophys. Acta 553, 107–131.
[10] Forte, J.G., Poulter, J.L., Dykstra, R., Rivas, J. and Lee, H.C. (1981) Biochim. Biophys. Acta 644, 257–265.
[11] Schrijen, J.J., Luyben, W.A.H.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1981) Biochim. Biophys. Acta 640, 473–486.
[12] Nandi, J., Meng-Ai, Z. and Ray, T.K. (1983) Biochem. J. 213, 587–594.
[13] Lorentzon, P., Eklundh, B., Brändström, A. and Wallmark, B. (1985) Biochim. Biophys. Acta 817, 25–32.