Binding Studies and Photoaffinity Labeling Identify Two Classes of Phencyclidine Receptors in Rat Brain†

Rachel Haring,‡ Yoel Kloog,‡ Asher Kalir,§ and Mordechai Sokolovsky*,‡

Laboratory of Neurobiochemistry, Department of Biochemistry, The George S. Wise Faculty of Life Sciences, and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

Received December 4, 1986; Revised Manuscript Received April 15, 1987

ABSTRACT: Binding and photoaffinity labeling experiments were employed in order to differentiate 1-(1-phenylcyclohexyl)piperidine (PCP) receptor sites in rat brain. Two classes of PCP receptors were characterized and localized: one class binds [^{3}H]-*N*-[1-(2-thienyl)cyclohexyl]piperidine ([^{3}H]TCP) with high affinity (K_d = 10–15 nM) and the other binds the ligand with a relatively low affinity (K_d = 80–100 nM). The two classes of sites have different patterns of distribution. Forebrain regions are characterized by high-affinity sites (hippocampus > frontal cortex > thalamus > olfactory bulb > hypothalamus), but some parts (e.g., hippocampus, hypothalamus) contain low-affinity sites as well. In the cerebellum only low-affinity sites were detected. Binding sites for [^{3}H]PCP and for its photolabile analogue [^{3}H]azido-PCP showed a regional distribution similar to that of the [^{3}H]TCP sites. The neuroleptic drug haloperidol did not block binding to either the high- or the low-affinity [^{3}H]TCP sites, whereas Ca^{2+} inhibited binding to both. Photoaffinity labeling of the PCP receptors with [^{3}H]AZ-PCP indicated that five specifically labeled polypeptides of these receptors (M_r 90 000, 62 000, 49 000, 40 000, and 33 000) are unevenly distributed in the rat brain. Two of the stereoselectively labeled polypeptides (M_r 90 000 and 33 000) appear to be associated with the high- and low-affinity [^{3}H]TCP-binding sites; the density of the M_r 90 000 polypeptide in various brain regions correlates well with the localization of the high-affinity sites, whereas the density of the M_r 33 000 polypeptide correlates best with the distribution of the low-affinity sites. The results are compatible with the existence of two classes of PCP receptors in the rat brain, each having a distinct polypeptide that carries the ligand recognition site and has a selective localization in the brain.

Phencyclidine (PCP)[1] is a psychotomimetic drug that can interact with a variety of enzymes (Maayani et al., 1974; Kloog et al., 1977; Johnson & Vickroy, 1981), receptors (Kloog et al., 1977; Vincent et al., 1978; Aronstam et al., 1980; Su et al., 1980; Oswald & Changeux, 1981; Haring et al., 1984), and ion channels (Albuquerque et al., 1981; Tourneur et al., 1982; Blaustein & Itzkowicz, 1983; Collingridge, 1985; Bartschat & Blaustein, 1986) as well as with a distinct population of receptors in the brain—the PCP receptors (Zukin & Zukin, 1979; Vincent et al., 1979). PCP receptors (K_d for PCP < 0.1 μM) are thought to mediate the behavioral effects of PCP, since the stereoselectivity and the potency of PCP analogues in displacing [^{3}H]PCP from its receptors correlate well with their relative potencies in several behavioral tests (Sturgeon et al., 1979; Contreras et al., 1986a). However, the diverse behavioral effects of PCP in man and in animals [for reviews see Domino and Luby (1981) and Gallant (1981)] seem to suggest that the drug would *show* relatively high affinity toward more than a single class of PCP-binding sites. This, however, was not observed in previous binding studies with radiolabeled PCP-receptor ligands such as [^{3}H]PCP (Vincent et al., 1979; Zukin & Zukin, 1979; Quirion et al., 1981; Vignon et al., 1982; Zukin et al., 1983; Haring et al., 1985, 1986), [^{3}H]-azido-PCP ([^{3}H]AZ-PCP) (Haring et al., 1985, 1986; Sorensen & Blaustein, 1986), and [^{3}H]TCP (Vignon et al., 1983, 1986; Sircar & Zukin, 1985; Contreras et al., 1986b). It was nevertheless demonstrated that [^{3}H]PCP can bind with low affinity (K_d = 3–5 μM) to a second class of sites in the brain (Mendelsohn et al., 1984; Murray & Leid, 1984). Binding of PCP analogues to neurotransmitter receptors, e.g., muscarinic (Kloog et al., 1977; Vincent et al., 1978), nicotinic (Kloog et al., 1977; Aronstam et al., 1980; Oswald & Changeux, 1981; Haring et al., 1983a, 1984), and opioid receptors (Vincent et al., 1978; Su et al., 1980), would occur at this concentration range. It is also important to note that drugs possessing psychomimetic effects like those induced by PCP, e.g., σ-opioids, can bind both to PCP receptors (with relatively low affinity) and with high affinity to the site of the σ agonist (+)SKF 10047 (Tam, 1985; Largent et al., 1986), known as the haloperidol-sensitive (+)SKF 10047 site (Tam & Cook, 1984). Similarly, the dissociative anesthetic [^{3}H]-dexoxadrol was found to bind to PCP receptors and to additional sites in the rat hypothalamus (Pilapil et al., 1985). Dexoxadrol is clearly a highly specific PCP-receptor ligand, and its inactive stereoisomer, levoxadrol (Teal & Holtzman, 1980; Hampton et al., 1982; Mendelsohn et al., 1984; Murray & Leid, 1984; Haring et al., 1985, 1986), binds to brain PCP receptors with an affinity 100–200 times lower (Teal & Holtzman, 1980; Hampton et al., 1982; Mendelsohn et al., 1984; Murray & Leid, 1984; Haring et al., 1985, 1986).

Thus, cross-interactions of PCP analogues and of σ-opioids or dexoxadrol with multiple receptors might account for the diverse behavioral effects of these drugs. Another possible

† This work was supported in part by the Julius Bär Foundation (Zurich) and by NIH Grant DABB 1RO1 DAO4168-01.

* Author to whom correspondence should be addressed.
‡ The George S. Wise Faculty of Life Sciences.
§ Sackler Faculty of Medicine.

[1] Abbreviations: PCP, 1-(1-phenylcyclohexyl)piperidine (phencyclidine); TCP, *N*-[1-(2-thienyl)cyclohexyl]piperidine; hydroxy-PCP, *N*-[1-(3-hydroxyphenyl)cyclohexyl]piperidine; SKF 10047, *N*-allylnormetazocine; AZ-PCP, *N*-[1-(3-azidophenyl)cyclohexyl]piperidine; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; NMDA, *N*-methyl-D-aspartate.

0006-2960/87/0426-5854$01.50/0

explanation posits the existence of two or more classes of brain PCP receptors. Recent photoaffinity labeling studies with [^{3}H]AZ-PCP and rat brain synaptosomal membranes revealed heterogeneity of the PCP receptors (Haring et al., 1985, 1986), while other studies have indicated specific blockade by PCP of different ion channels, namely, the voltage-sensitive noninactivating potassium channel (Albuquerque et al., 1981; Bartschat & Blaustein, 1986) and the ion channels that are coupled to the *N*-methyl-D-asparate (NMDA)/L-glutamate receptor (Anis et al., 1983; Collingridge, 1985; Idriss & Albuquerque, 1985). These phenomena appear to be consistent with the existence of different PCP-receptor types or classes. The present study provides evidence for the presence of two classes of PCP receptors in rat brain, each with its distinct regional distribution and specific association with a different polypeptide that carries its major ligand-binding sites.

Materials and Methods

Materials

PCP, AZ-PCP, and TCP were prepared as described previously (Haring et al., 1983a,b, 1984). [^{3}H]AZ-PCP (22.8 Ci/mmol) and [^{3}H]PCP (22.5 Ci/mmol) were purchased from Israel Nuclear Center, Negev, Israel. The purity of the drugs was >98%. [^{3}H]TCP (40Ci/mmol) was purchased from New England Nuclear, Boston, MA. Haloperidol was purchased from Sigma, St. Louis, MO.

Methods

Tissue Preparation. Synaptosomal membranes were prepared from the various brain regions, essentially as described by Zukin et al. (1974) and as detailed previously (Haring et al., 1986). Charles Rivers derived (CD) male rats (200–250 g) obtained from Levinstein's Farm (Yokneam, Isreal) were decapitated and the hippocampus, frontal cortex, thalamus, hypothalamus, cerebellum, and olfactory bulb rapidly dissected out and homogenized in 20 volumes of ice-cold 0.32 M sucrose in a glass homogenizer fitted with a Teflon pestle. The sucrose solution, as well as the other solutions used during preparation of synaptosomes, contained the following antiproteases: 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 3 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 5 units/mL aprotinin, and 5 μg/mL pepstatin A. The homogenate was centrifuged at 1000*g* for 10 min, the pellet was discarded, and the supernatant was centrifuged at 20000*g* for 20 min. The resulting pellet was resuspended in distilled water containing the above cocktail of antiproteases and dispersed in a Model W-10 sonicator (Heat Systems-Ultrasonics Inc.) at setting 7 for 20 s. The suspension was then centrifuged at 8000*g* for 20 min. The supernatant was collected and the upper coat of the pellet rinsed carefully with the supernatant fluid in order to collect the upper layer of the pellet. The combined supernatant was then centrifuged at 48000*g* for 20 min. The final crude synaptic membrane pellets were resuspended in 5 mM Tris-HCl buffer, pH 7.4, containing the cocktail of antiproteases, and used immediately either for binding or for photoaffinity labeling experiments.

Binding Assays. Aliquots of synaptosomal membranes (100–120 μg of protein) were incubated with 200 μL of 5 mM Tris-HCl buffer (pH 7.4) containing the indicated concentrations of [^{3}H]PCP, [^{3}H]TCP, or [^{3}H]AZ-PCP, at 25 °C, for 15 min. Under these conditions binding of the labeled ligands reached equilibrium; prolonging the incubation period to 30 or 60 min had no effect on the binding isotherms obtained. Nonspecific binding was determined in samples containing the labeled ligands and 100 μM unlabeled PCP, and corrections for adsorption to filters were made as described (Hampton et al., 1982). Reactions were terminated by the addition of 3 mL of ice-cold buffer and rapid filtration (<10 s) on Whatman GF/C glass filters presoaked in 0.05% poly(ethylenimine), pH 7.4, as described by Hampton et al. (1982). The filters were washed twice with 3 mL of ice-cold buffer and counted in 4 mL of scintillation liquid (Hydro-Luma, Lumac Systems). Nonspecific adsorbance of labeled drugs to the filters (in the absence of membranes) was low and was subtracted from total counts. All assays were carried out in triplicate together with triplicate samples containing 100 μM unlabeled PCP. Specific binding was calculated as the total minus the nonspecific binding. All binding experiments employing AZ-PCP or [^{3}H]AZ-PCP were carried out in the dark.

Photoaffinity Labeling. These experiments were performed essentially as described previously (Haring et al., 1986a). Aliquots (200 μL) of synaptosomal membranes (200–250 μg of protein), freshly prepared in the antiprotease buffer, were incubated with 2.4 mL of 10 mM sodium phosphate buffer, pH 7.4, containing 100 nM [^{3}H]AZ-PCP with or without the indicated concentrations of unlabeled drug, for 15 min at 25 °C in the dark. For nonspecific labeling 1 mM TCP was employed. Phosphate buffer was used since the efficiency of labeling was found to be greatly diminished when 50 mM Tris-HCl buffer was used.

Following incubation in the dark, samples were withdrawn for determination of total binding. The reaction mixture was then photolyzed with a long-wave ultraviolet spotlight lamp (Thomas Scientific Apparatus, Model B-100A, 366 nm) at a distance of 5 cm (1500 μW/cm^2) with continuous stirring for 5 min. After photolysis, membranes were precipitated and washed 4 times with Tris-HCl buffer (5 mM, pH 7.4) containing the cocktail of antiproteases. After precipitation and washing, the photoaffinity-labeled membranes were used for gel electrophoresis.

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). Membrane samples (150–200 μg of protein) were dissolved 1:2 (v/v) in 3× sample buffer (52.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol) and applied after 2 h to polyacrylamide slab gels (12.5%). Proteins were electrophoresed together with molecular weight standards (Pharmacia Fine Chemicals, low molecular weight proteins) at 15–20 mA/slab for 3 h and then stained with 0.2% Coomassie Brilliant Blue in 50% methanol and 7% acetic acid and destained in 7.5% acetic acid. Gels were sliced into 2-mm sections with a gel slicer, and each slice was digested in 5 mL of Lipoluma–Lumasolve–water (10:1:0.2) (Lumac Inc.) in a closed scintillation vial. Radioactivity was determined after 24 h by liquid scintillation spectrometry. Proteins were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Data Analysis. Individual binding curves were analyzed according to a curve-fitting routine (BMDPAR derivative free, nonlinear regression, 1982, Reagents of University of California, distributed by BMDP Inc., Los Angeles, CA) adapted for two-site analysis of ligand binding (Egozi et al., 1980). The nonlinear least-squares regression computer program and the criteria for best fit have been described previously (Baron et al., 1985). The analysis yields values for the proportion of the high-affinity sites as well as for the high- and low-affinity binding constants, which characterize binding to the high- and low-affinity sites.

Linear regression analysis was used to determine the correlation between the extent of specific labeling of an individual

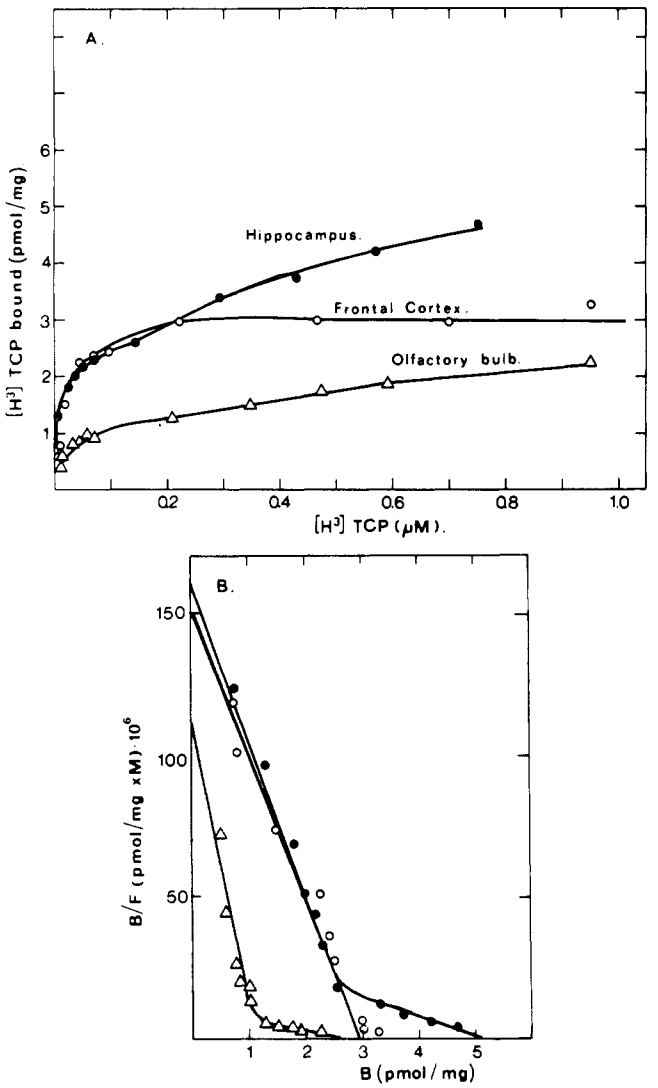

FIGURE 1: Binding of [^{3}H]TCP to PCP receptors of rat hippocampus, frontal cortex, and olfactory bulb. Binding was determined as described under Materials and Methods. (A) Specific binding plotted as a function of the free ligand concentration: (●) hippocampus; (○) frontal cortex; (△) olfactory bulb. (B) Scatchard plots of the same data.

labeled band and the densities of the high- and low-affinity [^{3}H]TCP-binding sites. Pearson's correlation coefficients and their significance (from the t distribution) were calculated according to Colton (1974).

RESULTS

Regional Distribution of [^{3}H]TCP-Binding Sites. Binding of [^{3}H]TCP to synaptosomal membranes prepared from six brain regions was measured over the concentration range of 1 nM–1 μM. Typical curves describing [^{3}H]TCP binding in hippocampus, frontal cortex, and olfactory bulb membranes are shown in Figure 1A, and the corresponding Scatchard plots are shown in Figure 1B. In the frontal cortex a single high-affinity (K_d = 10 nM) saturable component was detected, whereas in the hippocampus and in the olfactory bulb an additional saturable binding site (K_d = 80–120 nM) was observed. The results of these experiments and of similar experiments performed with hypothalamic, thalamic, and cerebellar membranes are summarized in Figure 2. As shown, the hippocampus, the frontal cortex, and the thalamus possess the highest densities of high-affinity [^{3}H]TCP sites, the olfactory bulb contains a moderate density, and the hypothalamus contains a very low density. No high-affinity sites were detected in the cerebellum. The low-affinity sites showed a

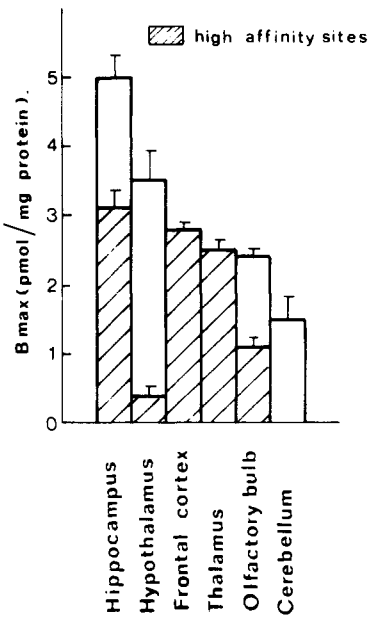

FIGURE 2: Regional distribution of high- and low-affinity [^{3}H]-TCP-binding sites. Data are represented by the mean values derived from three separate binding curves, as shown in Figure 1 (vertical bars represent the SD values). Each of the binding curves was computer-analyzed as described under Methods. The cross-hatched part of the bars represents the density of the high-affinity sites. The difference between B_{max} (the total binding capacity) and the density of the high-affinity sites is the density of the low-affinity sites (the open part of the bar). The respective dissociation constants (K_d high and K_d low) were 10–15 nM and 80–250 nM.

different distribution pattern: the highest density was observed in the hypothalamus, moderate densities were observed in the hippocampus, olfactory bulb, and cerebellum, and there was complete absence in the frontal cortex and in the thalamus.

Regional Distribution of [^{3}H]TCP-, [^{3}H]PCP-, and [^{3}H]-AZ-PCP-Binding Sites. The regional distribution pattern of binding sites was also measured by using the two PCP-receptor ligands, namely, [^{3}H]PCP and [^{3}H]AZ-PCP. The latter ligand was used for subsequent photoaffinity labeling experiments (see below). The regional distribution pattern of [^{3}H]TCP-binding sites measured at a single ligand concentration (75 nM) was found to be similar to that measured with both [^{3}H]PCP and [^{3}H]AZ-PCP at the same concentration (Figure 3). Under these experimental conditions, hippocampus receptors were highly occupied by all three ligands, while frontal cortex, thalamic, and olfactory bulb receptors were moderately occupied, and hypothalamic and cerebellar receptors were poorly occupied (Figure 3). In all brain regions, however, site occupancy was higher with 75 nM [^{3}H]TCP than with 75 nM [^{3}H]PCP or [^{3}H]AZ-PCP, probably because of the higher affinity of the first ligand (Vignon et al., 1983).

The distribution patterns thus described were to be expected in view of (a) the regional distribution of the high-affinity sites (Figure 2), of which 60–85% are occupied at 75 nM, and (b) the distribution of low-affinity sites (Figure 2), of which 20–33% are occupied. The low occupancy by the ^{3}H ligands observed in the hypothalamus (Figure 3) constitutes an exception and accounts for both the low densities of high affinity and the relatively low affinities toward the low-affinity sites in this brain region (250, 350, and 300 nM for [^{3}H]TCP, [^{3}H]PCP, and [^{3}H]AZ-PCP, respectively). The data, together with earlier findings on the competitive nature of PCP/TCP (Vignon et al., 1983, 1986; Haring et al., 1986) and of PCP/AZ-PCP (Haring et al., 1985, 1986; Sorensen & Blaustein, 1986), suggest that all three labeled ligands bind to both the high- and low-affinity sites.

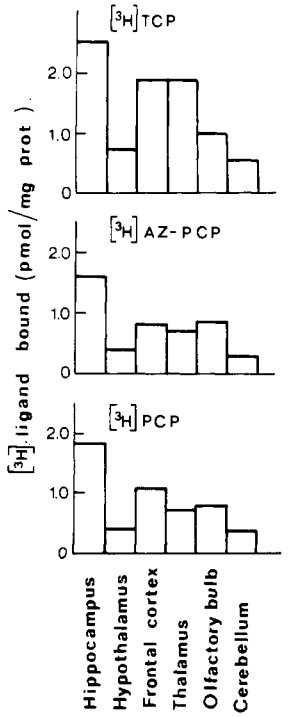

FIGURE 3: Comparison between the regional distributions of [^{3}H]TCP-, [^{3}H]PCP-, and [^{3}H]AZ-PCP-binding sites. Binding was determined as described under Materials and Methods using a single concentration (75 nM) of each of the radiolabeled ligands. Data points represent mean values of the specific binding (four determinations).

Effect of Haloperidol on the Binding of [^{3}H]TCP to the High- and Low-Affinity Binding Sites. Haloperidol has been previously shown to bind with high affinity (K_d = 5 nM) to the high-affinity (+)SKF 10047 binding sites (Tam & Cook, 1984; Tam, 1985; Largent et al., 1986). These sites also bind PCP analogues, although with relatively low affinity (Tam & Cook, 1984; Tam, 1985; Largent et al., 1986). We therefore decided to examine the effect of haloperidol on the high- and low-affinity PCP-binding sites in two typical brain regions, viz., the frontal cortex (high-affinity sites) and the hippocampus (high- and low-affinity sites). As shown in Figure 4, 5 μM haloperidol did not affect the binding of [^{3}H]TCP either to the high- or to the low-affinity binding sites. Thus, these sites appear to be distinct from the high-affinity (+)SKF 10047 sites.

Effects of Ca^{2+} on the High- and Low-Affinity PCP-Binding Sites. Previous studies have shown that divalent ions such as Ca^{2+} and Mg^{2+} inhibit the binding of [^{3}H]PCP to its brain receptors (Vignon et al., 1982). It was also reported that Ca^{2+} had no effect on the binding of (+)SKF 10047 to its high-affinity binding sites (Tam, 1985). We therefore examined the effects of Ca^{2+} on the two binding sites for [^{3}H]TCP. A typical Ca^{2+} inhibition curve obtained in hippocampal membranes is shown in Figure 5A. Fifty percent inhibition (I_{50}) occurred at a concentration of 1 mM Ca^{2+}. Similar results were also obtained when frontal cortex, cerebellar, thalamic, and hypothalamic preparations were used (not shown). Both the high- and the low-affinity binding sites were affected by the Ca^{2+} ions; in the hippocampus 2.5 mM $CaCl_2$ resulted in a 70% reduction in the high-affinity sites (from 3.1 to 1.0 pmol/mg protein) and in a 25% reduction in the low-affinity sites (from 1.9 to 1.4 pmol/mg of protein). Since the Ca^{2+}-induced effect (which is reversible) on [^{3}H]TCP binding is a reduction in the maximal binding capacity (Figure 5B), it is reasonable to assume that the inhibition by Ca^{2+} is of a noncompetitive nature.

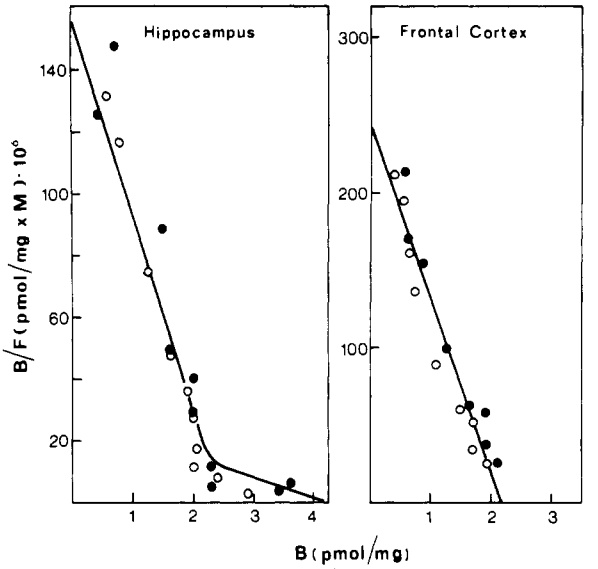

FIGURE 4: Scatchard plots of [^{3}H]TCP binding to rat hippocampus, cerebellum, and frontal cortex membranes in the presence of haloperidol. Binding was determined as described under Methods and in Figure 1 in the absence (●) and in the presence (○) of 5 μM haloperidol: (left) hippocampus; (right) frontal cortex.

Photoaffinity Labeling of PCP Receptor in the Presence and Absence of Ca^{2+}. Hippocampal or frontal cortex PCP receptors were photoaffinity-labeled with 100 nM [^{3}H]AZ-PCP, a concentration that allows partial occupation of both the high- and low-affinity binding sites (see above) (Haring et al., 1987). Experiments were performed in the absence and in the presence of various concentrations of Ca^{2+} ranging from 10 μM to 10 mM. The affinity-labeled polypeptides of the PCP receptors were separated by SDS–polyacrylamide gel electrophoresis. In agreement with previous results (Haring et al., 1985, 1986) and in the absence of Ca^{2+}, five major polypeptides were found to be specifically labeled with [^{3}H]AZ-PCP (M_r 90 000, 62 000, 49 000, 40 000, and 33 000) both in the frontal cortex and in the hippocampus. As shown in Figure 6, Ca^{2+} inhibited the labeling of all radioactive peaks in a dose-dependent manner. Fifty percent inhibition of the labeling occurred at 1–2 mM Ca^{2+}, i.e., at a concentration close to the I_{50} for the inhibition of [^{3}H]TCP binding (Figure 5A). The fact that Ca^{2+} inhibits the labeling of all five peaks is also in line with its inhibitory effect on the binding of [^{3}H]TCP to both the high- and low-affinity binding sites.

Correlation of the Distribution of High- and Low-Affinity [^{3}H]TCP Sites with the Distribution of [^{3}H]AZ-PCP-Labeled Polypeptides. The purpose of these experiments was to examine the relationship between the high- and low-affinity [^{3}H]TCP-binding sites and the polypeptides labeled with [^{3}H]AZ-PCP. Synaptosomal membranes prepared from each of the brain regions under study were labeled with 100 nM [^{3}H]AZ-PCP in the absence (total labeling) and in the presence of 1 mM TCP (nonspecific labeling). The photoaffinity-labeled polypeptides were separated by SDS–polyacrylamide gel electrophoresis. For each brain region the specific labeling found in each of the radioactive peaks was then calculated by subtracting the nonspecific labeling from the total labeling. The regional distribution of the labeled peaks was compared with that of the high- and low-affinity [^{3}H]TCP-binding sites in order to determine which labeled polypeptide correlates best with these binding sites. As shown in Figure 7A, good correlation was found between the regional distribution of the high-affinity [^{3}H]TCP-binding sites and the M_r 90 000 polypeptides in all brain regions examined. No

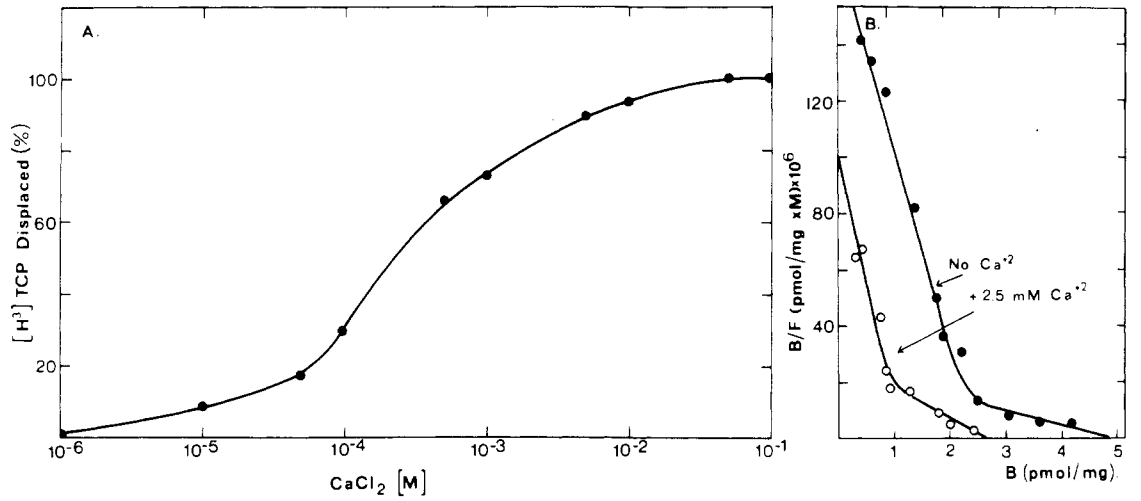

FIGURE 5: Effect of Ca^{2+} on [^{3}H]TCP binding to rat hippocampus membranes. (A) Specific binding of 50 nM [^{3}H]TCP was determined in the absence and in the presence of various concentrations of $CaCl_2$, as described under Methods. The percentage of inhibition was plotted as a function of $CaCl_2$ concentrations. (B) Scatchard plot of [^{3}H]TCP binding in the absence (●) and in the presence (○) of 2.5 mM $CaCl_2$. Binding of [^{3}H]TCP as a function of its concentrations was determined as described under Methods and in Figure 1.

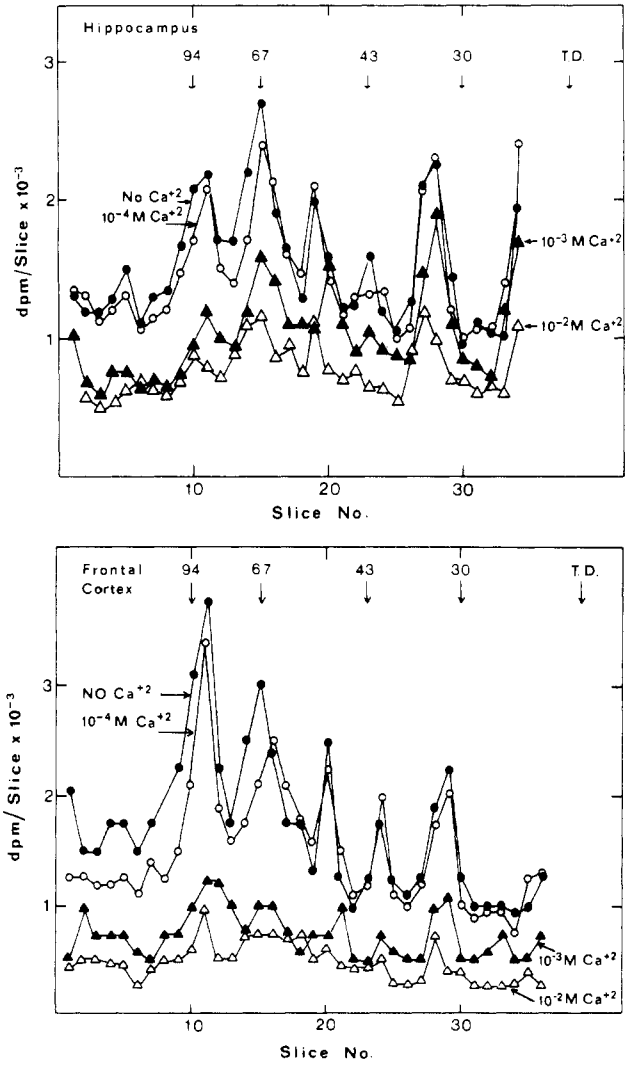

FIGURE 6: Effect of Ca^{2+} on [^{3}H]AZ-PCP photoaffinity labeling of rat brain PCP receptors. Photoaffinity labeling was performed as described under Methods in the absence of (●) and in the presence of 10^{-4} (○), 10^{-3} (▲), and 10^{-2} M (△) $CaCl_2$. The photolabeled receptors were separated on SDS–polyacrylamide gel (12.5%) as described under Methods. The labeling pattern in the presence of 1 mM TCP (not shown) was essentially the same as that observed in the presence of 10^{-2} M $CaCl_2$. Migration of molecular weight standards (in kilodaltons) is indicated by arrows: (T.D.) tracking dye; (upper) hippocampus; (lower) frontal cortex.

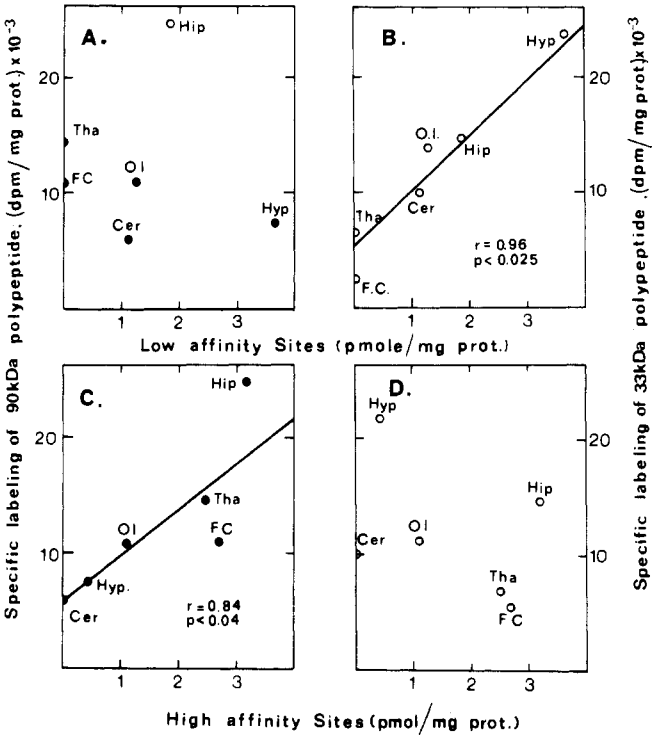

FIGURE 7: Regional distribution of [^{3}H]AZ-PCP-photolabeled polypeptides in relation to the distribution of high- and low-affinity [^{3}H]TCP-binding sites. The amount of specific labeling of the M_r 90 000 (90-kDa) polypeptide (A and C) and of the M_r 33 000 (33-kDA) polypeptide (B and D) in each of the brain regions is correlated with the density of the low-affinity binding sites (A and B) and with the density of the high-affinity binding sites (C and D). Specific labeling was calculated by subtracting the nonspecific labeling (in the presence of 1 mM TCP) from the total labeling as detected in SDS–polyacrylamide gels. Each point represents the mean values (three to six separate determinations) of the specific labeling per milligram of protein loaded on the gel. The values for the high-affinity and low-affinity [^{3}H]TCP-binding sites are taken from Figure 2 (r = Pearson's correlation coefficient).

correlation was observed between the distribution of this labeled polypeptide and the distribution of low-affinity [^{3}H]-TCP-binding sites (Figure 7B). However, the regional distribution of these low-affinity sites was positively correlated with the labeled M_r 33 000 polypeptide present (Figure 7C), while the distribution of the high-affinity [^{3}H]TCP-binding sites showed no such correlation (Figure 7D). We did not find

any correlation between the regional distribution of the M_r 62 000, 49 000, or 40 000 polypeptides and the density of [^{3}H]TCP sites. The amount of labeling of the M_r 49 000 and 40 000 polypeptides was low in all brain regions, amount to about 3–8% of the total specific labeling (Haring et al., 1985, 1986, 1987; see also Figure 6). The amount of labeling of the M_r 62 000 polypeptide was relatively high, but did not vary significantly among brain regions (values recorded were between 12×10^3 and 17×10^3 dpm/mg of protein) except for the cerebellum, in which no labeled M_r 62 000 polypeptide was detected.

Discussion

We describe here the distribution of two classes of [^{3}H]-PCP-binding sites in the rat brain. One class of sites, which binds PCP-receptor ligands with high affinity, appears to be associated with an AZ-PCP-labeled M_r 90 000 polypeptide and is mainly localized in forebrain regions such as the hippocampus, the olfactory bulb, the frontal cortex, and the thalamus. The second, lower affinity class of sites appears to be associated with an M_r 33 000 photolabeled polypeptide and is found both in forebrain and in lower brain regions. Binding of [^{3}H]TCP to either class of sites is inhibited by Ca^{2+} as well as by Na^+ and K^+ ions (Haring et al., 1987) and is not blocked by the neuroleptic drug haloperidol, suggesting that these sites are distinct from the high-affinity (+)SKF 10047 binding site (Tam & Cook, 1984; Tam, 1985; Largent et al., 1986).

The existence of two or more classes of PCP receptors was suggested by earlier observations in which polypeptides of the PCP receptors were identified by photoaffinity labeling with [^{3}H]AZ-PCP (Haring et al., 1985, 1986, 1987). Three observations were incompatible with the existence of a single receptor population: (1) of the five [^{3}H]AZ-PCP-labeled bands detected in the hippocampus, the labeling of only two (M_r 90 000 and 33 000) was stereoselectively inhibited by dexoxadrol (Haring et al., 1985, 1986); (2) the labeling of the M_r 90 000 polypeptide was more sensitive than that of the M_r 33 000 polypeptide to inhibition by TCP and PCP, while the labeling of the latter polypeptide was more sensitive to inhibition by (±)SKF 10047 and hydroxy-PCP (Haring et al., 1986); (3) variations in the proportions of the labeled polypeptides were clearly observed between the different brain regions (Haring et al., 1985). A single receptor population should have yielded a constant proportion between labeled components in all the brain regions.

In spite of these results, direct binding experiments with either [^{3}H]PCP (Zukin & Zukin, 1979; Vincent et al., 1979; Quirion et al., 1981; Vignon et al., 1982; Haring et al., 1985, 1986) or [^{3}H]AZ-PCP (Haring et al., 1985, 1986; Sorensen & Blaustein, 1986) did not indicate heterogeneity of PCP receptors. This discrepancy appears to be resolved by recent findings that both high- and low-affinity PCP-binding sites can be detected in the rat brain by [^{3}H]TCP (Vignon et al., 1986; Haring et al., 1987), [^{3}H]PCP, and [^{3}H]AZ-PCP (Haring et al., 1987), provided that low ionic strength buffers are used in the binding assays. Employing such conditions, Vignon et al. (1986) have demonstrated high-affinity sites (K_d = 50–80 nM) in lower brain regions of the rat. The results of the present study support the findings of Vignon et al. (1986) with regard to the selective localization of high-affinity [^{3}H]TCP-binding sites in forebrain regions and the presence of only low-affinity sites in the cerebellum. Extending the ligand concentration range in our experiments (1 nM–1 μM) also made it possible to detect low-affinity sites in some forebrain regions (hippocampus, olfactory bulb, hypothalamus) and confirmed that other forebrain regions (such as the thalamus and the frontal cortex) contain only high-affinity PCP receptors.

The regional distribution of the high-affinity [^{3}H]TCP-binding sites described here (Figure 2) resembles the previously described localization of [^{3}H]PCP (Vincent et al., 1979; Zukin & Zukin, 1979; Quirion et al., 1981) and [^{3}H]TCP (Sircar & Zukin, 1985; Vignon et al., 1986; Contreras et al., 1986b) binding sites (hippocampus > frontal cortex ≈ thalamus > olfactory bulb > hypothalamus > cerebellum). This resemblance can probably be explained by the use of relatively low ligand concentrations in autoradiographic studies (<20 nM), in which mainly the high-affinity PCP receptors are occupied. It is clear, however, that in brain regions containing a mixed population of PCP receptors (hippocampus, hypothalamus, and olfactory bulbs) the low-affinity receptors contribute significantly to the total receptor density. The regional distribution of these low-affinity PCP receptors (hypothalamus > hippocampus > cerebellum ≈ olfactory bulbs > frontal cortex and thalamus) is clearly different from that of the high-affinity receptors, suggesting that the two receptor populations are distinct. The different sensitivities of the high- and low-affinity PCP-binding sites to bivalent ions (Figure 5B) and to monovalent ions (Haring et al., 1987) are another distinguishing property. While both Ca^{2+} and Na^+ or K^+ exhibit noncompetitive inhibition of the binding of [^{3}H]PCP to its receptors (Vignon et al., 1982; Haring et al., 1987), it is evident that the high-affinity sites are more sensitive to the ions (Haring et al., 1987). Apparently, the effects of monovalent and bivalent cations on PCP receptors are different: Ca^{2+} ions appear to reduce the number of binding sites, whereas Na^+ or K^+ reduces the affinity of the PCP receptors (Vignon et al., 1982; Haring et al., 1987). In view of the reversibility of the Ca^{2+} effect on PCP receptors, it is likely that bivalent ions also reduce the affinity of the receptors but to a very low affinity state which is not detected under the experimental conditions used here. Monovalent ions, however, reduce the affinity of the high-affinity PCP receptors to a level that can be detected in the binding assays (Haring et al., 1987). Thus, Na^+ or K^+ converts the high-affinity [^{3}H]TCP- or [^{3}H]-PCP-binding sites to a lower affinity state that is indistinguishable from the preexisting low-affinity PCP receptors (Haring et al., 1987). At monovalent ion concentrations of 25 mM the inhibitory effect is restricted to the high-affinity sites, while at higher ion concentrations (100 mM) both high- and low-affinity sites are inhibited (Haring et al., 1987). These phenomena also indicate that each of the two PCP-receptor populations can exist in two different conformational states, which could be modified by both endogenous and exogenous factors. An important endogenous agent affecting PCP receptors could be L-glutamate, which was shown to enhance [^{3}H]TCP binding (Loo et al., 1986). Exogenous factors that play a part in the interconversions between conformational states of PCP receptors include the structure of the ligand, i.e., TCP vs. PCP, negative effectors such as ions, and positive effects such as the drug 2-amantadine (Quirion & Pert, 1982).

The presence of two distinct PCP receptor populations in the rat brain, together with the conformational constraints imposed by other membrane components possibly associated with them [e.g., a monovalent ion binding component, a potassium channel (Albuquerque et al., 1981; Bartschat & Blaustein, 1986), or the L-glutamate/NMDA receptor (Anis et al., 1983; Collingridge, 1985; Idriss & Albuquerque, 1985)], should be reflected in the labeling of polypeptides of these receptors. Indeed, such manifestations have been partially demonstrated in the present study and elsewhere. Thus, the

NMDA receptor antagonist APV (Haring et al., 1987) as well as Ca^{2+} ions (Figure 6) inhibits labeling of all polypeptides labeled by [^{3}H]AZ-PCP, and Na^+ or K^+ ions (25 mM) or tetraethylammonium (1 mM) selectivity inhibits labeling of the M_r 90000 polypeptide (Haring et al., 1987). The fact that inhibition by APV is nonselective, while that exerted by tetraethylammonium is selective, cannot yet be explained, as we have no data on the effects of these drugs on the binding of [^{3}H]PCP or [^{3}H]TCP to the high- and low-affinity sites. However, the inhibition of both high- and low-affinity sites by Ca^{2+} (Figure 5) adequately explains the nonselectiveness of this ion in the photoaffinity labeling experiment (Figure 6).

Several lines of evidence suggest that the high-affinity PCP receptors are associated with the M_r 90000 polypeptide and that the low-affinity receptors are associated with the M_r 33000 polypeptide: (1) the density of the M_r 90000 polypeptide in various brain regions correlates best with the distribution of high-affinity receptors, whereas the density of the M_r 33000 polypeptide correlates best with the distribution of the low-affinity receptors; (2) monovalent ions at concentrations of 25 mM selectively inhibit both binding of [^{3}H]PCP analogues to the high-affinity receptors and labeling of the M_r 90000 polypeptide by [^{3}H]AZ-PCP (Haring et al., 1987); (3) the greater affinity of PCP or of TCP toward the high-affinity sites than toward the low-affinity sites would be expected to result in selective inhibition of the labeling of a specific polypeptide. Indeed, the M_r 90000 polypeptide is clearly more sensitive than the M_r 33000 polypeptide to inhibition by both TCP and PCP (Haring et al., 1986). Similarly, the M_r 33000 polypeptide is more sensitive than the M_r 90000 to inhibition by (±)SKF 10047 (Haring et al., 1986), which has a relatively low affinity toward brain PCP receptors as compared to that of TCP or PCP (Zukin et al., 1983). It is important to note that the M_r 33000 polypeptide detected in SDS–polyacrylamide gel electrophoresis is composed of two isoelectric forms (Haring et al., 1986) and that both the M_r 90000 and the M_r 30000 polypeptides appear as broad bands on the gels (see also Figure 6). This could result from microheterogeneity among labeled polypeptides or from the presence in each band of labeled components, which are not associated with the PCP receptors. In either case, the correlation lines between the density of the labeled polypeptide and the densities of sites will not intercept at zero point of the ordinate (Figure 7). This phenomenon is now being studied in our laboratory. Also under investigation are the pharmacological profiles of the two classes of PCP receptors and their interactions with potassium-channel blockers, NMDA-receptor ligands, and σ-opioid, as well as the characterization of the three labeled polypeptides, i.e., 62, 50, and 40 kDa.

The physiological significance of the two PCP receptors is not yet known. However, their different localizations and the probable differences in their polypeptie composition would suggest that each of these receptors mediates a specific or separate function. This hypothesis could account for the various behavioral effects induced by PCP and its analogues, such as stereotyped behavior (Contreras et al., 1986a), ataxia (Sturgeon et al., 1979; Contreras et al., 1986a), increase of locomotor activity in rodents (Sturgeon et al., 1979), aggressiveness (Luby et al., 1962) and nystagmus (Gallant, 1981) in man, and anticonvulsant activity (Domino & Luby, 1981; Gallant, 1981). It would also be compatible with the differences observed in the doses of PCP needed to induce, for example, an increase in locomotor activity and ataxia (Sturgeon et al., 1979) and with the fact that PCP-receptor interactions are better correlated with stereotyped behavior than with ataxia (Contreras et al., 1986a).

ACKNOWLEDGMENTS

We thank Dr. M. Blaustein for providing a preprint of the work from his laboratory. We acknowledge with thanks the editorial assistance of Shirley Smith.

Registry No. PCP, 77-10-1; Ca, 7440-70-2.

REFERENCES

Albuquerque, E. X., Aguayo, L. G., Warnick, J. F., Weistein, H., Glick, S. D., Maayani, S., Itzkowicz, K. R., & Blaustein, M. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7792–7796.

Anis, N. A., Berry, S. C., Burton, N. R., & Lodge, D. (1983) *Br. J. Pharmacol. 79*, 565–575.

Aronstam, R. S., Eldefrawi, M. E., Eldefrawi, A. T., Albuquerque, E. X., Kim, K. F., & Triggle, D. J. (1980) *Mol. Pharmacol. 18*, 179–184.

Baron, B., Gavish, M., & Sokolovsky, N. (1985) *Arch. Biochem. Biophys. 240*, 281–296.

Bartschat, D. K., & Blaustein, M. P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 189–192.

Blaustein, M. P., & Itzkowicz, K. R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3855–3859.

Collingridge, G. L. (1985) *Trends Pharmacol. Sci. 74*, 407–411.

Colton, T. (1974) *Statistics in Medicine*, pp 196–212, Little, Brown and Co., Boston.

Contreras, P. C., Kenner, C. R., Jacobson, A. E., & O'Donnohue, T. L. (1986a) *Eur. J. Pharmacol. 121*, 9–18.

Contreras, P. C., Quirion, R., & O'Donohue, T. L. (1986b) *Neurosci. Lett. 67*, 101–106.

Domino, E. F., & Luby, E. D. (1981) in *PCP (Phencyclidine): Historical and Current Perspectives* (Domino, E. F., Ed.) pp 401–418, NPP Books, Ann Arbor, MI.

Egozi, Y., Kloog, Y., & Sokolovsky, M. (1980), in *Neurotransmitters and Their Receptors* (Littauer, U. Z., Dudai, Y., & Silman, I., Eds.) pp 201–217, Wiley, London.

Gallan, D. M. (1981) in *PCP (Phencyclidine): Historical and Current Perspectives* (Domino, E. F., Ed.) pp 437–447, NPP Books, Ann Arbor, MI.

Hampton, R. Y., Medzihardsky, J. H., Wodds, J. H., & Dahlstrom, P. J. (1982) *Life Sci. 30*, 2147–2154.

Haring, R., Kloog, Y., Kalir, A., & Sokolovsky, M. (1983a) *Biochem. Biophys. Res. Commun. 113*, 723–729.

Haring, R. Theomy, S., Kalir, A., & Sokolovsky, M. (1983b) *Arch. Toxicol. Suppl. 6*, 81–90.

Haring, R., Kloog, Y., & Sokolovsky, M. (1984) *J. Neurosci. 4*, 627–637.

Haring, R., Kloog, Y., & Sokolovsky, M. (1985) *Biochem. Biophys. Res. Commun. 131*, 1117–1123.

Haring, R., Kloog, Y., & Sokolovsky, M. (1986) *Biochemistry 25*, 612–620.

Haring, R., Kloog, Y., Harshak-Felixbrodt, N., & Sokolovsky, M. (1987) *Biochem. Biophys. Res. Commun. 142*, 501–510.

Idriss, M., & Albuquerque, E. X. (1985) *FEBS Lett. 189*, 150–156.

Johnson, K. M., & Vickroy, T. W. (1981) in *PCP (Phencyclidine): Historical and Current Perspectives* (Domino, E. F., Ed.) pp 191–205, NPP Books, Ann Arbor, MI.

Kloog, Y., Rehavi, M., Maayani, S., & Sokolovsky, M. (1977) *Eur. J. Pharmacol. 45*, 221–227.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Largent, B. L., Gundluch, A. L., & Snyder, S. H. (1986) *J. Pharmacol. Exp. Ther. 238*, 739–748.

Loo, P., Braunwalder, A., Lehmann, J., & Williams, M. (1986) *Eur. J. Pharmacol. 123*, 467–468.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Luby, E. D., Gottlieb, J. S., Cohen, B. D., Rosenbaum, G., & Domino, E. F. (1962) *Am. J. Psychiatry 119*, 61–67.
Maayani, S., Weinstein, A., Ben-Zvi., N., Cohen, S., & Sokolovsky, M. (1974) *Biochem. Pharmacol. 23*, 1263–1281.
Mendelsohn, L. G., Kerchner, G. A., Kalra, V., Zimmerman, D. M., & Leander, J. D. (1984) *Biochem. Pharmacol. 33*, 3529–3535.
Murray, T. F., & Leid, M. E. (1984) *Life Sci. 34*, 1899–1911.
Oswald, R., & Changeux, J. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3925–2929.
Pilapil, C., Contreras, P. C., O'Donohue, T. L., & Quirion, R. (1985) *Neurosci. Lett. 56*, 1–7.
Quirion, R., & Pert, C. B. (1982) *Experientia 38*, 955–956.
Quirion, R., Hammer, R. P., Herkenham, M., & Pert, C. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5881–5885.
Sircar, R., & Zukin, S. R. (1985) *Brain Res. 344*, 142–145.
Sorensen, R. G., & Blaustein, M. P. (1986) *J. Neurosci. 6*, 3676–3687.
Sturgeon, R. D., Fessler, R. G., & Meltzer, H. Y. (1979) *Eur. J. Pharmacol. 59*, 169–179.
Su, T. P., Cone, E. J., Shanon, H., & Vaulel, D. B. (1980) *Res. Commun. Subst. Abuse 1*, 85–98.
Tam, S. W. (1985) *Eur. J. Pharmacol. 109*, 33–41.
Tam, S. W., & Cook, L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5618–5621.
Teal, J., & Holtzam, S. G. (1980) *J. Pharmacol. Exp. Ther. 212*, 368–376.
Tourneur, Y., Romey, G., & Lazdunski, M. (1982) *Brain Res. 245*, 154–158.
Vignon, J., Vincent, J. P., Bidard, J. N., Kamenka, J. M., Geneste, P., Monier, S., & Lazdunski, M. (1982) *Eur. J. Pharmacol. 81*, 531–542.
Vignon, J., Chicheportiche, R., Chicheportiche, M., Kamenka, J. M., Geneste, P., & Lazdunski, M. (1983) *Brain Res. 280*, 194–197.
Vignon, J., Privat, A., Chandien, I., Thierry, A., Kamenka, J. M., & Chicheportiche, R. (1986) *Brain Res. 378*, 133–141.
Vincent, J. P., Karalovski, K., Geneste, P., Kamenka, J. M., & Lazdunski, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4678–4682.
Zukin, S. R., & Zukin, R. S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5372–5376.
Zukin, S. R., Young, A. B., & Snyder, S. H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4802–4807.
Zukin, S. R., Fitz-Syage, M. L., Nichtenhauser, R., & Zukin, R. S. (1983) *Brain Res. 258*, 277–284.

Catecholamine-Induced Desensitization of Adenylate Cyclase Coupled β-Adrenergic Receptors in Turkey Erythrocytes: Evidence for a Two-Step Mechanism

Jeffrey M. Stadel,* Richard Rebar, and Stanley T. Crooke
Department of Molecular Pharmacology, Smith Kline and French Laboratories, Philadelphia, Pennsylvania 19101
Received February 3, 1987; Revised Manuscript Received May 11, 1987

ABSTRACT: Preincubation of turkey erythrocytes with isoproterenol is associated with (1) 50–60% attenuation of agonist-stimulated adenylate cyclase activity, (2) altered mobility of the β-adrenergic receptor on sodium dodecyl sulfate–polyacrylamide gels, and (3) increased phosphorylation of the β-adrenergic receptor. Using a low-cross-linked polyacrylamide gel, the β-adrenergic receptor protein from isoproterenol-desensitized cells, labeled with ^{32}P or with the photoaffinity label ^{125}I-(*p*-azidobenzyl)carazolol, can be resolved into a doublet ($M_r \simeq 37\,000$ and $M_r \simeq 41\,000$) as compared to a single $M_r \simeq 37\,000$ β-adrenergic receptor protein from control erythrocytes. The appearance of the doublet was dependent on the concentration of agonist used to desensitize the cells. Incubation of erythrocytes with dibutyryl-cAMP did not promote formation of the doublet but decreased agonist-stimulated adenylate cyclase activity 40–50%. Limited-digestion peptide maps of ^{32}P-labeled β-adrenergic receptors using papain revealed a unique phosphopeptide in the larger molecular weight band ($M_r \simeq 41\,000$) of the doublet from the agonist-desensitized preparation that was absent in the peptide maps of the smaller band ($M_r \simeq 37\,000$), as well as control or dibutyryl-cAMP-desensitized receptor. These data provide evidence that maximal agonist-induced desensitization of adenylate cyclase coupled β-adrenergic receptors in turkey erythrocytes occurs by a two-step mechanism.

The biological effects of catecholamines are initiated by binding to cell surface receptors. The physiological responses resulting from β-adrenergic stimulation are mediated by adenosine cyclic 3′,5′-phosphate (cAMP)[1] which is produced from substrate ATP by the membrane-bound enzyme adenylate cyclase (Robison et al., 1971; Lefkowitz et al., 1983). Prolonged occupancy of β-adrenergic receptors by agonist leads to desensitization, i.e., an attenuated responsiveness of the adenylate cyclase to a fresh challenge of agonist (Lefkowitz et al., 1983; Harden, 1983). Studies of the β-adrenergic receptor–adenylate cyclase complex have provided insights into the molecular mechanisms underlying this regulatory process.

The β-adrenergic receptor–adenylate cyclase complex in avian erythrocytes has served as a model system in which to investigate receptor–cyclase coupling and regulation. Fol-

[1] Abbreviations: ^{125}I-PABC, ^{125}I-(*p*-azidobenzyl)carazolol; ^{125}I-CYP, ^{125}I-cyanopindolol; A, acrylamide monomer; B, bis(acrylamide); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; dbcAMP, dibutyryl-cAMP; ISO, (−)-isoproterenol; cAMP, adenosine cyclic 3′,5′-phosphate.

0006-2960/87/0426-5861$01.50/0