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CHARACTERISTICS OF THE BINDING OF PHENOXYBENZAMINE TO CALMODULIN

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Abstract—To determine the factors that influence the interaction between phenoxybenzamine and calmodulin, the binding of phenoxybenzamine to calmodulin was determined by equilibrium dialysis under a variety of experimental conditions. This interaction was found to be similar in some respects to the interaction between phenothiazines and calmodulin. It was saturable, with between 1 and 2 mol of phenoxybenzamine bound to 1 mol of calmodulin. It was also dependent upon temperature, the presence of a divalent cation such as calcium, and on pH, showing maximum binding at pH 6.5 with little binding at pH values below 4.2 or above 8.0. The site at which phenoxybenzamine bound to calmodulin appears to be similar to that at which certain antipsychotic agents bind, since several of them, including penfluridol, pimozide and spiroperidol, prevented the binding of phenoxybenzamine to calmodulin. However, in contrast to the reversible binding of most phenothiazines to calmodulin, phenoxybenzamine bound to calmodulin irreversibly. The binding of phenoxybenzamine to calmodulin was fairly selective in that other alpha-adrenergic agents such as prazosin, yohimbine and clonidine failed to bind to calmodulin when examined under the same experimental conditions. In addition, phenoxybenzamine showed little or no calcium-dependent binding to the S-100 protein, bovine serum albumin or cytochrome c. The irreversible complex between phenoxybenzamine and calmodulin may be useful for inhibiting certain calmodulin-dependent reactions and for studying the various biological functions of calmodulin.

Calmodulin is a widely-distributed, heat-stable, acidic, calcium-binding protein that is found in high concentrations in the mammalian brain [1]. Discovered independently by Cheung [2] and Kakiuchi and Yamazaki [3] as an activator of cyclic nucleotide phosphodiesterase, calmodulin has been shown to activate many other Ca^{2+}-dependent enzymes and to play a role in modulating a variety of cellular functions [4–6].

Early reports on the regulation of phosphodiesterase activity showed that the calmodulin-induced activation of this enzyme could be inhibited by phenothiazine antipsychotic drugs [7, 8]. The mechanism by which the phenothiazines inhibit the actions of calmodulin is by directly binding to calmodulin in a Ca^{2+}-dependent manner [9, 10]. Under ordinary conditions this binding is reversible, although it can be made irreversible by irradiating chlorpromazine and calmodulin with UV light [11].

Besides the phenothiazines, a variety of other agents belonging to diverse pharmacological and chemical classes have now been shown to interact with the Ca^{2+}–calmodulin complex, although there are marked differences in their affinities for calmodulin and in their potencies for inhibiting its activity [12]. These include antipsychotic drugs chemically different from the phenothiazines, tricyclic antidepressants, antianxiety agents [13], local anesthetics, antimalarials [14], anthistamines, certain peptides [15], muscle relaxant agents [16], and an opiate agonist [17]. Unfortunately, calmodulin-binding drugs, such as the phenothiazines and related agents, can interact with a variety of biochemical sites, making the interpretation of results obtained in more complex biological systems difficult. A recently described calmodulin inhibitor exhibiting greater specificity is the compound calmidazolium. This agent is a potent calmodulin antagonist that displays little affinity for several neurotransmitter receptors with which other classes of calmodulin binding drugs interact [18].

Although some reports suggested that hydrophobic interactions were the sole requirement for calmodulin inhibition [10], studies on the structure–activity relationships and pH dependency of calmodulin inhibitors showed that the binding of phenothiazines and related drugs to calmodulin involves both hydrophobic interactions between the phenothiazine nucleus and a non-polar region of calmodulin, as well as an electrostatic interaction between the positively charged amino group on the drug and a negatively charged residue on calmodulin [19, 20].

These structural characteristics are also shared by drugs of other pharmacological classes, including certain of the alpha-adrenergic blocking agents. Indeed it has been reported [21, 22] that the alpha-adrenergic antagonist phenoxybenzamine inhibits the calmodulin-induced activation of phosphodiesterase and that the mechanism by which

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phenoxybenzamine inhibits calmodulin activity is by irreversible binding directly to it. In the present study, we describe the selectivity of this binding and the factors that influence their irreversible interaction. The results show that, like the binding of phenothiazine antipsychotics to calmodulin, the phenoxybenzamine–calmodulin interaction was calcium dependent, pH dependent, temperature dependent, and saturable.

METHODS

Calmodulin was purified to homogeneity from bovine brain by the method of Teo *et al.* [23]. The binding of chlorpromazine and alpha-adrenergic agents to calmodulin was determined by equilibrium dialysis using the technique described by Levin and Weiss [9]. Calmodulin was dialyzed to equilibrium at 4° against radiolabeled chlorpromazine or alpha-adrenergic agents in a buffer containing 5 mM Tris–HCl (pH 7.0), 1 mM $MgCl_2$ and either 0.1 mM calcium or 0.3 mM EGTA.* The amount of drug bound to calmodulin was calculated as the difference between the radioactivity found inside the dialysis tube (which contained calmodulin) and that found in the dialysis bath. Calculations of the molar concentration of calmodulin are based on a molecular weight of 16,200 daltons.

To determine whether or not the binding of phenoxybenzamine to calmodulin is irreversible, calmodulin (20 μg/ml) was dialyzed to equilibrium at 4° in a solution of 5 mM Tris–HCl (pH 7.0) with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ containing either radiolabeled chlorpromazine or radiolabeled phenoxybenzamine. Radiolabeled chlorpromazine was present at a final concentration of 1 μM (1 μM cold chlorpromazine and 0.25 μCi for each vial); phenoxybenzamine was present at a final concentration of 0.2 μM (0.2 μM cold phenoxybenzamine and 0.25 μCi for each vial). After this first period of dialysis, some samples were kept in buffer containing calcium whereas others were transferred to a buffer containing either 0.3 mM EGTA or unlabeled chlorpromazine or phenoxybenzamine at 500 times the concentration of the radiolabeled drug. The samples were then dialyzed for 18 hr more. The amount of radiolabeled drug that remained in the dialysis tubing was then determined.

In the time–course study, calmodulin (20 μg/ml) and [^{3}H]phenoxybenzamine (0.2 μM) were incubated in 5 mM Tris buffer containing either 0.1 mM $CaCl_2$ or 0.3 mM EGTA. After different periods of time, the calcium-dependent binding was arrested by adding 0.3 mM EGTA and 100 μM unlabeled phenoxybenzamine. The samples were dialyzed overnight with several changes of buffer to remove the free drugs. At the end of the dialysis procedure, the radioactivity inside the dialysis tube was determined in a liquid scintillation spectrometer.

* Abbreviations: EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; ADTN, amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; BSA, bovine serum albumin; FNM, fluphenazine-*N*-mustard; CM calmodulin; PBZ, phenoxybenzamine; and CPZ, chlorpromazine.

Materials. [^{3}H]Phenoxybenzamine (13.6 Ci/mmol), [^{3}H]chlorpromazine (26.3 Ci/mmol), [^{3}H]prazosin (17.1 Ci/mmol), [^{3}H]yohimbine (87.6 Ci/mmol) and [^{3}H]clonidine (23.8 Ci/mmol) were obtained from the New England Nuclear Corp. (Boston, MA). The sources of other compounds were as follows: chlorpromazine, phenoxybenzamine and trifluoperazine from Smith, Kline & French Laboratories (Philadelphia, PA); sulpiride, yohimbine, triprolidine and pyrilamine from the Sigma Chemical Co. (St. Louis, MO); penfluridol, loperamide and spiroperidol from Janssen Pharmaceutical (Piscataway, NJ); pimozide from McNeil Pharmaceutical (Spring House, PA); (+)butaclamol and (−)butaclamol from Ayerst Laboratories (New York, NY); clonidine from Boehringer-Ingelheim, Ltd. (Ridgefield, CT); phenylephrine from Winthrop Laboratories (New York, NY); prazosin from Pfizer Laboratories (New York, NY); phentolamine from the Ciba Pharmaceutical Co. (Summit, NJ); metergoline from Farmitalia, Carlo Erba (Milano, Italy); methiothepine from Roche (Basel, Switzerland); and amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) from Burroughs Welcome (Triangle Park, NC). The S-100 protein from bovine brain was a gift of Dr. Stefano Alema of the Laboratorio di Biologia Cellulare (Rome, Italy). All other reagents and chemicals were obtained from general commercial sources.

RESULTS

Binding of alpha-adrenergic agents to calmodulin. Figure 1 shows the binding of chlorpromazine and different types of alpha-adrenergic agonists and antagonists to calmodulin. Of these compounds only chlorpromazine and phenoxybenzamine exhibited marked binding to calmodulin. This binding was evident only in the presence of calcium ions; there was no substantial binding in samples containing the calcium chelating agent EGTA. At a concentration of the radioligand of 1 μM, there was approximately 0.7 mol of chlorpromazine and 1.6 mol of phenoxybenzamine bound for each mole of calmodulin.

Irreversible binding of phenoxybenzamine to calmodulin. Table 1 compares the binding of chlorpromazine and phenoxybenzamine to calmodulin under different experimental conditions. As may be seen, the binding of chlorpromazine to calmodulin was calcium dependent and reversible. That is, far more chlorpromazine bound to calmodulin in the presence of calcium, and the drug readily dissociated from calmodulin in the presence of EGTA or high concentrations of unlabeled chlorpromazine. By contrast, although the formation of the binding of phenoxybenzamine to calmodulin was inhibited in the presence of EGTA (Fig. 1), once the binding occurred, the phenoxybenzamine–calmodulin complex could not be readily dissociated even when dialyzed against EGTA or an excess of unlabeled phenoxybenzamine (Table 1).

Figure 2 shows the binding of phenoxybenzamine to calmodulin as a function of increasing concentrations of phenoxybenzamine. As has already been reported for the antipsychotic drugs, the bind-

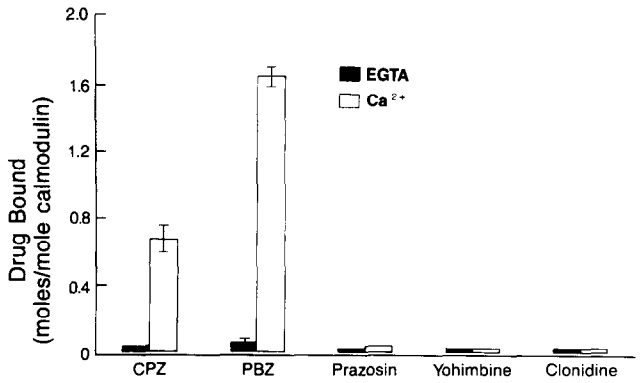

Fig. 1. Binding of alpha-adrenergic agents to calmodulin. Calmodulin (20 μg/ml) was dialyzed to equilibrium against 20 ml of 5 mM Tris–HCl (pH 7.0), 1 mM $MgCl_2$ and either $CaCl_2$ (0.1 mM) or EGTA (0.3 mM). Each radiolabeled drug was present at a concentration of 1 μM (1 μM drug + 1 μCi for each vial). Each value is the mean of six experiments. Vertical brackets indicate the standard error.

Table 1. Comparison of the binding of chlorpromazine and phenoxybenzamine to calmodulin

	Binding of drug to calmodulin (mmol drug bound/mol calmodulin)		
Drug	Dialyzed against Ca^{2+}	Dialyzed against EGTA	Dialyzed against EGTA + unlabeled CPZ (500 μM) or PBZ (100 μM)
CPZ	610 ± 20	21 ± 10	7.5 ± 1.6
PBZ	870 ± 30	840 ± 20	600 ± 30

Calmodulin (20 μg/ml) was dialyzed to equilibrium in Tris buffer containing either radiolabeled chlorpromazine (1 μM) or phenoxybenzamine (0.2 μM). Once the binding reached equilibrium, the buffer in the dialysis bath was changed, and the samples were further dialyzed in buffer containing either calcium, EGTA or EGTA plus unlabeled drugs. The radioactivity still bound to calmodulin was then determined. Each value is the mean ± SEM of six experiments.

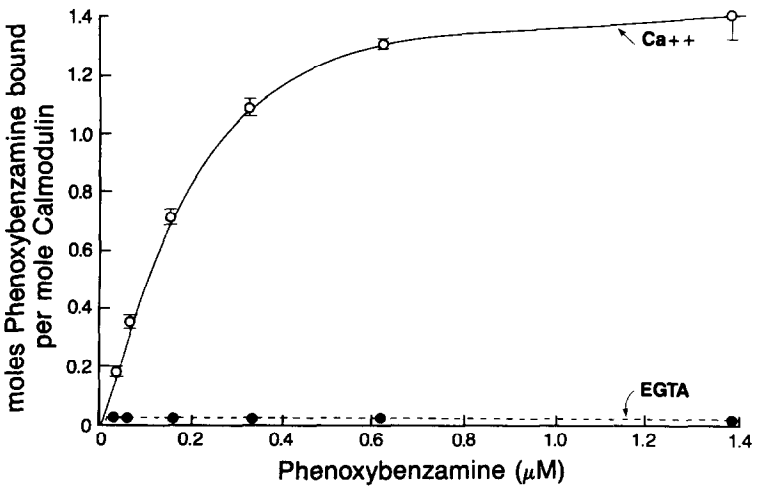

Fig. 2. Binding of phenoxybenzamine to calmodulin as a function of the concentration of phenoxybenzamine. Samples of calmodulin (20 μg/ml) were dialyzed to equilibrium against 20 ml of 5 mM Tris–HCl (pH 7.0) containing various concentrations of [^{3}H]phenoxybenzamine (0.05 to 1.4 μM) in the presence of Ca^{2+} (0.1 mM) or EGTA (0.3 mM). Each point represents the mean of six experiments. Vertical brackets indicate the standard error.

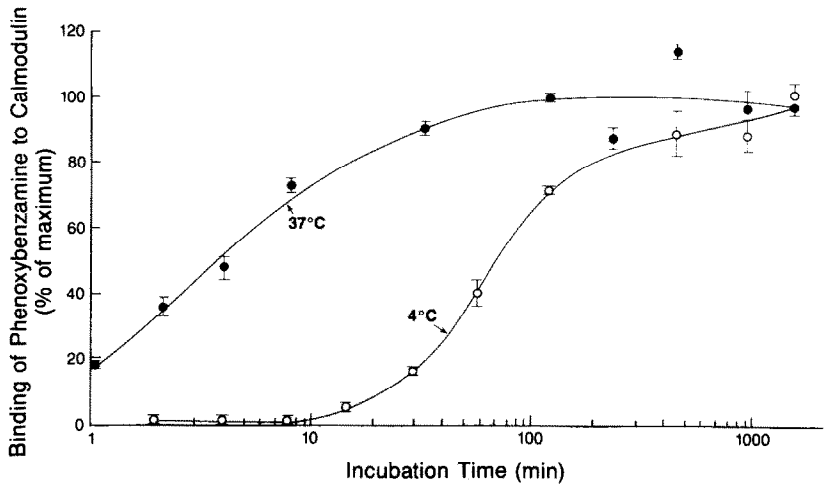

Fig. 3. Rate of binding of phenoxybenzamine to calmodulin as a function of temperature and time. Samples of calmodulin (20 μg/ml) were incubated at 4° and 37° in 5 mM Tris buffer, pH 7.0, with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ in the presence of radiolabeled phenoxybenzamine at a concentration of 0.2 μM (0.2 μM of nonradioactive drug + 0.25 μCi for each sample). After different times of incubation, the reaction was stopped by adding non-labeled phenoxybenzamine (0.1 mM) and EGTA (0.3 mM). Samples (0.5 ml for each dialysis tube) were then dialyzed overnight with several changes of buffer to remove the free phenoxybenzamine. Each point represents the mean value of three experiments. Vertical brackets indicate the standard error.

ing of phenoxybenzamine to calmodulin was calcium dependent with essentially no binding occurring when EGTA was present in the dialysis buffer. Since phenoxybenzamine binds to calmodulin irreversibly, it is not appropriate to determine the kinetic parameters (K_d and B_{max}) of the saturation isotherm. However, from the graph it is apparent that 50% of the maximum binding of the drug occurred at a concentration of approximately 0.2 μM and that at saturation there was about 1.4 mol of phenoxybenzamine bound for each mole of calmodulin. This is similar to the molar ratio of the binding of phenothiazine antipsychotics to calmodulin reported earlier [24].

Time and temperature dependency of the binding of phenoxybenzamine to calmodulin. To determine the effect of temperature on the rate of irreversible binding of phenoxybenzamine to calmodulin, solutions of calmodulin were incubated with radiolabeled phenoxybenzamine at 4° and 37°. After different time intervals, the reaction was arrested by adding EGTA (0.3 mM) and non-labeled phenoxybenzamine (100 μM), and the samples were dialyzed overnight against buffer to remove the free phenoxybenzamine. The radioactivity found in the dialysis tube at the end of the procedure was considered to be the amount of drug bound at the specific time when the reaction was stopped. Figure 3 shows that the irreversible binding of phenoxybenzamine to calmodulin was considerably more rapid when incubation was carried out at 37° rather than 4°. At 37° 50% of the maximum binding occurred in less than 3 min, whereas at 4°, 50% of the maximum binding was reached after about 1 hr.

Effect of pH on the binding of phenoxybenzamine to calmodulin. Figure 4 shows the influence of pH on the binding of phenoxybenzamine to calmodulin. The range of pH values was obtained using either citric acid–phosphate buffer for pH values from 3 to 7 or Tris–HCl buffer for pH values from 6 to 8.5. Overlapping pH values gave similar results regardless of the buffer used. The figure shows that the binding increased markedly above pH 4.2, reached a maximum between pH 6 and 6.5, and then started to decrease at pH values above 7.2.

Relative binding of phenoxybenzamine to calmodulin and other proteins. To determine the specificity and calcium dependency of the binding of phenoxybenzamine to calmodulin, the binding of phenoxybenzamine to calmodulin, the S-100 protein, bovine serum albumin and cytochrome *c* was determined in the presence of Ca^{2+} (0.1 mM) and in the absence of Ca^{2+} (0.3 mM EGTA). Of these proteins, only calmodulin displayed a high degree of calcium-dependent binding to phenoxybenzamine (binding in the presence of $Ca^{2+} = 0.62 \pm 0.02$ mol/mol calmodulin). There was no significant binding to calmodulin in the absence of Ca^{2+} and no significant binding to bovine serum albumin or to cytochrome *c* in the absence or presence of Ca^{2+}. The brain specific calcium-binding protein S-100 displayed some calcium-dependent binding to phenoxybenzamine (0.14 mol/mol calmodulin); however, this binding was substantially less than that observed with calmodulin, and some calcium-independent binding to the S-100 protein was also apparent (0.08 mol/mol calmodulin).

Displacement of phenoxybenzamine from calmodulin by various compounds. The abilities of several compounds of different chemical classes to displace [^{3}H]phenoxybenzamine from calmodulin are shown in Table 2. The antipsychotic agents, penfluridol, pimozide and spiroperidol, which were reported previously to displace trifluoperazine from

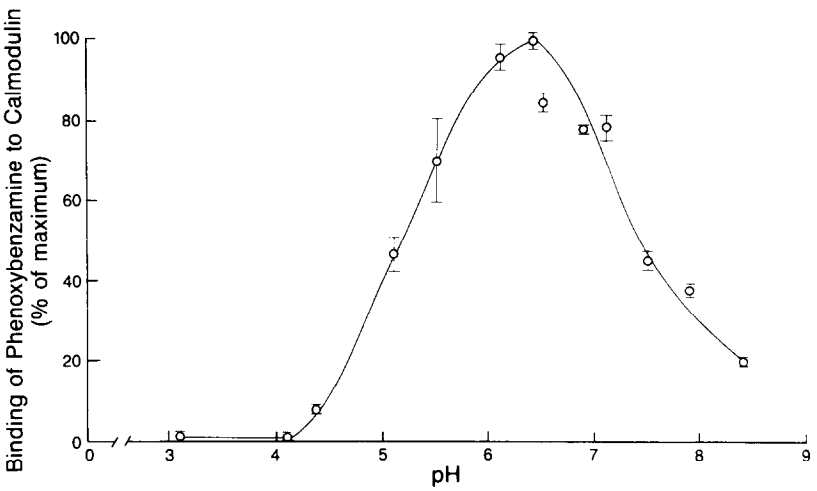

Fig. 4. Effect of pH on the binding of phenoxybenzamine to calmodulin. Calmodulin (20 μg/ml) was dialyzed to equilibrium against 20 ml of buffer containing [^{3}H]phenoxybenzamine (0.2 μM) in the presence of 0.1 mM $CaCl_2$ using either citric acid-phosphate buffer (pH 3 to 7) or Tris–HCl buffer (pH 6 to 8.5). Each point represents the mean value of three experiments. Vertical brackets indicate the standard error.

calmodulin [24], were among the most potent in inhibiting the binding of phenoxybenzamine. Methiothepine, metergoline, loperamide, pyrilamine and trifluoperazine were moderately potent, and a large number of other agents, including the dopaminergic agonist ADTN and some of the adrenergic agonists and antagonists, were relatively ineffective in preventing the binding of [^{3}H]phenoxybenzamine to calmodulin. Yohimbine, although it did not bind to calmodulin when present at 10 μM (Fig. 1), partially inhibited the binding of [^{3}H]phenoxybenzamine to calmodulin when present at 50 μM (Table 2).

Table 2. Displacement of [^{3}H]phenoxybenzamine from calmodulin by various compounds

Compound	% Displacement
Penfluridol	95
Phenoxybenzamine	92
Pimozide	80
Spiroperidol	79
Methiothepine	74
Metergoline	72
Loperamide	65
Pyrilamine	61
Trifluoperazine	57
Sulpiride	45
Yohimbine	41
Triprolidine	39
(+)Butaclamol	35
Clonidine	28
(−)Butaclamol	16
Phenylephrine	14
Prazosin	12
Phentolamine	4.0
ADTN	1.8

Calmodulin (20 μg/ml) was dialyzed to equilibrium against [^{3}H]phenoxybenzamine (0.2 μM) and the various drugs under study at a final concentration of 10^{-5} M. After 18 hr of dialysis in the presence of the labeled drug and the displacers, the dialysis tubes were transferred to vials containing 5 mM Tris–HCl (pH 7.0), 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ and dialyzed for 8 hr to remove the free phenoxybenzamine. Values are the means of three experiments.

It is surprising that the serotonin antagonists methiothepine and metergoline were both good inhibitors of the binding, although methiothepine has been reported to exhibit some antidopaminergic activity. Of particular interest are the effects of drugs such as loperamide, pyrilamine and triprolidine on the binding of [^{3}H]phenoxybenzamine. Loperamide, an opiate agonist, has been reported to inhibit the calmodulin-stimulated activity of phosphodiesterase although this property has been found to be independent of the opioid agonist activity. This anti-calmodulin effect is now confirmed in this study where loperamide displayed a relatively high potency in inhibiting the binding of [^{3}H]phenoxybenzamine to calmodulin. Pyrilamine and triphrolidine are two antihistamic agents with chemical structures similar to the general structure required for other calmodulin antagonists as proposed by Prozialeck and Weiss [19]. As expected, they apparently bind to calmodulin since they prevented the binding of [^{3}H]phenoxybenzamine to calmodulin.

DISCUSSION

A number of drugs of diverse chemical structures and pharmacological activities have been shown to inhibit the biological activity of calmodulin [25, 26]. Among these are certain of the alpha-adrenergic blocking agents [21, 22]. In particular, phenoxybenzamine and dibenamine were reported to be fairly potent irreversible inhibitors of the calmodulin-induced stimulation of the activity of phosphodiesterase [22]. The results presented in this report

show that phenoxybenzamine inhibited calmodulin by directly and irreversibly binding to it in a calcium-dependent manner.

The interaction between phenoxybenzamine and calmodulin is similar in some respects to the interaction between the phenothiazine antipsychotics and calmodulin. As in the case of the binding of phenothiazine antipsychotics to calmodulin [13], the interaction between phenoxybenzamine and calmodulin was dependent upon pH, temperature and calcium, and at saturation there were between 1 and 2 moles of drug bound for each mole of calmodulin. However, unlike the phenothiazines, which require photochemical activation to induce an irreversible binding [11, 27], phenoxybenzamine bound to calmodulin irreversibly without being subjected to ultraviolet irradiation. This is also in agreement with the reports that both compounds cause an irreversible inhibition of calmodulin-activated phosphodiesterase under conditions at which they irreversibly bind to calmodulin [11, 22].

The binding of phenoxybenzamine to calmodulin appears to be relatively specific for this alpha-adrenergic agent since the $alpha_1$-antagonist prazosin, the $alpha_2$-agonist clonidine, and the $alpha_2$-antagonist yohimbine failed to interact with calmodulin.

Studies of the abilities of various compounds to prevent the binding of phenoxybenzamine to calmodulin showed that the most effective agents were a group of structurally dissimilar antipsychotics and some structurally related, though pharmacologically dissimilar, compounds. This suggests that the site at which phenoxybenzamine binds on calmodulin may be similar to that at which the phenothiazines bind.

Another type of selectivity was demonstrated by experiments showing that phenoxybenzamine failed to bind to a number of other proteins, such as BSA and cytochrome *c*. Phenoxybenzamine, however, did bind slightly to the calcium binding protein S-100, although there was little calcium dependency for this binding. The specificity of the binding was further supported by its requirement for calcium and by its saturability, an important requisite for demonstrating a specific interaction between a drug and selective binding sites.

The mechanism by which phenoxybenzamine interacts with calmodulin is complex and may be explained, in part, by the chemical properties of phenoxybenzamine. In aqueous solutions phenoxybenzamine undergoes a complex series of decomposition reactions, initially cyclization to an ethyleneimonium ion [*N*-benzyl-*N*-(1-phenoxy-2-propyl)-ethyleneimonium] followed by hydrolysis of the cyclic intermediate which occurs at pH values above 6.5 [28]. Studies of the pH optimum for the binding of phenoxybenzamine with calmodulin showed that at pH values below 4.2 there was essentially no binding; the binding increased to a maximum at about 6.5 and then decreased to very little binding at pH values of 8.5. Since calmodulin has a pK_a of about 4.2 and phenoxybenzamine has a pk_a of about 5.0 [28], the results suggest that calmodulin must be negatively charged and phenoxybenzamine positively charged for the interaction to occur, a conclusion also drawn from studies of the interaction of phenothiazines with calmodulin [29]. The irreversible binding of phenoxybenzamine to calmodulin probably involves an alkylating interaction between calmodulin and the highly reactive ethyleneimonium intermediate which occurs at the tertiary amine on phenoxybenzamine at neutral or alkaline pH values [30]. However, the initial interaction between phenoxybenzamine and calmodulin appears to be more dependent on hydrophobic bonds and on the structure and ionization of phenoxybenzamine rather than on its alkylating properties since alkylating drugs such as EEDQ, chlorambucil and mechlorethamine are poor calmodulin inhibitors [22].

Irreversibly bound adducts of calmodulin, such as that formed by the interaction between phenoxybenzamine and calmodulin, may have several applications. A number of irreversibly bound calmodulin–phenothiazine adducts have been described recently [31–34], and some of these have been studied on certain calmodulin-dependent enzymes. The results suggest that these agents may be useful for selective pharmacological intervention of calmodulin activity. For example, Newton *et al.* [32, 33] prepared and studied a covalent complex of calmodulin with norchlorpromazine isothiocyanate. They found that this adduct inhibited the calmodulin-induced activation of phosphodiesterase and myosin light chain kinase but acted as a partial agonist with phosphoprotein phosphatase.

In our laboratory we have prepared and studied a complex formed by ultraviolet irradiation of calmodulin and chlorpromazine (CPZ–CM) [35]. This CPZ–CM complex inhibited the calmodulin-induced activation of phosphodiesterase at concentrations that had no effect on the basal activity of the phosphodiesterase. However, this complex failed to block the calmodulin-induced activation of ATPase at concentrations that almost totally blocked the calmodulin-induced activation of phosphodiesterase, suggesting that the complex has a degree of selectivity for certain calmodulin-sensitive enzymes. The results of these studies indicate that this CPZ–CM adduct acts not on calmodulin but rather on specific calmodulin binding sites of calmodulin-dependent enzymes.

The covalent interaction between phenoxybenzamine and calmodulin has been used by Lukas *et al.* [36] to prepare several adducts having different molar ratios of drug to calmodulin. These adducts of calmodulin and phenoxybenzamine have a reduced ability to activate phosphodiesterase or myosin light chain kinase.

These studies suggest that the different irreversibly bound adducts of calmodulin may be useful as tools to study the many biological functions of calmodulin and perhaps to selectively modulate the various calmodulin-mediated processes.

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