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[^{3}H]Phencyclidine: A probe for the ionic channel of the nicotinic receptor

(acetylcholine receptor/neuromuscular synapses/*Torpedo* electric organ/channel lifetime/[^{3}H]perhydrohistrionicotoxin)

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ABSTRACT To evaluate [^{3}H]phencyclidine ([^{3}H]PCP) as a probe for the ionic channel of the nicotinic receptor, the characteristics of its binding to electric organ membranes of *Torpedo ocellata* and its effects on frog sartorius muscle were studied. Similar to PCP, [^{3}H]PCP depressed the peak amplitude of end-plate current, caused nonlinearity in the voltage–current relationship at negative potentials, accelerated the decay time of the end-plate current, and shortened the channel lifetime. Thus, [^{3}H]PCP interacted with the ionic channel of the nicotinic receptor, although there were a few differences between its effect and that of PCP. Binding of [^{3}H]PCP to *Torpedo* membranes was to sites on the ionic channel of acetylcholine (AcCho) receptor because it was saturable, dependent upon protein concentration, and inhibited by drugs that interact with the ionic channel, and the initial rate of binding was potentiated by receptor agonists. Equilibrium binding of [^{3}H]PCP to *Torpedo* membranes was with two affinities, but in the presence of AcCho, [^{3}H]PCP binding was with a single affinity. The affinities of channel drugs obtained by inhibition of binding of [^{3}H]PCP and [^{3}H]perhydrohistrionicotoxin to *Torpedo* membranes were different, with correlation coefficients of 0.52 and 0.82 in the absence and presence of a receptor agonist, respectively; this suggests differences in their binding sites on the ionic channel of the AcCho receptor.

Phencyclidine [PCP; (1-phencyclohexyl)piperidine] is a general anesthetic and hallucinogen that has prolonged duration of action and is also psychotomimetic (1, 2). It potentiates the direct and indirect evoked muscle twitch, an effect that seems to be related to a marked blockade of potassium conductance (3). In addition, after an initial twitch potentiation the neuromuscular transmission is blocked as a result of a direct effect of PCP on the ionic channel of the acetylcholine (AcCho) receptor. PCP decreases the peak amplitude of the end-plate current (epc) in a voltage- and time-dependent manner, causes significant nonlinearity of the current–voltage relationship and marked acceleration of the decay time of both epc and miniature end-plate current (mepc), and concomitantly shortens the mean channel lifetime without alteration of the single channel conductance (4, 5). Moreover, PCP does not inhibit [^{3}H]AcCho or ^{125}I-labeled α-bungarotoxin (α-BGT) binding to the AcCho receptor sites in membranes from the electric organ of the electric ray *Torpedo ocellata* (4, 5). Thus, the effects of PCP resemble those of perhydrohistrionicotoxin (H_{12}-HTX), which is the saturated analog of histrionicotoxin, the spiropiperidine alkaloid isolated from the skin of the Colombian frog *Dendrobates histrionicus* (6, 7). [^{3}H]H_{12}-HTX has been utilized successfully to identify the ionic channel of the nicotinic receptor *in vitro* (7–9), but its supply is limited. This study was initiated to evaluate [^{3}H]PCP as a substitute for [^{3}H]H_{12}-HTX in biochemical studies of the ionic channel of the nicotinic receptor.

MATERIALS AND METHODS

Electrophysiological Techniques. Experiments were performed at room temperature (20–22°C) on sciatic nerve sartorius muscle preparations of the frog *Rana pipiens*, except that extrajunctional AcCho sensitivity measurements were made on 10- to 15-day denervated rat soleus muscles. The Ringer solutions used, nerve stimulation, intracellular recordings of the resting membrane potential, action potential, end-plate potential (epp), and miniature end-plate potential (mepp), delayed rectification, and microiontophoresis of AcCho were as described (6, 10, 11). The voltage-clamp circuitry and recording and analysis of the epc fluctuations in response to iontophoretic application of AcCho were similar to those described previously and were analyzed by an on-line PDP-11 computer. The drugs were superfused from micropipets by pressure with close application to the end-plate region.

Biochemical Techniques. Membrane preparation from the electric organ of *T. ocellata* was as described (8). [*piperidyl*-3,4-^{3}H(N)]PCP (48 Ci/mmol; New England Nuclear; 1 Ci = 3.7×10^{10} becquerels) was incubated with *Torpedo* membranes (2–10 μg of protein) in 50 mM Tris·HCl buffer (pH 7.4) for an appropriate time at room temperature; then the mixture was filtered with suction through Whatman GF/B glass fiber filters that had been dipped in 1% organosilate concentrate (Prosil-28). The filters were washed with 7 ml of the Tris buffer, and their radioactivity was measured in a toluene-based solution containing 4% BioSolv (Beckman). PCP binding curves were determined by dilution of the radiolabeled drug with unlabeled PCP. Theoretical binding curves were fitted to experimental data by iterative nonlinear least squares regression by using the MLAB system (12). Data were fitted to a mass action expression for the case of one or two receptor populations that bound the same ligand with unique affinities as follows: $B = N_1F/(K + F) + N_2F/(K_2 + F)$, in which B is the fraction of receptors

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Abbreviations: PCP, phencyclidine; AcCho, acetylcholine; H_{12}-HTX, perhydrohistrionicotoxin; α-BGT, α-bungarotoxin; epc, end-plate current; mepc, miniature end-plate current; epp, end-plate potential; mepp, miniature end-plate potential.

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occupied with ligand, F is the concentration of unbound ligand, N_1 and K_1 are the fraction of receptors and the dissociation constant (K_d) of the high-affinity receptor sites, and N_2 and K_2 are the corresponding variables of the low-affinity receptor sites.

RESULTS

Effect of [^{3}H]PCP on Muscle Twitch, Membrane Potential, Action Potential, and AcCho Sensitivity. Addition of PCP or [^{3}H]PCP to the frog sartorius muscle at concentrations of 50 and 100 μM induced potentiation and subsequent block of the indirect elicited muscle twitch; however, at these concentrations [^{3}H]PCP was less potent than PCP. [^{3}H]PCP caused no membrane depolarization and, like PCP, it prolonged the directly and indirectly elicited muscle action potential. These effects of [^{3}H]PCP and PCP (4) are most likely related to its reaction with the potassium channel. Exposure of the 15-day chronically denervated rat soleus muscle to [^{3}H]PCP (80 μM) for 30–60 min significantly reduced extrajunctional sensitivity to microiontophoretically applied AcCho, from a control value of 1463 ± 279 mV/nC (mean ± SD, 6 fibers per muscle) to 43 ± 17 mV/nC (16 fibers per muscle). Control denervated muscle fibers reacted to AcCho with regular and almost identical potentials even at higher rates of stimulation (e.g., 5 Hz); PCP-treated fibers showed decreased AcCho potential at pulse 5 or 6.

One muscle was incubated for 60 min with [^{3}H]PCP (100 μM), and then with PCP and α-BGT (5 μg/ml) for 30 min, and with PCP alone for 60 min. (The use of PCP to wash and the excess α-BGT was needed because of the scarcity of the radioactive compound and possible contamination of the suction system.) After washing for 4 hr with Ringer's solution free of PCP, the recovery of AcCho sensitivity (15–30 mV/nC) was the same as that obtained in muscles treated with α-BGT (5 μg/ml) for 30 min. Thus, similar to PCP (4, 5), [^{3}H]PCP did not react with AcCho receptor sites.

Effect of [^{3}H]PCP on epc and Synaptic Noise. Similar to PCP, [^{3}H]PCP (10–80 μM) decreased significantly the peak amplitude of the epc and caused marked nonlinearity in the current–voltage relationship, although at twice the concentrations of PCP (Fig. 1). It also did not affect significantly the equilibrium potential (null potential) of the epc (−1.01 and +0.71 mV). However, in contrast to PCP, there was no looping at hyperpolarizing membrane potentials and [^{3}H]PCP did not induce a time-dependent effect on the epc because use of a long conditioning pulse (3 sec) or a short conditioning pulse (<20 msec) to induce the epc gave similar effects. Use of 0.1–0.6% alcohol (the vehicle for [^{3}H]PCP) was the control and had no effect on epc. The minimal concentration of [^{3}H]PCP for effect on epc was 5 μM. The actions of [^{3}H]PCP were slowly reversed by continuous washing of the muscles with normal Ringer's solution for 2 hr.

The rise and decay times of the epc (τ_{epc}) were accelerated by treatment with [^{3}H]PCP for 30 min. τ_{epc} at −180 mV was accelerated from 3.85 msec in the control condition to 1.33 and 0.85 msec by 40 and 80 μM [^{3}H]PCP, respectively. The decay phase remained a single exponential function. [^{3}H]PCP significantly depressed the epc induced by microiontophoresis of AcCho to the end plate. [^{3}H]PCP at 5 μM shortened the mean (±SEM) channel life-time (τ_{AcCho}) from 1.70 ± 0.06 to 1.39 ± 0.05 msec and shortened τ_{mepc} from 2.05 ± 0.06 to 1.6 ± 0.09 msec but did not affect single channel conductance (range, 24.1 ± 0.7 to 22.4 ± 0.4 pS). It depressed the iontophoretically evoked epcs at lower concentrations compared to mepcs or the neurally evoked epcs (Fig. 2). Also, similar to PCP but at higher concentration, [^{3}H]PCP shortened epc and depressed the peak amplitude of epc to different extents (Fig. 2 *Inset*).

Binding of [^{3}H]PCP to *Torpedo* Membranes and Effect of Receptor Activation. In the absence of tissue, the filters bound 800–1500 cpm of [^{3}H]PCP, which represented about 1% of the filtered radioactivity. In presence of *Torpedo* membranes, the filters retained [^{3}H]PCP in a concentration-dependent manner up to at least 250 μg of protein (Fig. 3A). [^{3}H]PCP binding to these filters was further reduced by pretreatment of the filters with 1% organosilate solution to the point that such binding represented <0.5% of the filtered ligand. Inclusion of 5 mM amantadine [a competitive inhibitor of the [^{3}H]H_{12}-HTX (13)] decreased binding to the level observed in the absence of tissue, suggesting that most of this binding of [^{3}H]PCP in absence of amantadine was specific. Nonspecific binding was the same in the presence or absence of AcCho (Fig. 3A); >90% of the nonspecific binding was removed by a single wash with 5 ml of buffer. Several washings with buffer had very little effect on specific binding, suggesting that the rate of dissociation of [^{3}H]PCP was slow, thus making it possible to use the filter assay.

The addition of 1 μM AcCho (after inhibition of AcCho esterase with 0.1 mM diisopropyl fluorophosphate) greatly increased [^{3}H]PCP binding; the relationship of amount bound to protein concentration was linear up to 100 μg of protein (Fig.

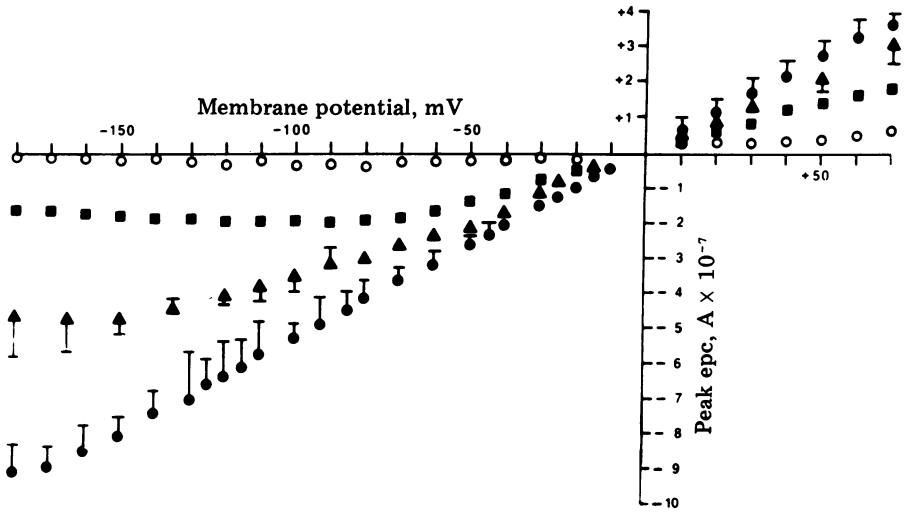

FIG. 1. The influence of membrane potential on the peak amplitude of the epc in frog sartorius muscle end plates in control (●) and after exposure for 30–60 min to [^{3}H]PCP at 10 μM (▲), 40 μM (■), and 80 μM (○). Each symbol represents the mean ± SEM of 8–12 single end plates.

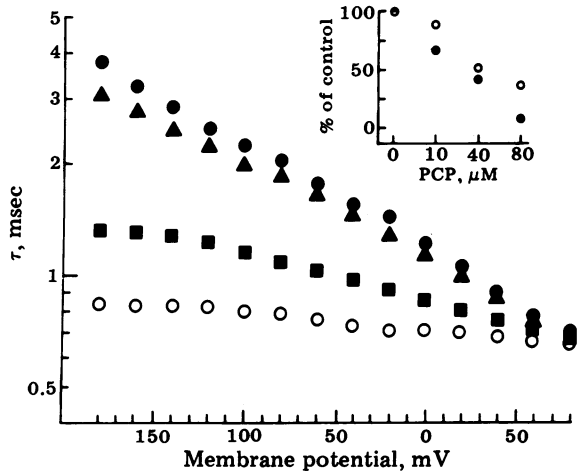

FIG. 2. Decay time constant (τ) of the epc of frog sartorius muscle end plates plotted as a function of membrane potential in absence (●) and in presence of [^{3}H]PCP at 10 μM (▲), 40 μM (■), or 80 μM (○). (*Inset*) Dose–effect relationships between the peak amplitudes (●) and τ (○) of the epc produced at −90 mV by three concentrations of [^{3}H]PCP. Each symbol is the mean of 8–12 end plates.

3A). Preincubation of *Torpedo* membranes with 1 μM AcCho before addition of [^{3}H]PCP depressed the initial binding rate in a time-dependent manner (Fig. 3A *Inset*). The effect of AcCho in potentiating the initial rate of [^{3}H]PCP binding was dose dependent, increasing with higher AcCho concentrations up to 300 nM AcCho (Fig. 3B), and was totally inhibited by 1 μM α-BGT.

Binding of [^{3}H]PCP to *Torpedo* membranes in absence of receptor activators reached equilibrium in 2 hr at all concentrations used. Binding of [^{3}H]PCP at equilibrium was saturable, but the binding curve was flattened over a considerable concentration range (Fig. 4A). A forced fit of the data to a one-population model (shown by the dashed line in Fig. 4A), giving a K_d of 0.65 μM, did not fit the data points well. A Scatchard plot of the data (Fig. 4B) showed a curvilinear function, which is indicative of either two populations of sites or negative cooperative interactions. The least squares computer fit to a two-population model indicated 47 ± 7% of receptors in a high-affinity state with a K_d of 0.10 ± 0.03 μM and 50 ± 6% as a low-affinity component population with a K_d of 3.1 ± 1.0 μM. The total number of [^{3}H]PCP binding sites was 0.65 nmol/mg of protein. This membrane preparation had 0.7 ± 0.15 nmol of [^{3}H]AcCho binding sites per mg of protein. The nature of the binding of [^{3}H]PCP changed in presence of 1 μM AcCho. The saturation isotherm (Fig. 5A) was fit reasonably well by a one-receptor population model, and nonlinear regression analyses indicated higher affinity and a K_d of 2.5 ± 0.1 × 10^{-7} M. In the Scatchard plot (Fig. 5B) the computer line drawn for a single noninteracting binding site population model did not fit all the points closely but the Hill coefficient of 1 (Fig. 5A *Inset*) may suggest a single population of sites.

Effect of Drugs on [^{3}H]PCP Binding. In order to determine the specificity of the observed [^{3}H]PCP binding, the effects of drugs that have been shown biophysically to block the ionic channel of the nicotinic receptor were studied. All of the channel drugs were effective in inhibiting [^{3}H]PCP binding with K_i values in the range 0.65–14 μM, except for procaine which had considerably lower affinity. The most effective drugs were PCP and H_{12}-HTX (K_i, 3.4 μM) followed by PCP methiodide and quinacrine and then the local anesthetics dimethi-

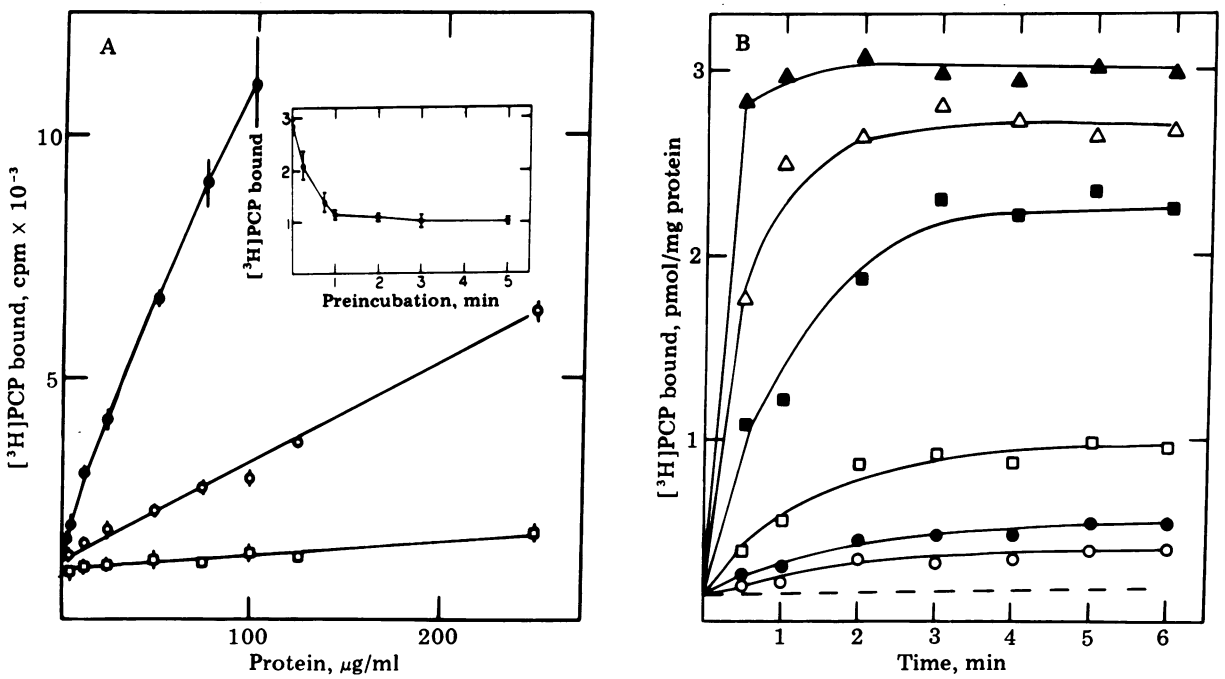

FIG. 3. Binding of [^{3}H]PCP (2 nM) to *Torpedo* membranes, and the effects of preincubation time and presence of AcCho. (*A*) Binding as a function of protein concentration in absence (○) and presence (●) of 1 μM AcCho. Total cpm in each assay tube, 235,000; binding of [^{3}H]PCP to filters in tissue blank controls, 1200 ± 212 cpm. Nonspecific binding (□) was determined by including 5 mM amantadine in a separate incubation and was unaffected by the presence of 1 μM AcCho. Reaction volume was 1 ml and binding was measured after 30 sec. (*Inset*) Influence of time of preincubation with AcCho on the initial rate of [^{3}H]PCP binding. *Torpedo* membranes were incubated for the indicated times with 1 μM AcCho before initiation of the binding reaction by addition of 2 nM [^{3}H]PCP. Binding is expressed in pmol/mg of protein. Data shown as mean ± SD of four experiments. (*B*) Influence of different concentrations of AcCho on the rate of [^{3}H]PCP binding. *Torpedo* membranes (20 μg/ml) were in 8 ml of incubation medium containing 50 mM Tris·HCl (pH 7.4) and the binding reaction was started by the simultaneous addition of [^{3}H]PCP (2 nM) and AcCho to the membranes. Total binding was determined by filtration of 1-ml aliquots at the indicated times. The final concentration of AcCho was as follows: 0 (○), 1 nM (●), 10 nM (□), 30 nM (■), 100 nM (△), and 300 nM (▲).

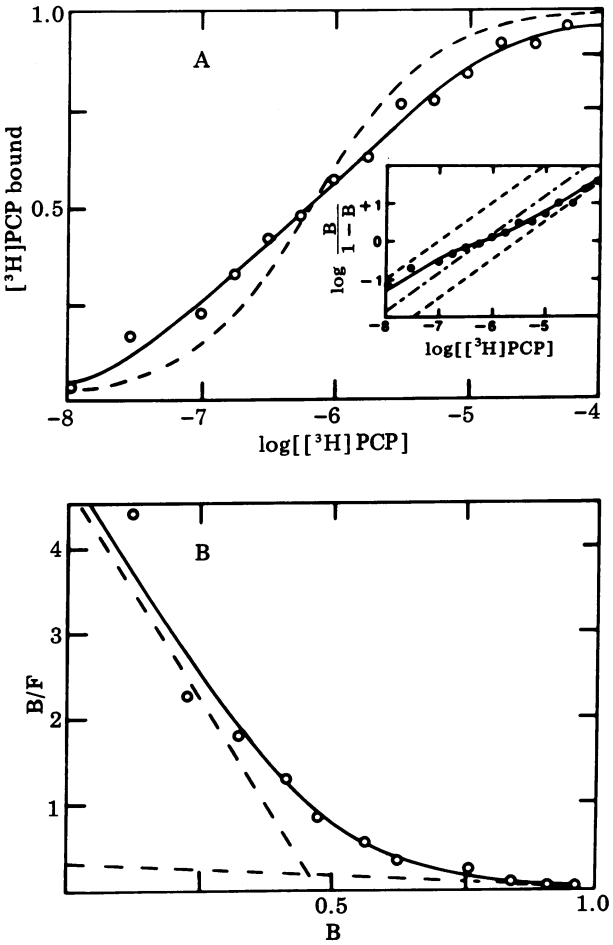

FIG. 4. Specific binding of [³H]PCP to *Torpedo* membranes determined after 2 hr of incubation. (*A*) Plot of specific binding (presented as fractional occupancy) as a function of PCP concentration (M). The solid and dashed lines represent least squares fit of the data to binding models incorporating two or one population of binding sites, respectively. Each point represents the mean ± SD of three determinations using the same tissue preparation. (*Inset*) Hill plot of the same binding data. The curve is drawn from a least squares fit of the data to a two-receptor population model; the dashed lines indicate the separate contribution of each binding site. The solid straight line is predicted from a least squares fit of the data to a single-receptor population model. (*B*) Scatchard plot of the same binding data. The curve is drawn from a least squares fit to a two-receptor population model, and the dashed lines indicate the separate contributions of each binding affinity. B, Amount of [³H]PCP bound presented as fractional occupancy; F, free [³H]PCP concentration (M).

soquin, tetracaine, dibucaine, piperocaine, and adiphenine (Fig. 6). Although the presence of AcCho potentiated the initial rate of [³H]PCP binding, and the affinity of PCP was altered, the change had negligible effects on these measurements because the concentration of PCP used (2 nM) was greatly below that of its K_d value (100–300 nM). The affinities were increased for certain drugs (PCP, dimethisoquin, dibucaine, piperocaine, and quinacrine), decreased for a few (procaine and amantadine), and unchanged for others (tetracaine, adiphenine, PCP methiodide). Ionic channel sites associated with the nicotinic receptor have been characterized by using [³H]H_{12}-HTX as a binding probe (8), and the affinities of the channel sites identified by the two probes can be compared (Fig. 6). The correlation between drug affinities, measured in the absence of an AcCho-receptor activator, was poor; the linear regression analysis on the negative logarithms of the inhibition constants

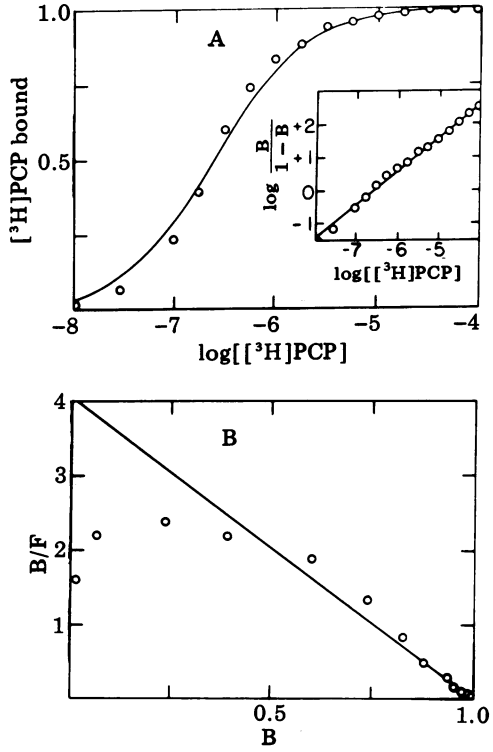

FIG. 5. Specific binding of [³H]PCP in presence of 1 μM AcCho. (*A*) Plot of specific binding as a function of PCP concentration. The line is drawn from a nonlinear least squares fit of the data to a single-site binding model. (*Inset*) Hill plot of the same data, with a line drawn according to the same analysis. (*B*) Scatchard plot of the same PCP binding data. The line is drawn as from the values generated in the fit of the data to a one-site model.

indicated a slope of 0.63, a Y intercept of 1.9, and a correlation coefficient (r) of 0.52. The correlation of the drug affinities measured in the presence of a receptor activator was somewhat better: $m = 0.79$, Y intercept = 1.1, and $r = 0.82$.

DISCUSSION

Several findings suggest that the binding of [³H]PCP to *Torpedo* membranes is to sites on the ionic channel of the AcCho receptor. Binding is dependent upon protein concentration (Fig. 3*A*), is saturable (Fig. 4), is stimulated by receptor activation (Fig. 3*B*), and is inhibited by drugs that have been shown biophysically to interact with the ionic channel in muscle endplates (Fig. 6). In addition, PCP inhibits [³H]H_{12}-HTX binding to the ionic channel of the AcCho-receptor (5) and does not inhibit [³H]AcCho or ^{125}I-labeled α-BGT binding to the AcCho receptor sites in these membranes (4).

The acceleration by [³H]PCP of the decay phase of epc and shortening of the single channel life-time are qualitatively similar to the effects of PCP (Figs. 1 and 2; Table 1) except for its lesser effectiveness and its inability to cause a time-dependent effect and looping of the epc peak amplitude. Nevertheless, it is evident that [³H]PCP interacts with the ionic channel of the nicotinic receptor. Its usefulness as a probe for this ionic channel depends, in part, on the concentration of these binding sites relative to other sites of possible [³H]PCP interactions, such as on the K^+ channel (3), and the amount of nonspecific binding to the glass filters, which may constitute a high percentage of the total binding observed in studies of brain (14, 15) or muscle membranes. Thus, although the assay is successful with *Torpedo electroplax* (≈1 nmol of sites per g of tissue), it is not applicable to brain or skeletal muscles where the concentration of sites is 4 orders of magnitude lower. Evidently the ratio of specific to

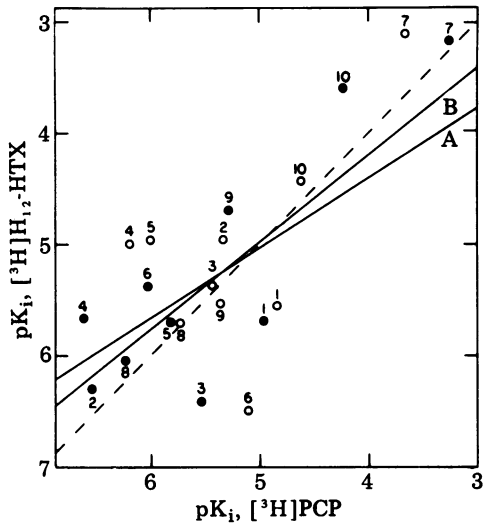

FIG. 6. Affinities of various drugs for the ion channel binding sites of *Torpedo* AcCho receptor identified by [^{3}H]PCP and [^{3}H]H$_{12}$-HTX binding. The negative logarithms of the apparent affinity constants (pK$_i$) in M are plotted. Affinities of the various drugs were measured in the absence (○) or presence (●) of 1 μM receptor agonist (AcCho in the case of [^{3}H]PCP, carbamoylcholine with [^{3}H]H$_{12}$-HTX). Broken line is line of identity for the pK$_i$s of the two sites. Lines A and B are drawn from linear regression analyses of the affinities determined in the absence and presence of receptor agonists, respectively. Drugs used: 1, adiphenine; 2, dibucaine; 3, dimethisoquin; 4, PCP; 5, PCP methiodide; 6, piperocaine; 7, procaine; 8, quinacrine; 9, tetracaine; 10, amantadine.

nonspecific binding ratio is enhanced greatly when [^{3}H]PCP binding is studied in presence of receptor agonists such as AcCho (Fig. 3*B*) or carbamaylcholine, as observed in the binding of [^{3}H]H$_{12}$-HTX (16). This effect is due to receptor activation because the presence of α-BGT inhibits it, and preincubation with AcCho decreases it (Fig. 3*A Inset*), which may be a result of receptor desensitization (17). The amount of [^{3}H]PCP bound at 30 sec reflects the sum of binding to open as well as closed channels and includes the effect of the receptor in the resting, active, and desensitized states. [^{3}H]PCP has been shown to bind to *T. electroplax* (18) and PCP also interacts with other ionic channels (4, 11). The demonstration of the allosteric effect of binding of agonists to the AcCho receptor on binding of [^{3}H]PCP proves the linkage of the two sites and strongly supports the suggestion that [^{3}H]PCP is binding to the ionic channel of the AcCho receptor.

There are many similarities between the binding of [^{3}H]-H$_{12}$-HTX and [^{3}H]PCP to *Torpedo* membranes. They bind to an equal number of sites and have similar drug specificities (8). PCP inhibits the binding of [^{3}H]H$_{12}$-HTX (5) and AcCho potentiates the binding of both (Fig. 3*B*; ref. 16). These argue in favor of the two ligands binding to the same ionic channel sites. However, there are many differences between their bindings. In absence of receptor agonist, [^{3}H]PCP binds with two affinities (Fig. 4) but [^{3}H]H$_{12}$-HTX binds with only one (16). The K_d values obtained directly for [^{3}H]PCP binding (0.1–0.03 ± 1.0 μM) are different from that (20 μM) obtained by PCP inhibition of [^{3}H]H$_{12}$-HTX binding to the same membranes (5). In the presence of receptor agonist, the K_d for [^{3}H]PCP binding is 0.25 μM; it is 2 ± 1.3 μM when obtained by inhibition of [^{3}H]H$_{12}$-HTX binding with PCP (5). Although channel-blocking drugs inhibit binding of [^{3}H]PCP and [^{3}H]H$_{12}$-HTX, they do so with different affinities such that the correlation coefficient is low (Fig. 6). Moreover, receptor activation increases the affinity of piperocaine for the [^{3}H]PCP binding site 10-fold but greatly decreases (by 90%) its affinity for the [^{3}H]H$_{12}$-HTX binding site (19). Despite these differences, the coupling between the receptor and channel sites is a proof of the identity of the binding sites and the similarities between the effects of the two ligands on muscles and on their binding to the *Torpedo* membranes support the conclusion that [^{3}H]PCP is a good substitute for [^{3}H]H$_{12}$-HTX.

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