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Interactions of Phencyclidine with Crayfish Muscle Membranes

Sensitivity to Calcium Channel Antagonists and Other Drugs

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SUMMARY

[^{3}H]Phencyclidine ([^{3}H]PCP) bound to crayfish abdominal muscle membranes at pH 7.4 with two affinities (K_d of 0.96 nM for 0.38 pmole/mg of protein, and 18.9 nM for 7.6 pmoles/mg of protein). Binding affinities increased at higher pH, suggesting that binding may be due mostly to the un-ionized form of [^{3}H]PCP. This high-affinity [^{3}H]PCP binding was sensitive to the actions of trypsin, protease, and dicyclohexylcarbodiimide, but insensitive to phospholipase A, concanavalin A, *N*-ethylmaleimide, and dithiothreitol. Calcium channel antagonists were most potent in inhibiting the high-affinity [^{3}H]PCP binding with the following descending order of potencies: bepridil > nicardipine = diltiazem = verapamil > cinnarizine > (+)-D-600 > (−)-D-600 > 4-NO_2-nifedipine > 2-NO_2-nifedipine. The binding was also highly sensitive to several PCP analogues, antipsychotics, piperocaine, and tilorone, and moderately sensitive to *d*-tubocurarine, atropine, imipramine, nortryptyline, and tetracaine. Although verapamil and nifedipine inhibited the action potential of crayfish muscle, PCP did not and actually prolonged slightly the falling phase of the action potential. Although it is unlikely that the [^{3}H]PCP binding protein in crayfish muscles is a Ca^{2+} channel, it is possible that it may be a K^+ channel.

INTRODUCTION

PCP[4] has varied pharmacological effects in humans and animals (1, 2). These effects may result from the interaction(s) of PCP with specific receptors. Indeed, [^{3}H]PCP binds to sites in mammalian brain (3–7), and the binding is displaced by several PCP analogues with rank orders of potency similar to theirs in altering animal behavior (3, 4, 6). These PCP binding sites also bind *sigma* opiates with high affinities and with potencies that correlate with their behavioral potencies (6–8), suggesting that the psychotomimetic effects of PCP and *sigma* opiates are related. Furthermore, these sites exhibit stereoselectivity not only for an enantiomeric pair of PCP analogues (9), but also for *sigma* opiates (10), and dissociative anesthetics (11) which are known to produce PCP-like discriminative stimuli. Whether these PCP-binding sites in the brain represent specific receptors for a yet-unidentified endogenous regulator substance in the nervous system, or a site of a known regulatory function, is not clear.

PCP has been shown to inhibit cholinesterases (12), muscarinic ACh receptors of brain and smooth muscle (13), the ionic channel of the nicotinic ACh receptor of skeletal muscle and *Torpedo* electric organ (14, 15), K^+ channels of skeletal muscles and rat brain synaptosomes (14, 16), and biogenic amine carriers responsible for the amine reuptake (17). Specific [^{3}H]PCP binding was also detected in other tissues such as kidney, liver and lung (18), and ileal smooth muscle (19), but the rank order of potency of a number of PCP analogues in inhibiting binding was different from that in brain.

Recently, we have detected high-affinity binding of [^{3}H]PCP to crayfish muscle membranes which was displaced effectively by a number of organic and inorganic Ca^{2+} channel antagonists (20). In the present study, we investigated in detail the properties of the high-affinity [^{3}H]PCP binding protein in crayfish muscles and determined the rank order of potency of several PCP ana-

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[4] The abbreviations used are: PCP, phencyclidine [1-(phencyclohexyl)piperidine] ACh, acetylcholine.

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logues, Ca^{2+} channel antagonists, and several other drugs. The relevance of this binding site to PCP pharmacological site(s) of action is discussed.

MATERIALS AND METHODS

Biochemical techniques

Tissue preparation. The abdominal muscle of the crayfish *Procambarus clarkii* (from NASCO, Fort Atkinson, Wisc.) was dissected from live animals, excised into small segments (0.5 cm long and 0.1 cm thick), and homogenized in 5 volumes of Van Harreveld's buffer (205 mM NaCl, 5.4 mM KCl, 13.6 mM $CaCl_2$, 2.6 mM $MgCl_2$, and 5 mM Tris-HCl, pH 7.4) using a Polytron and two 30-sec bursts (20). The homogenate was centrifuged at 100,000 × *g* for 30 min. The pellets were resuspended in 5 volumes of the same buffer and homogenized by the Polytron for 30 sec, then centrifuged at 1,000 × *g* for 10 min. The supernatant fraction was saved, and the pellet was resusupended in the same buffer, homogenized, and recentrifuged at 1,000 × *g*. The supernatant fractions were pooled and centrifuged at 100,000 × *g* for 30 min. The final pellet was resuspended in 5 mM Na_2HPO_4 (pH 7.4) and 0.2 M NaCl so that the protein concentration averaged 1–2 mg of protein per milliliter. This membrane preparation consisted mostly of vesicular membrane fragments, in addition to a fair amount of cellular debris as shown in electron micrographs of stained sections of the final pellet.[5]

Binding assay. Binding of [^{3}H]PCP (specific activity 48 Ci/mmole; New England Nuclear Corporation) to membrane preparations of crayfish abdominal muscles was measured by a filter assay. The tissue (100 μg of protein, unless otherwise stated) was added to a disposable culture tube containing 900 μl of 50 mM Tris-HCl (pH 7.4 except in experiments studying the effect of pH on binding) and 2 nM [^{3}H]PCP (90,000 cpm). The mixture was incubated for 30 min at 23°, unless otherwise stated, then filtered over GF/B Whatman glass-fiber filters, pretreated with 1% Prosil-28 (an organosilicone, PCR Research Chemicals, Gainesville, Fla.) and washed with 10 ml of cold buffer. Each filter was then placed in a 5-ml minivial, 4 ml of a toluene-based liquid scintillation solution were added, and its radioactive content was determined after a period of equilibration (at least 5 hr). Prosil treatment reduced [^{3}H]PCP binding to the filter, which at 2 nM reached only 1% of specific binding to 100 μg of protein. To determine the effect of various agents on [^{3}H]PCP binding, the desired concentration of the agent was added to the incubation buffer along with [^{3}H]PCP. All assays were performed in triplicate, and mean values (showing <10% SD) were used to obtain the dose-response relationships. The concentration that inhibited 50% of the binding (IC_{50}) was determined graphically from titration curves (log concentration-response functions).

Electrophysiological Techniques

Crayfish muscle. The solution used for the dissection and mounting of the crayfish muscle was of the following composition (millimolar): NaCl, 205; $CaCl_2$, 13.5; KCl, 5.5; $MgCl_2$, 2.6; $NaHCO_3$, 2.3. The muscle was then allowed to equilibrate for 1–2 hr in a solution containing 160 mM $SrCl_2$. The NaCl concentration was adjusted to compensate for the increase in the osmolarity of the solution, and the pH was adjusted to 7.5. Directly elicited action potential was recorded with one microelectrode inserted into a surface fiber while passing a 30- to 50-msec depolarization pulse through another microelectrode inserted into the same fiber at an interelectrode distance of 50 μm.

RESULTS

[^{3}H]PCP equilibrium binding. The binding of [^{3}H]PCP to crayfish muscle membranes was displaced equally well with several Ca^{2+} channel antagonists (20). Both verapamil and PCP at 1 μM were equal in displacing [^{3}H]PCP binding from the membranes, but only verapamil did not displace [^{3}H]PCP binding to the filters. Therefore, verapamil was selected to identify the specific [^{3}H]PCP binding (Fig. 1A). This verapamil-displaceable binding under equilibrium conditions (30-min incubation) was linearly related to protein concentration up to 300 μg/assay. Specific binding was saturable (Fig. 1A), and Scatchard analysis of saturation isotherms obtained in preliminary experiments gave an apparent single affinity with a K_d equal to 13.5 nM (20). However, increasing the number of [^{3}H]PCP concentrations tested gave a saturation isotherm whose Scatchard plot was curvilinear (Fig. 1B). Iterative analysis of the binding indicated a high-affinity site (K_d = 0.6 nM) present at 0.38 pmole/mg of protein and a lower-affinity site (K_d = 18.9 nM) present at 7.6 pmoles/mg of protein. The Hill coefficient of this binding isotherm was 0.91.

Kinetics of [^{3}H]PCP binding. The time course of specific PCP binding indicated that binding reached a steady state within 10 min at 23° (Fig. 2A). The observed association rate constant (k_{obs}) was calculated according to the equation

$$\ln \frac{B_e}{B_e - B_t} = k_{obs} \cdot t \qquad (1)$$

where B_e is the specific binding at equilibrium, and B_t is the specific binding at time t. It was obvious that there were two components, a fast component whose k_{obs} was 4.09 ± 1.02 min^{-1} (mean ± standard deviation, n = 4), and a slow component whose k_{obs} was 1.95 ± 0.52 min^{-1} (mean ± standard deviation, n = 4). This nonlinearity of the association curve could not be due to depletion of [^{3}H]PCP, since the amount bound at equilibrium represented <10% of the total radioactivity added in the assay. The association rate constants were determined from the following equation

$$k_{obs} - k_{-1} = k_{+1} \cdot [[^3\text{H}]\text{PCP}] \qquad (2)$$

where k_{-1} is the dissociation rate constant that is determined experimentally (Fig. 2B). Crayfish muscle membranes were incubated with [^{3}H]PCP (2 nM) for 30 min at 23°. Dissociation was accomplished by diluting the incubation mixture 50-fold with 50 mM Tris (pH 7.4, 23°) with or without unlabeled PCP (1 μM), then filtering at the indicated time intervals. The data points were plotted as a percentage of the specific binding at t = 0, and the dissociation rate constant (k_{-1}) was calculated from the relationship $k_{-1} = \ln 2/t_{1/2}$.

The semilog plot of [^{3}H]PCP dissociation also exhibited biphasic kinetics. The values of k_{-1} for the fast and slow components were 6.9 min^{-1} and 1.25 min^{-1}, respectively. The values of k_{+1}, calculated from the fast k_{obs}, and the slow k_{-1} ranged from 1.89 to 3.84 nmoles·min^{-1} and gave K_d values ranging from 0.15 to 0.26 nM. These K_d values are 4–6 times smaller than the high-affinity value (K_d = 0.96) determined from the saturation isotherm (Fig. 1B). However, using the slow k_{obs} gave K_d values ranging from 0.45 to 1.81 nM, which were closer to the value determined from equilibrium measurements. The fast rate of dissociation could not be used to calculate K_d values because it gave negative rates of association.

Another method of determining K_d is to measure binding at different [^{3}H]PCP concentrations and determine

[5] C. S. Hudson, personal communication.

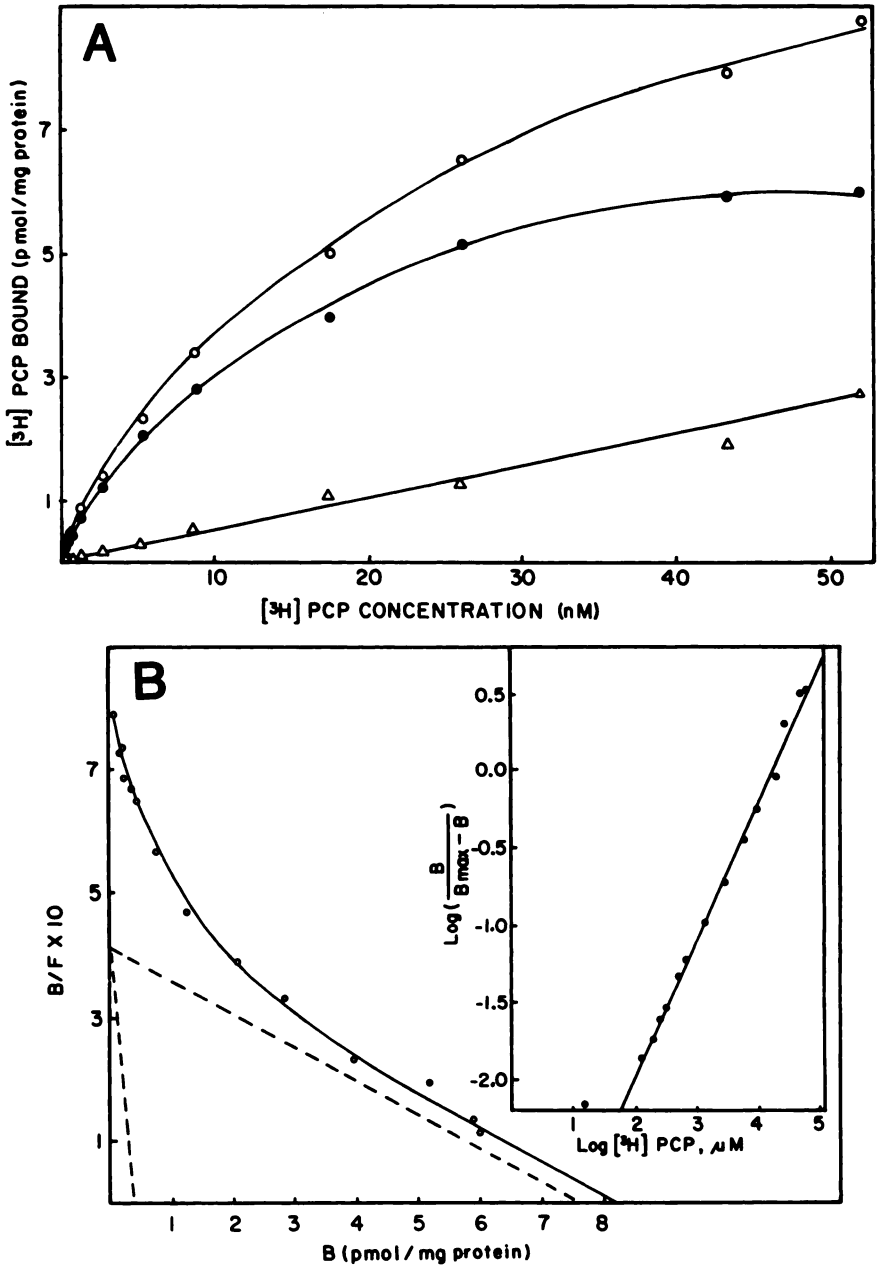

FIG. 1. *[^{3}H]PCP binding to crayfish muscle membranes*

A. Saturation isotherm of [^{3}H]PCP binding to crayfish muscle membranes (200 μg of protein/ml). Total binding of [^{3}H]PCP in the presence (Δ) and absence (○) of 1 μM verapamil; specific binding (●) is the difference between the two. The final volume of the incubation mixture was 1 ml, the buffer was 50 mM Tris (pH 7.4), and the incubation time was 30 min. B. Scatchard plot of the specific [^{3}H]PCP binding. *Broken lines* were drawn from B_{max} and K_d values obtained from iterative analysis. *F*, free ligand concentration (nanomolar. *Inset* is the Hill plot of the same data.

k_{obs} for each [^{3}H]PCP concentration. Then a plot of k_{obs} versus [^{3}H]PCP concentration will have a slope of k_{+1} ($k_{obs} = k_{+1}$[[^{3}H]PCP] + k_{-1}), and the intercept will be k_{-1}. When k_{obs} was determined at six [^{3}H]PCP concentrations ranging from 0.29 nM to 2.32 nM and plotted (Fig. 3), k_{+1} of 2.45 nM^{-1}·min^{-1} and k_{-1} of 2.54 min^{-1} were obtained. These values yielded a K_d value of 1.04 nM, which is similar to the K_d of the high-affinity component.

Effect of pH on [^{3}H]PCP binding. The pH of the buffer had a strong effect on [^{3}H]PCP binding to crayfish muscle membranes. Binding increased from pH 6 to pH 9, parallel to the degree of ionization of PCP (Fig 4). The binding decreased fast at pH values higher than 10, most likely due to protein denaturation. Similarly, the absence of PCP binding at pH values below 6 may be due in part to denaturation of the binding site at acidic pH values. Membranes that were incubated at pH 5 and 11 for 30 min and then washed in 50 mM Tris (pH 7.4) lost 27% and 88% of their ability to bind [^{3}H]PCP at pH 7.4, respectively. Since binding of 2 nM [^{3}H]PCP at pH 9 was almost double that at pH 7.4, it was important to determine whether that was due to increased affinity or increased number of binding sites. Scatchard analysis of

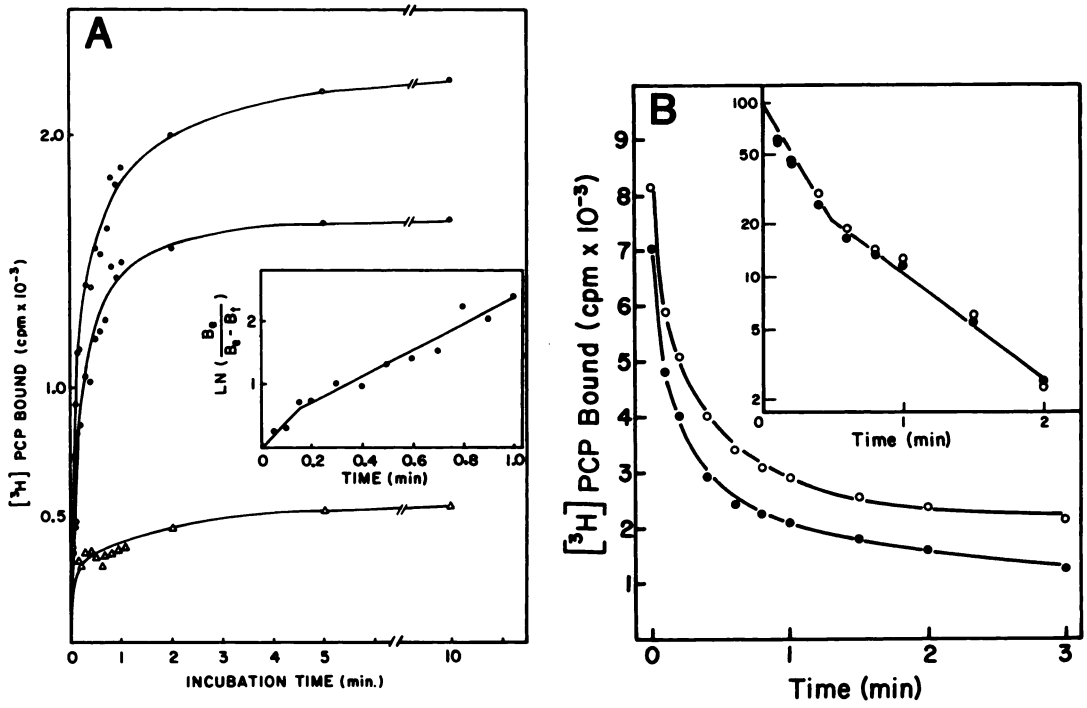

FIG. 2. *Time course of binding, and dissociation of bound [³H]PCP in crayfish muscle membranes*

A. Time course of [³H]PCP binding to crayfish membranes (40 μg of protein/ml). Binding of [³H]PCP (0.4 nM) in the presence (Δ) and absence (O) of 1 μM verapamil; specific binding (●) is the difference between the two. The reaction was terminated by filtration, and the filters were rinsed with 8 ml of ice-cold buffer. *Inset*, a linear transformation of the time course of specific [³H]PCP binding within the 1st min. B_e, binding at equilibrium; B_t, binding at different times. B. Course of dissociation of bound [³H]PCP from its binding site in crayfish muscle membranes. Tissue was incubated with [³H]PCP (2 nM) for 30 min at 23°, then dissociation was started by dilution into a 50-fold volume of 50 mM Tris with (●) or without (O) 1 μM unlabeled PCP. Each point represents the mean of three determinations. *Inset*, the semilogarithmic plot of the data from which $t_{1/2}$ values were calculated. (Ordinate shows percentage of bound [³H]PCP.)

binding isotherms at pH 7.4 and pH 9 indicated an increase in the affinity of both binding sites at higher pH (30-fold and 2.5-fold for high- and low-affinity sites, respectively) without a significant change in the total number of the two sites.

Effect of membrane treatment with enzymes on [³H] PCP binding. Membranes were incubated with one of three proteases (at 600 μg/200 μg of membrane protein) at 23° for 60 min, then for 10 min with 2 nM [³H]PCP before filtration. Binding was reduced to 47.2%, 82.8%, and 46.5% of control by exposure to trypsin, chymotrypsin, and protease, respectively. Phospholipase A, on the other hand, appeared without effect. Raising the ratio of trypsin to crayfish protein from 1.0 to 10 increased inhibition by only 3-fold (Fig. 5), and a time course study showed that proteolytic degradation of the binding site was rather slow ($t_{1/2}$ = 60 min). The crayfish muscle membranes that were preexposed for 60 min to trypsin (10 mg of trypsin/1 mg of membrane protein) lost 65% of their total specific [³H]PCP binding sites without change in the affinities of the remaining sites.

Effect of cations on [³H]PCP binding. Sodium ions were present in all assays at 10 mM, since the membranes were usually stored in 200 mM NaCl, and 50 μl of the membrane preparation were used in each assay (final volume of 1 ml of Tris buffer, 50 mM). This monovalent ion as well as K^+ and Li^+ at concentrations up to 100 mM had little effect on [³H]PCP binding at 2 nM. At 300 mM, the three cations inhibited binding by 20–30%. On the other hand, divalent cations were more inhibitory, with 10 mM Ca^{2+}, Mg^{2+}, and Sr^{2+} inhibiting 35, 30, and 46%, respectively, of [³H]PCP binding. Mn^{2+}, Co^{2+}, and La^{3+} were even more potent, with 1 mM causing 27, 73, and 72% inhibition, respectively. The divalent post-transition metals Cd^{2+} and Hg^{2+} and the metal ions Cu^{2+} and Ni^{2+} were most potent, inhibiting binding totally at 1 mM concentrations and 37, 25, 48, and 26%, respectively, at 0.1 mM.

Effect of temperature on [³H]PCP binding. The binding of [³H]PCP to crayfish muscle membrane was highest at 2° and lowest at 32°. In these experiments, [³H]PCP, verapamil, and the Tris buffer (50 mM) were pipetted into the incubation tubes, and the tubes were placed into baths of the appropriate temperatures. The membrane preparation was also brought to the appropriate temperature before addition to the incubation tubes. After 30 min, the amount bound was determined. An Arrhenius plot of the data showed an inflection at 12° and a linear decrease in binding up to 32° (Fig. 6). The lower binding at higher temperature resulted from loss of binding sites rather than a decrease in affinity (data not shown). It is important to note that the crayfish membrane preparations were stored in 5 mM phosphate and 200 mM NaCl surrounded by ice and kept in the refrigerator, so that it was kept at near zero but never froze. Under these storage conditions, the membranes did not lose binding activity for 1 week.

Sensitivity of [³H]PCP binding to drugs. The specific

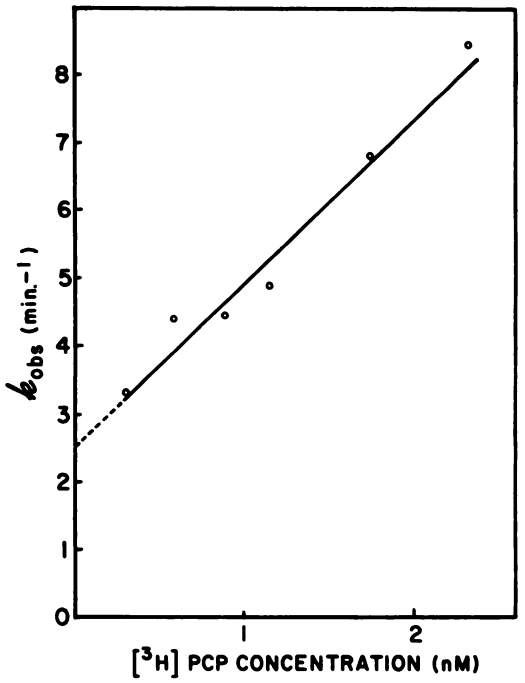

FIG. 3. *Effect of varying [^{3}H]PCP concentration on the initial rate of association and the calculated value of k_{obs}*

The k_{obs} values calculated at six concentrations (ranging from 0.29 nM to 2.32 nM) are plotted versus [^{3}H]PCP concentrations.

binding of [^{3}H]PCP was displaced totally by 1 μM unlabeled PCP. Other PCP derivatives also displaced [^{3}H]PCP binding but varied in potency (Table 1). TCPY, the thienylpyrrolidine analogue (IC_{50} 0.8 nM) had 1 order of magnitude higher potency than PCP, but the rest were less potent. The Hill slope values, calculated from dose-response curves, ranged from 0.87 to 0.93 for the six PCP analogues.

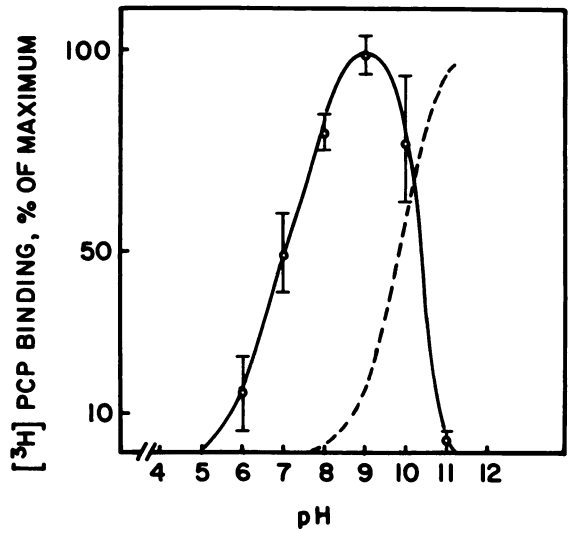

FIG. 4. *Specific binding of [^{3}H]PCP (2 nM to crayfish muscle membranes as a function of pH.*

Maximal binding is that measured at pH 9. The *broken line* shows the theoretical values of the percentage of nonionized form of PCP based on an estimated pK_a for PCP of about 9.8 (21). Each symbol and bar represent mean ± standard deviation of three experiments.

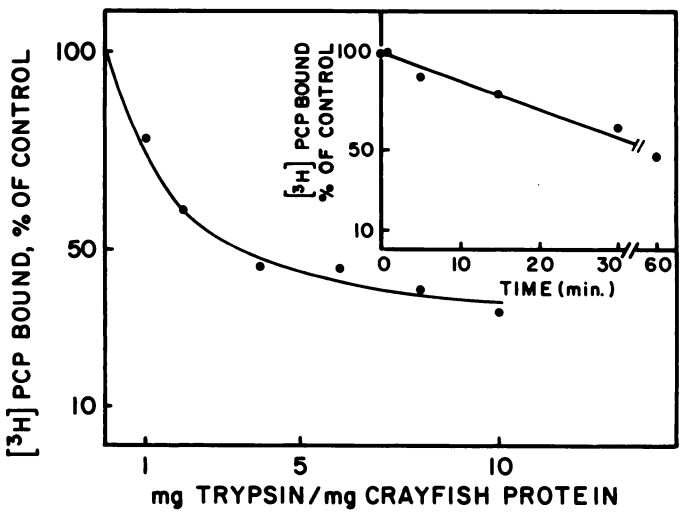

FIG. 5. *Effect of incubation of crayfish muscle membranes with trypsin on the specific binding of [^{3}H]PCP*

Membranes were incubated with different concentrations of trypsin for 60 min at 37°, then 100-μl aliquots were transferred into tubes containing [^{3}H]PCP (2 nM) and incubated for another 30 min at 23°. Another set of tubes containing 1 μM verapamil was used to measure nonspecific binding. *Inset* shows the time course of trypsin reduction of [^{3}H]PCP binding by pretreatment with 10 mg of trypsin per gram of membrane protein.

A number of neurotransmitters had no effect on [^{3}H]PCP (2 nM) binding to crayfish muscle membranes up to 100 μM, except for ACh, which inhibited it with an IC_{50} of 50 μM (Table 2). However, this effect might be due to the ammonium group, since several tetraalkylammonium compounds had similar potencies, and the effect of ACh was not potentiated in presence of 100 μM diisopropylfluorophosphate, the potent anticholinesterase.

As shown in Table 2, a large number of drugs belonging to diverse groups inhibited binding of [^{3}H]PCP to the crayfish muscle membranes at 2 nM. However, many of

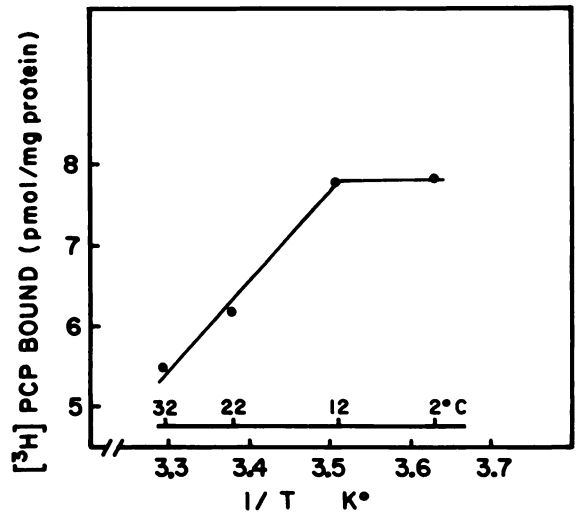

FIG. 6. *Arrhenius plot of the specific binding of 2 nM [^{3}H]PCP to muscle membranes (100 μg of protein/ml)*

Each symbol represents a mean of triplicate measurement.

TABLE 1

Comparison of the effect of PCP analogues on the binding of [^{3}H]PCP (2 nM) to membranes from crayfish muscle, Torpedo electric organ, and rat brain, and their relative potencies in producing PCP-like discriminative stimuli in rat

Drug	Name	Inhibition of [^{3}H]PCP binding IC_{50}			Relative potency rat discriminative stimuli
		Crayfish muscle[a]	Torpedo electric organ[b]	Rat brain	
			μM		
PCP	Phencyclidine	0.012	0.18	0.23[c]	1.0[e]
PCM	1-(1-Phenylcyclohexyl)morpholine	0.017	0.78	2.40[c]	0.1[e]
TCPY	1[1-(2-Thienyl)cyclohexyl]pyrrolidine	0.008	0.18	0.69[d]	0.87[e]
PCE	*N*-Ethyl-1-phenylcyclohexylamine	1.3	0.37	0.14[c]	5.79[e]
NSBPCA	*N*-(*s*-Butyl)-1-phenylcyclohexylamine	0.18	0.03	0.59[d]	0.58[d]
NMBPCA	*N*-(*n*-Butyl)-1-phenylcyclohexylamine	2.9	0.08	3.38[d]	0.30[e]
NIPPCA	*N*-(Isopropyl)-1-phenylcyclohexylamine	0.20	0.14	1.17[d]	2.86[d]

[a] Data from present work using 1 nM [^{3}H]PCP.
[b] Data from Eldefrawi *et al.* (15).
[c] Data from Zukin and Zukin (3).
[d] Data from Jasinski *et al.* (22).
[e] Data from Shannon (8).

those had very low potencies. On the other hand, a few inhibited the binding with potencies similar to, or even higher than, that of PCP. Among them were several antipsychotics, the antiviral drug tilorone, and the local anesthetic piperocaine. In addition to verapamil, several other Ca^{2+} channel antagonists were potent inhibitors of [^{3}H]PCP binding, but with varying potencies (Fig. 7). The IC_{50} values (micromolar) were as follows: bepridil, 0.007; diltiazem, 0.015; nicardipine, 0.015; verapamil, 0.02; cinnarizine, 0.15; (+)-D-600, 0.2; and (−)-D-600, 0.5; 4-NO_2-nifedipine, 2.5; 2-NO_2-nifedipine, 30. The 3-NO_2, 3-*O*-methyl, and 3-chloro analogues were equipotent to nifedipine (the 2-NO_2 derivative).

TABLE 2

Effect of drugs on the binding of 2 nM [^{3}H]PCP to crayfish muscle membranes[a]

Drug	IC_{50}	Drug	IC_{50}
	μM		μM
Neurotransmitters		Antidepressants	
Acetylcholine	50	Imipramine	0.15
Norepinephrine	>100	Nortriptyline	0.74
Dopamine	>100	Fluoxetine	6.0
5-Hydroxytryptamine	>100	Burpropion	12.0
Histamine	>100	Antipsychotics	0.03
GABA	>100	Chlorpromazine	0.04
Glutamate	>100	Trifluoperazine	0.02
Cholinergic drugs		Thiothixene	0.20
Carbamylcholine	58.0	Chlorprothixene	
Decamethonium	10.0	Antiarrhythmics	3.6
d-Tubocurarine	0.2	Procainamide	1.2
Atropine	0.1	Quinidine	1.6
Tetraalkylammoniums		Propranolol	
		Tranquilizers	100.0
Tetramethyl	>100	Diazepam	8.0
Tetraethyl	80.0	Flunitrazepam	
Tetrabutyl	40.0	Calcium antagonists	0.50
Tetrahexyl	5.0	(−)-D-600	0.02
Antiviral		Verapamil	0.007
Amantadine	10.0	Bepridil	
Tilorone	0.06	Local anesthetics	0.63
Antibacterial		Tetracaine	0.04
Neomycin	>100	Piperocaine	
Gentamicin	>100		
Polymyxin	>100		

[a] No inhibition occurred by 100 μM ethanol, 1-propanol, or 1-butanol, nor by 1 μM apamine.

Since many drugs, especially the Ca^{2+} channel antagonists, were dissolved in ethanol, and the alcohol was present at 1% concentration in the assay medium, its effect on [^{3}H]PCP binding was studied. Ethanol at concentrations up to 3% did not have any effect on the binding, but 1-propranol and 1-butanol decreased binding significantly at concentrations higher than 2%.

Effect of detergents on the binding of [^{3}H]PCP. To investigate the possibility of solubilizing the binding protein for eventual purification, we decided to determine the effect of detergents on binding. Several detergents inhibited [^{3}H]PCP binding totally when present at 1% in the assay medium during incubation. These included cholate, deoxycholate, sodium dodecyl sulfate, lysolecithin, and Trition X-100. At 0.1% concentrations, digitonin and Lubrol inhibited 50% and 28%, respectively,

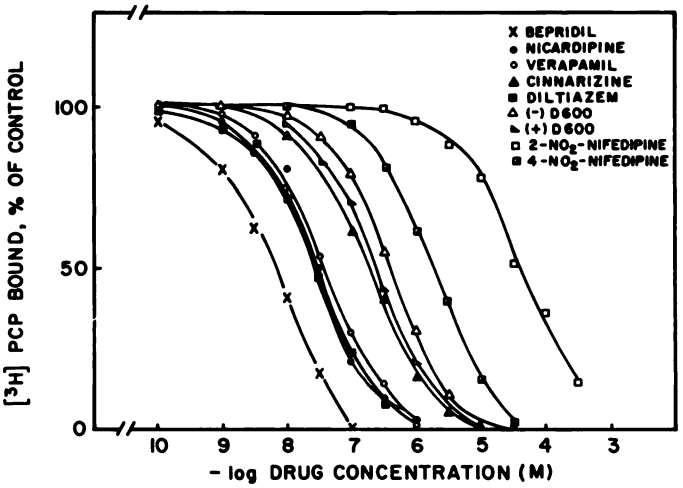

FIG. 7. *Inhibition of [^{3}H]PCP (2 nM) binding to crayfish muscle membranes by calcium channel antagonists.*
Each symbol represents a mean of triplicate measurements.

whereas the other detergents still inhibited all or most of the binding. When crayfish membranes were extracted in 0.5% Lubrol and then verapamil-sensitive [^{3}H]PCP binding was measured in the pellet and supernatant fraction by equilibrium dialysis, it was found that 20% of the binding was solubilized. Higher Lubrol concentrations increased the total soluble [^{3}H]PCP binding protein, but the verapamil-sensitive binding remained constant.

Effect of protein-modifying reagents. The crayfish membrane preparation was incubated with one of various reagents, and its binding of [^{3}H]PCP was then measured. It was totally insensitive to dithiothreitol up to 10 mM, suggesting that disulfide bonds are not present or that their presence does not contribute much to the binding site. However, the sulfhydryl reagent *N*-ethylmaleimide at 10 mM reduced binding by 20%, suggesting that sulfhydryl groups are present at or near the binding site and that their alkylation reduces [^{3}H]PCP binding. The carboxylic reagent dicyclohexylcarbodiimide at 1 mM concentration gave 37% inhibition and at 10 mM gave 46% inhibition. Washing the crayfish membranes in 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid twice by centrifugation had no effect on [^{3}H]PCP binding. Incubating the crayfish tissue for 1 hr with 1 mM concentration of ATP, GTP, cyclic AMP, dibutyryl cyclic AMP, or dibutyryl cyclic GMP had no effect on [^{3}H]PCP binding, and neither did incubation with cyclic AMP-dependent kinase. Concanavalin A in the concentration range 0.1–10 μM also had no effect on [^{3}H]PCP binding.

Effect of PCP, verapamil, and nifedipine on Ca^{2+} conductances in crayfish muscle membrane. Electrical properties of the cell membrane of the crayfish muscle were similar to those obtained under the same experimental conditions (160 mM $SrCl_2$) (23). For example, the values for the membrane potential, amplitude, threshold, and overshoot of the action potential were −85.5 ± 1.2, 53.2 ± 1.5, −71.5 ± 1.7, and 30 ± 1.0 mV, respectively (Fig. 8).

The values for the membrane potential, amplitude, and overshoot of the action potential obtained after 30 min of exposure to 100 μM PCP were −83.5 ± 1.5, 47.1 ± 2.1, and 24.3 ± 2.5 mV, respectively. The threshold value was unaltered, suggesting that the activation of the Ca^{2+} channel was also unaltered. The small decrease in the amplitude of the action potential produced by 100 μM PCP can be related to small decreases in Na^+ conductance, since the rate of rise of the action potential was decreased from 217 ± 15 V/sec to 190 ± 16 V/sec under the same conditions.

On the other hand, verapamil at concentrations that varied from 50 to 100 μM completely blocked the action potential in 30 min of exposure, suggesting that activation of the Ca^{2+} channels was blocked by this agent. Nifedipine at 100 μM reduced the amplitude of the action potential from 53.2 ± 1.5 to 23.4 ± 1.8 mV and the overshoot from 30 ± 1.0 mV to 4.2 ± 0.4 mV after a 30-min exposure (Fig. 8). The membrane potential, however, remained similar to control values (−89.5 ± 3 mv).

In addition, PCP at 50–100 μM produced a small but

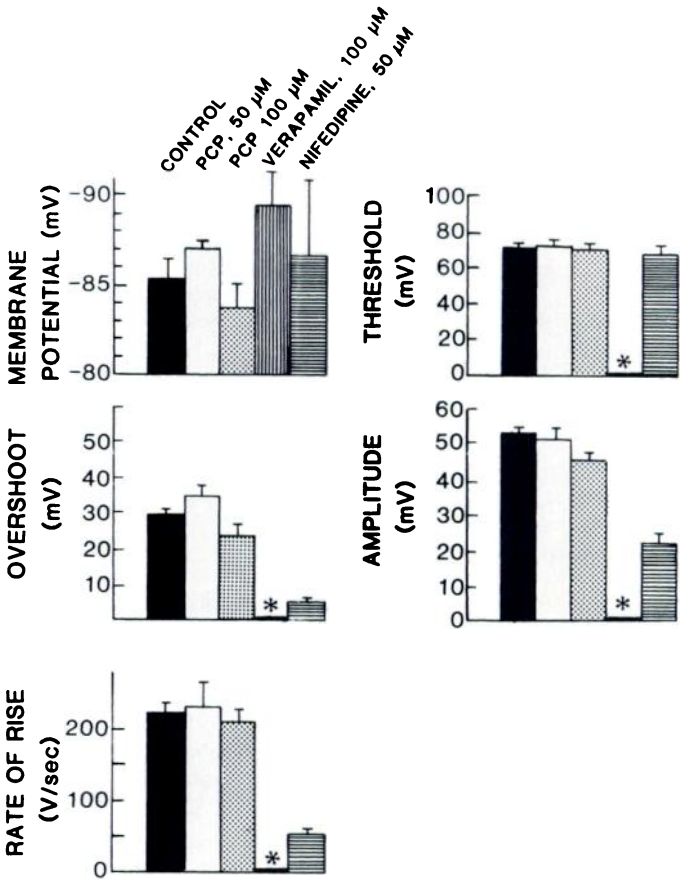

FIG. 8. *Effect of PCP, verapamil, and nifedipine on the action potential of the crayfish muscle evoked in a physiological solution containing strontium chloride*

The drugs did not cause any significant change in membrane potential except for nifedipine (*asterisk*) and verapamil, which significantly decreased the amplitude of the action potential and completely blocked it, respectively, after exposure to these agents for 30 min at 22°. Values are means, and each bar represents the standard deviation from at least 10 fibers in two muscles.

significant prolongation of the falling phase of the action potential, suggesting that it possibly blocked K^+ conductances (Fig. 9).

DISCUSSION

The crayfish abdominal muscle contains fairly high concentrations (8 pmoles/mg of protein; Fig. 1B) of protein(s) that bind(s) [^{3}H]PCP with high affinities (20). Binding is with two affinities, both of which are obtained whether verapamil or PCP (at 1 μM) is used to define specific binding, and both are inhibited totally by either drug. The effects of drugs and various treatments are tested on 2 nM [^{3}H]PCP binding, which consists of binding to about 60% of the high-affinity sites (0.24 pmole) and 6.5% of the low-affinity sites (0.7 pmole). Thus, binding of 2 nM [^{3}H]PCP represents 33% and 67% to the high- and low-affinity sites, respectively. The two binding affinities for [^{3}H]PCP (K_d 0.96 nM and 18.9 nM) may reflect binding to two independent sites, two sites that are negatively cooperative or to the same site on a protein that undergoes isomerization as shown for the muscarinic ACh receptor (24). It is unlikely that the two

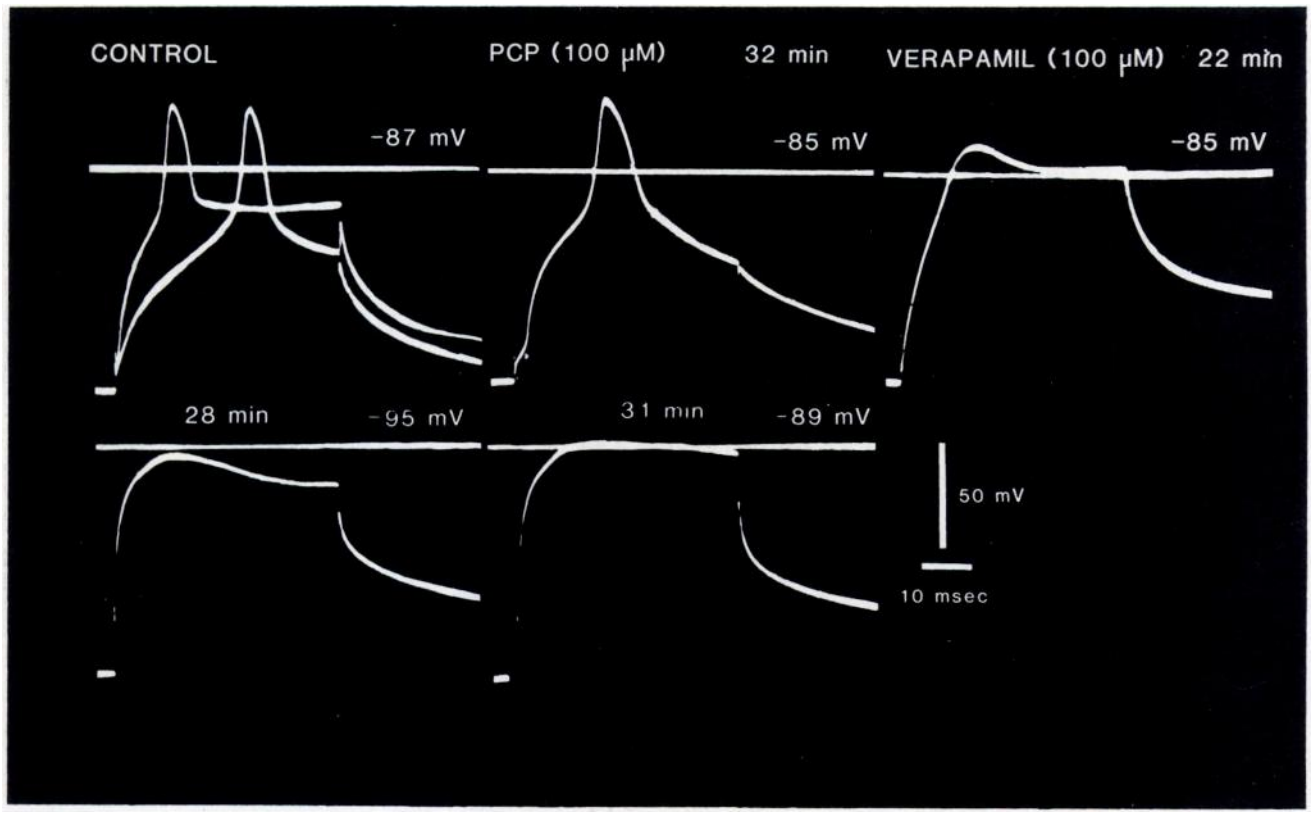

FIG. 9. *Effect of PCP and verapamil on the directly elicited action potentials of the extensor muscle of the crayfish carpopodite*
The *upper trace* represents the zero membrane potentials and the *lower trace* the single-action potentials evoked by a depolarizing pulse applied with a second electrode to the membrane. The control panel has two action potentials evoked at slightly different times, and in the presence of PCP there was a slight prolongation of the decay phase of the action potential, an effect which would be seen much more intensively if repetitive stimulation was applied to the preparation, indicating that the drug might be prolonging the decay phase of the action potential. The other traces show the effect of verapamil and the complete blockade of the action potential in the presence of these agents. The membrane potential was held −90 mV before stimulation; the negative number in the *upper right corner* was the true resting membrane potential of that particular fiber.

[^{3}H]PCP affinities observed reflect binding of ionized and nonionized forms of PCP, since raising the pH from 7.4 to 9 increases the affinities of both sites and the ratio of the concentration of the low- to high-affinity sites. These affinities are higher than the reported ones for the PCP-binding protein of mammalian brain (K_d values range from 46 nM (6), 85–150 nM (3, 7), and 250 nM (4): 2.5- to 13-fold higher for the low-affinity sites and 46- to 250-fold for the high-affinity sites. The increased affinity at pH 9 of [^{3}H]PCP binding to crayfish muscle (Fig. 4) (K_d of the low-affinity sites from 18.9 to 7.5 nM) is also observed in binding to ileal muscle, which increases 3-fold at pH 9 (19), and to the ionic channel of *Torpedo* ACh receptors, which increases 2.5-fold.[6] This pH effect suggests that [^{3}H]PCP binding is due mainly to the nonionic form.

There are several other differences between the [^{3}H]PCP binding protein in crayfish muscle and that in rat brain: the former is tolerant to monovalent cations and *N*-ethylmaleimide treatment, whereas the latter is highly sensitive in most studies (6, 7). Also, the potencies of PCP analogues in inhibiting [^{3}H]PCP binding to the crayfish proteins are different from theirs on the brain protein in most studies (Table 1). For example, PCE is 100 times less potent than PCP on crayfish muscle, whereas all of the other studies of [^{3}H]PCP binding to brain have shown that PCE was more potent (1.5–6 times) than PCP (3, 6). PCE is also more potent than PCP in behavioral assays (8). On the other hand, TCPY, which is more potent than PCP on crayfish muscle, is much less potent in brain assays (3, 6). The crayfish binding protein is also different from the well-characterized PCP-binding ionic channel of the nicotinic ACh receptor (Table 1) (18).

The good correlation found between the potencies of a number of PCP analogues in inhibiting binding of [^{3}H]PCP to rat brain and in eliciting a behavioral or pharmacological response (3, 4, 6) argues favorably for the physiological significance of the binding sites.

In rat brain, a number of lines of evidence suggest that PCP may be acting on biogenic amine carriers, namely (a) the blockade of reuptake of norepinephrine and possibly dopamine by PCP, (b) the antagonism of PCP effects by dopamine and *beta*-adrenergic receptor antagonists, and (*c*) the ineffectiveness of PCP after treating animals with 6-hydroxy-dopamine (which selectively destroys adrenergic neurons (25). Also, [^{3}H]PCP binding

[6] M. E. Eldefrawi, unpublished observations.

sites and dopamine receptors are reduced in animals chronically treated with PCP (26), an effect that is similar to that produced by inhibitors of biogenic amine uptake (27, 28). However, although biogenic amine carriers may be a target for PCP action in mammalian brain, they cannot be the target in crayfish muscle, since it does not receive adrenergic innervation; instead, it is innervated by excitatory (glutamergic) and inhibitory (γ-aminobutyric acid-ergic) neurons (29).

An interesting feature of the PCP-binding protein of crayfish muscle is its high sensitivity to Ca^{2+} channel antagonists (Fig. 7) (20). Since crayfish muscles have Ca^{2+} channels and have been used to study Ca^{2+} current conductances (23), it was suggested the PCP binding may be associated with Ca^{2+} channels (20). Inhibition of [^{3}H]PCP binding to brain by calcium antagonists led to similar conclusions (30). Indeed, if PCP bound to and activated rather than inhibited Ca^{2+} channels, it would cause increased transmitter release and synaptic activity. However, there are several observations that argue against the possibility that PCP binding sites are Ca^{2+} channels: the reversed potency [(+)-isomer > (−)-isomer] of the stereoisomeric pair of D-600 (31), the low affinity of nifedipine, which has a very high affinity for Ca^{2+} channels of smooth (32) and cardiac muscles (33), and most of all the lack of PCP effect on the verapamil-sensitive Sr^{2+} conductances in crayfish muscle (Figs. 8 and 9). However, there are many kinds of Ca^{2+} channels (34), and it is conceivable that PCP may be binding to a subclass of Ca^{2+} channel that is not identified by the biophysical techniques used here. Some of the Ca^{2+} channel antagonists, which are potent inhibitors of [^{3}H]PCP binding (Table 2), inhibit the slow Ca^{2+} channels of chick heart muscle cells (35). These Ca^{2+} channels are also blocked by millimolar concentrations of heavy metals such as Co^{2+}, Mn^{2+}, Cd^{2+}, and Cu^{2+}, which also totally block [^{3}H]PCP binding to crayfish muscles. It is important, however, to recall that many of these inorganic divalent cations also inhibit K^+ conductances (36). The possibility that the binding protein may be calmodulin is eliminated because of its heat sensitivity (20) (Fig. 6). Moreover, no [^{3}H]PCP binding to purified calmodulin (from Sigma Company) was observed.[6]

The above discussion suggests that the [^{3}H]PCP-binding protein in crayfish muscle is not a neurotransmitter receptor, Na^+ channel, or biogenic amine carrier (which are not present in these muscles) or calmodulin, and possibly not even a Ca^{2+} channel. Another proposed molecular target for PCP action is the voltage-sensitive K^+ channel. PCP produces a small but significant prolongation of the falling phase of the action potential (Fig. 9). However, a high concentration is required for its effect (50–100 μM). The drug profile of [^{3}H]PCP binding to crayfish muscle (Table 2) indicates that many drugs belonging to different classes are fairly potent in displacing [^{3}H]PCP. Many of these drugs, such as atropine, imipramine, tetraethylammonium, tetracaine, and verapamil, have been shown to inhibit K^+ conductances (36–39). It has been found that only PCP and behaviorally active PCP analogues, but not inactive ones, inhibit K^+ conductances in skeletal muscles and inhibit [^{3}H]PCP binding to rat brain synaptosomes (15). [^{3}H]PCP binding to rat brain synaptosomes is to a high-affinity site (K_d 60 nM) and is observed in the presence of 150 mM Na^+ and K^+; thus it is insensitive to monovalent cations. This binding correlates well with the ability of PCP (10–50 nM) to block depolaraization-activated flux of ^{86}Rb, possibly through K^+ channels (40). Indeed, blockade of presynaptic K^+ channels would lead to increased presynaptic depolarization and transmitter release, producing physiological effects similar to those produced by blockade of amine reuptake. In fact, it is possible that [^{3}H]PCP may bind to a K^+ channel and a biogenic amine reuptake carrier in brain with similar or close affinities.

In summary, crayfish muscle membranes contain (a) protein(s) that bind(s) [^{3}H]PCP with very high affinities and differ in certain of their drug specificities and sensitivity to ions from the [^{3}H]PCP-binding protein in rat brain. It is not known whether the two proteins carry the same function in the two tissues or are two functionally different proteins. The crayfish muscle protein is very sensitive to Ca^{2+} channel blockers, but is unlikely to be a Ca^{2+} channel. It is possible that it may be a K^+ channel, but more studies are needed to be certain about the physiological identity of this high-affinity PCP binding protein and the possibility of its occurrence in vertebrates as well.

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REFERENCES

1. Chen, G. The neuropharmacology of phencyclidine, in *PCP (Phencyclidine): Historical and Current Perspectives* (E. F. Domino, ed.). NPP Books, Ann Arbor, Mich., 9–16, (1981).
2. Domino, E F., and E. D. Luby. Abnormal mental states induced by phencyclidine as a model of schizophrenia, in *PCP (Phencyclidine): Historical and Current Perspectives* (E. F. Domino, ed.). NPP Books, Ann Arbor, Mich., 401–418 (1981).
3. Zukin, S. R., and R. S. Zukin. Specific [^{3}H]phencyclidine binding in rat central nervous system. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5372–5376 (1979).
4. Vincent, J. P., B. Kartalovski, P. Genete, J. M. Kamenka, and M. Lazdunski. Interaction of phencyclidine ("angel dust") with a specific receptor in rat brain membranes. *Proc. Natl. Acad. Sci. U. S. A.* **76**:4678–4682 (1979).
5. Vincent, J.-P., J. Vignon, B. Kartalovski, and M. Lazdunski. Binding of phencyclidine to rat brain membranes: technical aspects. *Eur. J. Pharmacol.* **68**:73–77 (1980).
6. Quirion, R., R. P. Hammer, Jr., M. Herkenham, and C. B. Pert. Phencyclidine (angel dush/o"opiate" receptor: visualization by tritium-sensitive film. *Proc. Natl. Acad. Sci. U. S. A.* **78**:5881–5885 (1981).
7. Zukin, S. R., M. L. Fitz-Syage, R. Nichtenhauser, and R. S. Zukin. Specific binding of [^{3}H]phencyclidine in rat central nervous tissue: further characterization and technical considerations. *Brain Res.* **258**:277–284 (1983).
8. Shannon, H. E. Evaluation of phencyclidine analogs on the basis of their discriminative stimulus properties in the rat. *J. Pharmacol. Exp. Ther.* **216**:543–551 (1981).
9. Quirion, R., K. C. Rise, P. Skolnick, S. Paul, and C. B. Pert. Stereospecific displacement of [^{3}H]phencyclidine (PCP) receptor binding by an enantiomeric pair of PCP analogs. *Eur. J. Pharmacol.* **74**:107–108 (1981).
10. Shannon, H. E. Phencyclidine-like discriminative stimuli of (+)- and (−)-N-alkylnormetazocine in rats. *Eur. J. Pharmacol.* **84**:225–228 (1982).
11. Hampton, R. Y., F. Medzihardsky, J. H. Woods, and P. J. Dahlstrom. Stereospecific binding of [^{3}H]phencyclidine in brain membranes. *Life Sci.* **30**:2147–2154 (1982).
12. Maayani, S., H. Weinstein, N. Ben-Zvi, S. Cohen, and M. Sokolovsky. Psychotomimetics as anticholinergic agents. 1. 1-cyclohexylpiperidine derivatives: anticholinesterase activity and antagonistic activity to acetylcholine. *Biochem. Pharmacol.* **23**:1263–1281 (1974).
13. Aronstam, R. S., E. X. Albuquerque, A. T. Eldefrawi, K. F. Jim, and D. J. Triggle. Sites of action of phencyclidine. III. Interactions with the muscarinic receptors. *Mol. Pharmacol.* **18**:179–184 (1980).
14. Albuquerque, E. X., M.-C. Tsai, R. S. Aronstam, B. Witkop, A. T. Eldefrawi, and M. E. Eldefrawi. Phencyclidine interactions with ionic channels of the

acetylcholine receptor and electrogenic membranes. *Proc. Natl. Acad. Sci. U. S. A.* **77**:1224–1228 (1980).
15. Eldefrawi, A. T., E. R. Miller, D. L. Murphy, and M. E. Eldefrawi. [^{3}H] Phencyclidine interactions with the nicotinic acetylcholine receptor channel and its inhibition by psychotropic, antipsychotic, opiate, antidepressant, antibiotic, antiviral, and antiarrhythmic drugs. *Mol. Pharmacol.* **22**:72–81 (1982).
16. Albuquerque, E. X., L. G., Aguayo, J. E. Warnick, H. Weinstein, S. D. Glick, S. Maayani, R. K. Ickowicz, and M. P. Blaustein. The behavioral effects of phencyclidines may be due to their blockade of potassium channels. *Proc. Natl. Acad. Sci. U. S. A.* **78**:7792–7796 (1981).
17. Smith, R. C., H. Y. Meltzer, R. C. Arora, and J. M. Davis. Effects of phencyclidines on [^{3}H]catecholamine and [^{3}H]serotonin uptake in synaptosomal preparations from rat brain. *Biochem. Pharmacol.* **26**:1435–1439 (1977).
18. Vincent, J. P., J. Vignon, B. Kartalovski, and M. Lazdunski. Compared properties of central and peripheral binding sites for phencyclidine. *Eur. J. Pharmacol.* **68**:79–82 (1980).
19. El-Fakahany, E., D. J. Triggle, A. T. Eldefrawi, and M. E. Eldefrawi. Distinction between high affinity [^{3}H]phencyclidine binding sites and muscarinic receptors in guinea pig ileum muscle. *J. Pharmacol. Exp. Ther.* (in press).
20. Eldefrawi, M. E., E. F. El-Fakahany, D. L. Murphy, A. T. Eldefrawi, and D. J. Triggle. High affinity binding of phencyclidine (PCP) to crayfish muscle. Displacement by calcium antagonists. *Biochem. Pharmacol.* **31**:2549–2551 (1982).
21. Geneste, P., J. M. Kamenka, S. N. Ung, P. Herrmann, R. Goudal, and G. Trouiller. Determination conformationnelle de derives de la phencyclidine en vue d'une etude structure-activite. *Eur. J. Med. Chem.* **14**:301–308 (1979).
22. Jasinski, D. R., H. E. Shannon, E. J. Cone, D. B. Vaupel, M. E. Risner, R. L. McQuinn, T. P. Su, and W. B. Pickworth. Interdisciplinary studies on phencyclidine, in *PCP (Phencyclidine): Historical and Current Perspectives* (E. F. Domino, ed.). NPP Books, Ann Arbor, Mich., 331–400 (1981).
23. Fatt, P., and B. L. Ginsborg. The ionic requirements for the production of action potentials in crustacean muscle fibers. *J. Physiol. (Lond.)* **142**:516–543 (1958).
24. Järv, J., B. Hedlund, and T. Bartfai. Isomerization of the muscarinic receptor-antagonist complex. *J. Biol. Chem.* **254**: 5595–5598 (1979).
25. Marwaha, J. Candidate mechanisms underlying phencyclidine-induced psychosis: an electrophysiological, behavioral, and biochemical study. *Biol. Psychol.* **17**:155–198 (1982).
26. Quirion, R., M. A. Bayorh, R. L. Zerbe, and C. B. Pert. Chronic phencyclidine treatment decreases phencyclidine and dopamine receptors in rat brain. *Pharmacol. Biochem. Behav.* **17**:699–702 (1982).
27. Langer. S. Z., M. Briley, S. Arbilla, R. Raisman, and M. Sette. Chronic antidepressant administration and changes in ^{3}H-imipramine binding and brain receptors, in *Advances in Pharmacology and Therapeutics*, Vol. 2: *Neurotransmitter Receptors* (Y. Yoshida, Y. Hagihara, and S. Ebashi, eds.). Pergamon Press, New York, 21–28 (1981).
28. Sulser, F., A. Janowsky, and R. Mishra. Up- and down-regulation of noradrenergic receptor systems in brain: molecular mechanisms and physiological significance, in *Advances in Pharmacology and Therapeutics*, Vol. 2: *Neurotransmitter Receptors* (Y. Yoshida, Y. Hagihara, and S. Ebashi, eds.). Pergamon Press, New York, 21–28 (1981).
29. Leake, L. D., and R. J. Walker. *Invertebrate Neuropharmacology*. John Wiley and Sons, New York (1980).
30. Quirion, R., and C. B. Pert. Certain calcium antagonists are potent displacers of [^{3}H]phencyclidine (PCP) binding in rat brain. *Eur. J. Pharmacol.* **83**:155–156 (1982).
31. Jim, K., A. Harris, L. B. Rosenberger, and D. J. Triggle. Stereoselectivity and non-stereoselective effects of D600 (methoxyverapamil) in smooth muscle preparations. *Eur. J. Pharmacol.* **76**:67–72 (1981).
32. Triggle, D. J. Calcium antagonists: basic chemical and pharmacologic aspects, in *New Perspectives on Calcium Antagonists* (G. B. Weiss, ed.). American Physiological Society, Bethesda, Md., 1–19 (1981).
33. Bolger, G. T., P. J. Gengo, E. M. Luchowski, H. Siegel, D. J. Triggle, and R. A. Janis. High affinity binding of a calcium channel antagonist to smooth and cardiac muscle. *Biochem. Biophys. Res. Commun.* **104**:1604–1609 (1982).
34. Hagiwara, S., and L. Byerly. Calcium channels. *Annu. Rev. Neurosci.* **4**:69–125 (1981).
35. Sperelakis, N. Effects of cardiotoxic agents on the electrical properties of myocardial cells, in Cardiac Toxciology (T. Balazs, ed.). C.R.C. Press, Boca Raton, Fla., 39–108 (1982).
36. Stefani, E., and D. J. Chiarandini. Ionic channels in skeletal muscle. *Annu. Rev. Physiol.* **44**:357–373 (1982).
37. Lapa, A. J., E. X. Albuquerque, and J. Daly. An electrophysiological study of the effects of d-tubocurarine, atropine, and α-bungarotoxin on the cholinergic receptor in innervated and chronically denervated mammalian skeletal muscles. *Exp. Neurol.* **43**:375–398 (1974).
38. Schauf, C. L., F. A. Davis, and R L. Kesler. Actions of the antidepressant drug imipramine on the voltage-clamped Myxicola giant axon. *J. Pharmacol. Exp. Ther.* **193**:669–675 (1975).
39. Posner, P., B. L. Miller, and C R. Lambert. The effects of verapamil on potassium fluxes in canine cardiac Purkinje fibres. *Eur. J. Pharmacol.* **34**:369–372 (1975).
40. Blaustein, M. P., and R. K. Ickowicz. Phencyclidine in nanomolar concentrations binds to synaptosomes and blocks certain potassium channels. *Proc. Natl. Acad. Sci. U. S. A.* **80**:3855–3859 (1983).

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