Difference between Two Isozymes of \((\text{Na}^+ + \text{K}^+)\)-ATPase in the Interaction with Omeprazole

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Abstract—The difference in functional SH groups between two isozymes \((\alpha^+\) and \(\alpha\) forms) of \((\text{Na}^+ + \text{K}^+)\)-ATPase was examined using omeprazole, a hydrophobic drug which was reported to modify SH groups of gastric \((\text{H}^+ + \text{K}^+)\)-ATPase. Omeprazole inhibited rat brain and kidney \((\text{Na}^+ + \text{K}^+)\)-ATPase activities in a time- and dose-dependent manner, and it inhibited incorporation of \([^{3}\text{H}]\text{NEM}\) into the catalytic subunit of the enzymes. The inhibition was greater in the brain enzyme than in the kidney enzyme. The inhibition of the brain enzyme showed a lag time, whereas the kidney enzyme was inhibited according to pseudo-first order kinetics. The inhibition by omeprazole of \(\text{Na}^+\)-dependent phosphorylation and \(\text{K}^+\)-stimulated phosphatase activity in the brain enzyme preparation was parallel with that of the overall \((\text{Na}^+ + \text{K}^+)\)-ATPase reaction, while the partial reactions of the kidney enzyme showed different sensitivities to inhibition by omeprazole. Furthermore, the inhibition by omeprazole of \([^{3}\text{H}]\text{NEM}\) reactivity in the brain \(\alpha^+\) form was greater in the presence of SDS than in the absence, whereas the inhibition in the brain and kidney \(\alpha\) forms was less in the presence of SDS than in the absence. These findings suggest that the isozymes of \((\text{Na}^+ + \text{K}^+)\)-ATPase differ in hydrophobicity of SH groups of their catalytic subunits.

There are two different molecular forms of \((\text{Na}^+ + \text{K}^+)\)-ATPase (EC 3.6.1.37) which are called \(\alpha^+\) and \(\alpha\) forms based on the difference in apparent molecular weight of the catalytic subunit (1–5). These isozymes have different affinities for cardiac glycosides and different reactivities to NEM (1, 6). We have studied the heterogeneity of \((\text{Na}^+ + \text{K}^+)\)-ATPase in the brain and heart (7–10): the recent findings suggest that the two isozymes of brain \((\text{Na}^+ + \text{K}^+)\)-ATPase differ in the interaction with phospholipids or in the lipid-environment (11) and in their conformational changes (12).

Recently, much attention has paid to substituted benzimidazoles such as omeprazole, a new class of drug that inhibits gastric acid secretion (13, 14). It has been recently demonstrated that the inhibitory effect of omeprazole on the \((\text{H}^+ + \text{K}^+)\)-ATPase may be due to its ability to react with SH groups of the enzyme (15–19). Furthermore, it is reported that the inhibitory effect of omeprazole on the enzyme may be mediated by the protonated form (15, 17): the treatment of omeprazole under an acid condition enhances the inhibitory potency. Keeling et al. (16) have recently shown that omeprazole and acid-treated omeprazole inhibit not only the gastric \((\text{H}^+ + \text{K}^+)\)-ATPase but also the kidney \((\text{Na}^+ + \text{K}^+)\)-ATPase. However, the precise mechanism for the inhibition of the \((\text{Na}^+ + \text{K}^+)\)-ATPase was not studied. We have been interested in omeprazole because it interacts with SH groups, and it, unlike NEM, is poorly soluble in water. In this paper, we examined the effect of omeprazole on rat brain and kidney \((\text{Na}^+ + \text{K}^+)\)-ATPases in order to study the hydrophobic environment of functional SH groups in two isozymes of \((\text{Na}^+ + \text{K}^+)\)-ATPase.

Abbreviations: SDS, sodium dodecyl sulfate; NEM, N-ethylmaleimide; SH, sulfhydryl.
This paper also reports that acid-treated omeprazole is a potent inhibitor of these enzymes.

Materials and Methods

Enzyme preparations and enzyme assays: (Na\(^++\)K\(^+-\))-ATPases were partially purified from rat cerebral cortex, medulla oblongata and kidney by a modification of the method of Jørgensen (20). In brief, a microsomal fraction was treated with SDS and subjected to discontinuous sucrose gradient centrifugation (21). (Na\(^++\)K\(^+-\))-ATPase activity was measured as previously reported (7, 21): the reaction was carried out at 37°C for 2 min in a medium of 0.1 ml containing 3 mM ATP, 3 mM MgCl\(_2\), 140 mM NaCl, 20 mM KCl, enzyme protein and ±1 mM ouabain. K\(^+\)-stimulated p-nitrophenyl phosphatase activity was assayed at 37°C in a medium of 0.2 ml containing 3 mM MgCl\(_2\), 3 mM p-nitrophenyl phosphate, 25 mM imidazole buffer (pH 7.25), enzyme protein and ±20 mM KCl. The reaction was stopped by addition of 1 ml of 0.3 M NaOH, and the absorbance at 410 nm was measured. ε was taken to be 18,300 M\(^{-1}\)cm\(^{-1}\). Yeast alcohol dehydrogenase was preincubated with the drug for 30 min at 25°C, and then the activity was determined by the method of Racker (22). Protein was assayed by the method of Lowry et al. (23) using bovine serum albumin as standard.

Preparation of acid-treated omeprazole: Omeprazole at 2–4 mM was incubated with an equivalence of 0.1 N HCl at 37°C for 15 min. The pH was then brought back to neutrality by addition of 1 N NaOH and 1 M imidazole buffer (pH 7.25), and the solution was used immediately as acid-treated omeprazole.

Treatment with omeprazole or acid-treated omeprazole: Unless otherwise indicated, the enzyme suspension (50 μg/mg) was incubated with omeprazole or acid-treated omeprazole in the presence of 20 mM imidazole buffer (pH 7.25) at 37°C for 30 min, and the reaction was terminated by addition of 2-mercaptoethanol (1 mM). The mixture was immediately centrifuged at 279,000 g for 15 min in a Beckman TL-100 ultracentrifuge, and the pellet was washed once. The resulting pellet was suspended in 0.16 M sucrose – 0.5 mM EDTA – 10 mM imidazole buffer (pH 7.25) for the assay.

Phosphorylation: Na\(^+\)-dependent phosphorylation of the enzyme was carried out in a volume of 0.1 ml at 0°C with 10 μM [γ-\(^{32}\)P]ATP, 3 mM MgCl\(_2\), 100 mM NaCl, 25 mM Tris-HCl (pH 7.25) and enzyme protein (about 10 μg). The reaction was terminated at 10–15 sec by addition of 0.5 ml of cold 5% (w/v) trichloroacetic acid containing 0.1 M sodium phosphate, and then 40 μg of 1 mg/ml bovine serum albumin was added to the mixture as carrier protein. The sample was filtered through a 0.45 μm membrane filter (Advantec Toyo, Japan) and washed four times with 3 ml of the trichloroacetic acid solution. The filter was dried and the radioactivity was counted.

Reaction with [\(^3\)H]NEM: The enzyme was incubated with [\(^3\)H]NEM (1 mM, 33 mCi/ mmol) in a medium containing 20 mM imidazole buffer (pH 7.25) and 1 mM EDTA in the presence and absence of 1% (w/v) SDS at 37°C for 30 min, and the reaction was terminated by addition of an excess of 2-mercaptoethanol. The two large subunits (α(+)) and α were separated by SDS-polyacrylamide gel electrophoresis (8). The gel was stained and destained, the bands were cut out with a razor blade, and the radioactivity was determined by liquid scintillation spectrometry after dissolving the gel with 30% H\(_2\)O\(_2\).

Chemicals: Vanadate-free ATP, ouabain and yeast alcohol dehydrogenase were obtained from Sigma Chemical Co. [γ-\(^{32}\)P]ATP and [\(^3\)H]NEM were from Amersham and New England Nuclear, respectively. Omeprazole used in this study was synthesized by the method reported by AB Hassle (Sweden) (24) and identified by elemental analysis (Anal. Calcd for C\(_{17}\)H\(_{19}\)N\(_3\)O\(_3\)S: C, 59.11; H, 5.54; N, 12.17; S, 9.28. Found: C, 59.11; H, 5.48; N, 12.15; S, 9.24) and m.p. (153–155°C). Omeprazole was dissolved in dimethylsulphoxide to a concentration of 100 mM. The solution was diluted with 20 mM imidazole buffer (pH 7.25) and used within 2 hr. The final concentration of dimethylsulphoxide in the reaction medium was less than 0.2% which had no effect on the enzyme activity.
All other reagents used here were of the highest purity commercially available.

Results

Brain (Na\textsuperscript{+}+K\textsuperscript{+})-ATPase preparation consists of both \( \alpha(+) \) and \( \alpha \) forms, and the kidney enzyme preparation contains only the \( \alpha \) form (1, 7). The percentages of the \( \alpha(+) \) in the cerebral cortex and medulla oblongata were determined to be about 75% and more than 95%, respectively, by SDS-polyacrylamide gel electrophoresis analysis of the phosphorylated intermediates with \([\gamma-\text{32P}]\text{ATP}\). Omeprazole inhibited rat cortical and kidney (Na\textsuperscript{+}+K\textsuperscript{+})-ATPases in a time-dependent manner (Fig. 1). The inhibition of kidney (Na\textsuperscript{+}+K\textsuperscript{+})-ATPase showed pseudo-first order kinetics, whereas there was a lag time in the inhibition of brain enzyme. A similar inhibition pattern which depended on the preincubation time was observed in the effect of acid-treated omeprazole on these enzymes (data not shown). The inhibition by omeprazole or acid-treated omeprazole of all the enzymes used here was dose-dependent (Fig. 2). These enzyme preparations differed a little in sensitivity to the drugs: medulla oblongata > cerebral cortex > kidney. In all the enzymes, the treatment of omeprazole under the acid conditions caused a 10-fold enhancement of the inhibitory potency.

Figure 3 shows the effect of omeprazole on sensitivity of the cortical (Na\textsuperscript{+}+K\textsuperscript{+})-ATPase to ouabain inhibition. In agreement with the previous reports (1, 7, 8, 11), high- (inhibition by 10\textsuperscript{-7}–5\times10\textsuperscript{-6} M ouabain) and low- (inhibition by 5\times10\textsuperscript{-6}–10\textsuperscript{-3} ouabain) affinity components of ouabain inhibition were observed in the control and treated enzymes. The treatment with omeprazole at 0.1 mM caused a small change in the dose response curve of ouabain inhibition: it decreased a proportion of the high affinity component and conversely increased that of the low affinity component.

A kinetic study on the effect of omeprazole on the brain (Na\textsuperscript{+}+K\textsuperscript{+})-ATPase was carried out (Fig. 4). Activation of the enzyme by ATP and K\textsuperscript{+} was shown as double reciprocal plots, and the square root of the reciprocal of velocity was plotted against the reciprocal of Na\textsuperscript{+} concentration for the Na\textsuperscript{+}}
activation to obtain a straight line (21). In all cases, the enzyme activity was inhibited in a noncompetitive fashion.

The effects of omeprazole on the partial reactions, \(\text{Na}^+\)-dependent phosphorylation and \(\text{K}^+\)-stimulated phosphatase, of the brain and kidney \((\text{Na}^++\text{K}^+)-\text{ATPases}\) were examined. The decreased enzyme activity was still observed after the preparation pretreated with omeprazole was washed, indicating the irreversibility of the action (Fig. 5). In the brain enzyme preparation, the \(\text{Na}^+\)-dependent phosphorylation was inhibited by omeprazole as much as the \(\text{K}^+\)-stimulated phosphatase activity, while the former reaction was inhibited less than the latter in the kidney enzyme.

Figure 6 shows that glutathione decreases

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**Fig. 2.** Effects of omeprazole and acid-treated omeprazole on the brain and kidney \((\text{Na}^++\text{K}^+)-\text{ATPase}\) activities. The enzyme preparations (50 \(\mu\text{g}\)/ml) from the cerebral cortex (circle) and medulla oblongata (square) and kidney (triangle) were preincubated with omeprazole (open) or acid-treated omeprazole (closed) for 30 min at 37°C, and the activities were determined. Each point is the mean ±S.E. of four to eight determinations. The control activities (mean±S.E.M.) were 7.38±0.62 (cerebral cortex), 6.36±0.54 (medulla oblongata) and 9.00±0.87 (kidney) \(\mu\text{mol/mg protein/min}\).

**Fig. 3.** Change in dose-response curve of the brain \((\text{Na}^++\text{K}^+)-\text{ATPase}\) activity versus ouabain concentration by omeprazole. The cortical enzyme was treated with (●) and without (○) 0.1 mM omeprazole for 30 min as described under Materials and Methods. The ATPase reaction was initiated by addition of the enzyme in the presence of various concentrations of ouabain and then terminated after 30 min. Protein concentration in the medium was adjusted to 2 \(\mu\text{g/ml}\) by addition of bovine serum albumin. Points are the mean ± S.E.M. of three to four determinations. The specific activities (\(\mu\text{mol}/\text{mg protein/min}\)) were 4.65 (control) and 0.56 (omeprazole treated).

**Fig. 4.** Effect of omeprazole on kinetics of activation of the brain \((\text{Na}^++\text{K}^+)-\text{ATPase}\) by ATP, \(\text{Na}^+\) and \(\text{K}^+\). The cortical enzyme was preincubated with (●) and without (○) 0.2 mM omeprazole for 30 min, and the activity was determined as described under Materials and Methods. Results are representatives of three separate experiments. \(\text{V}\) is expressed as \(\mu\text{mol/mg protein/min}\).

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the inhibitory effect of omeprazole or acid-treated omeprazole on the (Na⁺⁺K⁺⁺)-ATPase in a dose-related manner. The inhibition by
0.2 mM omeprazole or 20 μM acid-treated omeprazole was almost completely prevented by the simultaneous addition of about 10–20

Fig. 5. Effect of omeprazole treatment on the partial reactions of the brain and kidney (Na⁺⁺K⁺⁺)-ATPases. The cortical (A) and kidney (B) enzymes were pretreated with omeprazole at the indicated concentrations for 30 min and washed. The resulting preparations were used for each assay. Each point is the mean±S.E.M. of six to eight determinations with two to three different enzyme preparations. The control activities (mean±S.E.M.) were as follows: (Na⁺⁺K⁺⁺)-ATPase (○), 6.94±0.39 (brain) and 7.34±0.90 (kidney) μmol/mg protein/min; Na⁺⁺-dependent phosphorylation (●), 0.52±0.08 (brain) and 0.53±0.02 (kidney) nmol/mg protein; K⁺⁺-stimulated phosphatase (▲), 0.72±0.06 (brain) and 1.13±0.09 (kidney) μmol/mg protein/min.

Fig. 6. Effect of glutathione on the inhibition of rat brain and kidney (Na⁺⁺K⁺⁺)-ATPases by omeprazole or acid-treated omeprazole. The cortical (A) and kidney (B) enzymes were preincubated with 0.2 mM omeprazole (○) or 20 μM acid-treated omeprazole (●) in the absence and presence of glutathione for 30 min, and then the ATPase activities were determined. Each point is the mean±S.E.M. of three determinations. The control activities (mean±S.E.M., n=3) of the brain and kidney enzymes were 5.88±0.18 and 9.84±0.73 μmol/mg protein/min, respectively.
A similar antagonism was observed with other SH-reducing reagents such as dithiothreitol and 2-mercaptoethanol (data not shown). We also examined if these drugs inhibited another SH-enzyme, like alcohol dehydrogenase. Omeprazole and acid-treated omeprazole inhibited the enzyme activity with IC50 values of 0.63±0.06 mM and 8.0±0.2 μM (mean±S.E. of six determinations), respectively. The effect of omeprazole on SH groups of the catalytic subunits of rat brain and kidney (Na++K+)-ATPases was examined by determining incorporation of [3H]NEM into the catalytic subunit in the presence and absence of SDS (Table 1). The incorporation in the presence of SDS was higher than that in the absence in all catalytic subunits. Omeprazole treatment inhibited the incorporation into catalytic subunits of the enzymes both in the presence and absence of SDS. In the brain α(+) form, the inhibition in the presence of SDS was greater than that in the absence, while in the brain α form and kidney enzyme, the inhibition was less in the former than in the latter.

**Table 1.** The effect of omeprazole on the reactivity of catalytic subunits of the brain and kidney (Na++K+)-ATPases with [3H]NEM

|                |  [3H]NEM reactivity (% of control) |  |  |  |  |
|----------------|-----------------------------------|---|---|---|---|
|                | [3H]NEM | α(+) | +SDS | -SDS | α | +SDS |
| (A) Cortical enzyme |        |          |      |      |      |      |
| Omeprazole 0.1 mM | 48.3±1.3 | 27.2±3.6*** | 34.3±0.9 | 55.5±3.8*** |      |      |
| Omeprazole 0.2 mM | 19.0±2.6 | 8.0±0.5**  | 24.9±5.9 | 41.0±1.7 |      |      |
| (B) Kidney enzyme |        |          |      |      |      |      |
| Omeprazole 0.1 mM | 33.3±1.5 | 58.4±5.9** | 28.0±0.9 | 39.3±3.8* |      |      |

The cortical and kidney enzymes were treated with omeprazole and centrifuged. The treated enzymes were then reacted with [3H]NEM in the presence and absence of SDS, and the samples (15 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis as described under Materials and Methods. Results, shown as % of the control, represent the mean±S.E. of three determinations. The control incorporations (pmol/band) were as follows: without SDS, 51.4±0.6 (brain α(+)), 25.1±2.2 (brain α) and 31.8±0.9 (kidney α); with SDS, 131.8±4.2 (brain α(+)), 58.8±1.5 (brain α) and 65.2±3.6 (kidney α). *P<0.05, **P<0.02, ***P<0.001, compared to the corresponding value without SDS. In the cortical α form treated with 0.2 mM omeprazole, the incorporation was the lowest, and the S.E. was relatively large.

μM glutathione. A similar antagonism was observed with other SH-reducing reagents such as dithiothreitol and 2-mercaptoethanol (data not shown). We also examined if these drugs inhibited another SH-enzyme, like alcohol dehydrogenase. Omeprazole and acid-treated omeprazole inhibited the enzyme activity with IC50 values of 0.63±0.06 mM and 8.0±0.2 μM (mean±S.E. of six determination), respectively. The effect of omeprazole on SH groups of the catalytic subunits of rat brain and kidney (Na++K+)-ATPases was examined by determining incorporation of [3H]NEM into the catalytic subunit in the presence and absence of SDS (Table 1). The incorporation in the presence of SDS was higher than that in the absence in all catalytic subunits. Omeprazole treatment inhibited the incorporation into catalytic subunits of the enzymes both in the presence and absence of SDS. In the brain α(+) form, the inhibition in the presence of SDS was greater than that in the absence, while in the brain α form and kidney enzyme, the inhibition was less in the former than in the latter.

**Discussion**

Sweadner (1) has shown using [3H]NEM that two isozymes of (Na++K+)-ATPase differ in the number and reactivity of SH groups. In accordance with this, Urayama and Nakao (6) reported that the inhibition of the brain (Na++K+)-ATPase by SH-blockers such as NEM, showdomycin and dithiobis-nitrobenzoate was significantly stronger than that of the kidney enzyme. The study on the difference in SH reactivity between the isozymes may contribute to understanding of the difference in their structure and function. In this paper, the inhibition of the brain and kidney (Na++K+)-ATPases by omeprazole, a hydrophobic drug, was studied in detail.

The inhibitory effect of omeprazole on the brain and kidney (Na++K+)-ATPases might be mediated by a modification of functional SH groups of the enzyme, since the inhibition was antagonized by glutathione, and omeprazole inhibited the incorporation of [3H]NEM into the catalytic subunit of the enzyme. The idea was also supported by the finding that the drug inhibited alcohol dehydrogenase, a SH-enzyme. The effect of acid-treated omeprazole was also antagonized by glutathione, though its potency in inhibiting the enzyme activity was about 10 times greater than that of omeprazole. This finding...
suggests that omeprazole and acid-treated omeprazole have essentially the same mechanism for inhibition. However, it is unknown if the effect of omeprazole on the (Na\(^{+}\)+K\(^{+}\))-ATPase is mediated by the protonated form as reported for the (H\(^{+}\)+K\(^{+}\))-ATPase (15, 17). In view of the finding that acid-treated omeprazole is a potent inhibitor of (Na\(^{+}\)+K\(^{+}\))-ATPase, it may be of interest to study its pharmacological effect. In a preliminary experiment, we have found that acid-treated omeprazole at 1 µM has a positive inotropic action in isolated guinea pig atrium.

On the whole, the effect of omeprazole on the (Na\(^{+}\)+K\(^{+}\))-ATPase described here was similar to that on the (H\(^{+}\)+K\(^{+}\))-ATPase as previously reported (16). This might be expected to some degree in view of the similarity in structure and function between these two enzymes (25–28). It seemed that the α form of (Na\(^{+}\)+K\(^{+}\))-ATPase was more similar to the (H\(^{+}\)+K\(^{+}\))-ATPase than the α(+) form in the aspects of sensitivities of the partial reactions of the enzymes to omeprazole and the time course of the inhibition by the drug. Lorentzon et al. (17) reported that inhibition of the phosphoenzyme level of the (H\(^{+}\)+K\(^{+}\))-ATPase by omeprazole was less than those of the (H\(^{+}\)+K\(^{+}\))-ATPase and phosphatase activities, and that there was no lag time in the inhibition.

The inhibition by omeprazole of the brain (Na\(^{+}\)+K\(^{+}\))-ATPase was stronger than that of the kidney enzyme. The result suggests that omeprazole, like NEM (8), inhibits the activity of the α(+) form more than that of the α form. It should be noted that omeprazole affected the ouabain sensitivity of the brain enzyme less than NEM did: the high affinity component was the major one in the ouabain inhibition of NEM-treated cortical (Na\(^{+}\)+K\(^{+}\))-ATPase (8), while two components were still observed in that of the omeprazole-treated cortical enzyme. That is, the specificity for the α(+) form was much less in the effect of omeprazole than in that of NEM. The difference might be explained in part by the finding that the inhibition of the brain enzyme by omeprazole was accompanied by a lag time and the inhibition of the kidney enzyme showed pseudo-first order kinetics. It was reported that NEM inhibited the brain and kidney enzymes without a lag time (6). It is unlikely that the lag time is due to a conversion of omeprazole to the active form, since the effect was observed only in the brain enzyme. Therefore, the present study using a hydrophobic SH-reagent suggests that the two isozymes of (Na\(^{+}\)+K\(^{+}\))-ATPase differ in hydrophobic environment of SH groups essential for the catalytic activity. In this connection, there was a difference between the two isozymes in the effect of SDS on the inhibition of [\(^{3}\)H]NEM reactivity by omeprazole. SDS enhanced the inhibitory effect of omeprazole on [\(^{3}\)H]NEM reactivity in the α(+) form, whereas it decreased that of omeprazole on [\(^{3}\)H]NEM reactivity in the α form. The finding implies that the isozymes differ in membrane organization of their SH groups that interact with omeprazole.

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