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Photoaffinity Labeling of Calmodulin by Phenothiazine Antipsychotics

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SUMMARY

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Previous studies have shown that the binding of radiolabeled phenothiazine antipsychotics to calmodulin can be reversed by removing calcium or by dialyzing against an excess of nonradiolabeled phenothiazine. The present studies show that the binding of [^{3}H]-chlorpromazine or [^{3}H]trifluoperazine to calmodulin can be made irreversible by irradiating the samples with UV light. Like the reversible binding of these agents to calmodulin, the irreversible binding was enhanced by calcium. This calcium-dependent binding was saturable, with approximately one binding site per molecule of calmodulin; one-half maximal binding occurred at a chlorpromazine concentration of approximately 10 μM and at a trifluoperazine concentration of 5 μM. The irreversible binding of chlorpromazine to calmodulin probably involves the formation of a covalent bond, since extensive dialysis and treatment with denaturing agents failed to displace the bound chlorpromazine from calmodulin. Chlorpromazine displayed little or no irreversible calcium-dependent binding to bovine serum albumin, trypsin, histone, or hemoglobin. Nonphenothiazine antipsychotics, such as penfluridol and haloperidol, and a number of other centrally active drugs, such as diazepam, apomorphine, and dopamine, showed little or no irreversible binding to calmodulin. However, the nonphenothiazine antipsychotics did block the irreversible binding of chlorpromazine and trifluoperazine to calmodulin. Psychotropic drugs with little antipsychotic activity did not prevent the irreversible binding of the phenothiazines. UV-irradiated samples of calmodulin and chlorpromazine or trifluoperazine failed to activate phosphodiesterase even after extensive dialysis, indicating that the irreversible binding of the phenothiazines to calmodulin results in an irreversible inhibition of the biological activity of calmodulin. These results suggest that the irreversible binding of antipsychotics to calmodulin may account for some of the long-term actions of these drugs and may provide a means with which to study the location and turnover of this calcium-binding protein.

INTRODUCTION

Calmodulin is a ubiquitous, heat-stable, calcium-binding protein (1) that has been shown to activate several calcium-dependent enzymes, including phosphodiesterase (1, 2), adenylate cyclase (3, 4), (Ca^{2+} + Mg^{2+})-ATPase (5, 6), myosin light-chain kinase (7), and phospholipase A_2 (8). The calmodulin-induced activation of these enzymes is inhibited by a variety of antipsychotic drugs (9–12), suggesting that the inhibition of calmodulin-sensitive enzymes may provide a common mechanism for explaining some of the diverse biochemical actions of antipsychotics (12).

The mechanism by which antipsychotic drugs inhibit calmodulin-activated enzymes is through the direct binding of these drugs to calmodulin (12–15). These early studies showed that the binding of antipsychotic drugs to calmodulin could be reversed readily by chelating calcium or by dialyzing against an excess of nonlabeled drugs. However, the reports indicating that certain phenothiazine antipsychotics irreversibly inhibit ATPase activity (16) and irreversibly bind to bovine serum albumin (17) following photochemical activation suggested that photochemically activated phenothiazines might also bind irreversibly to calmodulin. Accordingly, we have studied the influence of UV irradiation on the binding of phenothiazine antipsychotic drugs to calmodulin.

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MATERIALS AND METHODS

Source of chemicals. [^{3}H]Chlorpromazine (26.5 Ci/mmole), [^{3}H]dopamine (18.8 Ci/mmole), [^{3}H]-haloperidol (13.1 Ci/mmole), and [^{3}H]-apomorphine (38.6 Ci/mmole) were purchased from New England Nuclear Corporation, Boston, Mass. Analysis of [^{3}H]chlorpromazine and [^{3}H]trifluoperazine by thin-layer chromatography showed the compounds to be greater than 95% pure. Additional drug samples were generously provided as follows: trifluoperazine, trifluoperazine sulfoxide, [^{3}H]trifluoperazine (38 mCi/mmole), chlorpromazine, and chlorpromazine sulfoxide by Smith Kline Laboratories (Philadelphia, Pa.); diazepam and [^{3}H]diazepam (7.4 mCi/mmole) by Hoffman LaRoche, Inc. (Nutley, N. J.); penfluridol by Janssen Pharmaceutica (Beerse, Belgium); and [^{3}H]penfluridol (46 mCi/mmole) by McNeil Laboratories, Inc. (Fort Washington, Pa.).

Assay of phosphodiesterase activity. An activatable form of phosphodiesterase was prepared from the soluble fraction of rat cerebrum according to the procedure previously described (2). Phosphodiesterase activity was measured by the luciferin-luciferase method (18) using 400 μM cyclic AMP as substrate.

Preparation and assay of calmodulin. Calmodulin was purified from bovine brain by the method of Teo *et al.* (19). Calmodulin activity was assessed by its ability to increase the activity of a calmodulin-deficient phosphodiesterase prepared from rat brain. One unit of calmodulin is defined as the amount necessary to produce 50% of the maximal activation of this calmodulin-deficient enzyme.

Irreversible binding of drugs to calmodulin. Calmodulin (15 μg) and various concentrations of radiolabeled drugs in 1 ml of 5 mM Tris-HCl buffer, pH 7.0, containing 1 mM $MgCl_2$ and either 0.1 mM $CaCl_2$ or 0.3 mM EGTA,[3] were incubated at 4° in a 5-ml glass culture tube. The samples were stirred vigorously with a Teflon stirring bar and were irradiated from above for 1 hr with a short wavelength (254 nm) UV lamp (General Electric Model G30T8) at a distance of 10 cm from the surface of the solution. Nonirradiated samples were stirred and incubated in the dark for 1 hr at 4°. Samples (0.5 ml each) were then placed in seamless cellulose tubing (Fisher Scientific Company, Pittsburgh, Pa.) having a molecular weight cutoff of approximately 12,000 and were dialyzed for 16–20 hr against 20 ml of 5 mM Tris-HCl buffer, pH 7.0, containing 1 mM $MgCl_2$ and either 0.1 mM $CaCl_2$ or 0.3 mM EGTA. Nonlabeled chlorpromazine (1 mM) or trifluoperazine (1 mM) was also included in the dialysis buffer when indicated. The 16 to 20-hr period of dialysis was sufficient to achieve equilibrium. Following dialysis, 0.3-ml portions of the solutions inside and outside the dialysis bag were transferred to plastic scintillation vials. Scintillation fluid (5 ml) containing 1 mM nonlabeled chlorpromazine or trifluoperazine was added and radioactivity was determined in a liquid scintillation spectrometer. The addition of nonlabeled drugs to the vials reduces variability in counting by displacing labeled drug that may be bound to the vials. The amount of bound radioligand was calculated as the difference between the counts inside the dialysis bag and the counts in the dialysis bath. Calcium-dependent binding was defined as the difference between the binding in the presence and absence of calcium.

[3] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

RESULTS

Irreversible binding of chlorpromazine to calmodulin. Figure 1 shows the influence of UV irradiation on the binding of [^{3}H]chlorpromazine to calmodulin. If the samples were not irradiated, less than 10 nmoles of chlorpromazine per milligram of calmodulin remained bound after dialyzing against calcium. The amount of chlorpromazine bound was reduced further by dialyzing against EGTA or 1 mM nonlabeled chlorpromazine. By contrast, if the samples of [^{3}H]chlorpromazine and calmodulin were irradiated by UV light, approximately 65 nmoles/mg of calmodulin remained bound after dialyzing against calcium. Furthermore, this binding could not be reversed by dialyzing against either EGTA or nonlabeled chlorpromazine.

Other experiments showed that urea (5 M) and hexadecyltrimethylammonium bromide (10 mM), agents which alter the ionic characteristics and tertiary structure of many proteins (20), reduced by 70–80% the binding of the phenothiazines to calmodulin if added before the samples were irradiated but failed to dissociate the

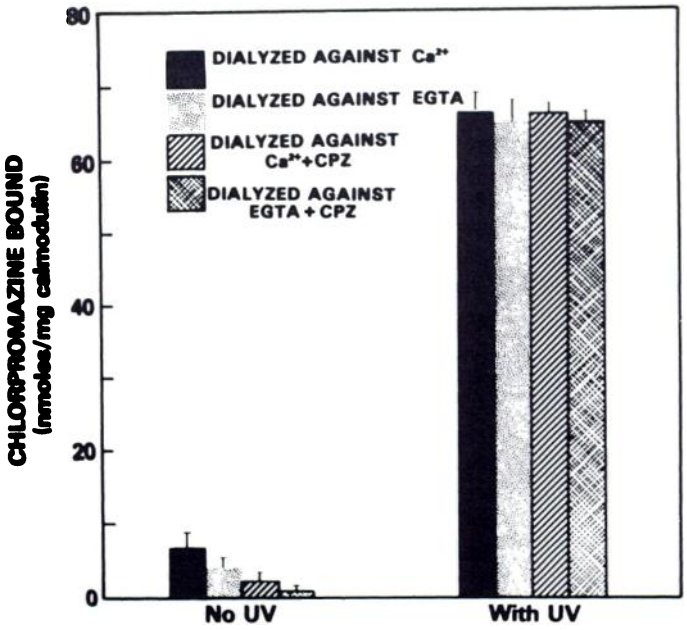

FIG. 1. *Effect of UV-irradiation on the reversibility of binding of [^{3}H]chlorpromazine to calmodulin*

Samples of calmodulin (15 μg) and [^{3}H]chlorpromazine (*CPZ*) (16 μM) in 1 ml of 5 mM Tris-HCl buffer (pH 7.0) containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ were incubated at 4° for 60 min in the presence or absence of UV light. Samples were then dialyzed for 16 hr against 5 mM Tris buffer containing 1 mM $MgCl_2$ and (a) 0.1 mM $CaCl_2$, (b) 0.1 mM EGTA, (c) 0.1 mM $CaCl_2$ and 1 mM nonlabeled chlorpromazine, or (d) 0.3 mM EGTA and 1 mM nonlabeled chlorpromazine. The radioactivity that remained bound to calmodulin was then determined. Each *bar* represents the mean of four samples. Vertical brackets indicate the standard error.

drugs from calmodulin if added after irradiation. Although it is still unclear whether these agents did, in fact, denature calmodulin, these results suggest that UV irradiation may induce the formation of a covalent bond between the phenothiazines and calmodulin.

Time course for the irreversible binding of chlorpromazine. Under the conditions described under Materials and Methods, the irreversible binding of chlorpromazine to calmodulin occurred rapidly and was essentially complete after 1 hr of UV irradiation (Fig. 2). Varying the distance between the sample and the light source from 2 to 30 cm appeared to have little effect on the amount of irreversible binding. In addition, no substantial differences in the irreversible binding was found between short (254 nm) and long (355 nm) wavelength (Mineralight, Model UVSL-25) light sources.

Effect of calcium on the irreversible binding of phenothiazines. Figure 3 shows the influence of calcium on the irreversible binding of chlorpromazine to calmodulin. The binding of chlorpromazine was much greater in the presence of calcium than in its absence, i.e., in the presence of EGTA. This calcium-dependent binding was saturable, with a maximum of approximately 50 nmoles of chlorpromazine bound per milligram of calmodulin, or about one binding site per molecule of calmodulin. One-half maximal binding occurred at a chlorpromazine concentration of approximately 10 μM. The irreversible, calcium-dependent binding of chlorpromazine to calmodulin increased linearly with increasing concentrations of calmodulin up to at least 40 μg of calmodulin per milliliter (data not shown).

The irreversible binding of trifluoperazine to calmodulin (data not shown) was also enhanced by calcium, and there was a maximum of one calcium-dependent binding site per molecule of calmodulin. One-half maximal binding of trifluoperazine occurred at a concentration of 5 μM.

Irreversible binding of various psychoactive drugs to calmodulin. Although several different classes of antipsychotic drugs bind reversibly to calmodulin (15) not all antipsychotic classes could be photoactivated to bind irreversibly to calmodulin. The phenothiazines, trifluoperazine and chlorpromazine, displayed the highest degree of irreversible binding to calmodulin (Fig. 4). By contrast, under the same conditions of irradiation and dialysis, nonphenothiazine antipsychotic drugs, such as the butyrophenone, haloperidol, and the diphenylbutylpiperidine, penfluridol, as well as other centrally active

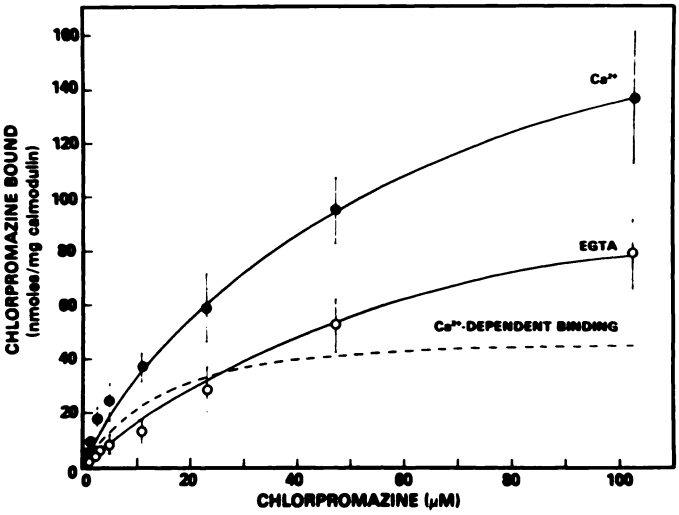

FIG. 3. *Influence of calcium on irreversible binding of chlorpromazine to calmodulin*

Calmodulin (15 μg/ml) in Tris buffer containing 1 mM $MgCl_2$ and either 0.1 mM $CaCl_2$ or 0.3 mM EGTA, was irradiated for 60 min in the presence of increasing concentrations of chlorpromazine. Following irradiation, samples were dialyzed against Tris buffer containing 1 mM $MgCl_2$, 0.3 mM EGTA, and 1 mM nonlabeled chlorpromazine. – – –, Calcium-dependent binding (the binding in the presence of calcium minus the binding in the presence of EGTA). Each *point* represents the mean of four samples. Vertical brackets indicate the standard error.

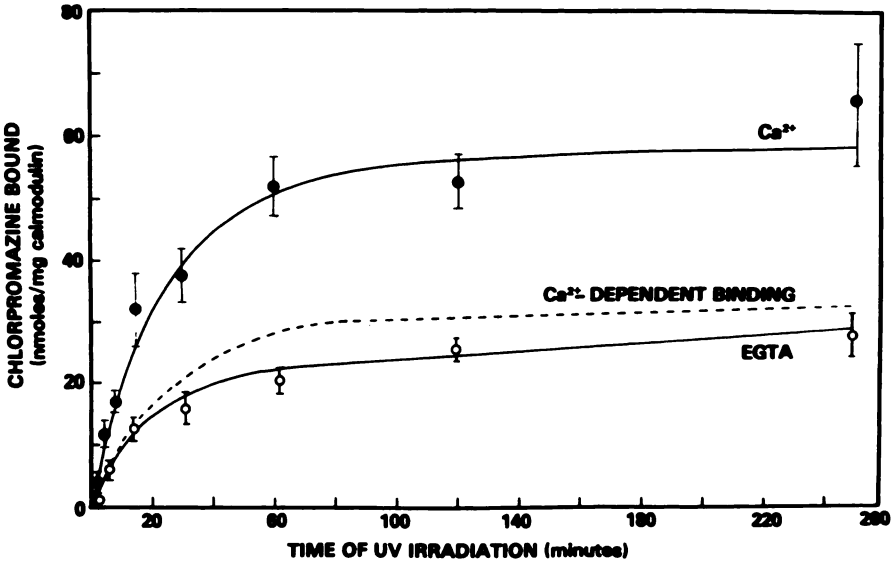

FIG. 2. *Irreversible binding of chlorpromazine to calmodulin as a function of time of irradiation*

Calmodulin (15 μg/ml) and [^{3}H]chlorpromazine (16 μM) were irradiated for varying lengths of time as described in legend to Fig. 1. Samples were then dialyzed against Tris buffer containing 1 mM $MgCl_2$, 0.3 mM EGTA, and 1 mM nonlabeled chlorpromazine, and the amount of radioactivity that remained bound to calmodulin was determined. – – –, Calcium-dependent binding (the binding in the presence of calcium minus the binding in the presence of EGTA). Each *point* represents the mean of three samples. Vertical brackets indicate the standard error.

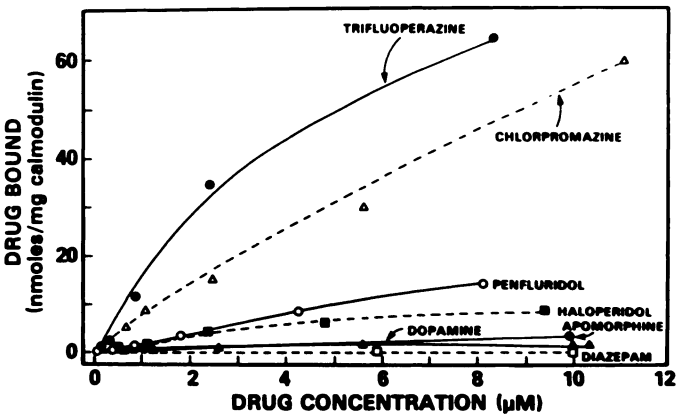

FIG. 4. *Selectivity of irreversible binding of various drugs to calmodulin*

Calmodulin (15 μg/ml) and varying concentrations (0.1–10 μM) of radiolabeled drugs were irradiated under UV light and were then dialyzed against Tris buffer containing 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1 mM nonlabeled drug.

compounds, such as dopamine, apomorphine, and diazepam, showed little or no irreversible binding to calmodulin.

Selectivity of the irreversible binding of chlorpromazine to various proteins. Figure 5 shows the irreversible binding of chlorpromazine to several proteins. Calmodulin showed the highest degree of irreversible binding of chlorpromazine. There was little or no irreversible binding of chlorpromazine to bovine serum albumin, histone, hemoglobin, or trypsin either in the presence or absence of calcium.

Inhibition of the irreversible binding of chlorpromazine by various drugs. Previous studies have shown that different chemical classes of antipsychotic drugs prevented the reversible binding of trifluoperazine to calmodulin (15). Therefore, we examined the ability of various drugs to inhibit the irreversible binding of chlorpromazine to this protein. Results in Fig. 6 show the effects of penfluridol on the irreversible binding of [^{3}H]-chlorpromazine to calmodulin. Penfluridol had little effect on nonspecific binding (i.e., binding in the presence of EGTA) but greatly reduced the calcium-dependent binding.

Table 1 shows the concentration of several psychoactive drugs needed to inhibit 50% of the irreversible, calcium-dependent binding of chlorpromazine to calmodulin (IC_{50}). The most potent compounds examined were the antipsychotic drugs, trifluoperazine and penfluridol. Chlorpromazine sulfoxide and trifluoperazine sulfoxide, pharmacologically inactive metabolites of chlorpromazine and trifluoperazine, and diazepam, an antianxiety drug, were 5–10 times less potent in preventing the binding of chlorpromazine to calmodulin.

Effects of the irreversible binding of phenothiazines to calmodulin on the biological activity of calmodulin. To determine whether the irreversible binding of phenothiazines alters the biological activity of calmodulin, the protein and phenothiazines were irradiated, dialyzed to remove reversibly bound drugs, and then examined for their ability to activate a calmodulin-dependent phosphodiesterase. Results presented in Fig. 7 show that UV irradiation of calmodulin alone did not affect its ability to activate phosphodiesterase. However, UV-irradiated samples of calmodulin and the phenothiazines failed to activate phosphodiesterase even after extensive dialysis, although similarly dialyzed, nonirradiated preparations of calmodulin and the drugs increased phosphodiesterase approximately 2.5-fold. Thus, it appears that the irreversible binding of the phenothiazines results in an irreversible inactivation of calmodulin.

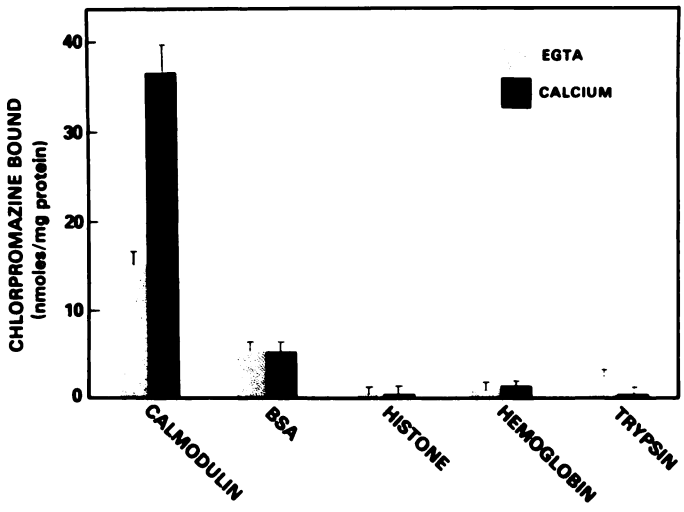

FIG. 5. *Selectivity of irreversible binding of chlorpromazine to various proteins*

Various proteins (15 μg/ml) were incubated and irradiated with [^{3}H]-chlorpromazine (16 μM) in Tris buffer containing either 0.1 mM $CaCl_2$ or 0.3 mM EGTA. Portions (0.5 ml each) were then dialyzed against Tris buffer containing 0.3 mM EGTA and 1 mM nonlabeled chlorpromazine. Each *bar* represents the mean of four samples. Vertical brackets indicate the standard error. *BSA*, Bovine serum albumin.

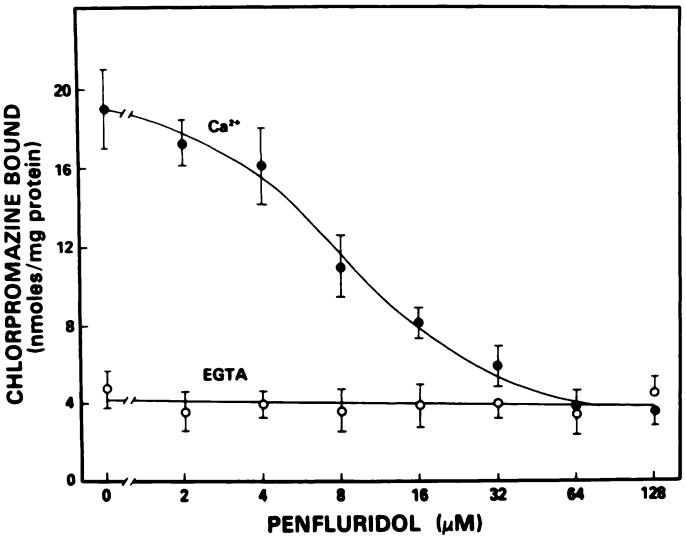

FIG. 6. *Inhibition of irreversible binding of [^{3}H]chlorpromazine to calmodulin by penfluridol*

Calmodulin (15 μg/ml) and varying concentrations of penfluridol in 1 ml of Tris buffer containing 1 mM $MgCl_2$ and either 0.1 mM $CaCl_2$ or 0.3 mM EGTA were preincubated at 4° for 30 min. [^{3}H]Chlorpromazine (final concentration 16 μM) was then added, and the samples were irradiated for 1 hr and dialyzed against Tris buffer containing 1 mM $MgCl_2$, 0.3 mM EGTA, and 1 mM nonlabeled chlorpromazine. Each *point* represents the mean of four samples. Vertical brackets indicate the standard error.

TABLE 1

Inhibition of irreversible binding of chlorpromazine to calmodulin by various drugs

Calmodulin (15 μg) and varying concentrations of drugs were preincubated at 4° for 30 min in 5 mM Tris buffer (pH 7.0) containing either 0.1 mM Ca^{2+} or 0.3 mM EGTA. [^{3}H]Chlorpromazine (final concentration 16 μM) was then added and the samples were irradiated with UV light as described under Materials and Methods. Portions were then dialyzed and counted for radioactivity. Calcium-dependent binding was determined from the difference between the amount of chlorpromazine bound in the presence of calcium and in the presence of EGTA. IC_{50} is the concentration of each drug that inhibited 50% of the calcium-dependent binding of chlorpromazine. All experiments were run in quadruplicate.

Drug	IC_{50}
	μM
Penfluridol	8
Trifluoperazine	10
Chlorpromazine sulfoxide	64
Trifluoperazine sulfoxide	90
Diazepam	110

DISCUSSION

Results presented in this report show that UV irradiation induces an irreversible binding of chlorpromazine and trifluoperazine to calmodulin. This irreversible binding is qualitatively similar, in many respects, to the reversible binding of the antipsychotic drugs to calmodulin (12, 15). For example, the irreversible binding of chlorpromazine is enhanced by calcium, is saturable with approximately one binding site per molecule of calmodulin, and shows one-half maximal binding at a concentration of chlorpromazine of approximately 10 μM. In addition, the irreversible binding of chlorpromazine is inhibited by the same compounds that block the reversible binding (15); the antipsychotic drugs trifluoperazine and penfluridol are more potent at blocking the irreversible calcium-dependent binding of chlorpromazine than are agents with little or no antipsychotic activity, such as trifluoperazine sulfoxide, chlorpromazine sulfoxide, and diazepam. Furthermore, like the reversible binding of the antipsychotic drugs (14), the irreversible binding of chlorpromazine appears to be relatively specific for calmodulin. Although chlorpromazine did display some irreversible binding to bovine serum albumin, this binding was much less than that seen with calmodulin, and more important, unlike the binding to calmodulin, the binding to albumin was not increased by calcium.

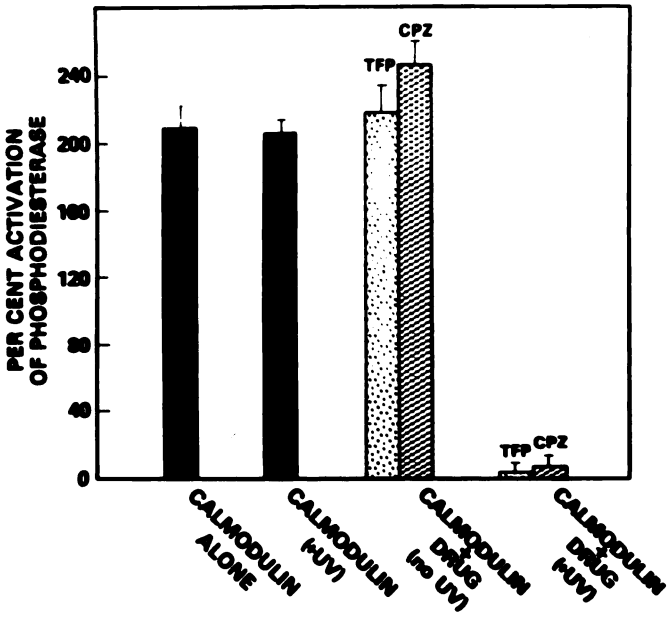

FIG. 7. *Irreversible inhibition of calmodulin-induced activation of phosphodiesterase following UV irradiation*

Calmodulin (15 μg/ml) was incubated in the presence or absence of 30 μM nonlabeled chlorpromazine (*CPZ*) for 1 hr at 4° with and without UV irradiation. Samples (1.8 ml) were then dialyzed for 20 hr (with three changes of buffer) against 500 ml of Tris buffer containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. Following dialysis the ability of these samples to activate a purified calmodulin-deficient phosphodiesterase was measured. Each *bar* represents the mean of three samples. Vertical brackets indicate the standard error. *TFP*, Trifluoperazine (10 μM).

Although the irreversible binding of chlorpromazine to calmodulin was qualitatively similar to the reversible binding of the antipsychotic drug to this protein, some quantitative differences were noted in the relative binding affinity of the drug for calmodulin, in the apparent number of drug binding sites on calmodulin, and in the relative ability of trifluoperazine and its sulfoxide analogue to prevent the binding of chlorpromazine to calmodulin. These quantitative differences between reversible and irreversible binding may have been caused by the photolability of the phenothiazines which may result in a change in their concentrations during UV irradiation. In addition, the products of the photochemical decomposition of these drugs might affect the apparent binding of the radioligands to calmodulin.

The chemical mechanisms involved in the irreversible binding of the phenothiazines to calmodulin and other proteins are unclear. The reactive species of the drug that binds to calmodulin apparently is not one of the end products of the photochemical decomposition of the phenothiazines, but rather is probably a short-lived intermediate such as a free radical, since little or no irreversible binding occurred when the phenothiazines were irradiated before they were added to calmodulin. Different types of free radicals have been postulated. Felmeister and Discher (21) showed that phenothiazines can generate semiquinone free radicals when irradiated by UV light. However, it is difficult to postulate a mechanism by which these radicals would bind irreversibly to proteins. Others (17, 22) have reported that photochemically activated chlorpromazine is subject to aromatic nucleophilic substitution at the position 2 by a free radical mechanism. Since calmodulin contains nucleophilic groups, this mechanism could account for the irreversible binding of chlorpromazine. However, it is difficult to explain the irreversible binding of trifluoperazine by this same mechanism, since the CFl_3 substituent at the position 2 of trifluoperazine would probably not be dissociated by UV irradiation as would the Cl substituent on chlorpromazine (22, 23).

Whatever the reactive species is, the photoactivated phenothiazine probably forms a covalent bond with calmodulin since extensive dialysis and treatment with agents such as EGTA, urea, and hexadecyltrimethylam-

monium bromide, which probably alter the tertiary structure and ionic characteristics of calmodulin, failed to reverse the binding of the phenothiazines to calmodulin. Furthermore the phenothiazines remained bound to calmodulin even after the drug-protein complex had been subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1).

Photoaffinity labeling has been used successfully for studying a variety of drug-receptor interactions (for review see ref. 24). Antipsychotic drugs apparently interact with several distinct binding or receptor sites in the brain (25, 26), one of these sites being calmodulin (1, 12, 15). Although it has not yet been determined whether photochemically activated phenothiazines bind to other types of antipsychotic binding sites, our data showing that the phenothiazines bind irreversibly to calmodulin suggest that chlorpromazine and trifluoperazine may be used as photoaffinity probes for this calcium-binding protein. Such photoaffinity probes should be useful in clarifying the nature of the receptor sites for antipsychotic drugs.

The binding of photochemically activated phenothiazines to tissue components has been studied previously in attempts to explain their biological activity. These findings indicated that photochemically generated phenothiazine derivatives interact with several biochemical sites, including membrane lipids (27), microsomal enzymes (28), DNA, serum proteins (17), and $(Na^+ + K^+)$-ATPase (16). In certain cases, such as the inhibition of $(Na^+ + K^+)$-ATPase and the binding to serum albumin, the interaction between the photoactivated phenothiazines and the proteins appeared to be irreversible.

The recent demonstration that phenothiazines bind irreversibly to calmodulin in crude fractions of brain homogenates as well as in purified preparations of calmodulin (1) suggests that phenothiazines may bind irreversibly to calmodulin *in vivo* and that this binding may account for some of their pharmacological effects. Furthermore, it is well known that phenothiazine antipsychotics and their metabolites remain in the body for extremely long periods of time following the cessation of drug treatment (29), indicating that these compounds are tightly bound to tissue constituents. Perhaps phenothiazines can be photochemically or metabolically activated *in vivo* to yield free radicals (30) that bind irreversibly to calmodulin. Since the irreversible binding of chlorpromazine and trifluoperazine results in an irreversible inactivation of calmodulin, the irreversible binding of phenothiazines *in vivo* could have important implications concerning the mode of action of these drugs.

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