Interaction of Phencyclidine with Voltage-Dependent Potassium Channels in Cultured Rat Hippocampal Neurons: Comparison with Block of the NMDA Receptor-Ionophore Complex

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Whole-cell voltage-clamp recording techniques were used to investigate the blockade of voltage-dependent K^+ channels by phencyclidine (PCP) in cultured rat hippocampal neurons. All recordings were carried out in the presence of tetrodotoxin (1–2 μM) to eliminate Na^+ currents. Step depolarization from a holding potential of −40 mV activated a slowly rising, minimally inactivating K^+ current (I_K). PCP (0.5–1000 μM) caused a reduction in the maximum conductance of I_K [IC_{50}(+30 mV), 22 μM] without altering its voltage dependency. The PCP block of I_K diminished at depolarized potentials. Analysis according to the scheme of Woodhull (1973) suggested that block occurs via binding to an acceptor site (presumably within the channel pore) that senses 40–50% of the transmembrane electrostatic field. PCP had no effect on the kinetic properties of I_K and the block failed to show use dependency, suggesting that PCP may bind to the I_K channel via a hydrophobic mechanism not requiring open channels. For comparison, we also investigated the effect of PCP on the transient K^+ current, I_A, activated by step depolarization following a 200 msec prepulse to −90 mV (20 mM tetraethylammonium was present in the bathing solution to reduce I_K). In contrast to the potent blocking action of PCP on I_K, the drug only affected I_A at high concentrations [IC_{50}(+30 mV), 224 μM]. At concentrations causing substantial block (300–500 μM), PCP produced an acceleration in the I_A inactivation rate, and, for brief (5–6 msec) depolarizing steps, the suppression of I_A was use dependent. These observations suggest that PCP block of I_A requires open channels. PCP reduced inward current responses induced by the excitatory amino acid agonist N-methyl-D-aspartate (NMDA) at substantially lower concentrations than those required for its effects on K^+ channels [IC_{50}(−60 mV), 0.45 μM]. The PCP-like dioxadrol stereoisomer dexoxadrol (10 μM) blocked NMDA-evoked inward current responses, while its behaviorally inactive enantiomer levoxadrol did not. Dexoxadrol and levoxadrol also blocked I_K in a stereoselective fashion (IC_{50}'s, 73 and 260 μM, respectively), whereas the sigma ligands (+)- and (−)-SKF 10,047 and (+)-3-[3-hydroxyphenyl]-N-(1-propyl)piperidine [(+)-3-PPP] had little effect on the current (IC_{50}'s, >300–500 μM). We conclude that PCP causes a selective, voltage-dependent block of I_K in hippocampal neurons via a PCP- and not a sigma-type acceptor site. The affinity of PCP for its acceptor site on I_K channels (corrected for the voltage dependence) is 13 times lower than its affinity for NMDA-receptor-associated channels. Therefore, at low doses, the behavioral effects of the drug are more likely to result from an interaction with NMDA receptor channels than voltage-dependent K^+ channels.

The dissociative anesthetic phencyclidine (PCP) blocks a wide variety of ligand- and voltage-gated ion channels in neural and muscle membranes (Albuquerque et al., 1981; Tourneur et al., 1982; Hadley and Hume, 1986; Lacey and Henderson, 1986). In particular, studies using Rb^+ flux measurements (Bartschat and Blaustein, 1986) and voltage-clamp techniques (ffrench-Mullen et al., 1988) have demonstrated that the drug produces a selective depression of current flow through sustained (minimally inactivating) voltage-dependent K^+ channels in mammalian brain (often referred to as "delayed rectifier" channels). During the past 2 decades, a conceptual framework for the mechanistic analysis of channel block has developed as a result of extensive studies on the interaction of reversible channel-blocking agents with voltage-dependent Na^+ channels and ACh-gated cation channels (see Woodhull, 1973; Adams, 1976; Yeh and Narahashi, 1977; Armstrong and Croop, 1982). These studies have led to the conclusion that many channel-blocking agents may act by binding specifically to an acceptor site within the channel pore and that this causes a temporary steric barrier to ion transit through the channel. Recently, it has been suggested that PCP may block nicotinic ACh receptor- (Oswald et al., 1984; Aguayo and Albuquerque, 1986) and N-methyl-D-aspartate (NMDA) receptor-coupled cation channels by such a mechanism (Honey et al., 1985). We have provided preliminary qualitative data in support of such a mechanism underlying the PCP block of voltage-dependent K^+ channels (ffrench-Mullen et al., 1988; Rogawski et al., 1988).

In the present report, we describe more detailed experiments on the interaction between PCP and voltage-dependent K^+ channels in cultured hippocampal neurons. We provide data on the specificity and affinity of the PCP acceptor site, on the voltage dependency of the block, and the effects of PCP on the kinetic properties of the currents. For comparison, we also furnish data on the affinity of PCP for NMDA receptor channels in the same cells. Our results lead to the conclusion that PCP blocks I_K

channels by binding to a stereoselective acceptor site that is likely to be within the channel pore. However, the affinity of PCP for its acceptor site on the I_K channel is an order of magnitude lower than its affinity for the NMDA receptor channel. In addition, the pharmacological specificity of the I_K binding site may differ from that of the behaviorally relevant PCP receptor. Therefore, we conclude that at low doses the behavioral effects of PCP are more likely to result from an interaction of the drug with excitatory amino acid-mediated neurotransmission than with voltage-dependent K^+ channels.

Materials and Methods

Cell culture. Neurons from 17- to 19-d-old Sprague-Dawley rat embryos were grown in dissociated culture according to methods described by Segal (1983). The cultures were prepared in modified Minimal Eagle's Medium supplemented with 10% horse serum, 10% fetal calf serum, and 1% glutamine (GIBCO, Grand Island, NY) and were incubated at 37°C in a humidified atmosphere containing 10% CO_2. Fresh medium was added every 4 d. Cells were used for electrophysiological recording 6–12 d after plating.

Whole-cell recording. All recordings were made at room temperature (19–25°C) on the stage of an inverted phase-contrast microscope (Nikon Diaphot). Prior to each experiment, the serum containing growth medium was replaced with recording medium containing (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 1.5; $MgCl_2$, 1; 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 5; D-glucose, 6. In some experiments, the following tetraethylammonium (TEA)-containing solution was used: NaCl, 130; KCl, 5; $CaCl_2$, 1.5; $MgCl_2$, 1; $CdCl_2$, 0.05; TEA-Cl, 20; HEPES, 5; D-glucose, 6. The solutions were adjusted to pH 7.4 with NaOH and to an osmolality of 325 mOsm/kg H_2O with sucrose and were filtered through a 0.22 μm membrane filter (Millipore Corp., Bedford, MA) immediately before use. Tetrodotoxin (1–2 μM) was always added to the recording solutions. For recording NMDA-activated inward currents, $MgCl_2$ was omitted from the extracellular medium.

Patch electrodes were prepared from 1.5 mm O.D. filament fused, borosilicate glass capillaries (Kwik-Fil TW150F, WPI Instruments, New Haven, CT) using a BB-CH programmable horizontal puller (Mecanex, Geneva). The electrodes were pulled in 2 stages, and, without further preparation, were filled with intracellular solution of the following composition (in mM): K-gluconate, 145; $CaCl_2$, 0.1; $MgCl_2$, 2; ethylene glycol *bis*(β-aminoethyl ether)-*N,N,N,N'*-tetraacetic acid (EGTA), 1.1 or K-*bis*-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), 5; HEPES, 5. In some experiments $CaCl_2$ was omitted. The pipette solution was adjusted to pH 7.2 with KOH and to an osmolality of 315 mOsm/kg H_2O with sucrose. Pipette-to-bath resistances prior to seal formation were typically 3–7 MΩ. Seal resistances were >10 GΩ. Liquid junction potentials between the electrode solution and the bath were minimized by placement of the reference electrode in a compartment filled with the pipette solution that was electrically connected to the tissue culture dish by an agar bridge.

Whole-cell currents were recorded with an Axopatch 1A patch-clamp amplifier (Axon Instruments, Burlingame, CA). After seal formation and prior to establishing a whole-cell recording, electrode capacitance was neutralized by using the capacitance compensation circuitry of the Axopatch. In the whole-cell mode, the Axopatch was further adjusted to correct for 80–85% of the series resistance. Voltage-step families were applied at a rate of 0.3–0.5 Hz.

Data acquisition and analysis. Voltages corresponding to the current signals were conditioned with the low-pass filter on the Axopatch set at a cutoff frequency of 10 kHz. The signals were digitized using a 12 bit (±10 V) analog-to-digital converter at a rate of 500 μsec/point and were stored on the Winchester disk of a minicomputer for later analysis with an automatic measuring program. Raw current traces in most cases are displayed without leak subtraction; however, all derived values were determined following linear leak subtraction accomplished by extrapolating the best-fit straight line to the current values obtained with voltage steps to between −80 and −50 mV. In the records shown in Figure 8, capacitance artifacts and leakage currents were digitally subtracted by addition to the raw data of an average and appropriately scaled current response to a 10 mV hyperpolarizing voltage step.

Except as noted, percent blockade was determined according to the formula $100 \times (1 - I_{drug}/I_{control})$, where $I_{control}$ is the current amplitude before drug application and I_{drug} is the current amplitude obtained ap-

proximately 2 min after the onset of the drug superfusion. For all dose–response data, K^+ current blockade was determined at +30 mV and NMDA-activated inward current blockade was determined at −60 mV. Data are expressed as means ± SEM. "95% C.L." refers to the 95% confidence limits of the derived value.

Fitting of dose–response data was carried out with the program ALLFIT as described by De Lean et al. (1978). Other nonlinear curve fitting was performed with an interactive mathematical modeling system (MLAB; Division of Computer Research and Technology, NIH) using a tuned version of the Marquardt-Levenberg iterative least-squares algorithm. Linear least-squares curve fitting and final plotting were performed on a microcomputer using the Grapher software package (Golden Software, Golden, CO). The Lineweaver-Burke analysis used for the determination of dissociation constants was carried out with the microcomputer programs accompanying Tallarida and Murray (1987). Statistical analyses were performed with Systat Version 4.0 (Systat, Inc., Evanston, IL).

Drug application. Drugs were dissolved in bathing medium and were applied by pressure ejection (typically 0.3–1 psi) from blunt glass micropipettes (tip diameter, 1–2 μm) positioned close to the cell membrane. It has previously been demonstrated that this method delivers a concentration of drug at the cell surface that is close to that in the pressure pipette (Choi and Fishbach, 1981). Therefore, drug concentrations reported are the uncorrected concentrations in the pressure pipette. A few experiments in which drugs were applied by bath perfusion gave comparable results to those obtained with the pressure-ejection technique. Except as noted, testing of the K^+ currents for block was begun 2–3 min following onset of the drug application. NMDA was applied by brief (100–300 msec) pressure pulses delivered to the cell at 30 sec intervals to avoid desensitization. Fractional block was determined after several NMDA applications when the reduction in peak current amplitude had attained a steady-state level.

Materials. PCP [1-(1-phenylcyclohexyl)piperidine HCl] and (+)- and (−)-SKF 10,047 [(+)- and (−)-*N*-allyl-*N*-normetazocine HCl] were obtained from the National Institute on Drug Abuse, Research Technology Branch, Research Triangle Park, NC. Dexoxadrol HCl and levoxadrol HCl were generous gifts of Drs. K. C. Rice, A. E. Jacobson, and A. Thurkauf, NIDDK. (+)-3-PPP [R(+)-3-(2-hydroxyphenyl)-*N*-propylpiperidine HCl] was from Research Biochemicals, Inc. (Natick, MA). All chemicals whose sources are not specified were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

The data presented in this report represent the results of whole-cell voltage-clamp recordings from 316 cultured rat hippocampal neurons.

PCP blocks I_K with greater potency than I_A

Step depolarization of hippocampal neurons for 200 msec from a holding potential of −40 mV to potentials between −30 and +40 mV resulted in the activation of a voltage-dependent outward current that rose to a plateau within 20–30 msec and showed no or minimal (<10%) inactivation during the remainder of the voltage command step (Fig. 1*A*, left). This sustained outward current (I_K) has previously been shown to be primarily due to activation of TEA-sensitive, voltage- but not Ca^{2+}-dependent K^+ channels (Segal and Barker, 1984; ffrench-Mullen et al., 1988). At a holding potential of −40 mV, the transient K^+ current, I_A, in cultured hippocampal neurons is inactivated and does not contribute to the total outward current (Segal et al., 1984). Upon repolarization, I_K deactivates within 50 msec. Analysis of tail currents in 5 cells showed a reversal potential of −60.2 ± 0.4 mV (range, −58 to −65 mV), and this is taken to be the equilibrium potential for K^+ currents under these recording conditions. The deviation of the tail current reversal potential from the calculated Nernst potential is likely due to K^+ accumulation near the external membrane face during the prolonged depolarizing steps, as well as errors caused by the liquid-junction potential generated at the bathing medium-pi-

pette solution interface and the Donnan potential at the electrode tip.

Superfusion with PCP caused a dose-dependent suppression of I_K (Figs. 1, right, and 3). At low concentrations (≤ 100 μM), 2–3 min was required to achieve a plateau in the blocking effect, whereas with higher concentrations a maximal response to the drug was obtained within 1 min. Figure 1A illustrates the effect of 20 μM PCP on I_K elicited at several step potentials (-10, 0, $+10$, $+20$, $+30$ mV) and Figure 1B (bottom) plots the leak-subtracted conductance at the end of the 200 msec step as a function of the step potential under control conditions and in the presence of PCP. PCP caused a reduction in the net I_K conductance without altering the voltage dependency of the current as determined by the $V_{1/2}$ values of the best-fit Boltzman curves to the data (control: 8.9 mV; PCP: 9.1 mV; see caption to Fig. 1). Figure 3A shows the percent block of I_K for various concentrations of PCP from 0.5 to 1000 μM. Assuming a reversible bimolecular binding reaction between PCP and an acceptor site on the I_K channel and, furthermore, that occupancy of the acceptor site results in channel block, the fractional block of the channel, B, can be described by a Langmuir adsorption isotherm:

$$B = 1/(1 + K_D/[\text{PCP}]) \qquad (1)$$

where K_D is the equilibrium dissociation constant of the binding reaction. This equation was modified as follows:

$$B = B_{max}/\{1 + (\text{IC}_{50}/[\text{PCP}])^n\} \qquad (1')$$

where B_{max} is a parameter ≤ 1 that accounts for the possibility that a fraction of the measured outward current represents contamination by drug resistant channels, IC_{50} is the concentration of drug that produces $B_{max}/2$ block, and n is an empirical parameter that determines the steepness of the dose–effect curve and considers the potential existence of heterogeneous binding sites. Only under conditions where $n = 1$, does the IC_{50} provide a true estimate of K_D. As illustrated in Figure 3A, the dose–effect curve for blockade of I_K is reasonably well fit by a curve defined by Eq. (1') with $B_{max} = 0.84 \pm 0.10$, $\text{IC}_{50} = 22 \pm 11$, and $n = 0.71 \pm 0.20$. The fact that $n < 1$ suggests the existence of 2 binding sites. Transforming Eq. (1) by taking the reciprocal of both sides results in a linear form in which K_D is the slope of the line obtained by plotting $1/B$ against $1/[\text{PCP}]$. Figure 3B shows the transformed dose–response data. The data are well fit by a straight line for PCP concentrations >10 μM giving a K_D at $+30$ mV of 25.7 ± 1.0 μM (95% C.L.: 22.9–28.5). However, as is apparent from the double-reciprocal plot, the points with very low concentrations of PCP (≤ 1 μM) deviate from the line, suggesting the presence of an additional higher-affinity acceptor site with $K_D \sim 7$ μM. This acceptor site produces a qualitatively minor contribution to the total outward current block produced by PCP and could reflect a second, high-affinity binding site on the I_K channel or the effect of PCP on a contaminating outward current species.

Conditioning hyperpolarization of hippocampal neurons to potentials more negative than -50 mV results in deinactivation of the A current (I_A), a rapidly activating and inactivating, TEA-insensitive K$^+$ current (Segal et al., 1984). We examined the effects of PCP on I_A by activating the current with a voltage-clamp protocol consisting of a 200 msec hyperpolarizing prepulse to -90 mV, followed by a 200 msec depolarizing step to various potentials between -50 and $+30$ mV. During the 2–3 sec rest period between the hyperpolarizing–depolarizing se-
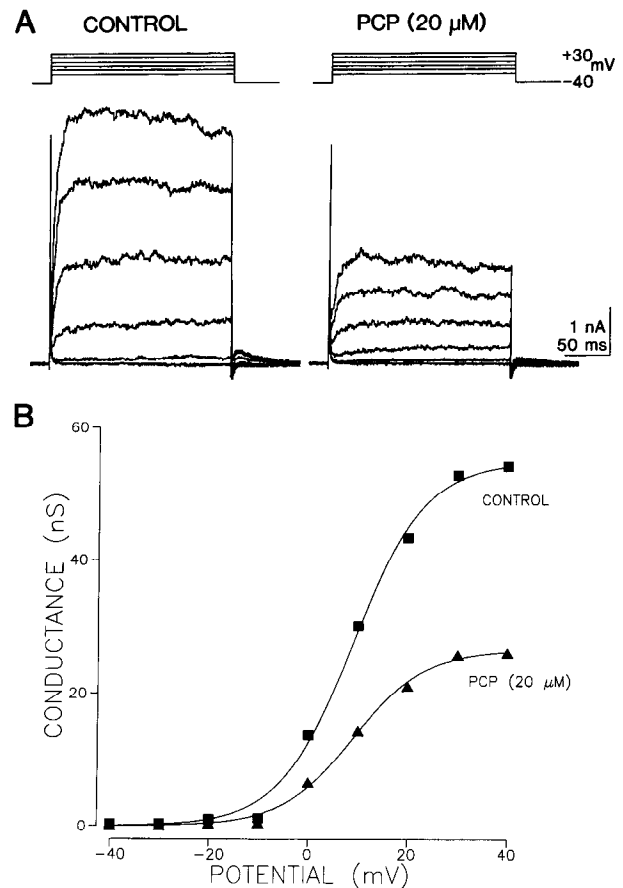

Figure 1. PCP-induced depression of the sustained voltage-dependent K$^+$ current (I_K). A, I_K was evoked with a family of 200 msec depolarizing steps applied before (left) and during (right) superfusion with 20 μM PCP. B, The I_K conductance for each step (plotted on lower graph) was determined from the data shown in A according to the relationship $G = I/(V - V_r)$, where I is the leak subtracted current at the end of the 200 msec voltage step, V is the step potential, and V_r is the reversal potential for K$^+$ currents as determined by tail current analysis (see text). The smooth curves were fitted to the data according to a Boltzman equation of the form $G = G_{max}\{1 + \exp[(V_{1/2} - V)/A]\}^{-1}$, where the parameter values ($\pm$SEM) before and during the drug treatment, respectively, were: G_{max} (pS) = 54.7 $\pm$ 1.4, 26.5 $\pm$ 0.7; $V_{1/2}$ (mV) = 8.9 $\pm$ 0.8, 9.1 $\pm$ 0.8; $A = 7.3 \pm 0.6$, 7.2 $\pm$ 0.6.

quences, the cells were held at -60 mV. TEA (20 mM) was present in the bathing medium to reduce I_K and allow I_A to be studied in relative isolation. In contrast to the potent blocking action of PCP on I_K at doses in the low micromolar range, the drug failed to affect I_A at doses ≤ 20 μM and caused only minimal blockade at 50 μM (Fig. 2A). Higher doses did cause a dose-dependent reduction in I_A (Fig. 2B) with an IC_{50} at $+30$ mV of 224 ± 32 μM (Fig. 3A). As was the case for I_K, there was no change in the voltage dependency for activation of I_A in the presence of PCP (see caption to Fig. 2).

PCP block of I_K is voltage dependent

The degree to which PCP blocks I_K diminishes at more positive membrane potentials. Voltage dependency of block data from experiments with 7 cells exposed to 20 μM are summarized in Figure 4. The upper panel shows the combined current-voltage relationship for I_K in these cells under control conditions and in the presence of PCP; the lower panel gives the percent block as a function of membrane potential. PCP is a charged molecule
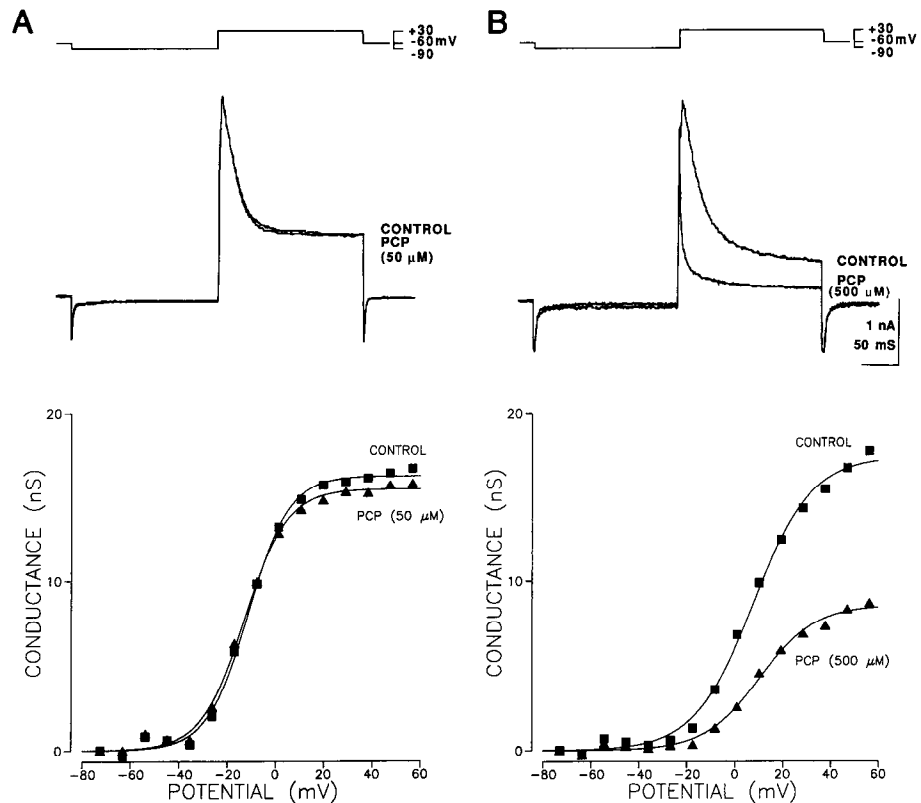
Figure 2. PCP-induced depression of the transient voltage-dependent K^+ current (I_A). I_A was evoked with a family of 200 msec depolarizing steps applied after a 200 msec conditioning prepulse to -90 mV. Cells were held at -60 mV between steps. TEA (20 mM) was added to the bathing medium to reduce I_K. Sample traces are shown for steps to $+30$ mV before and during superfusion with PCP (A, 50 mM; B, 500 μM). Graphs plot I_A conductance calculated according to the formula given in the caption to Figure 1, except that I is taken to be the peak current minus the current at the end of the 200 msec step. Smooth curves were fit with the Boltzman equation. The parameter values are as follows (*Control, PCP*): A, G_{max} (pS) $= 16.3 \pm 0.2$, 15.5 ± 0.2; $V_{1/2}$ (mV) $= -11.3 \pm 0.5$, -13.0 ± 0.6; $A = 8.5 \pm 0.5$, 8.9 ± 0.5; B, $G_{max} = 17.4 \pm 0.4$, 8.5 ± 0.2; $V_{1/2} = 7.8 \pm 1.0$, 10.3 ± 1.1; $A = 12.2 \pm 0.7$, 11.5 ± 0.9.

at physiological pH due to protonation of the piperidine ring nitrogen atom (pK_a, 9.4 at 25°C; Maayani et al., 1983). Therefore, if the PCP blocking site is within the electric field of the membrane, at positive membrane potentials, electrostatic forces would tend to drive the PCP molecule from the acceptor site and decrease the degree of block. The voltage dependency of block was further analyzed according to the approach originally described by Woodhull (1973). In this model, the ratio of the net (leak-subtracted) currents in the absence and presence of PCP, I_0 and I_{PCP}, can be related to the transmembrane voltage, V, by the relationship

$$I_0/I_{PCP} = 1 + \{[PCP]/K_D(0)\} \exp(-zFV\delta/RT), \qquad (2)$$

where $K_D(0)$ represents the dissociation constant of the PCP-acceptor site complex at zero transmembrane potential, δ is the fractional voltage drop experienced at the acceptor site, and z, F, R, T have their usual meanings. Equation (2) can be linearized by rearranging and taking the natural logarithms of both sides resulting in the following equation for a straight line:

$$\ln(I_0/I_{PCP} - 1) = \ln\{[PCP]/K_D(0)\} - (z\delta F/RT) \times V, \qquad (3)$$

in which $K_D(0)$ and δ can be determined from a plot of $\ln(I_0/I_{PCP} - 1)$ against V (cf. Coronado and Miller, 1979; Blatz and Magleby, 1984; Wong and Adler, 1986; Smart, 1987). Data similar to that of Figure 4 expressed in this fashion are shown in Figure 5A, where linear least-squares fits give $K_D(0)$ of 19.4 μM and δ of 0.49.

As an independent means of estimating δ, we utilized the following expression for the voltage dependency of the binding constant (also taken from Woodhull, 1973):

$$K_D(V) = K_D(0) \exp(\delta FV/RT). \qquad (4)$$

Plots of fractional block against the logarithm of the PCP concentration (10–300 μM; 5–8 cells at each of 5 concentrations) were made at potentials of -30 to $+30$ mV. The $IC_{50}(V)$ values as determined from the best-fit lines to the data were used as estimates of $K_D(V)$ and are plotted in Figure 5B. The fraction of the voltage drop at the blocking site, δ, determined by this method was 0.39, which is reasonably close to the value obtained with the previous analysis. According to Eq. (4), the dissociation constant increases e-fold per 66 mV increase in membrane potential. Correcting the $K_D(0)$ values determined in Figure 5A with Eq. (4) to $+30$ mV gives a value for $K_D(+30)$ of 30.4 μM, which is similar to the value obtained by the titration method for the major blocking site described above (Fig. 3B).

PCP alters the kinetic properties of I_K and I_A in different ways

Following step depolarization, I_K reaches a plateau in about 20 msec. The activation of the current occurred in 2 exponential phases as illustrated in Figure 6A. Depite the reduction in peak current observed with PCP, the drug did not alter the time course of activation. In the experiment illustrated in Figure 6A, I_K was activated by a voltage step from -40 to $+30$ mV. Superfusion with 20 μM PCP caused a 42% reduction in the plateau current; however, as is apparent from the normalized current records shown in Figure 6A, there was no change in the activation kinetics. A similar lack of effect on the activation kinetics was observed with steps to less depolarized potentials (0, $+10$, and $+20$ mV; 2 cells examined; data analysis not shown).

In some cells there was a minimal (<5–10%) time-dependent decay of I_K during the 200 msec voltage step, particularly at very depolarized potentials ($+20$ to $+30$ mV; cf. Fig. 1A, left). PCP caused no consistent increase in the decay rate, and, in
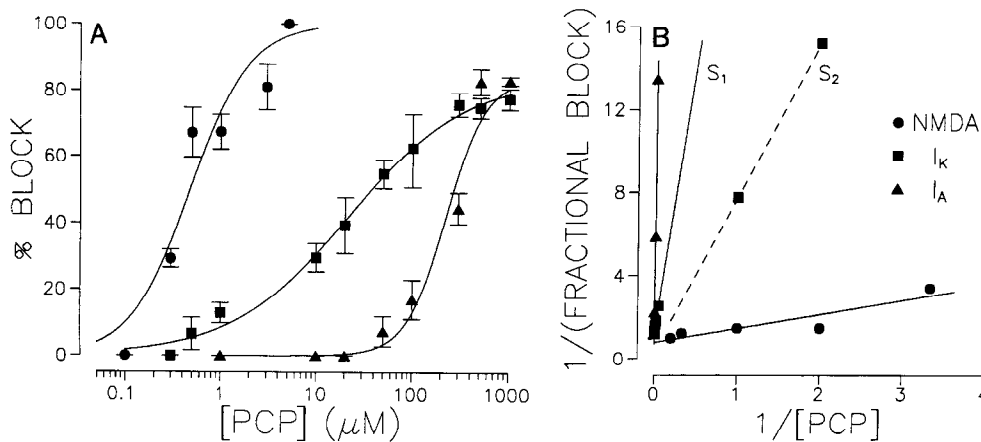
Figure 3. *A,* Dose–effect curves for PCP block of NMDA-evoked inward current responses (●), I_K (■), and I_A (▲). NMDA-evoked currents were recorded at −60 mV, whereas K⁺ currents were elicited with voltage steps to +30 mV. Percent block was determined according to the formula given in Materials and Methods. Testing for block was carried out 2 min after the onset of the PCP ejection. Each point represents the mean ± SEM of experiments on 4–8 cells. Smooth curves represent the best fits to the data according to Eq. (1′) with parameters as given in the text. *B,* Double-reciprocol plots of the data shown in *A* (except that fractional block was used). S_1 and S_2 represent 2 putative components of the I_K data. Some of the data were taken from ffrench-Mullen et al. (1988).

fact, in most cases, the drug reduced the decay rate (cf. Fig. 9*A*). We observed little use-dependent block of I_K. For example, in a cell exposed initially to 20 μM PCP and pulsed continuously at 0.5 Hz from −40 to +30 mV to activate I_K, there was a 33% block of the current at 3 min following onset of the drug perfusion. The PCP perfusion was then discontinued and the outward current was allowed to recover to its predrug amplitude. When PCP was again applied with the cell held *continuously* at −40 mV and tested with a single pulse only at 3 min, the current was reduced by 30%. A second cell was studied with 100 μM PCP using the same protocol except that repetitive activation was performed first. The initial trial gave 57% block at 3 min, while the second gave 54% block. Thus, we were unable to demonstrate significant use dependency for the PCP block of I_K. This observation in conjunction with the failure of PCP to cause pseudo-inactivation of I_K demonstrates that the binding of PCP to its acceptor site in the K⁺ channel occurs rapidly in comparison with the rate of channel activation or that the drug can bind to the blocking site while the channels are in their resting (closed) state.

I_A activates rapidly following depolarization (peak, 3–10 msec; +30 to −10 mV) and inactivates in an exponential fashion with a time constant of 10–40 msec (Segal et al., 1984). While the time to peak of I_A occurs slightly earlier at more depolarized step potentials, the decay rate of the current is independent of potential. The speed with which I_A reaches a peak precluded a detailed analysis of the effects of PCP on the activation kinetics of the current. Nevertheless, it is apparent from the records shown in Figure 2*B* that in the presence of concentrations of PCP which cause a substantial reduction in the current (>300 μM), the peak occurred earlier than normal (<3 msec). In addition, PCP caused a marked speeding of the I_A decay rate. As illustrated in Figure 6*B* (same cell as Fig. 2*B*), decay of I_A is normally well fit by a single exponential function. In the presence of PCP, however, inactivation occurs in 2 exponential phases. The initial phase is markedly faster than the control decay, whereas the late phase is slower. The apparent facilitation of A-current inactivation by PCP resembles the effects of various channel-blocking agents on voltage-dependent Na⁺ channels (Yeh and Narahashi, 1977; Cahalan and Almers, 1979; Kirsch et al.,
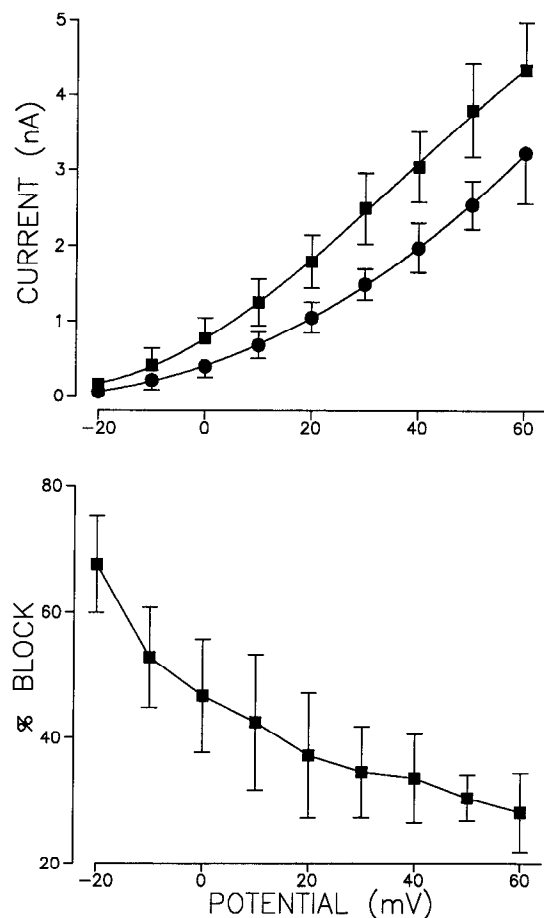

Figure 4. Voltage dependency for PCP block of I_K. *Upper panel,* Current–voltage relationship for I_K under control conditions (■) and in the presence of PCP (20 μM; ●). Each point represents the mean ± SEM of data from 7 cells. Error bars are not shown when they are smaller than the size of the symbols. *Lower panel,* Percent block was determined as described in Materials and Methods. The mean values were significantly different ($p < 0.02$) by a 1-way analysis of variance.

Figure 5. Analysis of the voltage dependency of block according to the method of Woodhull (1973). *A,* Data similar to that shown in Figure 4 were transformed as described in the text using Eq. (3). The *straight lines* were drawn by a linear least-squares method. The derived parameters are shown. *B,* Analysis of additional data (30 cells) as described in the text using Eq. (4).
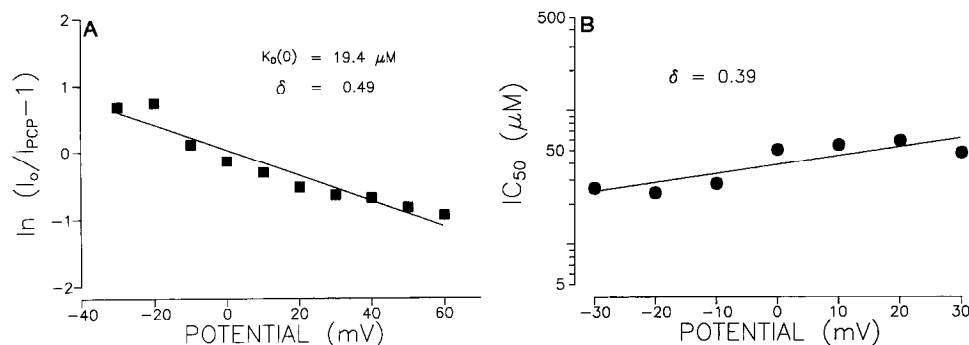
1980; Shapiro, 1977; Armstrong and Croop, 1982) and can be explained by a model in which the blocking drug is able to enter the channel only after it has been opened by depolarization. Alternatively, however, the drug could alter the inactivation characteristics of the I_A channel. To distinguish among these possible mechanisms, we further investigated the onset and recovery of the PCP block.

Block of I_A by high concentrations of PCP is use dependent

I_A was repetitively activated by brief (5–6 msec) depolarizing steps so that the A-current channels remained in the open state for only a short period of time during each step. The duration of these steps was sufficiently long to include the peak and the inital decay of the A current, but the inactivation period was markedly truncated. In 2 cells tested with this protocol, block occurred in a cumulative (use-dependent) fashion. This is illustrated in Figure 7, which shows the use-dependent reduction of the theoretical current amplitude (assuming maximum activation and no inactivation) estimated from exponential fits to the decay curve extrapolated to zero time. In the presence of PCP, where I_A decayed in 2 exponential phases (see Fig. 7, inset), we used the initial fast phase for the amplitude estimates. The use-dependent reduction in I_A illustrated in Figure 7 supports the concept that high concentrations of PCP block I_A by an open-channel mechanism. Note also in Figure 7 that full recovery from block occurs rapidly after cessation of the PCP superfusion.

PCP has a substantially higher affinity for NMDA-activated cation channels than for voltage-dependent K^+ channels

It has been well documented that PCP is a potent antagonist of inward current responses evoked by activation of NMDA-type excitatory amino acid receptor-coupled cation channels (Anis et al., 1983; Honey et al., 1985; Lacy and Henderson, 1986; Rothman, 1988). Moreover, it has been argued that PCP (and related drugs such as ketamine and MK-801) bind to an acceptor site within the ionophore of the NMDA receptor that is accessible only when the channel is in the agonist-activated state (Honey et al., 1985; MacDonald et al., 1987; Huettner and Bean, 1988; Mayer et al., 1988). To compare the affinities of the PCP acceptor site associated with the NMDA receptor channel with that of voltage-dependent K^+ channels, we examined the ability of PCP to block NMDA responses in hippocampal neurons under the same recording conditions as used for the studies of K^+ channel blockade, except that Mg^{2+}, which itself blocks the channel (Mayer et al., 1984; Nowak et al., 1984), was eliminated from the external medium. Inward current responses were evoked by brief (100 msec) pulses of NMDA (100 μM). As illustrated in Figure 8, PCP caused a reversible blockade of the responses to NMDA. The dose–effect relationship for concentrations of PCP from 0.1–5 μM is shown in Figure 3*A*. The best fit to the data using Eq. (1') gives an IC_{50} of 0.45 $\pm$ 0.08 μM. Since $n = 1.1 \pm 0.2$, the data suggest the existence of a single binding site on the NMDA channel and the IC_{50} can be interpreted as the dissociation constant (K_D) of this site.

Figure 6. Effect of PCP on I_K activation kinetics and I_A inactivation kinetics. *A,* I_K was elicited by step depolarization from −40 to +30 mV. Currents recorded before and during superfusion with 20 μM PCP were normalized to the peak current during the step (control: 1.7 nA; PCP: 1.0 nA). *Straight lines* give the best fits to the 2 exponential phases of the control data as determined by the method of peeling. *B,* I_A was elicited by step depolarization to +30 mV after a 200 msec prepulse to −90 mV. Currents recorded before and during superfusion with 500 μM PCP (less the steady-state current at the end of the 200 msec step) were normalized to the peak currents (control: 2.4 nA at 7.5 msec; PCP: 1.7 nA at 3.5 msec). Best-fit lines were determined as in *A.* Same cell as Figure 2*B.*
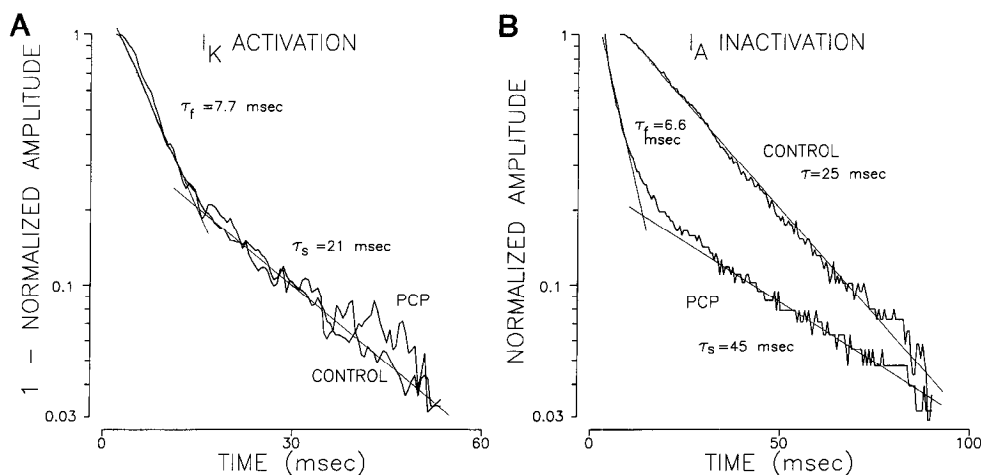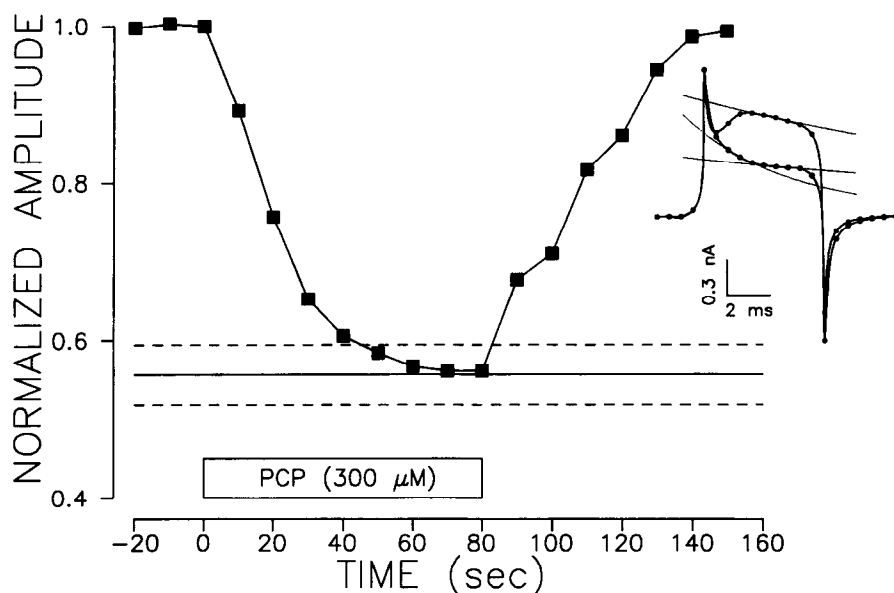
Figure 7. Use-dependent block of I_A. I_A was repetitively evoked at 10 sec intervals with 6 msec duration depolarizing steps to +30 mV after a 200 msec prepulse to −90 mV. The step protocol was applied before, during (indicated by *open bar*) and after superfusion with PCP (300 μM). Between steps, the cell was held at −60 mV. TEA (20 mM) was present in the bathing medium to reduce I_K. The control I_A theoretical maximal amplitude was estimated from exponential fits to the decay curve extrapolated to zero time (see *inset*). During PCP, the decay occurred in 2 exponential phases; the initial fast phase was used for the amplitude determinations. For each step, the I_A amplitude, normalized to the value at zero time (1.02 nA), is plotted against the time before (negative time values) or after the onset of the PCP superfusion. The *solid horizontal line* represents the steady-state block obtained with several long (200 msec) steps (same data as in Fig. 3); the *dashed horizontal lines* represent the SEM. *Inset*, Control current trace and the current trace 20 sec after onset of the PCP superfusion. The *thin smooth curves* show the exponential fits. Note that the current trace recorded during PCP is fit with 2 exponentials (see Fig. 6). Capacitance transients have not been subtracted.

We also evaluated the ability of the dioxadrol enantiomers dexoxadrol and levoxadrol to block NMDA-induced inward current. Dioxadrol is a chiral dioxolane analog whose dextrorotatory form, dexoxadrol, has pharmacological characteristics similar to PCP, whereas its levorotatory form, levoxadrol, is virtually devoid of PCP-like activity (see Discussion). As expected, dexoxadrol (10 μM) was a potent antagonist of NMDA responses, whereas levoxadrol at the same concentration was inactive (Table 1).

Dioxadrol enantiomers block I_K in a stereoselective fashion

Figures 9A–D compare the activity of the dioxadrol stereoisomers with that of PCP as blockers of I_K. At 100 μM, dexoxadrol causes a substantial block of I_K (53%; Fig. 9C), although the degree of block is less than that produced by PCP at the same concentration (77%; Fig. 9B). In contrast, levoxadrol is less potent in blocking I_K (14%; Fig. 9D). Dose–effect curves for

dexoxadrol and levoxadrol blockade of I_K and I_A are shown in Figure 10A. As is the case with PCP, dexoxadrol is a more potent blocker of I_K than of I_A. The dose–effect curves further illustrate the enantioselectivity of the I_K blockade; however, there is no such stereoselectivity for blockade of I_A.

K^+ channel blockade does not occur via sigma sites

To further characterize the pharmacological properties of the PCP-acceptor site on the voltage-dependent K^+ channels of hippocampal neurons, we examined the channel blocking potencies of several sigma site ligands. The sigma site was originally defined on the basis of the binding of the prototypical benzomorphan (+)-SKF 10,047 [(+)-N-allylnormetazocine] to brain membranes (Mendelsohn et al., 1985; Sircar et al., 1986). More recently, however, drugs such as (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine [(+)-3-PPP] have been discovered which bind with higher selectivity to the site (Largent et al., 1986,
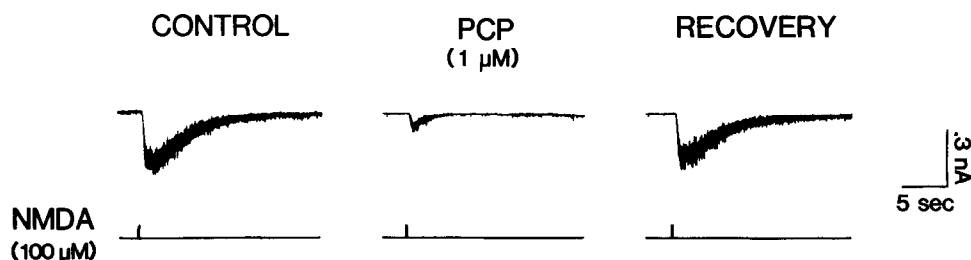

Figure 8. PCP-induced depression (68%) of inward current responses evoked by NMDA (100 μM; 100 msec application). NMDA was pulsed at 30 sec intervals during the PCP superfusion until no further decrement in the current was obtained. The record shown was obtained 90 sec following the onset of the superfusion.
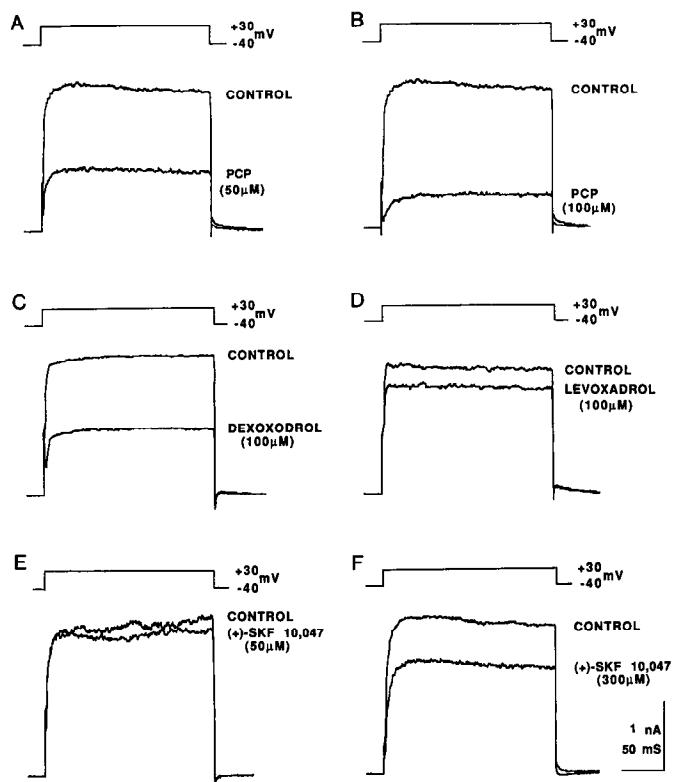
Figure 9. Comparison of the effects of PCP, the dioxadrol enantiomers dexoxadrol and levoxadrol, and (+)-SKF 10,047 on I_K. A and B are from experimetns on the same cell; C–F are from different cells. Percent block of the current at the end of the 200 msec step are as follows: A, 58; B, 77; C, 53; D, 8; E, 14; F, 28.

Table 1. Effect of dioxadrol enantiomers on NMDA-evoked inward current responses

Test drug	Percent block	
	(5 μM)	(10 μM)
Dexoxadrol	3.6 ± 3.5	76.0 ± 5.6
	(4)	(6)
Levoxadrol	0	0
	(3)	(4)

Inward current responses were elicited with 100 msec pressure pulses of NMDA (100 μM). The percent block was calculated as described in Materials and Methods 2–3 min after onset of the drug superfusion. The holding potential was −60 mV. The number of cells tested at each dose is shown in parentheses. Data are presented as the mean ± SEM.

1987). PCP has a moderate affinity for sigma binding sites (Largent et al., 1986; Contreras et al., 1988; Itzhak, 1988; Wong et al., 1988), and therefore, the K$^+$ channel blocking activity of PCP could result from its interaction with sigma sites. Figure 9, E, F, illustrates that (+)-SKF 10,047 is a very weak blocker of I_K. This is further demonstrated by the dose–effect curves shown in Figure 10B. Even at concentrations as high as 300–500 μM, (+)-SKF 10,047 and its enantiomer (−)-SKF 10,047 failed to block I_K by 50%, so that an IC_{50} could not be determined. Similarly, the selective sigma site ligand (+)-3-PPP only partially blocked I_K at doses as high as 300 μM. Since these drugs are more potent ligands for sigma sites than for PCP sites (Downes et al., 1986; Largent et al., 1986), it can be concluded that PCP does not block I_K channels via an acceptor site that is of the sigma type.

Discussion

As initially demonstrated by Bartschat and Blaustein (1986) on the basis of Rb$^+$ flux measurements in rat brain synaptosomes, our studies confirm that PCP is a potent and selective blocker of voltage-dependent K$^+$ channels in rat CNS neurons. At low concentrations (<50–100 μM), PCP selectively blocks the sustained voltage-dependent K$^+$ current (I_K) channel and only affects the transient K$^+$ current (I_A) channel at higher concentrations. Two previous voltage-clamp studies also reported that PCP can block voltage-dependent K$^+$ currents in cultured spinal cord (Aguayo and Alburquerque, 1987) and hippocampal (Rothman, 1988) neurons, yet failed to observe a selective effect

on I_K. However, in these studies PCP concentrations of 100 μM or more were used, and at these concentrations we also found that the drug depresses both I_K and I_A.

In addition to confirming that PCP blocks I_K and I_A channels with different potencies, the present study also suggests that the precise way in which PCP interacts with the 2 channel types may differ. PCP blocks I_K channels by binding to a site with a K_D of 26 μM. The voltage dependency of the PCP block indicates that the PCP acceptor site senses about 40–50% of the transmembrane electric field. It is likely that the acceptor site is within the channel pore. However, other interpretations of these data are possible. For example, the acceptor site could theoretically be outside the transmembrane electrostatic field if a membrane potential-dependent conformational change altered its binding affinity or it could inhibit ion transit through the channel by binding to an allosteric site within the electrostatic field but outside the pore per se. Block of I_K occurs more rapidly than the current activates and does not show use dependency. In addition, I_K channels recover from block even in the absence of voltage-dependent activation (unpublished observations), indicating that PCP is not trapped within the closed channel. These results suggest that block may occur through a hydrophobic pathway in which the drug is able to bind to closed channels (see Hille, 1977). Nevertheless, the possibility cannot be excluded that binding and unbinding are so rapid that neither pseudoinactivation nor use dependency is observed, despite an open channel-blocking mechanism.

In contrast to the results with I_K, PCP only weakly blocked I_A, but at high concentrations that caused a substantial reduction in the current there was a marked speeding of the decay rate. In addition, with repeated brief voltage steps, we observed cumulative block of the current which rapidly recovered upon cessation of the drug superfusion. These observations indicate that PCP block of I_A is use dependent and suggest that I_A channels must open before they can become blocked, so that block occurs via a hydrophilic pathway.

The mechanism by which PCP blocks I_K channels may also differ from the way in which PCP-related drugs interact with NMDA receptor channels. As discussed above, there is substantial evidence in favor of the concept that NMDA receptor channels must be in the agonist activated state for binding and block to occur. Moreover, the drug molecule can become trapped within the ionophore when the channels close so that they are only liberated upon reopening (MacDonald et al., 1987; Huettner and Bean, 1988). Thus, block of the NMDA receptor channel occurs via a hydrophilic mechanism like the block of I_A.
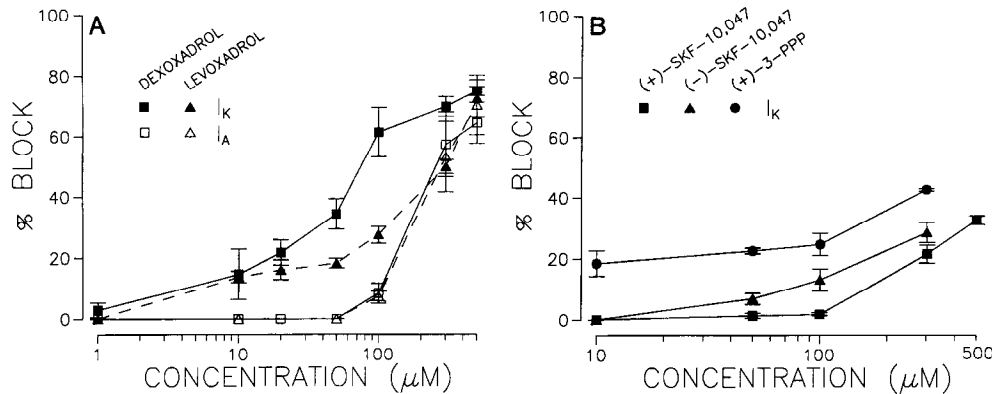
Figure 10. Dose–effect curves for block of I_K and I_A by (*A*) dioxadrol enantiomers dexoxadrol and levoxadrol, and (*B*) (+)- and (−)-SKF 10,047, and (+)-3-PPP. The experimental protocol and method of data analysis are the same as in Figure 7. IC_{50} values in *A* are as follows (μM): dexoxadrol I_K, 73; dexoxadrol I_A, 260; levoxadrol I_K, 285, levoxadrol I_A, 285. IC_{50} values for drugs shown in *B* are >300–500 μM. Each point represents the mean ± SEM of experiments with 4–5 cells.

Studies with ^{3}H-PCP and related radioligands have demonstrated the existence of high-affinity binding sites in brain membranes with a pharmacological profile for PCP-related drugs that corresponds with the behavioral activity of the drugs in inducing PCP-like behaviors (Vincent et al., 1979; Zukin and Zukin, 1979). These binding sites are present in high density in the rat hippocampus (Quirion et al., 1981; Largent et al., 1986). Recently, it was proposed that these binding sites represent the ionophore of the NMDA receptor channel (Fagg, 1987; Bonhaus and McNamara, 1988; Kloog et al., 1988; Wong et al., 1988). Moreover, although PCP does bind to sigma sites with moderate affinity, sigma-selective drugs such as (+)-3-PPP bind only very weakly to high-affinity PCP sites (Downes et al., 1986; Largent et al., 1986), indicating that PCP and sigma sites are pharmacologically distinct. Physiological studies in the hippocampal slice have demonstrated that PCP's block of excitatory transmission is probably not mediated by sigma sites (Coan and Collingridge, 1987; Malouf et al., 1988). [Although Coan and Collingridge reported that (±)-SKF 10,047 did block excitation, there was no difference in the potencies of the enantiomers, suggesting that the effect was mediated by the PCP-like activity of the drugs since PCP binding sites show less selectivity for the SKF 10,047 enantiomers than do sigma sites (Largent et al., 1986; Contreras et al., 1988; Sokolovsky and Kloog, 1988).] As is the case with the NMDA receptor channel, we have demonstrated that PCP's interaction with voltage-dependent K$^+$ channels does not occur via its sigma binding activity. However, are there pharmacological differences between the PCP binding site on the NMDA receptor channel and the site on K$^+$ channels? Clearly, PCP binds with greater affinity to the NMDA receptor channel. This is true even if the affinity for the I_K channel is corrected to −60 mV (where the block of the NMDA-activated current was determined) using Eq. (4). In this case, the theoretical K_D (−60 mV) for blockade of I_K would be ~6 μM, an order of magnitude greater than the K_D (−60 mV) for blockade of the NMDA receptor channel (0.45 μM). Moreover, the enantioselectivity of NMDA receptor channels for the dioxadrol stereoisomers may be greater than the stereoselectivity we have observed for I_K channels. In radioligand binding studies using PCP-related ligands, dexoxadrol has a 230- to 412-fold greater binding affinity than levoxadrol (Mendelsohn et al., 1984, 1985; Contreras et al., 1986; Largent et al., 1986; Fagg, 1987). Functional studies have demonstrated that levoxadrol is inactive in

blocking NMDA responses (Berry et al., 1984; Coan and Collingridge, 1987; Jones et al., 1987; Bourne et al., 1988; Kushner et al., 1988) and in inducing PCP-like behavioral effects (Snell and Johnson, 1985; Contreras et al., 1986). Our data (Table 1) confirm that dexoxadrol has far greater activity as an NMDA antagonist than does levoxadrol. In contrast, there was only a 4-fold difference between dexoxadrol and levodadrol in their IC_{50} values for blockade of I_K. These considerations indicate that the pharmacological specificities of the PCP site on the NMDA receptor channel and that on the I_K channel may differ, and, furthermore, suggest that the K$^+$ channel may not represent the behaviorally relevant site of PCP action. However, blockade of voltage-dependent K$^+$ channels may be important in mediating some of the pharmacological actions of PCP, such as its propensity to induce convulsions at high doses (Domino et al., 1983; Leccese et al., 1986).

Recently, Bartschat and Blaustein (1988) have reported the effects of dioxadrol stereoisomers and sigma ligands on sustained voltage-dependent K$^+$ channels in rat brain synaptosomes as determined by measurements of ^{86}Rb$^+$ efflux. While in general terms the results obtained with this technique are comparable to those we have obtained in the present electrophysiological study (see Bartschat and Blaustein, 1986), this more recent study has revealed some differences in the pharmacological properties of the channels as studied by the 2 techniques. In particular, Bartschat and Blaustein (1988) observed a greater potency difference between dexoxadrol and levoxadrol than was found in the present study, and, in addition, these workers reported that (+)-SKF 10,047 was significantly more potent than PCP in blocking the channels. The reason for the differences in the results obtained with the 2 methods is unclear. However, inasmuch as the pharmacological profile obtained in the flux studies does not correspond with either PCP [dexoxadrol > levoxadrol; PCP > (+)-SKF 10,047] or sigma [dexoxadrol ≈ levoxadrol, both weak; (+)-SKF 10,047 > PCP] type binding sites, the nature of the pharmacophore detected with this methodology requires further clarification.

In conclusion, while it is apparent that PCP can block a wide variety of different ion channel species, the present study demonstrates that the details of the interaction may differ substantially depending upon the specific channel type. In all cases which have been examined in sufficient detail, ion channel block by PCP appears to occur via binding of the drug molecule to a

site within the ionophore. However, the affinity of this inter-action differs among channel types, as does the requirement for channel opening and the tendency of drug molecules to become trapped within closed channels. These variations in blocking mechanism can be expected to contribute to the complex phar-macological profile of PCP.

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