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PRESENCE OF SIGMA AND PHENCYCLIDINE (PCP)-LIKE RECEPTORS IN MEMBRANE PREPARATIONS OF BOVINE ADRENAL MEDULLA

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Abstract—Receptor binding studies with [^{3}H]-(+)SKF-10047 were carried out to characterize the putative sigma (σ) and phencyclidine (PCP) receptors in membrane preparations of bovine adrenal medulla. Specific binding of the radiolabelled compound was observed after incubation with the membrane preparation at 37°, the equilibrium being reached at 20 min and the maximal binding being observed with 0.6 mg/ml protein. Saturation binding studies were performed at equilibrium (30 min at 37° with 0.5 mg/ml of membrane protein) in the presence of haloperidol (1 μM) or 1-[1-(2-thienyl) cyclohexyl] piperidine (TCP; 0.2 μM) to block σ or PCP receptors, respectively. The binding of [^{3}H]-(+)SKF-10047 was characterized by two distinct components. A high affinity binding site (haloperidol sensitive) had an apparent K_D of 8.3 nM and a B_{max} of 67 pmol/g protein. A lower affinity binding site (TCP sensitive) had an apparent K_D of 32.7 nM and a B_{max} of 83 pmol/g protein. The drug specificity of the high affinity binding site resembled that of the putative σ receptor, being potently inhibited by haloperidol and pentazocine. The binding pharmacology of the low affinity site resembled that of the phencyclidine receptor, being potently displaced by TCP and PCP. The binding of [^{3}H]-(+)SKF-10047 to both receptors showed marked stereoselectivity for the dextrorotatory (+) isomer of SKF-10047 and was insensitive to the receptor specific opioid ligands DAGO (μ), DSLET (δ) and U-69593 (κ). These data indicate that bovine adrenal medulla contains sigma and PCP-like receptors.

The existence of the sigma (σ) receptor was postulated by Martin *et al.* [1] on the basis of behavioral pharmacology of the drug *N*-allylnormetazocine (SKF-10047†) in the chronic spinal dog. In humans and other mammalian species, the psychomimetic syndrome induced by SKF-10047 and other benzomorphans such as cyclazocine resembles that elicited by PCP [2, 3]. The cross-binding activity of PCP and σ site-active drugs led to the presumption that both σ and PCP related compounds act through one common receptor [4]. Recently, radioligand binding studies with rat brain membrane preparations demonstrated that [^{3}H]-(+)SKF-10047 interacts with two receptors that are pharmacologically distinct from opioid receptors and selective for the dextrorotatory (+) isomer of SKF-10047 [5–7]. One high affinity site (σ) is sensitive to haloperidol whereas the lower affinity site selectively binds TCP (PCP site) [8] and is insensitive to neuroleptics [9]. Autoradiographic visualization of [^{3}H]-(+)SKF-10047 and [^{3}H]TCP binding sites is consistent with the concept of a dissociation of σ and PCP receptor binding sites [6, 7]. The specific involvement of the σ (haloperidol sensitive) site in the behavioral and neurosecretory activity of SKF-10047 has not been defined. Growing experimental evidence indicates that the PCP binding site is involved in both the interoceptive effects induced by PCP [10, 11] and the modulation of NMDA-evoked release of acetylcholine and/or catecholamines from rat striatum [12] and hippocampus [13–15].

Much less is known about the presence and the role of peripheral σ and PCP receptors but PCP has been shown to selectively inhibit nicotinic agonist-induced secretion of catecholamines from perfused bovine adrenal glands [16] and cultured adrenal chromaffin cells [17]. In agreement with other laboratories [18, 19], we have found that the bovine adrenal medulla contains all types of opioid receptors [20]. However, after blockade of all opioid receptor sites with a high concentration of [D-Ala^2,D-Leu^5]-enkephalin, there was some remaining binding activity for the racemer [^{3}H]-(±)SKF-10047. The residual binding activity was then attributed to the presence of σ-like receptors [18]. The present study was aimed at characterizing the adrenal medullary binding sites for the dextrorotatory (+) isomer of [^{3}H]-SKF-10047 in regard to the criteria of brain σ and PCP receptors.

MATERIALS AND METHODS

Materials. [^{3}H]-(+)SKF-10047 (40 Ci/mmol) was a product of New England Nuclear, Boston, MA. Haloperidol was obtained from the Sigma Chemical Co. (St. Louis, MO). Cyclazocine and pentazocine hydrochloride were products of Sterling Winthrop, Rensselaer, NY. DAGO, DSLET, thiorphan and

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† Abbreviations: SKF-10047, *N*-allylnormetazocine; PCP, phencyclidine, 1-(1-phenylcyclohexyl) piperidine; TCP, 1-[1-(2-thienyl) cyclohexyl] piperidine; DAGO, [D-Ala^2,Me-Phe^4,Gly-ol]-enkephalin; DSLET, [D-Ser^2,Thr^6]-Leu-enkephalin; U-69593, (5α,7α,8β)-(−)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)-1-oxaspiro-(4,5)-dec-8-yl)-benzeneacetamide; and NMDA, *N*-methyl-D-aspartate.

bestatin were purchased from Peninsula Laboratories, Belmont, CA. (+)SKF-10047 and (−)SKF-10047 were donated by Dr R. L. Hanks, NIDA, Rockville, MD. TCP and PCP were obtained from Dr H. W. Avdovich, Bureau of Drug Research, Ottawa, Canada.

Membrane preparation. Membranes were prepared from bovine adrenal medulla according to the method of Dumont and Lemaire [20]. Briefly, the medulla of bovine adrenal glands were dissected free of cortical tissue, minced with a Waring blender (2 × 20 sec) and homogenized with a glass–Teflon homogenizer in 10 vol. of ice-cold 50 mM Tris–HCl buffer (pH 7.4; buffer A). Particulate matter was removed by centrifugation at 1000 *g* for 30 min at 4° in a Sorvall SS34 rotor. The pellets were discarded, and the supernatant fraction was centrifuged at 26,000 *g* for 30 min at 4°. The resulting membrane pellets were resuspended in buffer A, incubated at 37° for 20 min, and recentrifuged at 26,000 *g* for 30 min. The final pellets were resuspended in buffer A at a concentration of 2 mg protein/ml (Lowry detection) [21] and frozen at −70°.

Binding assays. Thawed membrane suspension was diluted to a final concentration of 0.5 mg protein/ml. Typical binding assays were performed at 37° for 30 min with 2-ml aliquots of the membrane preparation in the presence of labelled and unlabelled drugs at the indicated concentrations. Incubations were terminated by placing the samples on ice for 15 min followed by filtration under reduced pressure through GF/B Whatman filters pretreated with 0.05% polyethylenimine [22]. Filters were washed with four 3-ml aliquots of ice-cold buffer A and placed in liquid scintillation vials along with 10 ml Aquasol (NEN). Radioactivity was measured in a Beckman LS 7800 liquid scintillation counter at 45% efficiency. Nonspecific binding of the tritiated ligand was determined in the presence of 2×10^{-5} M unlabelled (+)SKF-10047. Specific binding was defined as the difference between the total radiolabel bound and that bound in the presence of unlabelled (+)SKF-10047. Selective [^{3}H]-(+)SKF-10047 binding to PCP receptors was measured in the presence of 1 μM haloperidol to mask the σ binding sites. Selective [^{3}H]-(+)SKF-10047 binding to σ receptors was measured in the presence of 0.2 μM TCP to mask PCP binding sites. Binding data were analysed with the iterative curve-fitting computer program BDATA (EMF Software Inc., Knoxville, TN, USA) and the non-linear least-square computer-fitting program CDATA (EMF Software Inc.). All experiments were repeated two or three times in duplicate and the values represent the mean ± SD.

RESULTS

Binding dependence on time and protein concentration. The binding of [^{3}H]-(+)SKF-10047 was investigated as a function of time in order to determine when equilibrium was achieved (Fig. 1B). Equilibrium was reached rapidly at 37°, 75% of the specific binding being obtained at 10 min and with no statistically significant increases after 20 min. The amount bound was stable up to 40 min. The change in specific binding of radioligand was also measured as a function of protein concentration. Specific binding increased linearly as a function of protein concentration up to about 1.2 mg/2 ml (Fig. 1A). At higher concentrations, binding plateaued. Therefore, subsequent binding assays were performed at 0.5 mg protein/ml for 30 min at 37° as described in Materials and Methods. Denaturation of the tissue by boiling for 10 min before assay destroyed over 95% of the specific radioligand binding.

Characterization of two binding components. Saturable and reversible binding of [^{3}H]-(+)SKF-10047 was performed at 37° for 30 min. Analysis of saturation data in the presence of 0.2 μM TCP, utilizing the iterative curve-fitting program, revealed a high affinity component of specific [^{3}H]-(+)SKF-10047 binding with an apparent dissociation constant (K_D) of 8.3 ± 0.2 nM and a B_{max} of 67 ± 7 pmol/g protein (Fig. 2). In the presence of 1 μM haloperidol to mask the σ receptor, a lower affinity site was observed with an apparent K_D of 32.7 ± 2 nM and a B_{max} of 83 ± 6 pmol/g protein (Fig. 3).

Drug competition binding studies also indicated a discrimination of at least two sites labelled by [^{3}H]-(+)SKF-10047 in bovine adrenomedullary membranes (Fig. 4). At a 5 nM concentration of the radiolabelled ligand, competition binding studies with selective ligands, namely haloperidol (σ-site) [6] and TCP (PCP-site) [6], produced biphasic inhibition curves. The addition of low concentrations of haloperidol (10^{-9}–10^{-7} M) displaced 72% of the total [^{3}H]-(+)SKF-10047 bound while the remaining 28% of the bound radiolabel required higher concentrations for complete displacement (Fig. 4). The K_i values for the high and low affinity binding components were 3.14 and 2475 nM respectively (Table 1). In contrast, the addition of increasing concentrations of TCP revealed a 23% displacement of [^{3}H]-(+)SKF-10047 at low concentrations, while the remaining 77% of the radiolabel was displaced at concentrations of 10^{-7} to 10^{-5} M. TCP displayed K_i values of 0.88 and 1011 nM for its high and low affinity binding components respectively. The high affinity binding sites for haloperidol and TCP correspond more likely to sigma and PCP receptors respectively [6]. PCP showed marked selectivity for the PCP binding site with a K_i value of 1.3 nM as compared with 3778 nM for the σ receptor. The benzomorphans, pentazocine and cyclazocine, also produced biphasic inhibition patterns (Table 1). The high affinity component of pentazocine (K_i: 5.5 nM) corresponded to the σ site (76% displacement of total binding), whereas the high affinity component of cyclazocine (K_i: 2.75 nM) corresponded to the PCP site (18% displacement of total binding).

Specificity of [^{3}H]-(+)*SKF-10047 binding sites.* (+)SKF-10047 was more than 120 and 18.4 times as potent as the levorotatory (−) isomer at competing with [^{3}H]-(+)SKF-10047 for its PCP and σ receptors respectively (K_i of 570 and 160 nM as compared with >69,000 and 2,900 nM for the (−) form respectively; Table 2). The racemate (±)SKF-10047 was 4 times less potent than the dextroisomer at interacting with both receptors. In addition, the selective opioid receptor ligands DAGO (μ) [23], DSLET (δ) [24] and U-69593 (κ) [25] were ineffective in displacing the binding of [^{3}H]-(+)SKF-10047 (IC_{50} >

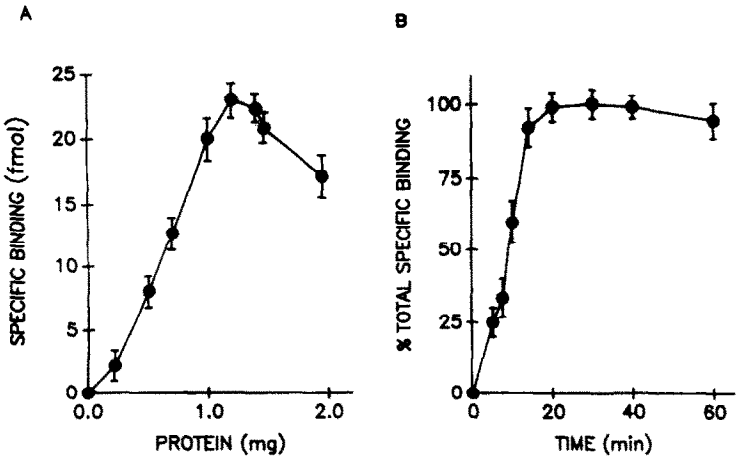

Fig. 1. [^{3}H]-(+)SKF-10047 binding as a function of time and protein concentration. (+)[^{3}H]SKF-10047 (5 nM) was incubated at 37° with increasing concentrations of adrenomedullary membrane protein for 30 min (A) or with 0.5 mg/ml of membrane protein for an increasing period of time (B). Specific binding was calculated as the difference between total and nonspecific binding measured in the presence of 20 μM unlabelled (+)SKF-10047. The maximal binding activity in B was 20 pmol/g protein. Results are the mean ± SD of two duplicated experiments (N = 4 samples per point).

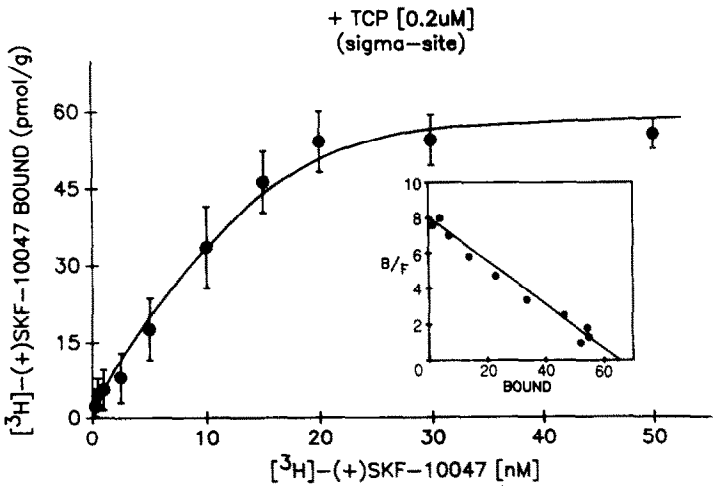

Fig. 2. Saturation curve and Scatchard plot (inset) of [^{3}H]-(+)SKF-10047 specific binding to bovine adrenal medulla membranes in the presence of 0.2 μM TCP. Binding of the tritiated ligand (1–50 nM) was carried out as described under Materials and Methods. The binding data were analysed by the BDATA Softwares program and resulted in a one-site best fit model. Results are the mean ± SD of two sets of duplicated experiments (N = 4 samples per concentration).

10,000 nM; Table 2).

DISCUSSION

The adrenal medulla is known to contain a large variety of opioid peptides [26] and opioid receptors [18–20]. The presence of the non-opioid σ receptor was first suggested by the observation of a receptor site for [^{3}H]-(+)SKF-10047 that was insensitive to a high concentration (5 μM) of [D-Ala^2,D-Leu^5-Enk (DADLE) [20]. At this concentration, DADLE is known to mask all opioid receptor sites [27]. The present data provide direct biochemical evidence for the existence of selective receptors for [^{3}H]-(+)SKF-10047 in bovine adrenal medulla. [^{3}H]-(+)SKF-10047 is known to bind to two specific sites in the brain: the σ and PCP receptors [5–7]. The adrenomedullary high affinity binding site observed after the blockade of the PCP receptor with TCP corresponds more likely to the σ receptor, since it is sensitive to haloperidol and pentazocine, two selective σ ligands [6, 28]. The adrenomedullary low affinity binding site for [^{3}H]-(+)SKF-10047 observed after the blockade of the σ receptor with haloperidol can be equated with the brain PCP receptor, being more sensitive to TCP and PCP, two compounds

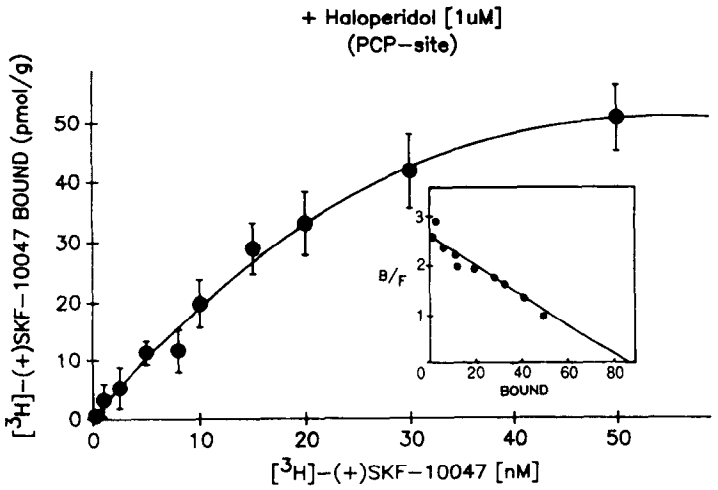

Fig. 3. Saturation curve and Scatchard plot (inset) of [^{3}H]-(+)SKF-10047 binding to bovine adrenal medulla membranes in the presence of 1 μM haloperidol. Binding of the tritiated ligand was carried out as described in the legend of Fig. 2. Results are the mean ± SD of two sets of duplicated experiments (N = 4 samples per concentration).

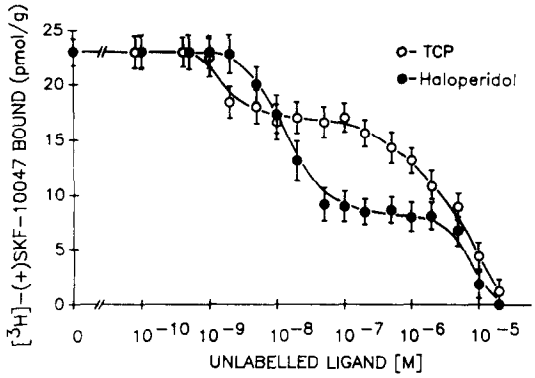

Fig. 4. Inhibition of [^{3}H]-(+)SKF-10047 binding by increasing concentrations of haloperidol and TCP. The abilities of the two compounds to compete with the binding of [^{3}H]-(+)SKF-10047 (5 nM) were determined by using sixteen concentrations of the unlabelled ligands as described in Materials and Methods. Results are expressed as the mean ± SD of two sets of duplicated experiments (N = 4 samples per concentration).

Table 1. Relative potency of various compounds in displacing the binding of [^{3}H]-(+)SKF-10047 to sigma and PCP receptors

Compound	Sigma K_i (nM)	PCP K_i (nM)
Haloperidol	3.14 ± 0.44	2475 ± 312
Pentazocine	5.5 ± 2.1	1449 ± 220
TCP	1011 ± 190	0.88 ± 0.28
PCP	3778 ± 600	1.3 ± 0.4
Cyclazocine	677 ± 80	2.75 ± 1.5

Values represent the means ± SD of three sets of duplicated experiments. [^{3}H]-(+)SKF-10047 binding was performed with a 5 nM concentration of the radiolabelled ligand as described in Materials and Methods. Competitive displacers were used at sixteen concentrations between 10^{-11} and 10^{-5} M. Biphasic competition curves were obtained, and the σ and PCP binding components were analysed with the non-linear least-square computer-fitting program CDATA. The standard deviation is derived from individual curves analysed separately.

that possess a high affinity for the PCP receptor [8, 29]. The particular high affinity of cyclazocine for the PCP-like receptor of the adrenal medulla is in agreement with its potent displacement of PCP-like drugs from brain PCP receptors [4, 29], although this compound possesses some selectivity for the brain σ receptor [30].

Initially, σ sites were defined under an opioid receptor classification as "σ opioid receptors" and SKF-10047 was the prototypic ligand [1]. Later on, a clear distinction was made between the activities of the isomers of SKF-10047. (−)SKF-10047 possessed opioid and psychotomimetic properties and bound with high affinity to μ and κ opioid receptors [30]. The dextrorotatory (+) isomer possessed psychotomimetic effects that were not antagonized by naloxone [31, 32]. Therefore, (+)SKF-10047 was proposed to interact with a novel type(s) of binding site(s) (σ receptor or others) that was insensitive to naloxone and could no longer be considered as an opioid binding site. Competition binding studies to membrane preparations of bovine adrenal medulla indicated that the binding of [^{3}H]-(+)SKF-10047 is insensitive to the (−) isomer of SKF-10047 as well as to prototypic ligands for μ (DAGO), κ (U-69593) and δ (DSLET) opioid receptors (Table 2). The ratio between the K_i of (−)SKF-10047 and (+)SKF-10047 for the adrenomedullary PCP-like receptor was more pronounced than that for the adrenal σ receptor (121

Table 2. Stereospecificity and selectivity of the binding of [^{3}H]-(+)SKF-10047 to membrane preparations of bovine adrenal medulla: Competition binding studies

Compound	PCP/σ sites IC_{50} (nM)	σ site (+0.2 μM TCP) K_i (nM)	PCP site (+1 μM haloperidol) K_i (nM)
(+)SKF-10047	128 ± 26	160 ± 38	570 ± 62
(−)SKF-10047	2,700 ± 109	2,900	> 69,000
(±)SKF-10047	482 ± 65		
DAGO	> 10,000		
DSLET	> 10,000		
U-69593	> 10,000		

Competition binding studies were performed in the presence of 0.2 μM TCP or 1 μM haloperidol to block PCP or σ sites or without discrimination of the two binding sites (PCP/σ sites). Values are expressed as the means ± SD of three sets of duplicated experiments. Binding experiments were performed as described in Materials and Methods.

as compared with 18.1, Table 2). Largent *et al.* [6] have also indicated that rat brain σ and PCP receptors interact stereospecifically with the (+) form of SKF-10047, but the stereospecificity seen in the brain was greater for the σ site (12.5 times) than for the PCP site (2 times).

Adrenal σ and PCP-like receptors resemble the brain σ and PCP receptors since they can be differentiated in the same way by their respective affinities and densities for the tritiated ligand. Thus, the σ site displays a high apparent affinity (K_D: 8.3 nM) and a low density (B_{max}: 67 pmol/g protein) while the PCP site has a lower apparent affinity (K_D: 32 nM) and a higher density (B_{max}: 83 pmol/g protein). Similar characteristics have been described for brain σ and PCP sites although rat brain receptors have been reported to have lower affinities (K_D of 42 and 615 nM) and higher densities (B_{max} of 300 and 1330 pmol/g protein) [6]. The adrenal PCP-like receptors are similar to the brain PCP receptors since they are both insensitive to low concentrations of haloperidol. They differ, however, in that high concentrations of haloperidol (> 1 μM) block the binding of [^{3}H]-(+)SKF-10047 to adrenal PCP sites (Fig. 4), whereas no such effect was observed in the brain [6, 33].

The findings that PCP and other dissociative anaesthetics can inhibit the secretion of catecholamines from perfused adrenal glands [16] and isolated adrenal chromaffin cells [17] suggest a physiological role for adrenal σ and/or PCP-like receptors. PCP and some other dissociative analgesics cause a dose-dependent inhibition of secretion evoked by nicotinic receptor stimulation. However, the basal secretion and secretion evoked by K^+ depolarization were not affected by the presence of PCP-like compounds. In the brain, the role of σ and/or PCP receptors is supported by the recent discovery of endogenous ligands that can selectively stimulate σ [34, 35] and PCP [33, 36] receptors. The existence of endogenous ligands for adrenal σ and PCP-like receptors is still unknown and constitutes a subject for further investigation.

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