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Inhibitory Sites on Sodium- and Potassium-Activated Adenosine Triphosphatase for Chlorpromazine Free Radical and Ouabain

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SUMMARY

The effects of chlorpromazine free radical and/or ouabain on ($Na^+ + K^+$)-ATPase activity [Mg^{2+}-dependent, ($Na^+ + K^+$)-activated ATP phosphohydrolase, EC 3.6.1.3], on free sulfhydryl concentration, and on the ability to bind ^{3}H-ouabain were studied in deoxycholate- and sodium iodide-treated rat brain microsomal fractions to determine the inhibitory sites of these compounds on ($Na^+ + K^+$)-ATPase. The treatment of the enzyme preparation with chlorpromazine free radical resulted in inhibition of ($Na^+ + K^+$)-ATPase activity and a proportionate decrease in free sulfhydryl concentration. Chlorpromazine, per se, affected neither free sulfhydryl concentration nor enzyme activity. Incubation of the enzyme with ouabain, NaCl, $MgCl_2$, and Tris-ATP at 37° for 20 min resulted in irreversible inhibition of the enzyme activity. Free sulfhydryl concentration was unaffected. Prior treatment with ouabain did not prevent chlorpromazine free radical from reacting with sulfhydryl groups of the enzyme preparation. Prior treatment with chlorpromazine free radical also did not prevent ouabain from reacting with the enzyme but did prevent *p*-hydroxymercuribenzoate from reacting with the inhibited enzyme, indicating that chlorpromazine free radical and *p*-hydroxymercuribenzoate inhibit the enzyme by interacting with free sulfhydryl groups whereas ouabain reacts at a different site. The ability of chlorpromazine free radical to inhibit ^{3}H-ouabain binding on ($Na^+ + K^+$)-ATPase was considerably less than its ability to reduce ($Na^+ + K^+$)-ATPase activity. We conclude that chlorpromazine free radical inhibits enzyme activity by interacting with sulfhydryl groups on the ($Na^+ + K^+$)-ATPase whereas ouabain is bound to a different site on the enzyme.

INTRODUCTION

Previous studies indicated that the semiquinone free radical of chlorpromazine is a potent inhibitor of microsomal ($Na^+ + K^+$)-ATPase [Mg^{2+}-dependent ($Na^+ + K^+$)-activated ATP phosphohydrolase, EC 3.6.1.3] (1) and that the mechanism by which chlorpromazine free radical inhibits this enzyme system is different from that of ouabain (2), a known inhibitor of the ($Na^+ + K^+$)-ATPase (3). A kinetic study of the interaction between ($Na^+ + K^+$)-ATPase and chlorpromazine free radical or ouabain suggested that these two inhibitors might inhibit different steps of the ATPase reaction (2). The ($Na^+ + K^+$)-ATPase reaction is believed to be a multiple-step reaction involving a number of different active sites for the substrate and activators (3–6).

In the present studies, we have explored the possibility that chlorpromazine free radical and ouabain interact with the en-

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zyme at different sites and have attempted to differentiate these inhibitory sites.

METHODS

Male Sprague-Dawley rats weighing 200–300 g were used. The preparation of the enzyme from the brain microsomal fraction by deoxycholic acid and NaI treatment was described previously (2).

All assays for ATPase activity were performed with 16 μg of enzyme protein, either treated or control, in an incubation volume of 1.0 ml. The incubation mixture contained 5.0 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.5), and various concentrations of inhibitors as indicated, with or without 100 mM NaCl and 15 mM KCl. After a 5-min incubation period at 37°, Tris-ATP was added to a final concentration of 5.0 mM. The incubation was performed for an additional 10 min at 37°. All incubations were performed in the dark. The reaction was terminated by the addition of 1.0 ml of ice-cold 15% trichloracetic acid with rapid mixing. The mixture was centrifuged for 15 min at 1000 × *g*. A 1.0-ml aliquot of the supernatant solution was assayed for inorganic phosphate by addition to a test tube containing 1.0 ml of distilled water and 2.0 ml of color reagent described by Bonting *et al.* (7). After 5 min, the absorbance was measured at 750 mμ. Na_2HPO_4 solutions, 0.05–0.2 mM, were used as the standard. Mg^{++}-ATPase activity, assayed in the absence of NaCl and KCl, was subtracted from the total ATPase activity, assayed in the presence of NaCl and KCl, to calculate $(Na^+ + K^+)$-ATPase activity.

Free sulfhydryl groups of enzyme protein were determined spectrophotometrically by Ellman's method (8) as modified by Hoffman and Discher (9). An aliquot of enzyme suspension containing 0.32–0.5 mg of protein was mixed with 1.0 ml of 0.1 M sodium phosphate buffer (pH 8.0) containing 0.2% sodium dodecyl sulfate. Distilled water was added to a total volume of 3.5 ml. After the mixture became clear, 0.2 ml of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 0.1 M sodium phosphate buffer (pH 7.0) was added, and after 20 min absorbance was measured at 412 mμ. The free sulfhydryl concentration was estimated from the molar extinction coefficient of *p*-nitrothiophenol anion, which was 13,600 M^{-1} cm^{-1} (8).

The binding of radioactive ouabain by the $(Na^+ + K^+)$-ATPase preparation was assayed by incubating 0.2 mg of enzyme protein with 0.1 μM ouabain containing tracer amounts of tritiated ouabain (New England Nuclear Corporation; specific activity, 3.7 Ci/mmole) in a ratio of 1 nmole of ouabain per milligram of protein. The incubation was performed for 10 min at 37° in the presence of 80 mM NaCl, 2.0 mM $MgCl_2$, 2.0 mM Tris-ATP, and 50 mM Tris-HCl buffer (pH 7.5) in a total volume of 2.0 ml. After the incubation, the mixture was centrifuged at 100,000 × *g* for 30 min. The supernatant solution was decanted, and the centrifuge tube was rinsed with distilled water. One-half milliliter of 0.2 N KOH was added to the pellet, the tube was heated in a boiling water bath until the pellet was dissolved, and the mixture was then diluted 4-fold with distilled water. A 1-ml aliquot of this solution and 0.5 ml of the supernatant solution were assayed for radioactivity in a liquid scintillation system using 10 ml of Bray's solution (10). Counting efficiency was monitored by the external standard channel ratio method.

The semiquinone free radical of chlorpromazine was generated in the chlorpromazine-enzyme mixture by ultraviolet irradiation as described previously (2). Solid semiquinone free radical of chlorpromazine was obtained by acid treatment of a mixture of chlorpromazine and chlorpromazine sulfoxide and subsequently purified as described previously (2).

The results were analyzed for significance by Student's *t*-test for group comparison (11).

RESULTS

Effect of chlorpromazine free radical and ouabain on free sulfhydryl groups and $(Na^+ + K^+)$-ATPase activity. The effects of chlorpromazine free radical on free sulfhydryl groups [SH] and $(Na^+ + K^+)$-ATPase activity are shown in Table 1. The enzyme preparation was exposed to ultraviolet light for 4 min or kept in the dark for comparable periods in the presence or ab-

TABLE 1

Effect of chlorpromazine free radical on free [SH] and $(Na^+ + K^+)$-ATPase activity

The enzyme preparation (64 μg of protein per milliliter of 100 mM Tris-HCl buffer, pH 7.5; total volume, 25 ml) was treated as indicated and subsequently centrifuged at 100,000 × *g* for 30 min. The pellet was resuspended with 10 ml of 10 mM Tris-HCl buffer, pH 7.3, and centrifuged again at 100,000 × *g* for 30 min. The pellet was resuspended with 1.5 ml of 10 mM Tris-HCl buffer, pH 7.3. After the determination of protein concentration, free [SH] was determined with 0.5 mg of protein. ATPase activities were also estimated using 16 μg of protein in the total incubation volume of 1.0 ml. Values are the means ± standard errors of five experiments.

Chlorpromazine (5.0 μM)	Ultraviolet exposure	$(Na^+ + K^+)$-ATPase activity	Free [SH]
		μmoles P_i/mg protein/10 min	*nEq/mg protein*
−	−	33.3 ± 1.8	65.9 ± 4.8
−	+	31.3 ± 2.6	65.5 ± 5.5
+	−	26.3 ± 1.1[a]	66.6 ± 3.8
+	+	12.2 ± 1.8[b]	40.3 ± 1.8[b]

[a] Significantly different ($p < 0.01$) from control (line 1).

[b] Significantly different ($p < 0.01$) from controls (lines 1–3).

sence of 5.0 μM chlorpromazine, washed twice by centrifugation, and resuspended to remove excess chlorpromazine. This procedure has been shown previously not to remove the inhibitor bound to the enzyme (2). The final suspension was analyzed for protein concentration and, after appropriate dilution, free [SH] and $(Na^+ + K^+)$-ATPase activity were assayed. Ultraviolet exposure of the enzyme preparation, in the absence of chlorpromazine, had little effect on free [SH] and on $(Na^+ + K^+)$-ATPase activity (line 2). Chlorpromazine, without ultraviolet exposure, also had no effect on free [SH] although it reduced $(Na^+ + K^+)$-ATPase activity (line 3). This inhibition in the absence of ultraviolet exposure was not observed in previous studies (1, 2) or in subsequent ones. However, in this series, in which both $(Na^+ + K^+)$-ATPase activity and free sulfhydryl groups were assayed simultaneously, it was not possible to perform the experiment in total darkness. The difference between lines 3 and 4 is due to the ultraviolet light-induced formation of the free radical form of chlorpromazine (1, 2). It is clearly shown that chlorpromazine free radical decreases free [SH] and inhibits $(Na^+ + K^+)$-ATPase activity significantly.

The relationship between the decrease in free [SH] and $(Na^+ + K^+)$-ATPase activity induced by chlorpromazine free radical is shown in Fig. 1, in which free [SH] is plotted against the $(Na^+ + K^+)$-ATPase activity of enzyme preparations treated with chemically prepared solid chlorpromazine free radical. A significant linear correlation was observed

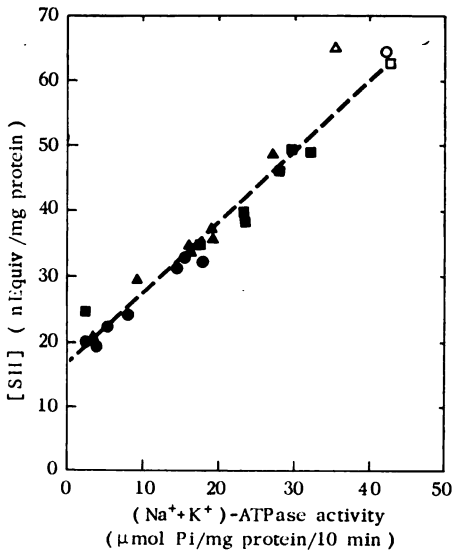

FIG. 1. *Free [SH] and $(Na^+ + K^+)$-ATPase activity of enzyme preparation treated with chlorpromazine free radical*

Various amounts of solid chlorpromazine free radical were added to 5–7 ml of the enzyme suspension (0.2 mg of protein per milliliter of 100 mM Tris-HCl buffer, pH 7.5) and mixed vigorously. Two 2.0-ml aliquots containing 0.4 mg of protein each were assayed for free [SH]. ATPase activity was also assayed using 16 μg of protein in a total incubation volume of 1.0 ml. Each point represents the result of an individual experiment run in duplicate. Three different enzyme preparations were used and are represented by different symbols. The open symbols indicate the control values for each enzyme preparation. The orthogonal regression line was fitted by the method of least squares.

between these two parameters ($r = 0.97$; $p < 0.001$). From extrapolation of the regression line, 16 ± 2.1 nEq of [SH] per milligram of protein, or 25% of the total [SH], are unrelated to the ($Na^+ + K^+$)-ATPase activity and nonreactive to chlorpromazine free radical under these experimental conditions. Thus, chlorpromazine free radical seems to inhibit ($Na^+ + K^+$)-ATPase activity by reacting with essential sulfhydryl groups.

The effects of ouabain on free [SH] and ($Na^+ + K^+$)-ATPase activity are shown in Table 2. The enzyme preparation was incubated for 20 min at 37° in the presence or absence of 20 μM ouabain and subsequently washed twice by centrifugation and resuspension to remove excess ouabain. Protein determinations were performed to assure an equal amount of protein in each of the preparations prior to estimation of ($Na^+ + K^+$)-ATPase activity and free [SH] concentration. When enzyme preparations were incubated with ouabain in the absence of NaCl, $MgCl_2$, and ATP, free sulfhydryl concentrations and ($Na^+ + K^+$)-ATPase activities after excess ouabain had been removed (line 2) were similar to those of controls (line 1). However, when the enzyme and ouabain were incubated together in the presence of 80 mM NaCl, 2.0 mM $MgCl_2$, and 2.0 mM Tris-ATP, the mean ($Na^+ + K^+$)-ATPase activity was significantly reduced after washout whereas free [SH] was unchanged (line 4). Incubation of the enzyme preparation with NaCl, $MgCl_2$, and ATP in the absence of inhibitor failed to influence either the free [SH] or ($Na^+ + K^+$)-ATPase activity, indicating that the preliminary incubation with NaCl, $MgCl_2$, and ATP per se had little effect on the enzyme. Thus, while ouabain inhibited ($Na^+ + K^+$)-ATPase activity, it had no effect on enzyme free [SH].

TABLE 2

Effect of ouabain on free [SH] and ($Na^+ + K^+$)-ATPase activity

The enzyme preparation (64 μg of protein per milliliter of 100 mM Tris-HCl buffer, pH 7.5; total volume, 25 ml) was incubated for 20 min at 37° with or without 20 μM ouabain and/or 80 mM NaCl, 2.0 mM $MgCl_2$, and 2.0 mM Tris-ATP, and was subsequently washed twice before assay (see the legend to Table 1). After the determination of protein concentration, free [SH] was determined with 0.5 mg of protein. ATPase activities were also estimated using 16 μg of protein in the total incubation volume of 1.0 ml. Values are the means ± standard errors of five experiments.

Ouabain (20 μM)	Na^+, Mg^{++}, ATP	($Na^+ + K^+$)-ATPase activity	Free [SH]
		μmoles P_i/mg protein/10 min	*nEq/mg protein*
−	−	24.8 ± 1.7	56.6 ± 3.9
+	−	25.6 ± 1.8	57.1 ± 3.7
−	+	25.0 ± 3.1	50.9 ± 3.6
+	+	14.1 ± 1.1[a]	56.9 ± 3.6

[a] Significantly different ($p < 0.01$) from controls (lines 1–3).

A separate series of experiments were performed in order to demonstrate that ouabain is actually bound to the washed enzyme after incubation in the presence of NaCl, $MgCl_2$, and ATP. Tritiated ouabain (0.04 μM) was incubated with the enzyme (64 μg of protein per milliliter of 100 mM Tris-HCl buffer, pH 7.5) in the presence of 80 mM NaCl, 2.0 mM $MgCl_2$, and 2.0 mM Tris-ATP for 20 min at 37°. After repeated washout of the excess ouabain, under the the same condition as above, the 100,000 × *g* sediment contained 132 ± 2 pmoles of ouabain per milligram of enzyme protein, whereas only 0.3 ± 0.1 pmole of ouabain per milligram of enzyme protein was found in the sediment when the enzyme and ouabain were incubated in the absence of NaCl, $MgCl_2$, and ATP (mean ± standard error of four experiments). Protein yield was identical under both experimental conditions. It should be noted, however, that this experiment was designed primarily to show the requirements for ouabain binding and the actual binding of ouabain to the enzyme preparation after repeated washout. The ouabain concentration used, 0.04 μM, does not inhibit ($Na^+ + K^+$)-ATPase activity significantly. Thus, the value of 132 pmoles of ouabain bound per milligram of enzyme protein does not represent the maximal capacity of this enzyme preparation to bind ouabain. To avoid nonspecific binding, a rather low concentration of ouabain was used.

Failure of interaction between chlorpromazine free radical and ouabain on $(Na^+ + K^+)$-ATPase activity. In order to investigate the inhibitory sites, the interaction between inhibition by chlorpromazine free radical and ouabain of $(Na^+ + K^+)$-ATPase activity was studied. First, the inhibitory effect of chlorpromazine free radical was compared in control and ouabain-treated enzymes (Table 3). The enzyme was incubated with 80 mM NaCl, 2.0 mM $MgCl_2$, and 2.0 mM Tris-ATP with or without 40 μM ouabain. After washing of the enzyme by repeated centrifugation and resuspension to remove excess ouabain, the final enzyme suspension, 160 μg of protein per milliliter, was exposed to ultraviolet light in the presence or absence of 8.0 μM chlorpromazine. Ouabain treatment alone inhibited $(Na^+ + K^+)$-ATPase activity by 25.9 ± 4.2% (mean ± standard error of six experiments) and failed to change free [SH] (line 3). Chlorpromazine free radical inhibited $(Na^+ + K^+)$-ATPase activity and free [SH] of control and ouabain-treated enzyme preparations to the same extent, in terms of percentage inhibition (lines 2 and 4 compared with lines 1 and 3, respectively), indicating that the ouabain treatment did not block the inhibitory effect of chlorpromazine free radical on $(Na^+ + K^+)$-ATPase and on free [SH].

To study further the interaction between inhibition by chlorpromazine free radical and ouabain of $(Na^+ + K^+)$-ATPase activity, and to explore the nature of the inhibitory site for chlorpromazine free radical, the effects of ouabain and *p*-hydroxymercuribenzoate on chlorpromazine free radical-treated enzyme were compared (Table 4). The enzyme was first exposed to ultraviolet light in the absence (control enzyme preparation) or presence (chlorpromazine free radical-treated enzyme preparation) of 2.5 μM chlorpromazine. Both control and chlorpromazine free radical-inhibited enzyme preparations were assayed for ATPase activity in the presence or absence of the inhibitors ouabain and *p*-hydroxymercuribenzoate. Treatment of the enzyme preparation with chlorpromazine free radical reduced its activity by 45.0 ± 7.0% (mean ± standard error of eight experiments). In the control enzyme preparation, 1.0 μM ouabain inhibited $(Na^+ + K^+)$-ATPase activity by 60%. *p*-Hydroxymercuribenzoate at 0.5 and 0.2 μM caused 54 and 25% inhibition, respectively. This is in good agreement with the calculated inhibition of this enzyme by the organic mercurial. These enzyme preparations contain approximately 64 nEq of [SH] per milligram of protein (Fig. 1). Since each 1.0 ml of incubation mixture contained 16 μg of enzyme

TABLE 3

Effects of ouabain and chlorpromazine free radical on free [SH] and $(Na^+ + K^+)$-ATPase activity

The enzyme preparation (64 μg of protein per milliliter of 100 mM Tris-HCl buffer, pH 7.5; total volume, 25 ml) was incubated for 20 min at 37° with 80 mM NaCl, 2.0 mM $MgCl_2$, and 2.0 mM Tris-ATP with or without 40 μM ouabain, and was subsequently washed twice (see the legend to Table 1). The washed enzyme suspension (160 μg of protein per milliliter of 100 mM Tris-HCl buffer, pH 7.5) was exposed to ultraviolet light in the presence or absence of 8.0 μM chlorpromazine before assay. Free [SH] was determined with 0.32 mg of protein. ATPase activities were also estimated using 16 μg of protein in the total incubation volume of 1.0 ml. Values are the means ± standard errors of six experiments.

Prior treatment with ouabain	Chlorpromazine (8.0 μM)	Ultraviolet exposure	$(Na^+ + K^+)$-ATPase activity	Inhibition due to chlorpromazine free radical	Free [SH]
			μmoles P_i/mg protein/10 min	*%*	*nEq/mg protein*
−	−	+	25.9 ± 3.1		48.1 ± 2.9
−	+	+	12.6 ± 0.8[a]	50.6 ± 4.7	34.3 ± 1.9[a]
+	−	+	19.3 ± 2.7		47.6 ± 2.8
+	+	+	9.7 ± 0.8[b]	50.3 ± 4.6	33.5 ± 2.5[b]

[a] Significantly different ($p < 0.01$) from control (line 1).

[b] Significantly different ($p < 0.01$) from ouabain-treated enzyme (line 3).

TABLE 4

Effect of ouabain or p-hydroxymercuribenzoate (HMB) on ($Na^+ + K^+$)-ATPase inhibited by chlorpromazine free radical

The enzyme suspension (32 μg of protein per milliliter of 100 mM Tris-HCl buffer, pH 7.5; total volume, 25 ml) was exposed to ultraviolet light in the absence (for control enzyme preparation) or presence (for enzyme preparations inhibited by chlorpromazine free radical) of 2.5 μM chlorpromazine and was subsequently washed twice (see the legend to Table 1). After protein determination, the ATPase activity was estimated in the presence of inhibitors. The enzyme concentration was 16 μg of protein per milliliter. Values are the means ± standard errors of eight experiments.

Enzyme and inhibitor	($Na^+ + K^+$)-ATPase activity	Inhibition	
	μmoles P_i/mg protein/10 min	*μmoles P_i/mg protein/10 min*	%
Control			
No inhibitor	26.0 ± 3.0		
Ouabain (1.0 μM)	10.6 ± 1.7	15.4 ± 1.8	59.8 ± 4.8
HMB (0.2 μM)	20.4 ± 3.6	5.6 ± 1.1	25.1 ± 7.0
HMB (0.5 μM)	12.1 ± 2.3	14.0 ± 2.1	53.5 ± 5.7
Chlorpromazine free radical-treated			
No inhibitor	13.7 ± 1.6	(12.3 ± 2.9)[a]	(45.0 ± 7.0)[a]
Ouabain (1.0 μM)	6.3 ± 0.6	7.4 ± 1.5[b]	51.1 ± 4.7[b]
HMB (0.2 μM)	7.2 ± 0.8	6.5 ± 1.3[b]	44.7 ± 5.8[b]
HMB (0.5 μM)	1.8 ± 0.3	11.9 ± 1.4[b]	87.2 ± 1.9[b]

[a] Inhibition due to chlorpromazine free radical compared to untreated control.

[b] Inhibition due to inhibitors (ouabain and p-hydroxymercuribenzoate) compared to enzyme preparation treated with chlorpromazine free radical.

protein, the amount of free [SH] was approximately 1 nEq. Thus, 0.5 and 0.2 μM p-hydroxymercuribenzoate, or 0.5 and 0.2 nmole of mercurial in a 1.0-ml incubation mixture, could titrate 50 and 20% of [SH] groups, respectively.

In the control enzyme preparation, 1.0 μM ouabain and 0.5 μM p-hydroxymercuribenzoate had similar inhibitory effects on ($Na^+ + K^+$)-ATPase activity under these experimental conditions, whereas 0.2 μM mercurial was significantly less effective ($p < 0.01$). In the chlorpromazine free radical-treated enzyme preparation, however, 0.2 μM p-hydroxymercuribenzoate had an inhibitory effect similar to that of 1.0 μM ouabain, and 0.5 μM p-hydroxymercurbenzoate was significantly more effective ($p < 0.01$). Furthermore, 1.0 μM ouabain inhibited the control and chlorpromazine free radical-treated enzyme preparations similarly in terms of percentage inhibition but dissimilarly in terms of micromoles of P_i per milligram of protein per 10 min. On the other hand, the inhibition of both preparations of ($Na^+ + K^+$)-ATPase by 0.2 and 0.5 μM p-hydroxymercuribenzoate was similar in terms of micromoles of P_i per milligram of protein per 10 min but dissimilar in percentage terms. Thus, it is apparent that 0.5 and 0.2 μM p-hydroxymercuribenzoate selectively titrated 0.5 and 0.2 nEq of free [SH] on the enzyme molecule which had not been inactivated by prior treatment with chlorpromazine free radical. These results indicate that ouabain can react with the free radical-inhibited enzyme. On the other hand, p-hydroxymercuribenzoate is unable to react with the enzyme previously exposed to chlorpromazine free radical.

Effect of prior treatment with chlorpromazine free radical on binding of ^3H-ouabain by ($Na^+ + K^+$)-ATPase. To determine whether chlorpromazine free radical and ouabain bind to ($Na^+ + K^+$)-ATPase at different sites, the ability to bind ^3H-ouabain was studied in enzyme preparations that had been treated with chlorpromazine free radi-

cal (Fig. 2). The enzyme suspension was exposed to ultraviolet light for 4 min with different concentrations of chlorpromazine (0–20 μM) before ATPase activity and ^{3}H-ouabain binding were assayed. As an additional control, 40 μM chlorpromazine was added to the enzyme suspension, and the suspension was kept in the dark until assay. During the incubation of ouabain with the brain microsomal fraction treated with deoxycholic acid, it was assumed that metabolism was minimal and all radioactivity assayed was ^{3}H-ouabain. In exploratory experiments, the amount of ouabain bound to the enzyme was halved when the amount of enzyme in the mixture was reduced by one-half, whereas the amount of ouabain bound to the enzyme was 80% of the control value when the concentration of ouabain was reduced by one-half. Thus, the available enzyme protein, not the available amount of ouabain, was the limiting factor under the present experimental conditions. The binding was also specific, since in the absence of ATP only a minimal amount of ouabain was bound to the enzyme.

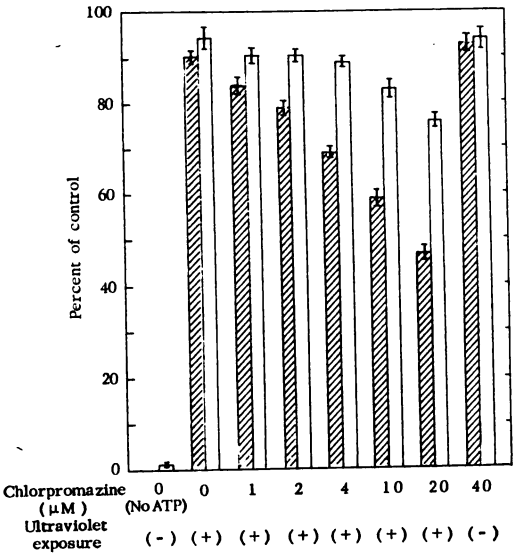

FIG. 2. *$(Na^+ + K^+)$-ATPase activity and ouabain binding in chlorpromazine free radical-treated enzyme preparation*

The enzyme suspension (0.2 mg of protein per milliliter of 100 mM Tris-HCl buffer, pH 7.5; total volume, 3.0 ml) was treated as indicated. "Chlorpromazine (μM)" indicates the concentration of chlorpromazine added to the enzyme suspension before ultraviolet exposure. ATPase activity was assayed after appropriate dilution. Each incubation mixture contained 16 μg of protein per milliliter. Two-tenths milligram of enzyme protein was also incubated with 0.1 μM ouabain and tracer amounts of tritiated ouabain for 10 min at 37° in the presence of 80 mM NaCl, 2.0 mM $MgCl_2$, 2.0 mM Tris-ATP, and 50 mM Tris-HCl buffer, pH 7.5, in a total volume of 2.0 ml. After centrifugation at 100,000 × *g* for 30 min, the pellet and supernatant fraction were assayed for radioactivity. All values are expressed as a percentage of those for the control enzyme preparation, to which no chlorpromazine was added and which was assayed without ultraviolet exposure. Shaded columns represent $(Na^+ + K^+)$-ATPase activity, and open columns indicate the ^{3}H-ouabain bound to the enzyme. "No ATP" (first column at left) indicates that Tris-ATP was omitted from the incubation mixture for the ouabain binding study. Values are the means ± standard errors of four experiments.

Chlorpromazine free radical, but not chlorpromazine itself, inhibited both $(Na^+ + K^+)$-ATPase activity and ouabain binding in a dose-dependent manner. However, the inhibition of ouabain binding was significantly less than that of ATPase activity. No significant reduction of ^{3}H-ouabain binding was observed when the enzyme preparation was first exposed to ultraviolet light in the presence of up to 4.0 μM chlorpromazine, whereas $(Na^+ + K^+)$-ATPase activity was significantly reduced ($p < 0.01$) (Fig. 2).

DISCUSSION

$(Na^+ + K^+)$-ATPase has been shown to contain sulfhydryl groups essential for its enzymatic activity (12–16). Sulfhydryl groups are also known to be effective hydrogen donors and to react rapidly with free radicals (17). In the present experiments the free radical produced by the ultraviolet irradiation of chlorpromazine decreased the free [SH] of the enzyme preparation and concomitantly inhibited $(Na^+ + K^+)$-ATPase activity. Seventy-five per cent of the total sulfhydryl groups of the enzyme preparation reacted with chemically produced free radical of chlorpromazine and totally inhibited the enzyme activity.

Because sulfhydryl groups are known to react with free radicals, it was of some importance to establish whether or not the

interaction between chlorpromazine free radical and sulfhydryl groups is a general or a more specific effect. While the present study indicates that chlorpromazine free radical can combine with sulfhydryl groups other than those associated with (Na^+ + K^+)-ATPase, earlier studies have demonstrated the complexity of this interaction. Oxidation per se is not a factor in the free radical inhibition of the enzyme, since agents such as metabisulfite and ascorbate failed to reactivate the inhibited enzyme. Reactivation, however, was observed on treatment with cysteine, dithiothreitol, or NaCl. It may be concluded from these observations that chlorpromazine free radical binds to, but does not oxidize, free sulfhydryl groups.

While it is true that the free radical of chlorpromazine could have interacted nonselectively with sulfhydryl groups and thereby inhibited a wide variety of enzymes, some specificity of the free radical has already been demonstrated. Mg^{++}-ATPase activity is much less sensitive (2), as are NADH–cytochrome *c* oxidoreductase and cholinesterase activities measured in the same rat brain fraction.[1]

Ouabain did not decrease free [SH] when it inhibited (Na^+ + K^+)-ATPase activity significantly. These data suggest that chlorpromazine free radical and ouabain react with chemically different sites on (Na^+ + K^+)-ATPase. Since our enzyme preparation contained 60 nEq of free [SH] per milligram of protein, it is possible that the interaction of ouabain with free [SH] would have been undetected. Nor do the present studies with chlorpromazine free radical differentiate between the sulfhydryl groups essential and nonessential for (Na^+ + K^+)-ATPase activity. While there is a correlation between the chlorpromazine free radical-induced inhibition of (Na^+ + K^+)-ATPase activity and the enzyme free sulfhydryl concentration, it is still possible that chlorpromazine free radical interacts with sulfhydryl groups unnecessary for enzyme catalysis.

The present data clearly indicate that chlorpromazine free radical and ouabain do not compete for the same enzyme site in inhibiting (Na^+ + K^+)-ATPase activity. Both the chlorpromazine-enzyme complex (2) and the ouabain-enzyme complex have been shown to be stable (5, 18, 19). The irreversibility of the *p*-hydroxymercuribenzoate–[SH] complex is also recognized (2, 16). Therefore, under the present experimental conditions, chlorpromazine free radical, ouabain, or *p*-hydroxymercuribenzoate bound to the enzyme is in a nonequilibrium state with respect to its concentration in the incubation medium. Furthermore, these inhibitors are capable of total inactivation of the enzyme if the concentrations are adequate (2, 5, 13). Thus, it is reasonable to assume that the enzyme molecule bound to chlorpromazine free radical, ouabain, or *p*-hydroxymercuribenzoate loses its activity completely and that the percentage inhibition of enzyme activity represents the proportion of inhibitor-bound, inactivated enzyme molecules in the total enzyme population. This may be visualized as the inhibitor titrating the active sites on the enzyme. Under these circumstances, one would expect that, if the two inhibitors interact with the enzyme at the same site, sequential treatment of the enzyme with these inhibitors would result in the second inhibitor titrating only those sites not inactivated by the first inhibitor (Fig. 3, bar *C*). This was the case with the sequential treatment of the enzyme with chlorpromazine free radical and *p*-hydroxymercuribenzoate (compare bars *C* and *E*, Fig. 3). On the other hand, if two inhibitors interact with the enzyme at different sites and therefore are not competitive, their sequential incubation with the enzyme would result in the second inhibitor titrating active sites of normal and inactivated enzyme molecules indiscriminately. This was the case with the sequential treatment of the enzyme with chlorpromazine free radical and ouabain (compare bars *D* and *E* of Fig. 4). Since ouabain bound to the enzyme molecule previously inactivated by chlorpromazine free radical did not contribute to the reduction in enzyme activity, the second inhibitor resulted in a lower rate of inhibition of the treated enzyme than control enzyme in terms of micromoles per

[1] Unpublished observations.

milligram of protein per minute. The alternative explanation for the reduced inhibition is that the second inhibitor reacting with the same site on the enzyme may have replaced the first inhibitor bound to the enzyme. If

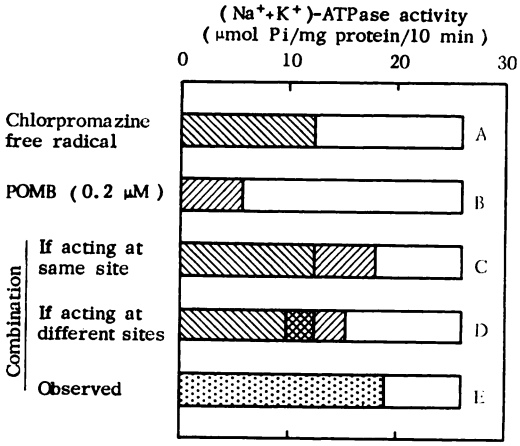

FIG. 3. *Possible and actual interactions between chlorpromazine free radical and p-hydroxymercuribenzoate (POMB) with $(Na^+ + K^+)$-ATPase*

The data for bars *A*, *B*, and *E* are taken from Table 4. Shaded areas represent inhibited enzyme activity. Open areas represent $(Na^+ + K^+)$-ATPase activity observed (*A*, *B*, and *E*) or to be expected (*C* and *D*).

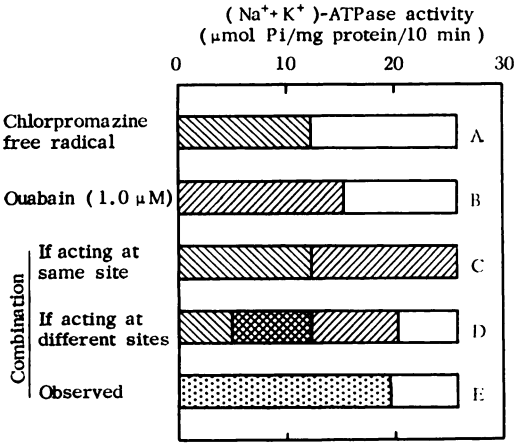

FIG. 4. *Possible and actual interactions between chlorpromazine free radical and ouabain with $(Na^+ + K^+)$-ATPase*

The data for bars *A*, *B*, and *E* are taken from Table 4. Shaded areas represent inhibited enzyme activity. Open areas represent $(Na^+ + K^+)$-ATPase activity observed (*A*, *B*, and *E*) or to be expected (*C* and *D*).

the second inhibitor had a significantly higher affinity for the enzyme than the first inhibitor, this may have occurred. This possibility, however, is excluded in the case of chlorpromazine free radical and ouabain, since sequential treatment of the $(Na^+ + K^+)$-ATPase in reverse order gave similar results: inhibition by the second inhibitor was always the same in control and in inhibited enzyme preparations in terms of percentage inhibition (Table 3). The possible and actual interactions between the various agents are shown in Figs. 3 and 4.

The relationship between inhibitory sites on $(Na^+ + K^+)$-ATPase for chlorpromazine free radical and ouabain is further substantiated by the ^{3}H-ouabain binding studies. If chlorpromazine free radical and ouabain bind to the enzyme at the same site or at closely related sites, the relative inhibition of $(Na^+ + K^+)$-ATPase activity and ouabain binding should be identical, since the enzyme inactivated by chlorpromazine free radical cannot bind ouabain further. Schwartz *et al.* (20) have shown that ouabain binds selectively to $(Na^+ + K^+)$-ATPase. If the binding sites for chlorpromazine free radical are different from the binding sites of ouabain, chlorpromazine free radical should not interfere with ouabain binding while inhibiting ATPase activity. The present data indicate that the binding sites for both inhibitors are not identical or even very close, since chlorpromazine free radical bound to the enzyme did not reduce the binding of ouabain proportionally to $(Na^+ + K^+)$-ATPase activity, although there was a minimal reduction in ^{3}H-ouabain binding at high chlorpromazine concentrations.

Although a linear relationship was observed between the decrease of free [SH] and the inhibition of $(Na^+ + K^+)$-ATPase activity, this does not imply the presence of a single sulfhydryl group on each active site of the enzyme. Such a relationship has also been reported with several enzymes which have multiple sulfhydryl groups per active site (21–26). Since the present enzyme preparations contain approximately 64 nEq of sulfhydryl groups per milligram of protein and the molecular weight of $(Na^+ + K^+)$-ATPase is estimated to be 250,000 (27), each

enzyme molecule would contain 16 sulfhydryl groups, assuming that the distribution is uniform among enzyme protein and non-enzyme contaminating protein. The present results showed that 25% of these sulfhydryl groups were unrelated to (Na^+ + K^+)-ATPase activity and nonreactive to chlorpromazine free radical. These data, however, do not indicate whether this was due to the difference in chemical structure surrounding these sulfhydryl groups or resulted from the fact that these sulfhydryl groups become accessible only after denaturation of the enzyme protein.

The hypothesis that the semiquinone free radical is the active form of the tranquilizing agent, chlorpromazine, is an attractive one. If one assumes that 50 mg of chlorpromazine in a 70-kg man can elicit a pharmacological response, the estimated concentration of chlorpromazine in the brain would be between 10^{-6} and 10^{-5} M, assuming a 4-fold concentration of the drug in the brain (28). The inhibition of a key metabolic function by such concentrations of chlorpromazine has not been demonstrated (29). It is also possible that the free radical could be generated at a specific site within the brain. It has been shown that the free radical can be formed enzymatically by the oxidative metabolism *in vivo* (30) or nonenzymatically by melanin (31). If it were generated by these or some other mechanism in the brain near a key metabolic process at a specific anatomical site, this could perhaps lead us closer to an explanation of the mechanism of action of the drug. The rapid dissipation of the free radical (2) could provide an alternative explanation for its selectivity. At least, such a hypothesis would provide an answer to two aspects of the action of chlorpromazine, its potency and its selectivity.

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