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HIGH AFFINITY BINDING OF PHENCYCLIDINE (PCP) TO CRAYFISH MUSCLE. DISPLACEMENT BY CALCIUM ANTAGONISTS

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Phencyclidine [1-(phenylcyclohexyl)piperidine hydrochloride] (PCP) was introduced ≈30 years ago as a general anesthetic (1) and is relatively safe and is still used as a veterinary anesthetic. However, because of its psychotomimetic effects, ease of synthesis and administration, it is one of the most widely used drugs of abuse (2,3). PCP produces different pharmacologic effects in different animal species at different doses (4). In humans, subanesthetic doses cause hypertension, tachycardia, gait ataxia, hostile behavior, auditory hallucinations and paranoid delusions (5). Its chronic use produces schizophrenia-like symptoms and intensifies those of schizophrenics (6,7). The multiple pharmacologic effects are underscored by the number of targets with which PCP interacts. Two biochemical approaches have been used to identify the primary target(s) for PCP actions. One was to study high affinity [^{3}H]PCP binding to brain membranes without identifying definitively the function of the binding proteins (8). The other was to study its binding to, or effect on, identified vital proteins of nerves and muscles. PCP was found to bind or interact with high affinities to biogenic amine uptake carriers (9,10), butyrylcholinesterase (11), muscarinic receptors (12,13) and the ionic channel of the nicotinic acetylcholine (ACh) receptor (14,15). The highest affinities reported for [^{3}H]PCP binding ranged from 100 to 1000 nM. In the present study, we report on binding of [^{3}H]PCP that is one order of magnitude higher affinity. It is to a protein in crayfish abdominal muscles, and the binding is inhibited most potently by Ca^{2+} antagonists.

Materials and Methods. Membrane preparation. The abdominal muscle of live crayfish (from NASCO, Fort Atkinson, WI) was cut up in small segments (0.5 cm long and 0.1 cm thick), and was 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 7.3) (16) using a polytron and two 30 sec bursts. The homogenate was centrifuged at 100,000 x g for 30 min. The pellets were resuspended in 5 volumes of the above buffer and homogenized by the polytron for 30 sec, then centrifuged at 1000 x g for 10 min. The supernatant fraction was saved and the pellet was resuspended in the same buffer, homogenized and centrifuged at 1000 x g. The supernatant fractions were pooled and centrifuged at 100,000 x 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 (17) averaged 1-2 mg protein/ml.

Binding assay. Binding of [^{3}H]PCP (sp. act. 48 Ci/mmole, NEN) to membrane preparations of the crayfish abdominal muscles was measured by a filter assay. The tissue (100 μg protein) was added to a disposable culture tube containing 900 μl of 50 mM Tris-HCl, pH 7.4, and 2 nM [^{3}H]PCP (≈90,000 cpm). The mixture was incubated for 30 min, unless otherwise stated, then filtered over GF/B Whatman glass fiber filters pretreated with 1% Prosil (an organosilicone) and washed with ≈10 ml of cold buffer. The filters were then placed in 5 ml glass minivials and 4 ml of a toluene-based liquid scintillation solution added and their radioactive content determined after a period of equilibration (usually 5 hr). To determine the effect of a drug on [^{3}H]PCP binding, the desired concentration of the drug was added to

the incubation buffer along with [^{3}H]PCP. The concentration that inhibited 50% of the binding (IC_{50}) was determined graphically, and the inhibition constant (K_i) was calculated from the relationship $K_i = IC_{50}$ (1 - f), where f = fractional occupancy at the [^{3}H]PCP concentration used in the assay.

Results. Our initial experiments showed that [^{3}H]PCP in the nM range bound to crayfish muscle membranes, and the binding was linear with tissue concentration up to 250 µg protein. Almost 90% of the [^{3}H]PCP binding at 2 nM was inhibited with 1 µM PCP, verapamil and trifluoperazine. Other drugs tested, but which gave less inhibition at 1 µM (between 50 and 90%), include imipramine, nortryptiline, chlorpromazine, quinidine, tetracaine and propranolol. The cholinergic drugs carbamylcholine and decamethonium had no effect at 1 µM, but caused significant inhibition at 100 µM. Subsequently verapamil was used to identify the specific binding of [^{3}H]PCP by subtracting total binding (i.e. in absence of verapamil) from nonspecific binding (i.e. in presence of 1 µM verapamil). This specific verapamil-sensitive [^{3}H]PCP binding to crayfish muscle membranes was saturable (Fig. 1A). The Scatchard plot of the specific binding obtained from two experiments (Fig. 1B) gave an apparent dissociation constant (K_d) of 13.5 x 10^{-9} M and a maximum number of sites of 8 pmoles/mg protein. Since the affinity of [^{3}H]PCP for the channel (i.e. allosteric) sites of the nicotinic ACh-receptor was increased by binding of ACh to the receptor sites, we tested the effect of several neurotransmitters on [^{3}H]PCP binding to crayfish muscle membranes. Concentrations ranging from 1 to 100 µM of GABA, glutamate, norepinephrine, dopamine or histamine did not affect [^{3}H]PCP binding, but 100 µM ACh or serotonin inhibited [^{3}H]PCP slightly (≈20%).

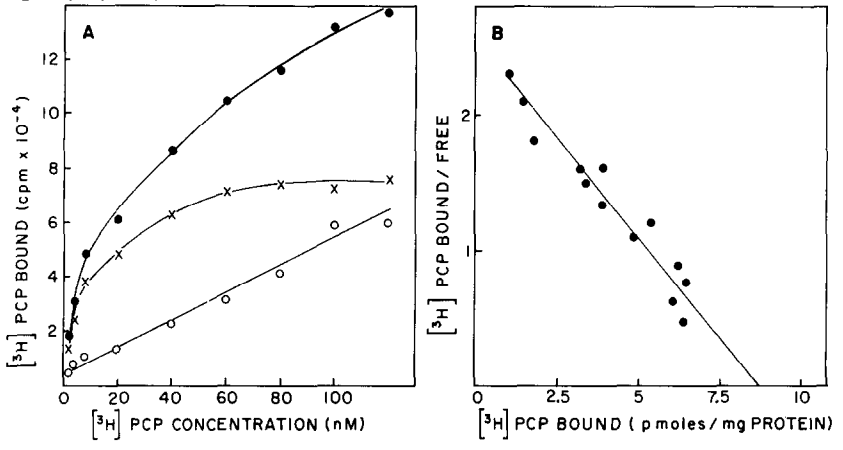

Fig. 1. Binding of [^{3}H]PCP to crayfish muscle membranes. A. Total binding (•), binding in presence of 1 µM verapamil (o), calculated specific binding (x). Binding was to membranes containing 60 mg protein. B. Scatchard plot of specific [^{3}H]PCP binding. Free ligand concentration is expressed in nM. Symbols are means of triplicate experiments, with standard deviations <10%.

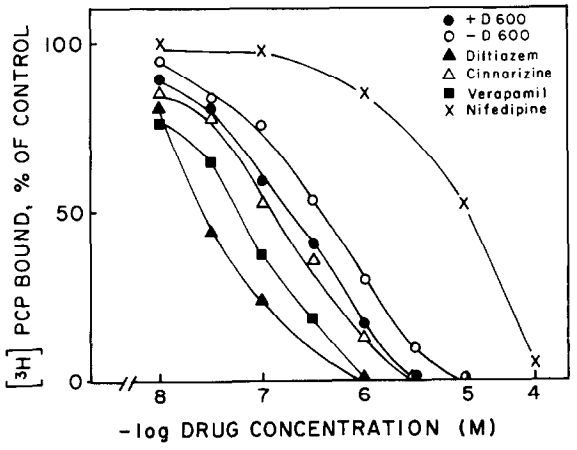

Fig. 2. The effects of calcium antagonists on the specific binding of [^{3}H]PCP (2 nM) to crayfish muscle membranes. Standard deviations <10%.

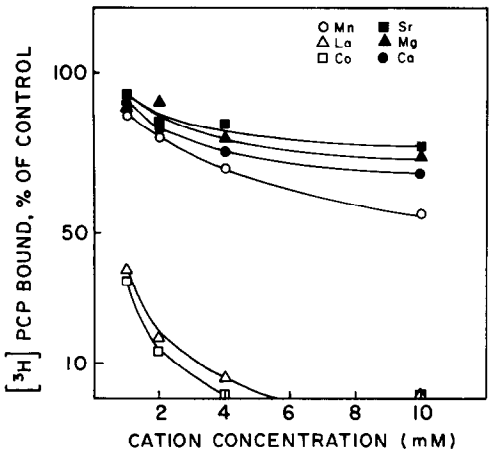

Fig. 3. The effects of cations on the specific binding of [^{3}H]PCP (2 nM) to crayfish muscle membranes. Standard deviations <10%.

A very potent group of drugs in inhibiting the binding of [^{3}H]PCP to crayfish muscle was the Ca^{2+} antagonists (Fig. 2). The most effective was diltiazem, followed by verapamil, which at 1 μM inhibited all specific binding. At 10 μM, cinnarizine, +D600 and -D600 inhibited all specific [^{3}H]PCP binding, but nifedipine was much less effective.

Binding of [^{3}H]PCP to the crayfish muscle was reduced slightly by increasing the ionic strength of the incubation media. NaCl and KCl up to 50 mM had no effect, but at 100 mM both salts reduced specific [^{3}H]PCP binding by ≈35%. Ca^{2+}, on the other hand, caused significant inhibition (≈25% at 10 mM). Thus, we tested the effect of several divalent cations and found that Co^{2+} and La^{3+} were the most potent, since at 4 and 5 mM, respectively, they inhibited all specific binding (Fig. 3).

Exposure of the membranes to 95°C for 2 min eliminated the binding totally. Similarly at pH 5 or below binding disappeared. In addition, incubation of the membrane with trypsin (0.1 mg trypsin/mg of protein) for 1 hr reduced the binding by 55%. However, incubation with chymotrypsin or phospholipase A was less effective.

Discussion. The data suggest that crayfish muscles contain high concentrations (8 pmoles/mg protein) (Fig. 1)) of a protein (because of its sensitivity to temperature, pH and trypsin) that binds [^{3}H]PCP with very high affinity. The apparent K_d is 13.5 nM, which we believe is an upper limit since preliminary results from experiments using less tissue concentrations and measurements of rates of association and dissociation suggest that the true K_d is much lower than the apparent one. The affinity of [^{3}H]PCP for these sites is one to two orders of magnitude higher than its affinities for the channel sites of the nicotinic ACh-receptor (K_d = 0.1 μM) or the "specific" [^{3}H]PCP binding sites of rat brain membranes (K_d = 0.25 μM) (8,15). The absence of stimulation of this [^{3}H]PCP binding by several neurotransmitters (e.g. GABA, glutamate, ACh, dopamine, serotonin or norepinephrine) suggests that either the [^{3}H]PCP binding sites are not associated with a neurotransmitter receptor, or that if [^{3}H]PCP is binding to a channel coupled to a receptor, there is no allosteric interaction between the receptor and channel. The crayfish muscle does contain GABA receptor, which is coupled to a Cl^- channel (18) and should also contain glutamate receptors which are usually coupled to a nonspecific cation channel (19).

The [^{3}H]PCP binding protein in crayfish muscle may be a calcium channel or a Ca^{2+}-binding protein as suggested by the strong inhibition of its binding of [^{3}H]PCP with organic (Fig. 2) and inorganic (Fig. 3) Ca^{2+} antagonists (20). The muscle membrane of the crayfish fibers is used as a model Ca^{2+} membrane, which has early Ca^{2+} inward currents (21,22); thus Ca^{2+} channels are present in its plasma membranes. These channels may have the observed binding sites for [^{3}H]PCP, but there also is no a priori reason why other Ca^{2+}-binding proteins in crayfish muscle may not be the ones involved. The heat sensitivity of the binding site would argue against its being a calmodulin-like calcium-binding protein. Also, the reversed potency ((+)isomer>(-)isomer) of the stereoisomeric pair of D-600, the specific calcium channel blocker (23), argues against the site being a Ca^{2+} channel. Interestingly, many of the drugs that inhibit this [^{3}H]PCP binding also inhibit the slow Ca^{2+} channels of chick heart muscle cells (24). Examples of these drugs are local anesthetics, neuroleptics, antidepressants, antiarrhythmic drugs and Ca^{2+} antagonists.

Another possibility for the identity of the [^{3}H]PCP binding protein in crayfish muscle is that it is a Ca^{2+}-activated K^+ channel (25). Our earlier studies suggested that PCP interfered with K^+ conductances in nerve and muscle (26). Calcium antagonists may inhibit such conductances by inhibiting binding of Ca^{2+} to regulatory sites on these K^+ channels.

The significance of this [^{3}H]PCP binding is its very high affinity. If a similar protein is present in mammalian brain and muscle, it would represent an excellent target for PCP action. Another interesting aspect is its high affinity for calcium antagonists, which suggests that interference with Ca^{2+} function is a mechanism of PCP action. Identification of this protein will be established through studies of radiolabeled ion flux and the effects of PCP and Ca^{2+} antagonists on it. It is possible that the primary target(s) for PCP is(are) ionic channel(s). A good correlation of the potencies of PCP and analogs in inhibiting binding of [^{3}H]PCP to, and function of, this protein with their potencies in modifying a certain animal behavior (27) would suggest that interference with the protein's function is responsible for this action of PCP. In attempting to understand the functional significance of PCP interference with the Ca^{2+}-binding protein, one wonders what the behavioral pharmacologic effects would be when comparable concentrations of PCP are administered to crayfish and if a similar molecular target is present in mammalian brain.

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