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BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERIZATION OF SIGMA BINDING SITES IN NCB-20 CELLS AND STUDY OF THE INHIBITION OF AGONIST-STIMULATED PHOSPHOINOSITIDE HYDROLYSIS BY SIGMA COMPOUNDS

by

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I. GENERAL INTRODUCTION

1. BACKGROUND

A. Historical Background of the Sigma Receptor

The original work that lead to the postulation of the existence of a "sigma receptor" was conducted in the early 1970's and published in a classical paper entitled "The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog" (Martin WR et al). The purpose of these studies was to characterize the pharmacological properties of morphine and its congeners. The investigators were searching for additional evidence to support the hypothesis that the actions of agonists were mediated by interaction with several categories of receptors eliciting distinguishable pharmacological syndromes. In the chronic spinal dog model, spinal cord transection (T10) ensures that the animals are immobile and that their reflexes are preserved without disturbances by higher brain structures -- in this way, the pharmacological effects of the drugs tested can be observed clearly and precisely.

Through this model system, Martin et al observed three distinct syndromes which they attributed to the interaction of agonists at three separate receptors (mu, kappa, and sigma). Morphine was designated the prototype agonist for the mu receptor subtype. The morphine syndrome was manifested by constriction of the pupils (miosis), slow heart beat (bradycardia), drop in body temperature (hypothermia), indifference to painful stimuli (analgesia), and a general indifference to environmental stimuli. A second syndrome was that represented by the effects of the opiate alkaloid, ketocyclazocine, acting at the designated kappa receptor. The ketocyclazocine syndrome was characterized by depression of the flexor reflexes and pronounced sedation, but without production of bradycardia. SKF-10,047 (N-allylnormetazocine) was the prototype for the third syndrome whose features prompted Martin and coworkers to postulate the existence of a novel receptor they termed "sigma" (after the first initial of the prototypic drug). In contrast to morphine and

ketocyclazocine, the benzomorphan drug SKF-10,047 caused a widening of the pupils (mydriasis), a large increase in pulse rate (tachycardia) and, most interestingly, a behavioral syndrome that can be characterized as mania with a delirium-like state that included head tossing and tracking.

Findings of the unusual behavioral effects of the synthetic benzomorphan, SKF-10,047, had been previously documented. Keats and Telford conducted studies on a number of benzomorphan analogs in human beings in search of potent, nonaddicting analgesics. In the evaluation of several pharmacological parameters, SKF-10,047 was found to produce definite analgesia, but its debilitating behavioral side effects prevented the researchers from being able to establish its dose equivalent to morphine. In the end, the studies had to be abandoned due to the severity of the psychotomimetic effects of SKF-10,047 which included hallucination, disorientation, and depersonalization phenomena. In 1970, Haertzen documented the effects of SKF-10,047-like benzomorphan drugs in humans showing that overall functioning was poorer than for LSD, barbiturates, or alcohol because they combined the depressant and disorienting feelings associated with barbiturate and alcohol use, but without producing the desirable euphoria.

The strong psychotomimetic effects of SKF-10,047 and benzomorphan relatives have fascinated researchers and incited significant interest in thoroughly characterizing the sigma receptor, particularly since the behavioral syndromes seen with these drugs parallel those seen in mentally ill patients. As a result, the sigma receptor is one of two receptor sites currently viewed as potential mediators of the behavioral effects of phencyclidine (PCP) and certain benzomorphan (sigma) opiates (Sonders et al, 1988; Deutsch et al).

There has been a strong incentive to elucidate the molecular actions of sigma-specific compounds in hopes of gaining a better understanding of some of the molecular abnormalities responsible for the hallucinations characteristic of such diseased states as schizophrenia. Implicit in the undertakings of research in this area is an article of faith that etiological factor(s) of a biochemical nature are at the root of diseases like

schizophrenia. However, one must bear in mind the multifactorial nature of this syndrome as well as reckon with the likely heterogeneity of its biochemical pathology and the role of genetic predisposition (Rodnight; Barnes; Byerley et al). The central nervous system (CNS) is, after all, an integrative one, and devoting energies to the search for the "ultimate culprit" may appear rather simplistic. In essence, however, the goal is to try to identify the relevant pieces of the puzzle from which we may begin to assemble a more coherent picture of this behavioral syndrome. It is hoped that in the process, much will also be learned about the workings of the brain in the non-diseased state.

As will be reviewed in the following sections, significant progress has been made, since its postulated existence, in establishing the sigma receptor as a novel site with distinct pharmacological and biochemical characteristics. While its exact physiological role in the CNS has not yet been clearly defined, this is an area of active pursuit in the sigma field and which has recently yielded several intriguing clues.

B. The Phencyclidine Psychosis

In addition to the sigma receptor, the phencyclidine (PCP) receptor has also been hypothesized to mediate the effects of a variety of psychotomimetic drugs. Just as for the sigma binding site, interest in the PCP receptor was largely generated by the fascinating behavioral effects of the prototypic compound. Originally introduced in the 1950's, PCP ([1-(1-phencyclohexyl)piperidine]) was used experimentally as a general anaesthetic and was initially praised as such for its lack of concomittant respiratory or cardiovascular depressive side effects. However, it was eventually taken out of use due to adverse psychotic reactions experienced by 10 to 20% of the patients upon recovery from anaesthesia (post-anaesthesia emergence reactions) (Aniline and Pitts).

PCP has since resurfaced as a drug of significant abuse on the streets, appreciated by some for its induction of a unique brand of drunkenness, heightened sensitivity to stimuli, dissociation, mood elevation, and tranquilization. These desirable attributes, unlike the negative aspects,

were not experienced at every incidence of drug use. The negatives most commonly reported include perceptual disturbances, restlessness, disorientation, and anxiety. PCP is a dangerous drug capable of precipitating harmful and life-threatening pharmacological effects covering a broad range of severity ranging from disorientation to violent and unmanageable behavior to inductions of a comatose state and unresponsiveness to deep pain. These expressed behaviors parallel the degree of PCP intoxication. In some cases, intoxication has progressed to a schizophreniform psychosis of prolonged duration (lasting days or weeks despite abstinence) (Luisada and Brown). It appears that schizophrenics are at greater risk than other subpopulations for psychiatric morbidity after PCP intoxication. PCP has been observed to intensify thought disorders and affective expression in schizophrenia patients. This observation lends further credence to the general perception of the existence of a biochemically significant link between psychotomimetic effects of PCP and behavioral hallmarks of schizophrenia. As a result, PCP psychosis in humans is currently viewed as a good model for studying schizophrenia.

C. Identification of the PCP Receptor

One of the complicating factors in the investigation of the biochemical basis of the actions of PCP is its multifaceted neurochemical effects. Findings in the literature include inhibition of catecholamine and indole amine uptake in brain tissue by PCP (Smith et al), its actions as an anticholinesterase (Maayani et al), its blockade of potassium channels (Albuquerque et al; Blaustein and Ickowics; Bartschat and Blaustein) and interactions with nicotinic acetylcholine receptor channels (Eldefrawi et al), and muscarinic acetylcholine receptors (Aronstam et al). The discovery of specific and saturable [^{3}H]PCP binding sites in rat brain (Zukin and Zukin, 1979) was instrumental in focusing much of the research in this area. The significance of these high affinity binding sites (K_D = 150 nM) is seen by the good correlation between the pharmacological profile of drugs effective at displacing [^{3}H]PCP binding and their rank order of potency in discriminative stimulus and animal behavioral tests (Mendelsohn et al, 1984; Hayes and Blaster; Vignon et al, 1986). It has

been demonstrated that in addition to the PCP-like arylcyclohexamine derivatives, certain drugs belonging to the benzomorphan class (like (+)SKF-10,057 and (+)cyclazocine) are also effective displacers of [^{3}H]PCP binding. Indeed, the sigma and PCP binding sites are recognized almost equally well by PCP and some benzomorphan drugs with submicromolar affinity. However, the PCP binding site has a higher affinity for PCP and its analogs than for benzomorphan opiates whereas the converse is true for the sigma binding site. The principal pharmacological distinction between these two sites is the high sensitivity of the sigma site to haloperidol and several other classes of neuroleptics, whereas the PCP site is insensitive to these drugs.

Major advancements in the characterization of the PCP site have come with the development of the more potent and selective tritiated PCP derivative, N-(1-[2-thienyl]cyclohexyl)3,4-[^{3}H]piperidine ([^{3}H]TCP) (Vignon et al, 1983.). [^{3}H]TCP has proven to be a useful tool in the autoradiographic localization of PCP receptors in the rat brain (Contreras et al, 1986; Largent et al, 1986a). Recent anatomical localization by means of autoradiography studies have firmly established that PCP and sigma sites are physically separate entities, with different regional distibution in the brain (Gundlach et al; Largent et al, 1986a; McLean and Weber). PCP sites are distributed mainly in the thalamus, cortex, and striatum, whereas sigma sites are more concentrated in hypothalamus, limbic forebrain, midbrain, cerebellum. Sigma receptors have also been localized on human peripheral blood leukocytes (Wolfe et al, 1988), rat spleen (Wolfe et al, 1987), and in the gastrointestinal tract of the guinea pig (Roman et al, 1989a). [^{3}H]TCP binding sites are notably absent from these tissues.

Biochemical studies conducted in our laboratory provide further evidence for distinct PCP and sigma receptors. We have reported the synthesis and characterization of an affinity label for sigma-type receptors called DIGIT (di-ortho-tolyl-guanidine-isothiocyante) (Adams et al). Guinea pig brain membranes treated with nanomolar doses of DIGIT followed by extensive washing exhibit a dose-dependent, irreversible reduction of sigma-selective radioligand binding. Equilibrium saturation binding

experiments show that the inhibition of binding to sigma receptors by DIGIT pretreatment is attributable to an irreversible reduction in the affinity (increase in K_D) of sigma receptors. Importantly, DIGIT pretreatment leaves binding at PCP receptors unaffected. Similar results have since been seen in studies conducted with an isothiocyanate derivative of phencyclidine, Metaphit (Rafferty et al). Pretreatment with Metaphit produced differential effects on these two classes of receptors; while it acted as an irreversible, noncompetitive inhibitor at PCP receptors, this compound produced an irreversible, competitive inhibition at sigma receptors (Bluth et al).

D. Functional Role of the PCP Receptor

A series of electrophysiological and biochemical studies on the PCP receptor have resulted in significant progress in elucidating a major aspect of the mechanism of action of PCP. There is convincing evidence for the functional interaction of PCP with the N-Methyl-D-Aspartate (NMDA) receptor-ion channel complex. The NMDA receptor is a subtype of glutamate receptors distinguished from kainic acid (KA)-type and quisqualate (QA)-type glutamate receptors in its drug specificity profile, the conductance properties of its associated channel, as well as its distribution throughout the brain (Ascher and Nowak; Cotman et al; Cotman and Iversen). Outstanding features of the synaptic transmission of NMDA receptors include a voltage-dependent Mg^{2+} block, Ca^{2+} permeability of its ion channel, and dependence on the occupation of the neurotransmitter binding site by an agonist to effect channel opening ("agonist dependency"/"use dependency"). Early clues to the alliance between PCP and the NMDA receptor-ion channel complex emerged from electrophysiological observations of the inhibition of NMDA-evoked depolarizations by PCP and ketamine (a structural analog) (Martin and Lodge; Fagg). The rank order of PCP-like compounds in antagonizing NMDA-induced excitation correlates well with the rank order of potency for these drugs in PCP radioreceptor binding assays (Church et al).

The determination of the nature of this antagonism was facilitated by the availability of selective, competitive antagonists for the NMDA receptor

(Watkins and Olverman). PCP and its analogs act by binding to a site within the lumen of the NMDA-associated channel in an agonist-dependent manner thereby causing a noncompetitive antagonism of NMDA-type responses (Kemp et al, 1987; MacDonald; Fagg). Alluring evidence generated from work with the PCP receptor-specific compound, MK-801, suggests that, by acting as open channel blockers, these compounds may function as potent anticonvulsants (Troupin; Wong et al) and neuroprotective agents against ischemia-induced injury (Simon et al). The question arises as to whether or not MK-801 can trigger psychotomimetic effects in humans at doses comparable to therapeutic levels established in animal models. If so, then the NMDA receptor-ion channel complex would indeed be seriously implicated as the functionally relevant entity of PCP psychoses. There is reason to speculate on the possibility that MK-801 may bring about psychotomimetic effects at higher doses in humans given its behavioral PCP-like effects in pigeons, rats, and rhesus monkeys (Koek et al). It is not clear, however, just how predictive these animal behavioral models are of psychoses in humans.

E. Nomenclature of Sigma and PCP Binding Sites

The significant pharmacological cross-reactivity between the PCP and sigma binding sites, as well as the similarity in the behavioral effects precipitated by their respective prototypic drug, lead some to believe early on that both sigma- and PCP-related compounds act through a single, common binding site to exert their behavioral effects, a site that was termed the sigma/PCP receptor (Zukin and Zukin, 1981; Mendelsohn et al, 1985). This terminology seems clearly inappropiate given the unequivocal identification of the PCP and sigma sites as two distinct entities. The currently proposed nomenclature for these two sites as simply the "PCP receptor" and the "sigma receptor" arises from a classification scheme based on ligand selectivity pattern, behavioral and biological effects, mechanism of action, identification of antagonist, and putative endogenous ligands (Quirion et al, 1987) -- though several of these criteria have not been estasblished for one or either of these two sites. This terminology does away with any specific reference to opioid

receptors or NMDA receptor complex and it has been suggested that newly discovered subtypes of these receptors be classified using numbers.

While this consensus avoids some of the confusion previously found in the literature, a question still raised is whether these two sites should be referred to as "receptors" at all (Kemp et al, 1988) since, by definition, a "receptor" must be shown to link its binding site to a transduction mechanism by which agonist binding elicits a response (Bowman et al). It should therefore be pointed out that the term "receptor" is rather loosely applied in conjunction with these two sites given our current state of knowledge.

2. PHARMACOLOGICAL CHARACTERIZATION OF THE SIGMA BINDING SITE

A. The Development of Sigma-specific Radioligands for Binding Assays

In order to systematically investigate the role of sigma receptors in normal and abnormal brain function, it is imperative that a spectrum of well-characterized, highly selective, and potent sigma-receptor ligands be available for use in receptor binding assays and bioassays. The initial attempts at developing such ligands were met with some difficulty, however. It was found that tritium-labeled SKF-10,047 could not be used to selectively label sigma receptors due to cross-reactivity at another site -- apparently the mu opioid receptor (Pasternak et al). It was subsequently found that inclusion of an excess of unlabeled ligand to saturate the mu, kappa, and delta opiod receptor binding sites revealed a single component of high affinity (K_D value in the low nanomolar range), specific [^{3}H]SKF-10,047 binding, corresponding to the sigma receptor (Su). However, a more significant breakthrough came with the demonstration that selectivity for the sigma receptor could be achieved by using the dextrorotatory isomer instead of racemic [^{3}H]SKF-10,047 (Tam, 1983; Tam and Cook; Martin et al, 1984; Tam, 1985). In addition to the evidence that stereoisomers of [^{3}H]SKF-10,047 bind to different sites in brain, behavioral experiments by Khazan and collaborators and Brady et al have shown that it is indeed the (+) isomer of SKF-10,047 that is responsible

for the psychotomimetic effects of this drug. The use of (+)[^{3}H]SKF-10,047 as a sigma-specific radioligand has been rendered less valuable, however, since its cross-reactivity with the phencyclidine receptor (albeit with lower affinity) has been revealed.

More recently, a chemically distinct and potent sigma-receptor radioligand has been developed. Solomon Snyder's group has described that sigma receptors in the rat CNS can be more selectively labeled with (+)[^{3}H]3-(3-hydroxyphenyl)-N-(1-propryl)piperidine ((+)[^{3}H]3-PPP)(Largent et al, 1984), a putative dopamine autoreceptor agonist . This radioligand binds saturably and with relatively high affinity (K_D = 30 nM) to a single population of binding sites in rat brain homogenates exhibiting the hallmarks of sigma pharmacology.

Research conducted in our own laboratory has lead to the development of another ligand that has proven very useful as a moleculer probe for the sigma receptor. During a search for potential sigma receptor-active compounds, it was discovered that certain symmetrically substituted guanidines possess sigma binding activity based on their ability to compete with (+)[^{3}H]SKF-10,047 binding from guinea pig brain membrane binding sites. The structures of these compounds was determined in collaboration with the laboratory of Dr. John Keana in Eugene, Oregon. A tritium-labeled derivative of one of these compounds, 1,3-di(2-[5-^{3}H]tolyl)guanidine ([^{3}H]DTG), was then synthesized and characterized (Weber et al). We've reported that [^{3}H]DTG binds saturably and with high affinity to an apparent homogeneous population of sites in guinea pig brain membrane homogenates and slide-mounted rat and guinea pig brain sections (K_D = 28 nM and B_{max} = 84 pmol/g of brain tissue -- original wet weight). The drug selectivity profile of [^{3}H]DTG closely correlates with that of (+)[^{3}H]3-PPP in guinea pig brain membrane homogenates ($r = 0.95$; $P < 0.00001$). This disubstituted guanidine is an attractive ligand in several respects. The simplicity of its structure facilitates its synthesis as well as that of derivatives containing additional functional groups for the development of irreversible ligands as tools for receptor purification. As will be further delineated in the sections ahead, DTG is also a useful starting compound for the development of numerous congeners with

varying affinities and selectivities for sigma and phencyclidine receptors, while being, itself, essentially inactive at the phencyclidine receptor (hence its usefulness as a radioligand). Availability of this sigma-receptor probe should therefore facilitate the identification of physiological, biochemical, and pharmacological properties of sigma receptors in brain.

B. Hallmarks of the Sigma Receptor Drug Selectivity Profile

Development of the highly specific and potent sigma receptor radioligands cited above has permitted the establishment of a unique drug specificity profile for this binding site. Its prominent characteristics include: 1) preference for the dextrorotatory isomer of benzomorphan compounds (i.e. (+)pentazocine, (+)cyclazocine, and (+)N-allylnormetazocine); 2) insensitivity to naloxone; 3) high affinity for the potent, clinically useful antipsychotic, haloperidol; 4) insensitivity to dopamine and apomorphine; and 5) stereoselectivity for (-) butaclamol, which is opposed to that of the dopamine D_2 receptor (Seeman; Su; Tam and Cook; Martin et al, 1984; and Largent et al, 1984). Thus, the sigma site is distinct from the phencyclidine and dopamine receptors, and the original designation of the sigma site as an opioid receptor subtype, as suggested by Martin WR et al, has proven to be misleading by virtue of the fact that its stereoselectivity and insensitivity to naloxone are the reverse of the hallmarks of classical opiate receptor pharmacology.

An important finding in the characterization of the sigma site is its high affinity for the neuroleptic, haloperidol, one of the primary drugs used in the treatment of schizophrenia. Secondary to its antipsychotic effects, however, haloperidol produces tardive dyskinesia (Snyder and Largent). Both the therapeutic and undesirable side effects of this drug have been attributed to its blockade of dopamine receptors. It has been shown, however, that radioactively labeled haloperidol interacts with both dopamine D_2 receptor sites and sigma sites (Tam and Cook). This evidence not only supports the postulated physiological relevance of the sigma site, but it brings into question which of the two receptor types is responsible for mediation of the observed behavioral effects of haloperidol. A related

finding is that the sigma receptor binds all synthetic opiate drugs known to have hallucinogenic and psychotomimetic effects. The intriguing possibility exists, therefore, that sigma receptor antagonism may be an effective therapeutic strategy for at least some types of human psychoses (Deutsch et al; Snyder and Largent).

C. Structural Determinants of the Sigma Binding Site

Recent studies of structure-activity relationships have been conducted to develop receptor models for the sigma binding site. Conformational analysis by computer-assisted molecular modeling techniques reveal a high percentage of molecular volume coincidence upon superimposition of haloperidol, (+)SKF-10,047, (+)3-PPP and DTG on the coordinates of a sigma receptor model (Manallack and Beart; Manallack et al). The receptor site topographies of PCP and sigma sites can be distinguished and it has therefore been reasoned that the models can be used to predict the activity of drugs with PCP and/or sigma affinity. The common pharmacophore for the sigma site is a 3- or 4-phenylpiperidine ring system and a lipophilic N-substituent (Largent et al, 1987). A similar model has also been presented by Manallack et al, featuring a "primary pharmacophore" and "lipophilic cleft" along with a hypothesized narrow hydrophobic pocket to accomodate the primary pharmacophore of the proposed model. Conformational calculations indicate a fairly wide range of tolerance for distances between the primary aromatic ring and the amine nitrogen, which may account for the potency at sigma sites of structures with considerable diversity.

In conjunction with our collaborators, we have synthesized a number of DTG analogs and evaluated their respective structure-activity relationships in *in vitro* radioligand binding displacement assays with guinea pig brain homogenates, using the sigma-specific radioligands [^{3}H]DTG and (+)[^{3}H]3-PPP, and the PCP receptor-specific compounds [^{3}H]TCP and [^{3}H]MK-801 (Scherz et al). It was found that a wide array of structural variations are associated with high affinity binding at the sigma site. All of the compounds tested (more than 70 DTG congeners) were several orders of magnitude more potent in sigma receptor than in

the PCP receptor binding assays, with a few exceptions. Our data are largely consistent with the models discussed above.

Separate studies have shown that certain alterations to the parent DTG molecule generate compounds with increased affinity for PCP receptors -- a somewhat predictable result given the considerable cross-reactivity of many sigma ligands with PCP receptors and vice versa. Interestingly, these molecules also behave as noncompetitive NMDA receptor antagonists with neuroprotective properties in an *in vitro* assay (Keana et al). Hence, these results identify a new class of drugs for development as potential therapeutic agents against excitatory amino acid-induced neuronal cell death.

3. THE SIGMA RECEPTOR: AN ALTERNATIVE STRATEGY FOR THE DEVELOPMENT OF NOVEL ANTIPSYCHOTIC DRUGS

A. The Hypothesis of Hyperdopaminergic Transmission and Schizophrenia

The effectiveness of neuroleptics to attenuate certain symptoms of schizophrenia correlates well with their affinity for the dopamine D_2 receptor. As a result, a hypothesis for hyperdopaminergic transmission in schizophrenia has been formulated (Seeman; Rodnight; Snyder and Largent). It appears, furthermore, that blockade of D_2 receptors accounts for both the therapeutic and extrapyramidal side effects (EPS) commonly associated with neuroleptic treatment. Postsynaptic stimulation of D_2 receptors in the limbic system is believed to mediate the cognitive and affective symptoms of disease, whereas stimulation of these sites in the striatum is thought to play a role in the debilitating side effects. These two separate limbs of dopamine (DA) transmission can be modeled in animal behavioral paradigms. Pharmacologists have traditionally assessed the antipsychotic potential of test compounds on the basis of antagonism of behavioral effects of the dopamine agonist, apomorphine. Potency for antagonism of apomorphine-induced fighting in paired male rats and climbing behavior in mice shows a good correlation with drug antipsychotic efficacy. On the other hand, antagonism of apomorphine-

induced stereotypic behaviors in rodents by test drugs is predictive of EPS and the emergence of tardive dyskinisia.

B. The Development of Atypical Antipsychotics

Given the predictive value of the aforementioned behavioral paradigms, it has been reasoned that a compound exhibiting selective blockade of climbing and aggressive behavior without antagonism of stereotyped behaviour (such as sniffing, gnawing, grooming, and rearing of hind legs) might be an ideal therapeutic drug. The pharmacology of BW234U, or rimcazole (cis-9-[3-(3,5-dimethyl-1-piperazinyl)propyl]carbazole dihydrochloride), fits just that criteria (Ferris et al, 1982). Rimcazole is an atypical antipsychotic in that it appears to be devoid of EPS liability and does not act directly with D_2 receptors. Evaluation of this compound in clinical trials has shown promising therapeutic effects in at least some patients without evidence of EPS (Chouinard and Annable; Schwarcz et al). Remarkably, rimcazole is inactive at a variety of receptor sites, including dopamine and PCP, while displaying moderate affinity against sigma-specific (+)[^{3}H]3-PPP binding (IC_{50} = 1.5 uM, Snyder and Largent) and (+)[^{3}H]SKF-10,047 binding (IC_{50} = 500 nM; Ferris et al, 1986) in rat brain tissue. Thus, it seems likely that the antipsychotic behavioral profile of rimcazole is mediated by binding at sigma receptors (it should be noted that development of rimcazole as a novel antipsychotic has been terminated because of grand mal seizures seen in several patients).

Enthusiasm for the development of antipsychotic agents not based exclusively on blockade of central dopaminergic receptors has been strengthened by the discovery of additional sigma-preferring compounds with antipsychotic potential, the most potent and selective of which is one developed by the Brystol Myers Company, BMY 14802 (Taylor et al; Taylor and Dekleva). Just as with rimcazole, BMY 14802 was identified on the basis of its desirable behavioral profile in animal models and was subsequently shown to be a high affinity ligand for the sigma site (K_D value < 100 nM). Similarly, BMY 14802 is devoid of activity at the PCP site and shows little affinity for the dopamine D_2 receptor.

C. Evidence for a Relationship Between Sigma Receptor Activation and Dopaminergic Transmission

While these new-generation antipsychotics do not appear to interact directly with D_2 receptors, a functional relationship between sigma sites and dopaminergic transmission can not be ruled out. In fact, there is mounting evidence on a variety of fronts to support such a link. An autoradiographic study of the distribution of specific [^{3}H]DTG binding sites in the nigrostriatal system of the cat shows a high density of haloperidol-displaceable sites in a discrete subdivision of the substantia nigra pars compacta, which also has heightened D_2-related and reduced D_1-related ligand binding (Graybiel et al). This sigma-rich zone is a pharmacologically and connectionally distinct subdivision of the substantia nigra leading the authors to recognize the possibility that sigma ligands may act selectively on a subset of dopaminergic pathways interconnecting the midbrain and the stiatum. Of course, anatomical localization does not necessarily imply cellular localization, but the data nonetheless suggest a means by which neuroleptics with sigma affinity might interact with dopaminergic systems in a functional way.

Electrophysiological evidence for a sigma-dopaminergic system link can also be found. Using single cell recording techniques to study the influence of sigma receptor activation on dopamine neurons in vivo, Steinfels and Tam showed that intravenous administration of (+)3-PPP dose-dependently inhibited the firing rate of DA neurons, whereas similar administration of BMY 14802 caused the opposite effect. The (+)3-PPP-induced inhibitory effects were completely and directly reversed by BMY 14802; pretreatment with BMY 14802 decreased the potency of (+)3-PPP. This indicates apparent modulation of DA neuronal activity via sigma-receptors, thereby potentially bypassing the EPS effects associated with direct interaction with D_2 sites.

The nigrostriatal (A9) DA system is the candidate pathway for the production of the adverse side effects of classical antipsychotic drugs, whereas the mesolimbic and mesocortical (A10) DA systems are credited with the mediation of their therapeutic effects (Mathysse; Stevens;

Snyder et al). Thus, electrophysiological models can be applied for evaluation of the therapeutic potential and EPS liability of test compounds. In accordance with such models, BMY 14802 was found to exert a differential effect on A9 and A10 DA systems (not involving interaction at DA receptors) in a way that reflects its reduced potential to cause EPS (Wachtel and White). An indirect influence on DA cellular firing has also been invoked for the actions of rimcazole (Piontek and Wang), and SKF10-047 (Freeman and Bunney) in electrophysiological studies.

4. BIOCHEMICAL AND MOLECULAR PROPERTIES OF SIGMA RECEPTORS

The development of a photoaffinity ligand for the sigma receptor would clearly be advantageous in furthering our understanding of the molecular properties of this site. To this end, a tritium-labeled, photosensitive azide derivative of DTG was synthesized and characterized in our laboratory (Kavanaugh et al, 1988). This photoaffinity ligand, 1-(4-azido-2-methyl[6-^{3}H]phenyl)-3-(2-methyl[4,6-^{3}H]phenyl)guanidine, or [^{3}H]N_3DTG, was found to bind specifically and saturably to the same sites as those recognized by [^{3}H]DTG (the correlation of K_i values of various drugs in the two binding assays was quite high, with $r = 0.97$). Incubation of guinea pig brain membranes with [^{3}H]N_3DTG in the absence or presence of excess unlabeled drug followed by exposure to UV light resulted in the selective labeling of a single polypeptide of Mr 29,000 as revealed by $NaDodSO_4$/Polyacrylamide gel electrophoresis (SDS/PAGE). In these covalent labeling studies, the membrane suspensions are filtered unto glass fiber filters (to minimize labeling of nonspecific sites) prior to UV exposure. When irradiated membranes were solubilized from the filters with $NaDodSO_4$ or sodium cholate and then subjected to gel permeation chromatography under nondenaturing conditions, this 29 kDa polypeptide is eluted as part of a complex with Mr = 150,000. When the material comprising the 150 kDa peak was concentrated and subjected to SDS/PAGE analysis, a single peak of radioactivity corresponding to Mr 29,000 was detected; no higher molecular weight band could be seen. These results, taken together, imply that covalent labeling with [^{3}H]N_3DTG identifies the putative binding subunit of the sigma-type receptor which is apparently a

membrane-bound entity since solubilization with detergent is required for its isolation.

It was reasoned that it may be possible to identify other subunits comprising the native complex by developing a second sigma-specific photoaffinity label structurally different from [^{3}H]N_3DTG. Efforts were therefore made to synthesize a tetraflouroarylazido derivative of [^{3}H]NH_2DTG. Unfortunately, once this particular ligand was purified and assayed in radioreceptor binding assays, it became clear that its high nonspecific binding (close to 90%) precluded its usefulness as a purification tool.

Further biochemical characterization of sigma sites has been made possible by conducting experiments with sodium cholate-solubilized membrane preparations. Having established that the solubilization protocol generated sigma binding sites with near identical pharmacological specificity as those seen with particulate membranes (Kavanaugh et al, 1989), photoaffinity labeling experiments were conducted with solubilized brain membrane preparations. [^{3}H]N_3DTG was found to specifically label a single polypeptide, Mr 29,000, identical in size to that labeled in intact membranes. When solubilized guinea pig brain membranes were incubated with [^{3}H]DTG, (+)[^{3}H]3-PPP, or (+)[^{3}H]SKF-10,047, and then passed over a Sepharose CL-6B chromatography column for sizing analysis, the putative native complex eluted as a peak of radioactivity corresponding to Mr 669,000 which could be completely displaced by incubation with 10 uM haloperidol. Therefore, these data support the hypothesis that all three ligands bind to the same macromolecular complex in membranes. It is not clear how the 150 kDa complex identified in solubilized, photoaffinity-labeled membranes is related to the 669 kDa macromolecular complex.

Isolation and purification of the 29 kDa protein is an area of active pursuit in our laboratory. The initial attempts at purifying this protein from guinea pig brain membranes involving a combination of techniques including electroelution, ion-exchange and hydrophobic interaction chromatography of N_3DTG-labeled guinea pig brain membranes, however,

did not prove to be successful. Since conditions for solubilization of the active sigma receptor complex had by then been established in our laboratory, a strategy was devised based on the purification of the sigma receptor (or subunit(s) thereof) from solubilized guinea pig brain membranes by affinity chromotography. The affinity column was prepared by coupling NH_2DTG to the activated ester of Affigel (this reaction was monitored by including an aliquot of [^{3}H]NH_2DTG). It was apparent from [^{3}H]DTG binding assays that sigma-specific radioligand binding sites were being lost to the column after loading of the sample overnight. However, a series of elution schemes was tried and the results indicated that we were unsuccessful in specifically eluting protein from the column. The current strategy being employed involves purification of the receptor from solubilized guinea pig liver homogenates by affinity chromatography, but using a different sigma ligand coupled to the solid matrix (i.e. perphenazine coupled to activated sepharose). It has been found that solubilized protein which does not bind to this column is devoid of sigma-specific radioligand binding activity. Protein bound to the affinity column can be eluted with DTG, and sigma-specific radioligand binding recovered following dialysis (to remove DTG). Thus, this protocol appears promising as a means of purifying the sigma receptor.

Evidence in the literature can also be found suggesting that different sigma-active ligands can be differentially affected by protein modifying treatments, and that binding of sigma-receptor ligands may be regulated by guanine nucleotides and ions. Thus, Bowen et al (1989) have proposed a multi-site model of the sigma receptor based on studies probing the effects of irradiation of rat brain membranes with light of 254 nm on the binding parameters of the selective sigma radioligands (+)[^{3}H]3-PPP, [^{3}H]DTG, and (+)[^{3}H]SKF-10,047 and their displacement by unlabeled compounds. They concluded that, since the binding charateristics of haloperidol, DTG, and (+)3-PPP were affected similarly by irradiation treatment, they likely interacted with a particular, shared domain of the sigma receptor. This domain is supposedly different from that recognized by (+)SKF10,047 and (+)pentazocine, since UV irradiation failed to cause a significant change in their ability to displace radioligand binding. Itzhak and Khouri (1988) have shown that (+)[^{3}H]3-PPP displays agonist-like

binding properties as evidenced by its sensitivity to GTP. The biphasic nature of the displacement of specific (+)[^{3}H]3-PPP binding by (+)cyclazocine in rat brain membranes can be altered by including the GTP analog, Gpp(NH)p, in the competition binding assays (Itzhak). A recent study by Beart et al, investigating the regulation of central sigma-binding sites, also demonstrates the ability of GTP to influence the interconversion of high and low affinity sites seen in the displacement of (+)[^{3}H]3-PPP by (+)SKF10,047 and (±)cyclazocine.

Just what these data tell us about the physiologically relevant state of the sigma receptor can not be definitively surmised at this point. To be sure, it is important that these initial findings be reproduced by other investigators. In addition, it is of paramount importance that "agonists" and "antagonists" at this site be clearly defined to permit deciphering of the apparent complexities of the regulation of sigma receptor activity.

5. PHYSIOLOGICAL FUNCTION OF THE SIGMA RECEPTOR

The characterization of the actions of sigma ligands in behavioral and pharmacological studies has seen much progress, but there have been far fewer reports on the functional role of the sigma receptor. Although it is possible to infer a role for the sigma receptor in these studies, the question of its biological significance is still left unanswered. Elucidation of the physiological role of the sigma receptor would be significantly facilitated by the identification of veritable receptor antagonists or endogenous ligand(s), neither of which has been achieved to date. While there is evidence for the existence of an endogenous sigma-specific ligand, or "sigmaphin" (Quirion et al, 1984; Sonders et al, 1986; Su et al, 1986; Contreras et al, 1987; Su and Vaupel; Chavkin and Neumaier), its purification and structural determination remain unresolved. It has been reported that progesterone (Su et al, 1988) and neuropetide Y (Roman et al, 1989b) are naturally occurring compounds with strong affinities for sigma sites (K_i = 268 nM and IC_{50} = 10 nM for displacement of (+)[^{3}H]SKF-10,047 by progesterone and neuropeptide Y, respectively). It is a matter of speculation, however, as to whether or not

interaction with the sigma receptor by any of these candidate endogenous ligands is responsible for mediation of their actions.

It is imperative that the effects of sigma-active compounds (active with respect to binding affinity for the sigma site) be shown to be actually mediated by sigma receptors to establish the biological relevance of these receptors. Several studies can now be found in the literature whose primary objectives have been to verify or rule out sigma receptor involvement in a variety of experimental paradigms. To date, a role for sigma receptors has been implied in neural regulation of motor behavior (Bowen et al, 1988b; Walker et al), inhibition of phosphoinositide hydrolysis (Bowen et al, 1988a), modulation of NMDA-evoked responses (Malouf et al; Campbell et al, manuscript submitted), and modulation of transmitter release (Campbell et al, 1987; Vaupel and Su; Campbell et al, 1989; Campbell et al, manuscript submitted).

Walker et al chose to investigate the possible role of sigma receptors in the motor effects of antipsychotic drugs. To accomplish this, they microinjected various sigma and control compounds into the red nucleus of the rat brain and measured postural effects (i.e. torticollis/rotation of the head). They found that DTG, haloperidol, and (+)SKF-10,047, elicited torticollis in a dose-dependent, reversible manner and with a rank order of potency corresponding to their binding affinities at the sigma site. These effects were observed at relatively low concentrations (doses ranged from 1.5 nmol to 18.6 nmol microinjected in a volume of 0.5 ul over 72 seconds). A control, non sigma-active antipsychotic drug, devoid of motor effects in humans, was also tested and failed to produce dystonia. PCP was also without effect. When the investigators microinjected DTG into the substantia nigra (an area recognized as having a role in the control of movement), stimulation of circling behavior was observed. These results suggest a biological role for the sigma receptor in the control of movement. In corroboration, Bowen et al (1988b) have reported that a strain of mutant rats which spontaneously develop severe dystonia also exhibit markedly abnormal sigma-receptor binding characteristics compared to control animals (the authors note that the binding parameters

for dopamine, beta-adrenergic and muscarinic cholinergic receptors are unaffected).

Campbell et al (1987) have shown that (+)3-PPP dose-dependently, rapidly, and reversibly causes an augmentation of the electrically-evoked twitch in the mouse vas deferens. The maximum effect was 251% of control and half-maximal effects (EC_{50}) were achieved at 57 uM (+)3-PPP. When norepinephrine content of the bathing solution was measured after a 20 minute exposure to 200 uM (+)3-PPP, a three-fold increase over control was noted. Thus, it appears in this system that (+)3-PPP acts as an agonist to enhance the release of norepinephrine evoked by electrical stimulation. Intracellular recordings in CA1 pyramidal cells indicate that (+)3-PPP (1 uM - 1 mM) enhances the NMDA-induced excitatory cellular response (Malouf et al). The observed enhancement by 1 uM (+)3-PPP is antagonized by 10 uM DTG, although the nature of this antagonism has yet to be defined. Further proof of sigma receptor involvement in these studies requires the testing of more sigma drugs.

Studies conducted with a well-characterized bioassay, the guinea pig longitudinal muscle/myenteric plexus (LMMP) preparation, have revealed the presence of functional sigma receptors responsible for the inhibition of electrically- or agonist-induced contractions of the LMMP by sigma-active compounds (Campbell et al, 1989; Campbell, manuscript submitted). Sigma compounds were effective at concentrations in the low micromolar range. Their potency in the bioassay strongly correlated with their IC_{50} values against [^{3}H]DTG binding. Two novel sigma-inactive DTG congeners were included in this study, bridge-DTG and dimethylguanidine; both congeners failed to influence stimulated contractions. The responses evoked by serotonin (5-HT) and NMDA at their respective receptors were noncompetitively inhibited by sigma compounds; these drugs caused a reduction in maximum contractile response in a dose-dependent manner. Sigma compounds did not appear to act directly on the muscle itself. Additional data suggest a neuronal mechanism for sigma receptor function whereby interaction of "agonists" at the sigma receptor leads to a reduction in acetylcholine release. Unlike all other sigma ligands tested, (+)SKF-10,047 and (+)cyclazocine potentiated contractions of the

stimulated muscle strips. However, the potentiation was not sensitive to the presence of naloxone (an opioid receptor antagonist) or 500nM DTG and was due to a leftward shift of the dose response curve in the case of 5-HT.

To what extent the inhibitory action mediated by the sigma receptor in the ileum is predictive of a drug's antipsychotic efficacy still needs to be determined. Perhaps an understanding of the biochemical pathway(s) affected by sigma receptor activation would help delineate the precise mechanism by which sigma compounds alter neurotransmission and help reconcile some of the apparently contradictory observations (for example, (+)3-PPP is observed to cause an increase in stimulated norepinephrine release in the mouse vas deferens and a decrease in acetylcholine release in the guinea pig LMMP; DTG acts as an antagonist of the actions of (+)3-PPP on NMDA-evoked responses in CA1 neurons and yet appears to be an agonist in the negative modulation of PI hydrolysis; haloperidol is viewed as an antagonist at the sigma receptor as judged by its insensitivity to Gpp(NH)p in drug displacement assays, and as an agonist in effecting down regulation of sigma receptors after chronic treatment (Matsumoto) and in inhibiting agonist-stimulated PI turnover (Bowen et al, in press).

Conceivably, some of the qualitative differences in the effects of sigma ligands that are seen between various experimental protocols are a function of differences in neuronal input, or the nature of the experiment (i.e. *in vitro* versus *in vivo* studies) and that, at the cellular level, there is a common, principal sigma receptor-mediated event(s) underlying these disparate effects. The identity of the particular biochemical pathway that may be modulated has remained largely unknown. Recently, however, work from the laboratory of Dr. Wayne Bowen suggests that sigma receptors negatively modulate agonist-stimulated phosphoinositide (PI) hydrolysis (Bowen et al, 1988). In this initial study, the rank order of potency for the sigma ligands (+)pentazocine, DTG, haloperidol, and dextrallorphan to inhibit carbachol-stimulated PI turnover in rat brain synaptoneurosomes correlated well with their affinity at sigma receptors. Though preliminary, these findings support the notion that sigma

receptors are somehow linked to the phosphoinositide signaling system in an inhibitory mode.

6. THESIS OBJECTIVES

The principal objectives of this study were: 1) to characterize the sigma binding site in NCB-20 cells with sigma-specific radioligands in solubilized and particulate cell membranes; 2) to characterize the sigma binding protein in NCB-20 cells with the photoaffinity label, [^{3}H]N_3DTG; 3) to characterize the effect of sigma compounds on the phosphoinositide (PI) hydrolysis signaling system in NCB-20 cells; and 4) to investigate the mechanism by which sigma drugs modulate PI turnover. The importance of this work lies in its potential for increasing our understanding of the biological role of the sigma receptor in normal cellular function. It may then be possible to elucidate or propose a pathogenic mechanism of psychosis in humans.

Prior to the initiation of these studies, it had already been reported in the literature that specific sigma receptor binding sites could be found on membranes of a neuroblastoma-brain hybrid cell line, NCB-20 cells (Largent et al, 1986a; Kushner et al), as evidenced by labeling with (+)[^{3}H]SKF-10,047 and (+)[^{3}H]3-PPP. We therefore chose to extend characterization of sigma sites in NCB-20 cells using two selective radioligands developed in our laboratory, 1,3-di(2-^{3}H]tolyl)guanidine) ([^{3}H]DTG) (Weber et al), and its photosensitive azide derivative, 1-(4-azido-2-methyl[6-^{3}H]-phenyl)-3-(2-methyl[4,6-^{3}H]phenyl)guanidine ([^{3}H]N_3DTG) (Kavanaugh et al, 1988). Biochemical and pharmacological studies were conducted on both particulate and solubilized NCB-20 cell membranes and compared with results obtained in our laboratory with guinea pig brain membranes, the source of tissue for much of the early work on the identification and characterization of sigma binding sites.

Preliminary studies published by Bowen et al (1988a), using rat brain synaptoneurosomes, indicated that sigma ligands can modulate the accumulation of inositol phosphates stimulated by the cholinergic agonist, carbachol. These findings formed the basis for our studies of the

interactions of sigma ligands with the phosphoinositide cellular signalling system in NCB-20 cells. We were interested in investigating the nature of these interactions and the possibility that they may represent sigma-receptor mediated events. It was reasoned that identification of the biochemical pathway(s) modulated by sigma receptor activation might provide an effective framework for studying and deciphering more precisely the mechanism(s) by which sigma-specific ligands exert their biological effects. A clonal neurotumor cell line should provide a useful model for studying molecular/cellular mechanisms of action, because it represents a homogeneous population of cell types and is therefore devoid of the complexity of cell-cell interactions of the intact CNS.

II. MATERIALS AND METHODS

Cell culture: NCB-20 cells were grown as monolayer cultures at 37^{0}C in Dulbecco's modofied Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS) or 5% fetal calf serum (FCS)/5% NCS (no differences were observed in binding parameters or growth characteristics between cells grown in either condition) in the presence of HAT from SIGMA (final concentrations: 100 uM hypoxanthine, 400 nM aminopterin, and 16 uM thymidine). Frozen stocks of cells were prepared in DMEM/10% FCS/HAT/10%DMSO, stored as 1 ml aliquots in freezing vials (NUNC), and placed in liquid nitrogen. Cell stocks were maintained in culture by passaging in T80 flasks (NUNC) from 4 to 6 weeks at which point they were replaced by freshly thawed cells from frozen stocks. For harvesting, cells were subcultured into 150-mm culture plates (NUNC) for 3 days to a density of approximately 2 x 10^7 cells/150-mm plate (90% confluency). Cells were easily harvested by briefly rinsing each plate with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline to detach the cells from the plate, followed by resuspension in growth medium. The cells collected were pelleted and stably stored at -70^{0}C until crude cell membranes were prepared from them.

Crude cell membrane preparation and solubilization: Frozen cell pellets were thawed and cells pre-swelled in 10 vol of ice-cold 50 mM Tris/HCl

(pH7.4) for 20 minutes on ice. Cells were then homogenized with a polytron homogenizer and the homogenate spun for 10 min at 45,000 x g at 4°C in a Sorvall RC5B centrifuge. The pellet was resuspended in Tris, homogenized, and spun down again in the same manner. The final pellet was resuspended in Tris and membrane protein concentration was determined by dye-binding protein assay (Bradford) available from Bio-Rad, using bovine serum albumin as the standard. The yield approximated 2.5 mg membrane protein/2 $x10^7$ cells. The membranes were usually freshly prepared for use in radioligand binding assays, although they could be stored in the refrigerator and used in binding assays over the course of three days without detectable loss of radioligand binding.

For solubilization with the ionic detergent sodium cholate, membranes were prepared according to the published protocol of Kavanaugh et al (1989). Cells membrane suspensions were prepared as indicated above, with the addition of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA), and resuspended at a final concentration of 10 mg/ml. Sodium cholate (1 M) was added to give a final concentration of 20 mM Na-cholate/50 mM Tris (pH7.4) and cell membranes were solubilized at 4°C for 2 hrs by stirring with a Teflon stir bar. Unsolubilized material was pelleted by ultracentrifugation at 100,000 x g for 60 min in a pre-cooled Ti50 rotor. The supernatant was filtered through a 0.22 um filter. On average, 18-30% of membrane protein was solubilized by this procedure.

Radioligand binding assays: Sigma receptor binding assays were conducted using the radioligands [^{3}H]DTG (46 Ci/mmol; custom synthesis, Amersham, Buckinghamshire, U.K.) and (+)[^{3}H]3-PPP (98 Ci/mmol, New England Nuclear). To a 12 x 75 mm polystyrene culture tube was added 200 ul of membrane suspension, 25 ul of [^{3}H]DTG or (+)[^{3}H]3-PPP to yield a final concentration of 1.9 nM, and 25 ul of unlabeled drug or buffer (50 mM Tris/HCl, pH 7.4). The protein concentration in the 250 ul final incubation volume was 800 ug/ml and corresponded to a concentration within the linear range for specific binding. Nonspecific binding was defined as that remaining in the presence of 10 uM haloperidol for both radioligands. Specific binding constituted 90-95% of total binding for both radioligands.

The assays were conducted in duplicate or triplicate replicates and incubated for 90 min at room temperature (both radioligands showed stable binding for at least 3 1/2 hrs at this temperature). Incubations were terminated by addition of cold buffer and rapid filtration through Whatman GF/B glass-fiber filters under vacuum, using a 48-well cell harvester (Brandel, Gaithersburg, MD), to separate bound from free ligand (no. 32 glass-fiber filters from Schleicher and Schuell were found to be equally effective). The filters were washed twice with 2 ml of buffer. Total filtration and washing time did not exceed 20 sec. Each filter was dissolved in scintillation fluid (Cytoscint, from Westchem, San Diego, CA) and radioactivity was measured by liquid scintillation spectrometry at a counting efficiency of approximately 50%.

Sigma radioreceptor binding assays in solubilized cell membranes were performed as for membrane-bound receptor except that glass-fiber filters were pretreated at 4°C with the cationic polymer polyethylenimine (PEI) to immobilize the soluble receptors to the filter and minimize nonspecific binding (Bruns et al). The material solubilized in 20 mM Na-cholate/50 mM Tris was first diluted with Tris to give a final concentration of 5 mM Na-cholate to optimize conditions for radioligand binding experiments.

For muscarinic receptor binding assays, (-)[^{3}H]QNB was used (46 Ci/mmol, New England Nuclear) at a final concentration of 0.1 nM in 50 mM Tris, pH 7.4. Nonspecific binding was defined as that remaining in the presence of 10 uM atropine and equalled approximately 25% of total radioligand binding. Assays were conducted as described above for [^{3}H]DTG and (+)[^{3}H]3-PPP binding except that final concentration of membrane protein was 400 ug/ml, corresponding within the linear range for specific binding.

Equilibrium binding analysis: Drug displacement curves of [^{3}H]DTG and (+)[^{3}H]3-PPP binding were composed of ten concentrations of unlabeled displacing drug, typically ranging from 300 pM to 10 uM, for displacement of binding of a fixed concentration of radioligand. The inhibition constant (K_i) for each drug was obtained by analyzing the data using the EBDA (McPherson) and LIGAND (Munson and Rodbard) programs on an IBM Personal Computer AT.

The EBDA program conducts a linear regression of log-logit transformed displacement data (a plot of log [%bound / (100- % bound)] against [displacer]) to obtain initial parameter estimates. These estimates are then used as starting points for the iterative part of the program which generates more accurate estimates of the binding parameters, including slope factor (Hill coefficient) and IC_{50} value. The displacement curve is then viewed as a Hofstee plot which provides a visually clearer indication of the presence of receptor subtypes (Molinoff et al). The Hofstee diagram is a plot of % displaced against (% displaced / [displacing ligand]). Segments of this plot can be specified for linear regression analysis in the case of marked curvi-linear relationship of the data. In this way, adequate initial estimates can be provided for testing the goodness-of-fit of models involving displacement of radioligand from one or two sites. The weighted non-linear curve fitting program, LIGAND, was used to analyze the data from radioligand binding experiments to obtain K_i and slope values and test model hypotheses. The criteria for rejecting or accepting a particular model is based on the generated probability values and the degree of standard error associated with the final parameter estimates.

Competition studies of (-)[^{3}H]QNB binding were constructed as for sigma-specific radioligands, except the concentrations of displacing drugs covered a higher range (typically from 1 uM to 1 mM). IC_{50} values were determined by interpolation from diplacement-curve plots on semilogarithmic graph paper and K_i values were calculated using the Cheng-Prusoff equation: $K_i = IC_{50} / [1 + ([trace]/K_D)]$, where K_D = 129 pM.

Equilibrium saturation studies with (+)[^{3}H]3-PPP and [^{3}H]DTG were performed such that a set amount of radioligand was added to each tube and the concentration of ligand increased by adding progressively larger amounts of unlabeled ligand. Effectively, a greater amount of radioligand is being added but of progressively lower specific activity. Nonspecific binding is determined at the first concentration of radioligand (highest specific activity). The data were first processed by EBDA for the construction of Scatchard and Hill plots, both of which are

transformations of the data obtained when the amount of binding is measured as a function of the concentration of unbound radioligand (a saturation curve). LIGAND files of the data were also produced to analyze the goodness-of-fit of a one-site vs two-site model of radioligand binding.

According to the Scatchard equation ($[Bd]/[F] = - [Bd]/K_D + B_{max}/K_D$), a plot of the concentration of ligand bound, [Bd], versus the concentration of the receptor-ligand complex divided by the concentration of free ligand, [Bd]/[F], has a slope equal to the negative reciprocal of the equilibrium dissociation constant, $-1/K_D$, and the intercept on the abscissa corresponds to the concentration of binding sites (or "B_{max}" value, when the concentration of binding sites is defined in units of moles/mg of protein). The shape and/or slope of Scatchard plots from data generated under steady state conditions are a function of the definition of nonspecific binding used, the accuracy of the measurement of free ligand concentration, the degree of homogeneity of receptor sites and purity of the radioligand, the extent of cooperative interactions, and the formation of ternary complexes (Molinoff et al; Bennett and Yamamura).

Once the concentration of binding sites has been measured, then the saturation curve can also be depicted as a Hill plot which measures the log [Bd/B_{max}-Bd] as a function of the log [F]. The slope of this plot equals 1.0 when the binding reaction follows the principles of mass action (no cooperativity in binding), and a slope of 1.0 is always associated with a linear Scatchard plot (although this does not necessarily imply that binding is a simple bimolecular reaction).

(-)[^{3}H]QNB saturation binding studies were performed by incubating a given receptor population with increasing amounts of radioligand (final concentrations of 50 pM to 6 nM), nonspecific binding being estimated at each concentration of radioligand. The data were processed and analyzed, as for (+)[^{3}H]3-PPP and [^{3}H]DTG binding, by EBDA and LIGAND computer programs.

Kinetics of [^{3}H]DTG binding: For experiments on the kinetics of [^{3}H]DTG binding, association and dissociation time course experiments in NCB-20 cell and guinea pig brain membranes were conducted at 4^{0}C to obtain greater accuracy of measurements at earlier time points. The kinetic association constant (k_1) was calculated applying pseudo-first order conditions (only a small fraction (<10%) of the ligand is bound at equilibrium) (Weiland and Molinoff). k_1 was determined from the pseudo-first order linear plot of the time course: ln $[LR]_e$ / ($[LR]_e$ - [LR]) vs t, where $[LR]_e$ = the concentration of ligand-receptor complex at equilibrium, and [LR] = the concentration of ligand-receptor complex at time t. In this instance, the slope, k_{obs}, of the pseudo-first order plot is equal to k_1 x ($[L]_t[R]_t/[LR]_e$), where $[L]_t$ = the total concentration of ligand, and $[R]_t$ = the total concentration of binding sites (measured independently by Scatchard analysis). The dissociation rate was determined by addition of excess DTG (5 uM) at various time points after equilibrium was reached. The dissociation constant, k_{-1}, is equal to the negative slope of the linear plot ln [LR] / $[LR]_0$ vs t, where $[LR]_0$ = the concentration of the drug-receptor complex just prior to the addition of a competing ligand (i.e. 5 uM DTG).

Photoaffinity labeling with [^{3}H]N_3DTG: The cell membrane suspensions were prepared as described above with a final protein concentration of 800 ug/ml. The presence of protease inhibitors (1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and bacitracin at 2.5 mg/ml) throughout the membrane preparation and incubation had no effect on labeling efficiency or specificity. The procedure followed for photoaffinity labeling was that described by Kavanaugh et al (1988). Typically, 1-ml aliquots of membrane suspensions at 1 mg/ml were incubated in the dark for 2 hr at room temperature with 10 nM [^{3}H]N_3DTG (about 1 x 10^6 cpm) alone or in the presence of various unlabeled drugs to determine specificity of photoaffinity labeling. The suspensions were then rapidly filtered through a 2.4-cm Whatman GF/B glass-fiber filter by using a Hoefer (San Francisco) FH224V filtration apparatus and washed with two 5-ml aliquots of Tris. The filters were then placed on ice and exposed to long wavelength (366 nm) UV light by using a 500-W UV lamp (Sunjet 400T deluxe, Electrolux-Kern GmbH, Gottingen, F.R.G.) at a distance of 10 cm for 15 min. Membrane proteins were solubilized from the filters by

incubating filters in either 0.2% $NaDodSO_4$ or 50% acetonitrile/0.1% TFA (in preparation for SDS/PAGE) or 20 mM sodium cholate in Tris (in preparation for gel permeation studies) for 4 hr at 4°C in 20-ml borosilicate glass vials with constant shaking.

$NaDodSO_4$/PAGE and Sepharose CL-6B Chromatography: Following photoaffinity labeling, $NaDodSO_4$-solubilized membranes were lyophilized and dissolved in sample buffer before loading and running on a 12% discontinuous acrylamide gel system (Laemmli) under reducing conditions. Gels were electrophoresed at 30 mA for 4 hr followed by fixing in a solution of 50% (vol/vol) methanol and 10% (vol/vol) acetic acid and staining with 0.25% Coomassie blue R-250. After destaining overnight (in a solution of 10% methanol/7% acetic acid), the gels were impregnated with EN^{3}HANCE (New England Nuclear) for 1 hr at room temperature before soaking in 20% MeOH and 5% glycerol in H_2O for 1 hr at room temperture with shaking. Gels were then dried down and exposed to x-ray film (Kodak X-Omat AR) for an appropiate amount of time (days to weeks) at -70°C before developing. Gel permeation chromatography of photoaffinity-labeled, cholate-solubilized membrane proteins was done on a 2x100 cm Sepharose CL-6B column (Pharmacia) equilibrated at room temperature in Tris containing 20 mM sodium cholate. The samples were eluted at a flow rate of 20 ml/hr. Fractions (2.5-ml) were collected, and aliquots (1-ml) dissolved in Aquasol (New England) or Ecolite (ICN Radiochemicals) scintillation fluids, and radioactivity counted. Alternatively, solubilized receptor preparations containing approximately 2.0 mg of protein/ml in 50 mM Tris(pH7.4) with 20 mM cholate was incubated with 1.0 uCi/ml of [^{3}H]DTG for 2.5 - 3.0 hr at 25°C. The sample (4-ml) was then applied to a 2 x 100-cm Sepharose CL-6B column equilibrated in 50 mM Tris with 20 mM cholate at 4°C. The column was eluted at a flow rate of 20 ml/hr, fractions collected, and aliquots counted.

Labeling cell membrane phosphoinositides and extraction of water-soluble inositol phosphates: Cells were maintained at 37°C until the extraction of inositol phosphates was begun. On day 1, cells were seeded in Falcon 6-well plates at a density of 5.0 x 10^5 cells per well in DMEM/10%FCS/HAT. On day 2, 2.5 uCi [^{3}H]myo-inositol (New England Nuclear) was added to

each well to label membrane phosphoinositides and stimulation of phosphoinositide (PI) turnover was begun the following day. Typically, cells were preincubated with Li^+ in the labeling medium for 15 min prior to the addition of test drug (i.e sigma compound). Five min later, cells were exposed to agonist (carbachol or bradykinin) for 20 min before extraction of water-soluble inositol phosphates was begun. The procedure followed for isolation of inositol phosphates was that described by Muldoon et al with few alterations. Stimulation of PI hydrolysis was stopped by removal of agonist upon aspiration of medium, and cells were then rinsed twice with 2 ml of ice-cold physiological salt solution (PSS: 118 mM NaCl, 4.7 mM KCl, 3.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10 mM glucose, and 20 mM Hepes, pH7.4) and extracted with 1 ml of 0.2M formic acid for 20 min on ice. Acid extracts were transfered to 12x75 mm tubes. Wells were rinsed with 2 ml of ice-cold H_2O and the rinses pooled with the extracts. Extracts were neutralized with the addition of 500 ul of 0.4 M ammonium hydroxide to each tube. Samples were either immediately run over ion-exchange columns for separation of inositol phosphates, or stored for up to three days at 4^0C prior to being chromatographed.

Separation of polyinositol phosphates: The columns were made from Dowex-1 ion exchange resin (Sigma 1-8x400; basic anion exchange group is trimethylbenzyl ammonium) which was first cleaned and converted to the formic-acid conjugated form. Columns were set up in 3 cc syringes, each with 1-ml bed volume, and thoroughly washed with H_2O prior to application of the samples. Bound inositol phosphates were eluted from the column with increasing concentrations of ammonium formate according to Berridge et al. Briefly, after loading samples on to the columns, the columns were rinsed with H_2O to remove free inositol, and "IPA" wash buffer (5 mM disodium tetraborate/60 mM sodium formate) to remove glycerophosphoinositol. The inositol phosphates were then sequentially eluted with increasing concentrations of ammonium formate (0.2 M to 1.1 M) in 0.1 M formic acid. Since no changes in the levels of $[^3H]InsP_2$, $[^3H]InsP_3$, or $[^3H]InsP_4$ could be detected between control and carbachol-stimulated cells, the columns were subsequently eluted only with 2 x 2-ml of 0.2 M ammonium formate/0.1 M formic acid to measure

[^{3}H]InsP_1 levels. 0.5 ml aliquots from the collected fractions were counted in Cytoscint scintillation fluid. Columns were re-used after washing with 8 ml of 2.0 M ammonium formate/0.1 M formic acid and rinsing extensively with H_20. Columns were re-used up to 5 times with no detectable difference in elution profile.

III. RESULTS

1. PHARMACOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF SIGMA BINDING SITES IN NCB-20 CELLS

A. Brief Introduction

The haloperidol-sensitive sigma binding site is one of two receptor candidates for the mediation of the central actions of certain psychotropic drugs (Deutsch et al; Sonders et al, 1988; Snyder and Largent). The other postulated site is the phencyclidine (PCP) receptor. Biochemical and anatomical studies have demonstrated that, although many ligands crossreact with both sites, the sigma binding site and the PCP receptor are two separate entities (Gundlach et al; Largent et al, 1986a; Adams et al). Pharmacologically, the sigma binding site displays high affinity for benzomorphan drugs with preference for their dextrorotatory isomers, potent binding by the neuroleptic drug haloperidol, and insensitivity for the opioid antagonist naloxone (Su; Tam, 1983; Martin et al, 1984; Tam and Cook). Benzomorphan drugs and PCP and its analogs have been shown to produce psychotomimetic symptoms in humans similar to those of schizophrenia (Keats and Telford; Heartzen; Aniline and Pitts). The importance of the sigma site is underscored by the discovery of potent sigma-selective agents with antipsychotic potential (Deutsch et al).

While significant progress has been made in characterizing its pharmacology and biochemistry, the physiological role of the sigma binding site in the central nervous system is less clear. Recent studies indicate that sigma activation can modulate neurotransmitter release in mouse and guinea pig peripheral tissues (Campbell et al, 1987; Campbell

et al, 1989), interfere with central control of motor activity in rats (Walker et al), and interfere with stimulated phosphoinositide hydrolysis in rat brain synaptoneurosomes (Bowen et al, 1988a). It would seem useful to develop an *in vitro* cell culture system to investigate the functional role of the sigma site and its mode of action. Identification of an appropriate cultured cell line should provide a relatively simple model for such studies.

NCB-20 cells represent a hybrid neurotumor cell line obtained by Sendai virus-induced fusion of N18TG2 cells (a mouse neuroblastoma clone) with fetal Chinese hamster brain cells dissociated from 18-day embryos (Minna et al). Haloperidol-sensitive sigma sites have been shown to be present in NCB-20 cells as evidenced by the drug selectivity profile of (+)[^{3}H]3-PPP (Largent et al, 1986b) and (+)[^{3}H]N-allylnormetazocine ((+)[^{3}H]SKF-10,047) binding (Largent et al, 1986b and Kushner et al, respectively) in cell membranes. These cells appear to be devoid of [^{3}H]TCP binding sites which makes them desirable since sigma receptor function can likely be examined in isolation from PCP receptor activity. In addition, NCB-20 cells have served as a useful model in the study of various nerve cell functions (MacDermott et al; Berry-Kravis et al, 1984; Berry-Kravis and Dawson, 1985; Berry-Kravis et al, 1988; Chuang and Dillon-Carter; Zhu and Chuang). We have conducted studies to extend characterization of the sigma site in NCB-20 cells using the selective radioligand [^{3}H]DTG (Weber et al) as well as its photosensitive azide derivative, [^{3}H]N_3DTG (Kavanaugh et al, 1988). We report our data compiled from work with particulate as well as solubilized NCB-20 cell membranes.

B. Equilibrium Saturation Binding Analysis of [^{3}H]DTG and (+)[^{3}H]3-PPP Binding in NCB-20 Cell Membrane Suspensions

Equilibrium saturation binding curves for [^{3}H]DTG and (+)[^{3}H]3-PPP in NCB-20 membrane suspensions reveal binding to saturable, high affinity sites (fig. 1). Scatchard analysis of the equilibrium binding data (fig. 1, inset) reveals linear plots for [^{3}H]DTG and (+)[^{3}H]3-PPP binding with K_D values of 62 nM and 66 nM, respectively (table 1), which is quite consistent with

Figure 1. Scatchard analysis (inset) of equilibrium saturation binding curves for [^{3}H]DTG(A) and (+)[^{3}H]3-PPP(B) binding in NCB-20 cell membrane suspensions (values = mean ± s.e.m.; n = 5 - 6).

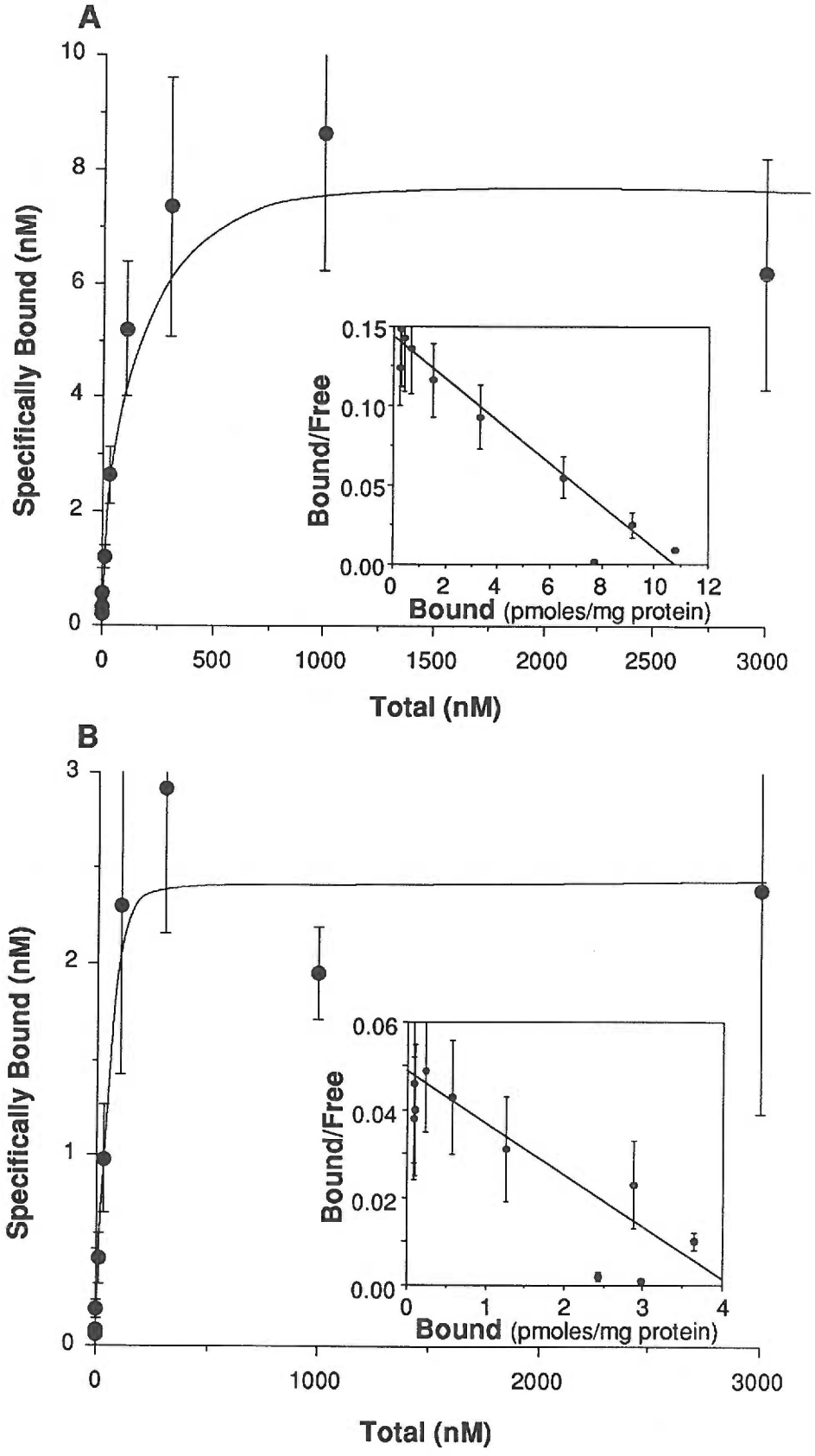

A
Specifically Bound (nM)
Total (nM)
Bound/Free
Bound (pmoles/mg protein)
B
Specifically Bound (nM)
Total (nM)
Bound/Free
Bound (pmoles/mg protein)

Table 1. Scatchard analysis of [^{3}H]DTG and (+)[^{3}H]3-PPP binding in NCB-20 cell membranes

membrane preparation	[^{3}H]DTG		(+)[^{3}H]3-PPP	
	K_D (nM ± s.e.m.)	B_{max} [a]	K_D (nM ± s.e.m.)	B_{max} [a]
Particulate	62 ± 7	10.6 ± 3.5	66 ± 9	3.8 ± 1.0
Solubilized	116 ± 26	7.0 ± 1.8	146 ± 59	7.9 ± 1.9

Each experiment was performed in triplicate; n = 4-5.

[a] $\left(\frac{\text{pmol}}{\text{mg protein}} \pm \text{s.e.m.}\right)$

those previously reported in other tissues (Largent et al, 1984; Weber et al). Analysis of the binding data with the curve-fitting program LIGAND (Munson and Rodbard) showed higher compatibility with a one-site model of binding for both sigma-specific radioligands. Noticeably, the data indicate a disparate maximum number of specific binding sites (B_{max}) for [^{3}H]DTG and (+)[^{3}H]3-PPP binding (10.6 ± 3.5 and 3.8 ±1.0 pmol/mg cell membrane protein, respectively).

Perhaps [^{3}H]DTG recognizes a pool of sites, or an additional receptor state, unbound by (+)[^{3}H]3-PPP, with equivalent potency as the sites it shares with (+)[^{3}H]3-PPP. Should this be the case, Scatchard analysis would fail to distinguish two K_D values for [^{3}H]DTG binding and would still generate the monophasic plot seen . In an effort to resolve this discrepancy, the characteristics of [^{3}H]DTG binding were examined in the presence of the sigma ligand (+)pentazocine (Fig. 2 and table 2). The concentration of 300nM for (+)pentazocine was chosen on the basis of its effectiveness at displacing greater than 80% of specific (+)[^{3}H]3-PPP binding, while interfering with only 20% of specific [^{3}H]DTG binding (see fig. 7). Presumably, under these conditions, the sites shared by both sigma radioligands would be almost fully masked, leaving the remaining [^{3}H]DTG sensitive sites for analysis. When equilibrium saturation binding analyses are performed for [^{3}H]DTG binding in the presence of 300nM (+)pentazocine, the K_D value is two fold greater compared to control. A two-fold difference in affinities for [^{3}H]DTG between the presumed two sites in this model is not large enough to be discriminated by Scatchard analysis, which may explain, at least in part, the apparent conflicting results ([^{3}H]DTG and (+)[^{3}H]3-PPP, both sigma-specific radioligands, apparently each recognizing a single population of sites, but disparate number of sites).

A few more equilibrium saturation binding experiments were conducted in our attempts to characterize the nature of the interaction between [^{3}H]DTG- and (+)[^{3}H]3-PPP-labeled sites (Table 2). Scatchard analysis of (+)[^{3}H]3-PPP binding in the presence of DTG resulted in a 100% increase in its K_D value compared to control without a significant change in B_{max}. This is indicative of competitive inhibition between these two ligands.

Figure 2. Scatchard analysis of [^{3}H]DTG binding in NCB-20 cell membranes in the absence or presence of 300 nM (+)pentazocine. Closed circles = in the absence of (+)pentazocine; open circles = in the presence of (+)pentazocine (values = mean ± s.e.m.; n = 4 - 6).

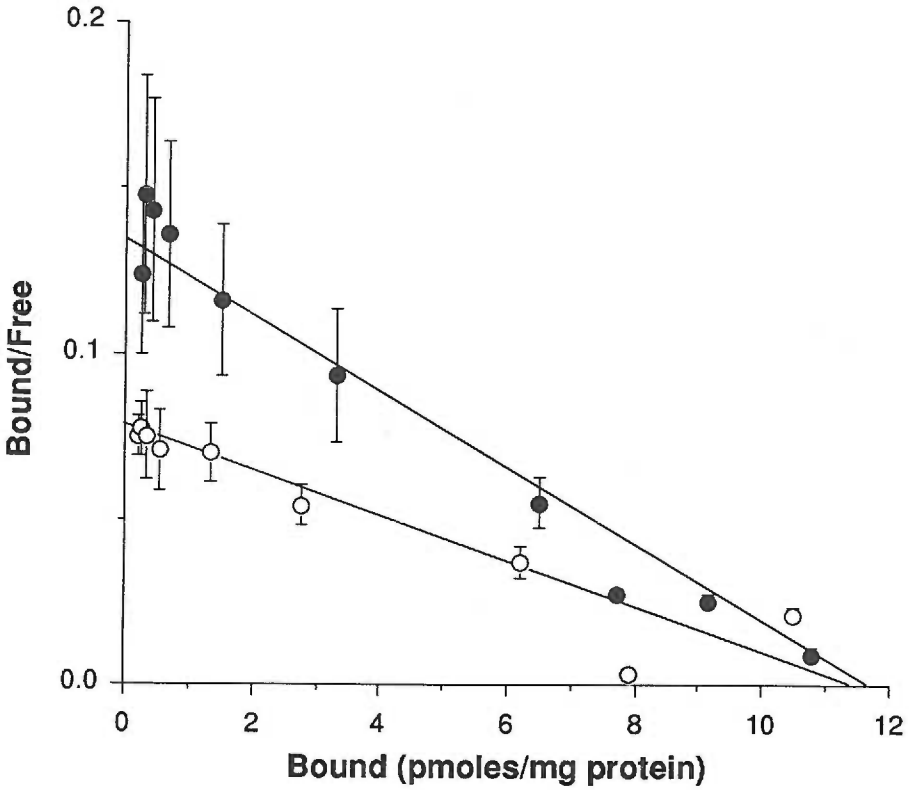

0.2
0.1
0.0
Bound/Free
0
2
4
6
8
10
12
Bound (pmoles/mg protein)

Table 2. Scatchard Analysis of [^{3}H]DTG and (+)[^{3}H]3-PPP Binding in NCB-20 Cell Membranes in the Absence or Presence of an Additional Sigma Compound

A. Condition	[^{3}H] DTG K_D*	[^{3}H] DTG B_{max}*
Control	100 ± 15	100 ± 33
300nM (+)pentazocine	197 ± 30	160 ± 19

n=4-6

B. Condition	[^{3}H] DTG K_D*	[^{3}H] DTG B_{max}*
Control	100 ± 15	99 ± 16
100 nM (+) 3-PPP	85 ± 23	69 ± 11
700 nM (+) 3-PPP	131 ± 22	62 ± 9

n=3

C. Condition	(+)[^{3}H]3-PPP K_D*	(+)[^{3}H]3-PPP B_{max}*
Control	100 ± 10	98 ± 4
100 nM DTG	124 ± 25	79 ± 15
300 nM DTG	199 ± 22	93 ± 10

n=3

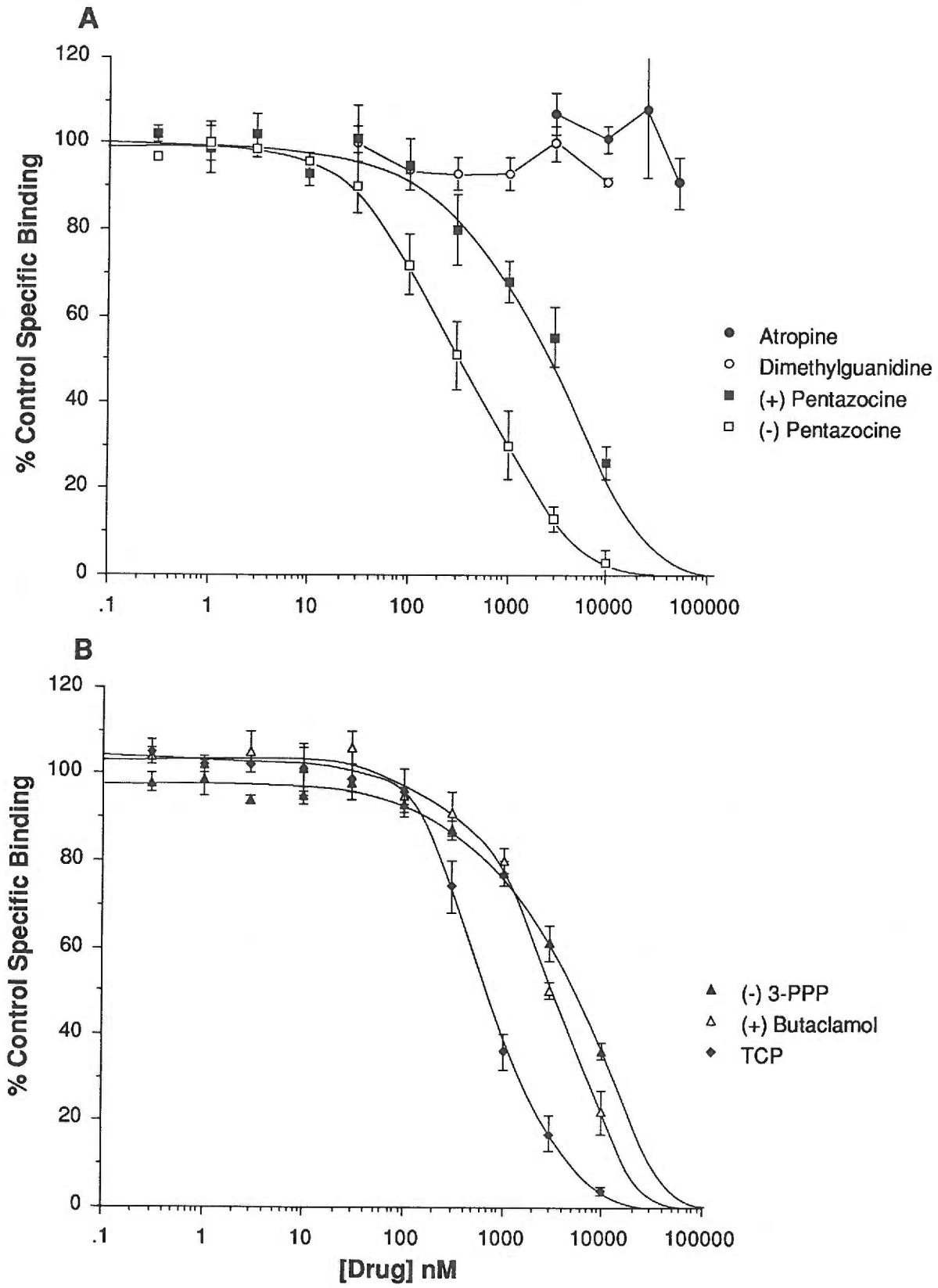
* (% control ± s.e.m.)

However, it should be noted that this change in the K_D for (+)[^{3}H]3-PPP binding required the presence of 300nM DTG, a concentration approximately four fold greater than its own affinity. Theoretically, one would have expected such a competitive interaction to be seen at doses of DTG more equivalent to its own K_D value (i.e. 67 nM). When the reverse is done, and [^{3}H]DTG saturation binding is measured in the presence of 100nM or 700nM (+)3-PPP, an approximate 30% reduction in the maximum number of [^{3}H]DTG binding sites is seen -- hence, this inhibition appears to be noncompetitive in nature.. Taken together, these data indicate that the interaction between these two ligands is complex in nature. It appears that (+)3-PPP and DTG interact competitively at a common site and that binding of (+)3PPP at this site noncompetitively inhibits binding of DTG at a second, unidentified site. The shared site apparently has a drug specificity profile which resembles the pharmacology of the prototypical sigma binding site described in other tissues.

C. Drug Specificity of [^{3}H]DTG and (+)[^{3}H]3-PPP Binding in Particulate NCB-20 Cell Membranes

The equilibrium binding curves for displacement of specific [^{3}H]DTG binding in NCB-20 membranes are displayed in figures 3 and 9A and the results of drug specificity testing are listed in Table 3. In particular, the pharmacological profile for displacement of [^{3}H]DTG binding indicates that the benzomorphan opiates (i.e. (+)SKF-10,047, (+)cyclazocine, and (+)pentazocine) are strikingly poor displacers of [^{3}H]DTG binding in membranes prepared from NCB-20 cells (K_i values in the low micromolar range). The decrease in potency is of greater magnitude for the (+) benzomorphan isomers to the point of reversing the stereoselectivity for dextrorotatory enantiomers charateristic of the sigma binding site. Thus, (-)SKF-10,047, (-)cyclazocine, and (-)pentazocine have inhibition constant values (K_i) from four to twenty times lower than that of the respective (+)enantiomers. Figure 4A illustrates the correlation between the pharmacological profile for [^{3}H]DTG binding in NBC-20 cells compared to that in guinea pig brain membranes (IC_{50} values were used in this analysis instead of K_i values in order to directly compare data from NCB-20 cells with those of our previously published results in guinea pig brain (Weber

Figure 3. (A - E) Binding curves for displacement of specific [^{3}H]DTG binding in NCB-20 particulate membranes by various compounds (values = mean ± s.e.m.; n = 2 - 4).


A
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% Control Specific Binding
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Atropine
Dimethylguanidine
(+) Pentazocine
(-) Pentazocine
B
120
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% Control Specific Binding
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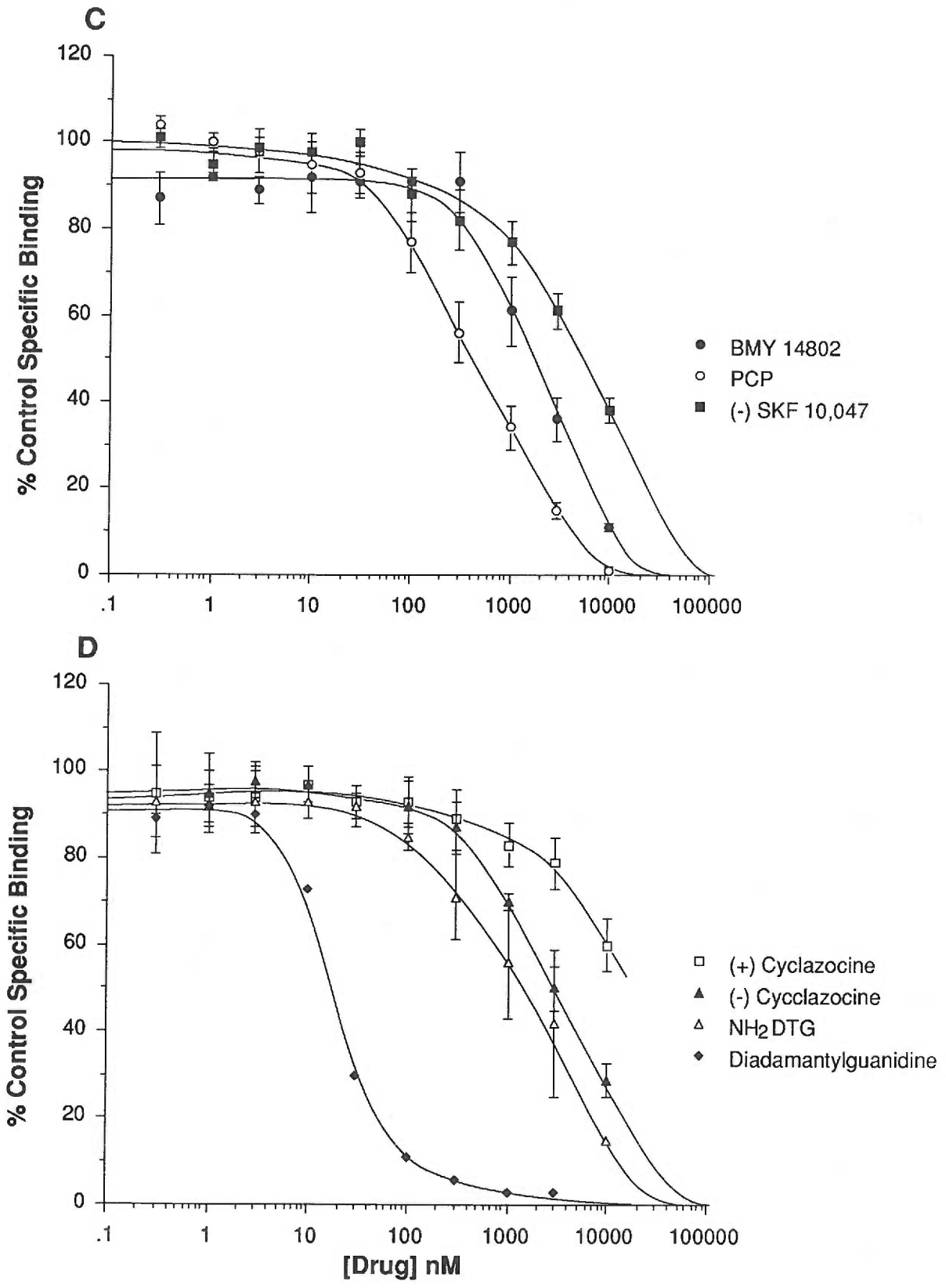
[Drug] nM
(-) 3-PPP
(+) Butaclamol
TCP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BMY 14802
PCP
(-) SKF 10,047
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% Control Specific Binding
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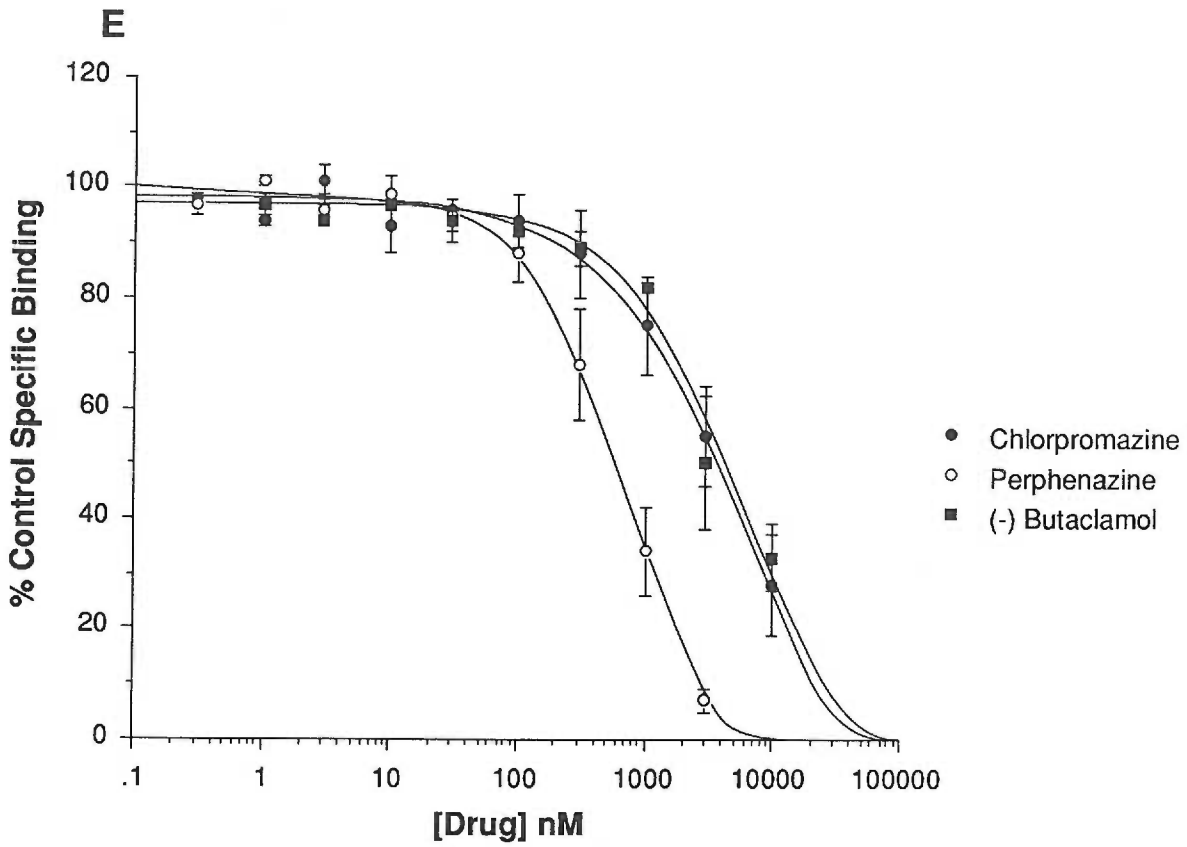
[Drug] nM
(+) Cyclazocine
(-) Cycclazocine
NH2 DTG
Diadamantylguanidine


E
120
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.1
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100
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10000
100000
[Drug] nM
Chlorpromazine
Perphenazine
(-) Butaclamol

Table 3. Displacement of [^{3}H]DTG and (+)[^{3}H]3-PPP binding in NCB-20 cell membranes

	[^{3}H]DTG		(+)[^{3}H]3-PPP	
Drug	**K_i (nM ± s.e.m.)**	**slope (± s.e.m.)**	**K_i (nM ± s.e.m.)**	**slope (± s.e.m.)**
DTG	62 ± 7 [a]	0.96 ± .05	128 ± 17	1.05 ± .14
(+)3-PPP	790 ± 184	0.88 ± .04	66 ± 9 [a]	0.93 ± .13
(-)3-PPP	3500 ± 600	0.95 ± .15	649 ± 98	0.82 ± .10
(+)SKF10,047	> 10,000	-----	22 ± 1	1.09 ± .05
(-)SKF10,047	2900 ± 1100	0.66 ± .14	1648 ± 360	0.87 ± .06
Haloperidol	532 ± 212	0.64 ± .10	43 ± 21	1.01 ± .13
(+)Cyclazocine	> 10,000	-----	100 ± 9	1.01 ± .09
(-)Cyclazocine	2650 ± 536	0.83 ± .04	320 ±224	1.11 ± .06
(+)Pentazocine	3300 ± 1600	0.84 ± .09	53 ± 5	1.04 ± .06
(-)Pentazocine	153 ± 46	0.97 ± .01	63 ± 15	0.96 ± .04
Perphenazine	357 ± 5	1.16 ± .02	93 ± 12	1.31 ± .02
(+)Butaclamol	2600 ± 107	0.87 ± .14	2150 ± 750	1.12 ± .05
(-)Butaclamol	3900 ± 2100	0.91 ± .03	458 ± 48	1.16 ± .02
Chlorpromazine	5500 ± 1500	1.10 ± .03	1550 ± 50	1.23 ± .05
TCP	604 ± 116	1.11 ± .04	296 ± 75	0.91 ± .02
PCP	600 ± 192	0.90 ± .03	865 ± 35	0.77 ± .29
NH_2DTG	2700 ± 1700	1.10 ± .30	6200 ± 4800	0.85 ± .15
DiAdguanidine [b]	11	1.20	250	0.83
Dimethylguanidine	>250,000	-----	>250,000	-----
BMY 14802	2200 ± 1100	1.22 ± .29	850 ± 2	1.23 ±.28
Atropine	>200,000	-----	>250,000	-----

The average displacement of specific [^{3}H]DTG binding at 10uM in NCB-20 cell membranes was less than 100% for the following drugs: (+)SKF10,047=35%, (-)SKF10,047=57%, (+)cyclazocine=39%, (-)cyclazocine=67%, (+)pentazocine=68%, (+)butaclamol=75%, (-)butaclamol=80%, and chlorpromazine=67%. Each experiment was run in triplicate; n = 2 - 4 (except diadamantylguanidine; n = 1).

[a] K_D value.

[b] DiAdguanidine = Diadamantylguanidine.

Figure 4. (A)Correlation plot of IC_{50} values for displacement of specific [^{3}H]DTG binding in NCB-20 cell vs guinea pig brain membranes.

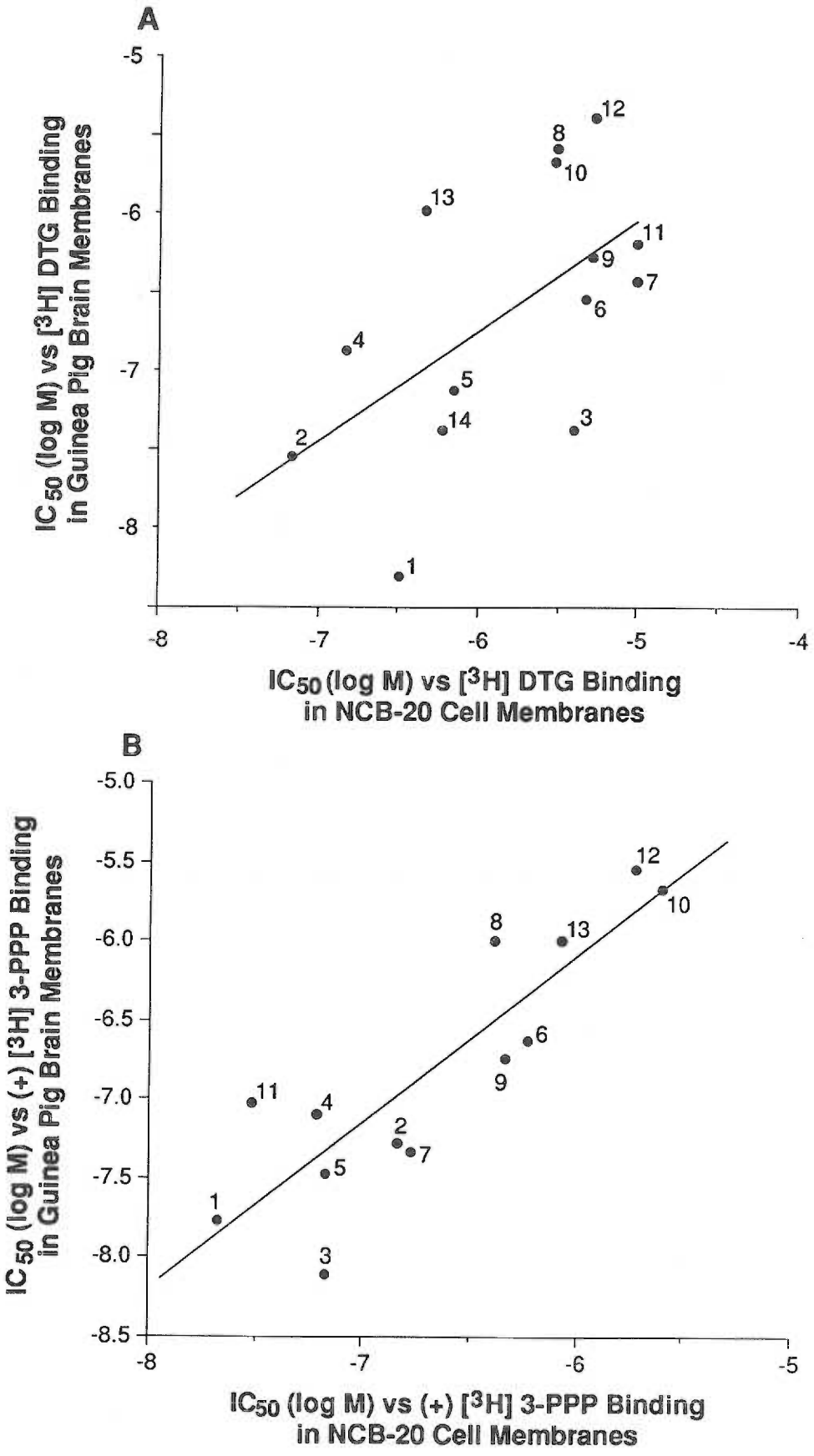
r = 0.58($P<0.05$) when the IC_{50} values for (+)cyclazocine and (+)SKF-10,047 vs [^{3}H]DTG binding in NCB-20 cell membranes are each set at 10 uM

r = 0.59($P<0.05$) when (+)cyclazocine and (+)SKF-10,047 are excluded from the analysis.

(B)Correlation plot of IC_{50} values for displacement of specific (+)[^{3}H]3-PPP binding in NCB-20 cell vs guinea pig brain membranes.

r = 0.87($P<0.001$)

1. haloperidol; 2. DTG; 3. (+)pentazocine; 4. (-)pentazocine; 5. (+)3-PPP; 6. (-)3-PPP; 7. (+)cyclazocine; 8. (-)cyclazocine; 9. (-)butaclamol; 10. (+)butaclamol; 11. (+)SKF-10,047; 12. (-)SKF-10,047; 13. PCP; 14. perphenazine.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5
14
3
2
1
IC50 (log M) vs [3H] DTG Binding in Guinea Pig Brain Membranes
IC50 (log M) vs [3H] DTG Binding in NCB-20 Cell Membranes
B
-5.0
-5.5
-6.0
-6.5
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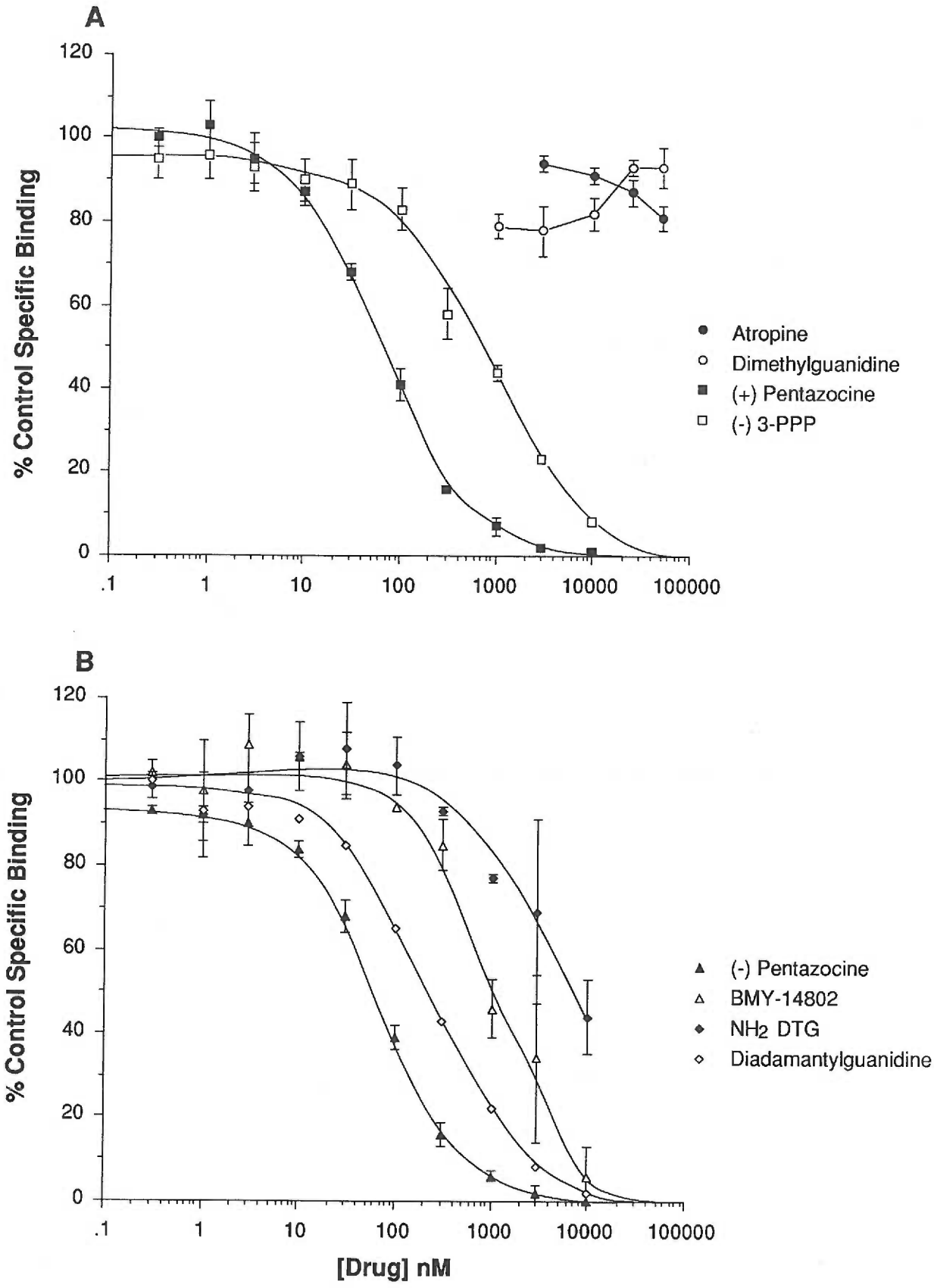
7
5
1
3
IC50 (log M) vs (+) [3H] 3-PPP Binding in Guinea Pig Brain Membranes
IC50 (log M) vs (+) [3H] 3-PPP Binding in NCB-20 Cell Membranes

et al). The correlation coefficient equals 0.58 ($P<0.05$) when the IC_{50} values for (+)SKF-10,047 and (+)cyclazocine against [^{3}H]DTG binding in the cell membranes are set at 10 uM. When these two benzomorphan compounds are excluded from the linear regression analysis, $r=0.59$ ($P<0.05$). Noticeably, several of the compounds assayed in cell membranes (i.e. (+) and (-)SKF-10,047, (+) and (-)cyclazocine, (+)pentazocine, (+) and (-)butaclamol, and chlorpromazine) failed to completely inhibit specific [^{3}H]DTG binding at a concentration of 10 uM, the highest concentration examined.

Of the compounds tested, only DTG and two of its congeners, diadamantylguanidine and aminoDTG (NH_2DTG), were significantly more potent displacers of [^{3}H]DTG than (+)[^{3}H]3-PPP binding in NCB-20 cells. The DTG congener, dimethylguanidine, was inactive in displacing either sigma-specific radioligand (K_i > 250uM). This is in agreement with findings in guinea pig brain membranes (Campbell et al, 1989). PCP was relatively equipotent in its displacement of [^{3}H]DTG and (+)[^{3}H]3-PPP binding in NCB-20 cells.

In contrast, sigma compounds compete very well with (+)[^{3}H]3-PPP for its specific binding in cell membrane preparations (K_i values in the low nanomolar range; Figs. 5 and 9B, and Table 3). Whereas DTG is relatively equipotent in its displacement of either sigma-specific radioligand, (+)3-PPP is approximately 10-fold less potent in displacing [^{3}H]DTG as compared with (+)[^{3}H]3-PPP binding on cell membranes. The tissue-specific differences for (+)[^{3}H]3-PPP binding are not as pronounced as for [^{3}H]DTG binding. In comparison with studies in guinea pig brain (Weber et al), the stereoselectivity of (+) and (-)cyclazocine, (+) and (-)pentazocine, and (+) and (-)butaclamol for the (+)[^{3}H]3-PPP site is significantly reduced in the cell membranes. However, in general, the prototypical sigma drugs remain potent displacers of (+)[^{3}H]3-PPP binding in NCB-20 cells. The correlation between the drug specificity profile for (+)[^{3}H]3-PPP binding in guinea pig brain versus NCB-20 cell membranes is 0.87 ($P<0.001$) (Fig 4B), which is significantly stronger than that obtained for [^{3}H]DTG binding in the same two tissues.

Figure 5. (A - E)Binding curves for displacement of specific (+)[^{3}H]3-PPP binding in NCB-20 cell membrane suspensions by various compounds (values = mean ± s.e.m.; n = 2 - 4).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Atropine
Dimethylguanidine
(+) Pentazocine
(-) 3-PPP
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[Drug] nM
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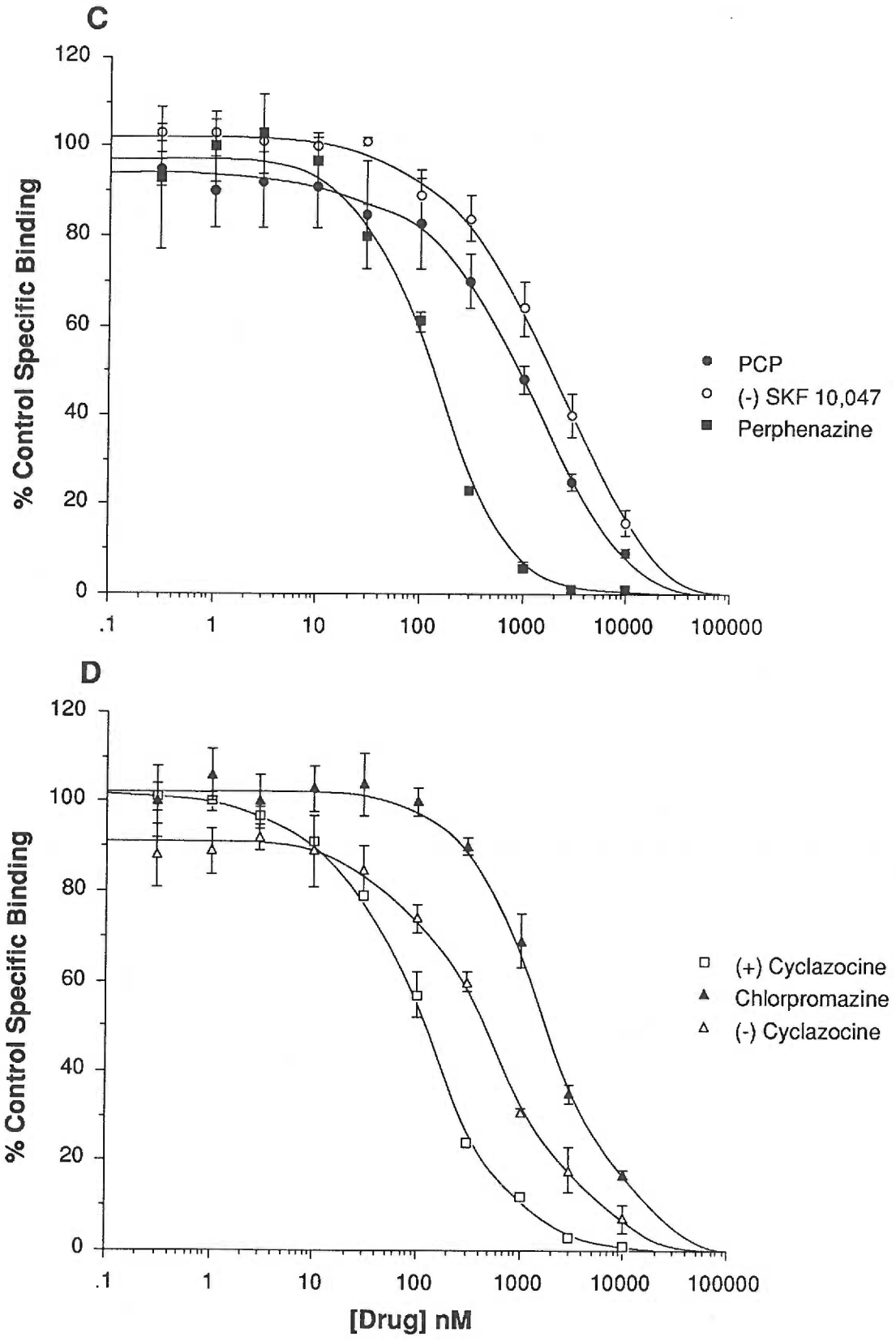
BMY-14802
NH2 DTG
Diadamantylguanidine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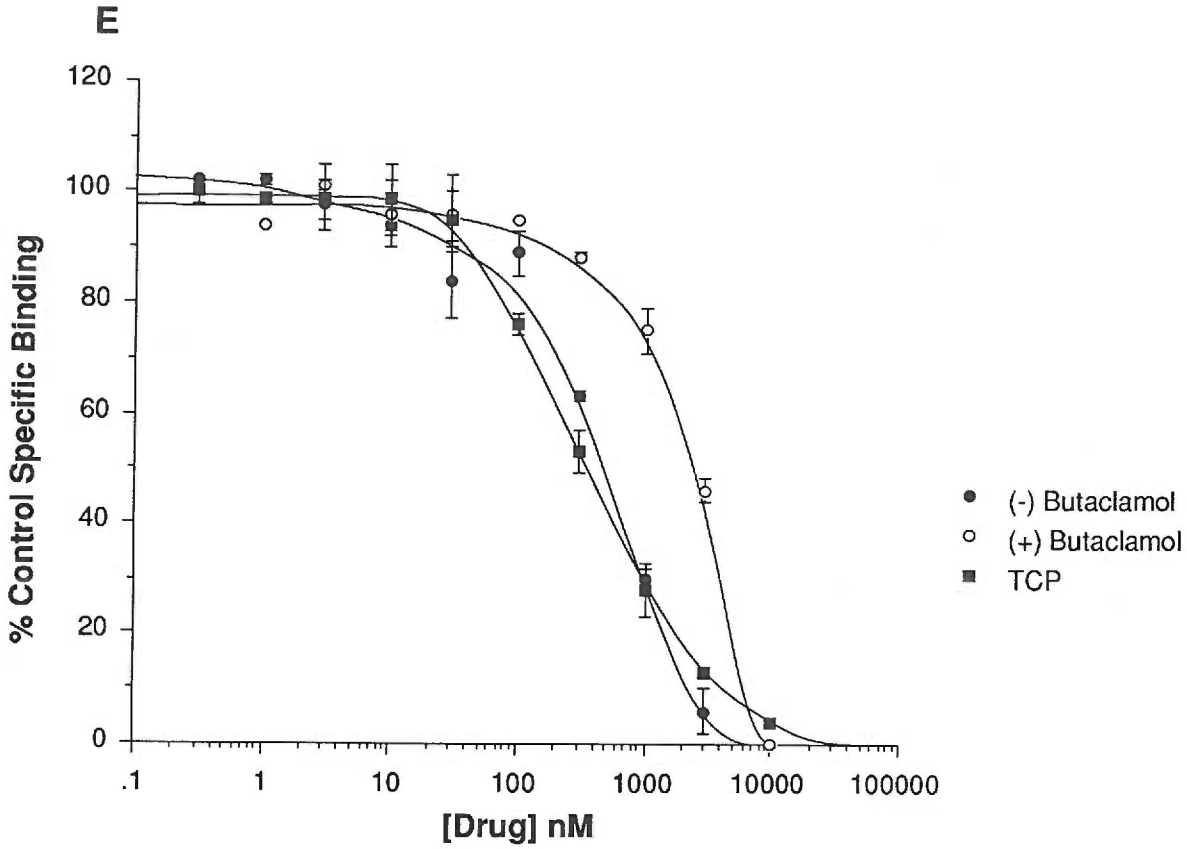
[Drug] nM
(+) Cyclazocine
Chlorpromazine
(-) Cyclazocine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% Control Specific Binding
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10000
100000
[Drug] nM
(-) Butaclamol
(+) Butaclamol
TCP

It is interesting to compare the extent of the correlation between the drug specificity profiles for [^{3}H]DTG and (+)[^{3}H]3-PPP binding in NCB-20 cells with the analogous correlation on guinea pig brain membranes. This correlation is very poor in cell membranes compared to brain membranes when the same fourteen drugs are used in the linear regression analysis in these two tissues; r=0.11 (P>0.1) for NCB-20 cell membranes, whereas r=0.95 (P<0.00001) for guinea pig brain membranes (compare fig. 6 and Appendix fig. ii). When (+)SKF-10,047 and (+)cyclazocine are excluded, thus reducing the linear regression to a twelve point analysis, the r value is increased from 0.11 (P>0.1) to 0.49 (P=0.1) in NCB-20 cells. The correlation between the drug specificity profiles for [^{3}H]DTG and (+)[^{3}H]3-PPP binding in NCB-20 cells is greatly improved when all twenty drugs listed in Table 3 are included in the analysis (Fig 6; r = 0.61 (P<0.01) when the K_i values for dimethylguanidine, (+)SKF-10,047, and (+)cyclazocine vs [^{3}H]DTG binding are set at 250 uM, 10 uM, and 10uM, respectively). Excluding (+)SKF-10,047 and (+)cyclazocine from this analysis leads to a stronger correlation (r=0.80, P<0.001).

The inhibition curves generated from NCB-20 particulate cell membranes were monophasic for both radioligands (Figs 3, 5, and 9A,B). Analysis of the data using the LIGAND program indicates that the one-site model is a statistically better fit overall for each displacer against each radioligand. The low Hill slope values for some of the drugs (notably, for (-)SKF-10,047 and haloperidol vs [^{3}H]DTG) suggest, however, that a greater number of points needs to be included in the displacement curves in order to obtain more reliable estimates of parameter values for the two-site model. Alternatively, the low Hill slope values may indicate the existence of allosteric interactions between the displacer and radioligand. When the displacement of [^{3}H]DTG binding by haloperidol is conducted in the presence of 300nM (+)pentazocine (a dose seen to inhibit 85% of specific (+)[^{3}H]3-PPP binding and only 20% of specific [^{3}H]DTG binding), the slope of this displacement curve is increased from 0.64 $\pm$ 0.10 to 0.90 $\pm$ 0.02 and the affinity of haloperidol against [^{3}H]DTG changes from 532 $\pm$ 212 nM to 173 +/- 12 nM (Fig. 7) as revealed by LIGAND analysis of the data.

Figure 6 Correlation plot of K_i values of various drugs against [^{3}H]DTG vs (+)[^{3}H]3-PPP specific binding in NCB-20 cell membrane suspensions.

r = 0.61($P<0.01$) when all twenty drugs are included in the linear regression analysis (the K_i values for (+)SKF-10,047 and (+)cyclazocine vs [^{3}H]DTG are each set at 10 uM; the K_i value for dimethylguanidine against each radioligand is set at 250 uM).

r = 0.31($P>0.1$) when dimethylguanidine is excluded.

r = 0.80($P<0.001$) when (+)cyclazocine and (+)SKF-10,047 are excluded.

r = 0.60($P=0.01$) when dimethylguanidine, (+)cyclazocine, and (+)SKF-10,047 are excluded.

r = 0.11($P>0.1$) when only drugs 1 -14 are included in the analysis (these are the same 14 drugs compared in guinea pig brain membranes (Appendix fig ii). This r value changes to 0.49($P=0.1$) when (+)cyclazocine and (+)SKF-10,047 are excluded.

1. haloperidol; 2. DTG; 3. (+)pentazocine; 4. (-)pentazocine; 5. (+)3-PPP; 6. (-)3-PPP; 7. (+)cyclazocine; 8. (-)cyclazocine; 9. (-)butaclamol; 10. (+)butaclamol; 11. (+)SKF-10,047; 12. (-)SKF-10,047; 13. PCP; 14. perphenazine; 15. chlorpromazine; 16. TCP; 17. NH_2DTG; 18. BMY 14802; 19. diadamantylguanidine; 20. dimethylguanidine.

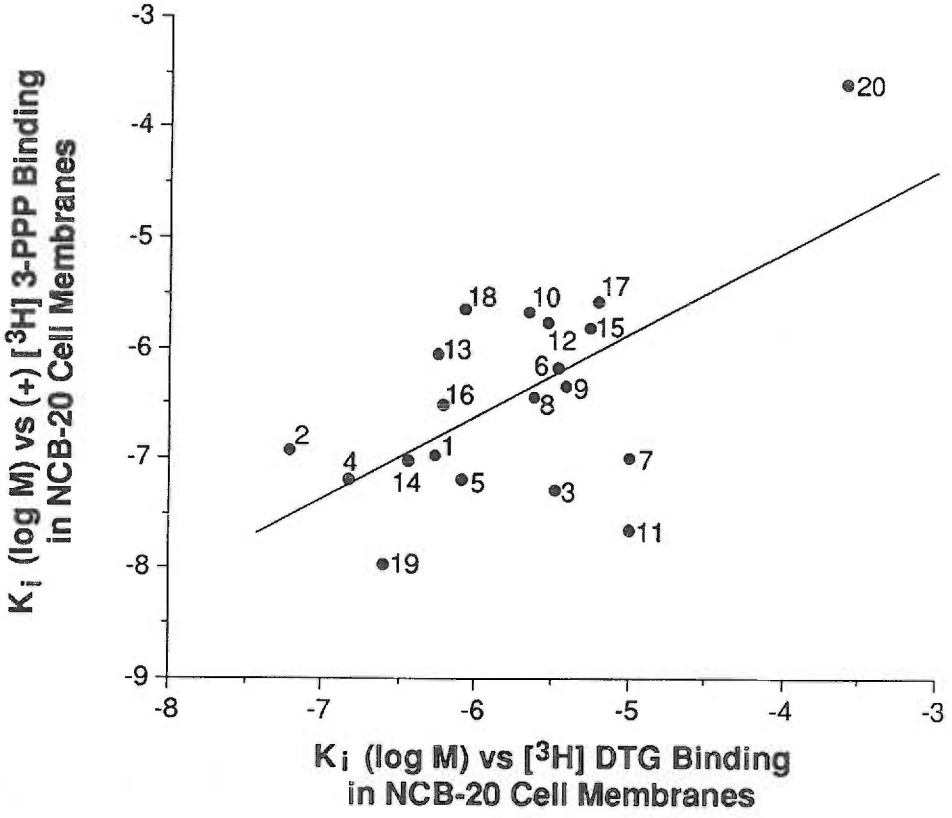
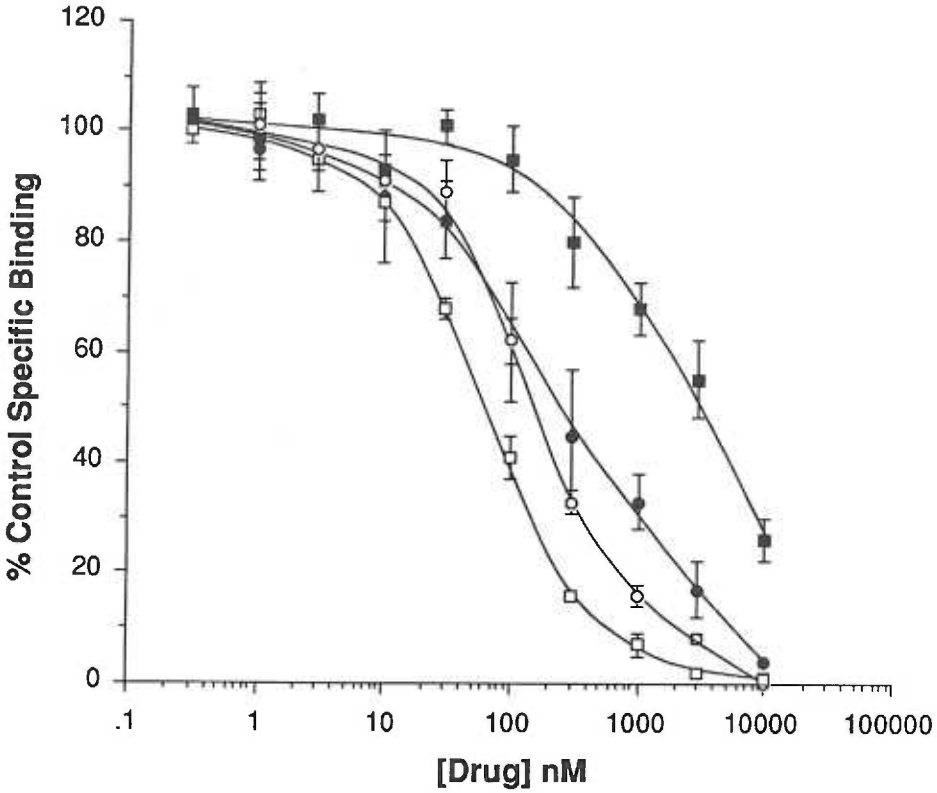

Ki (log M) vs (+) [3H] 3-PPP Binding in NCB-20 Cell Membranes
Ki (log M) vs [3H] DTG Binding in NCB-20 Cell Membranes
-3
-4
-5
-6
-7
-8
-9
-8
-7
-6
-5
-4
-3
20
18
10
17
15
12
13
6
9
16
8
2
1
4
7
14
5
3
11
19

Figure 7. Displacement of [^{3}H]DTG specific binding by haloperidol in NCB-20 cell membranes in the absence or presence of 300 nM (+)pentazocine. Open boxes = (+)pentazocine vs (+)[^{3}H]3-PPP; closed boxes = (+)pentazocine vs [^{3}H]DTG; open circles = haloperidol vs [^{3}H]DTG in the presence of 300 nM (+)pentazocine; closed circles = haloperidol vs [^{3}H]DTG in the absence of (+)pentazocine.

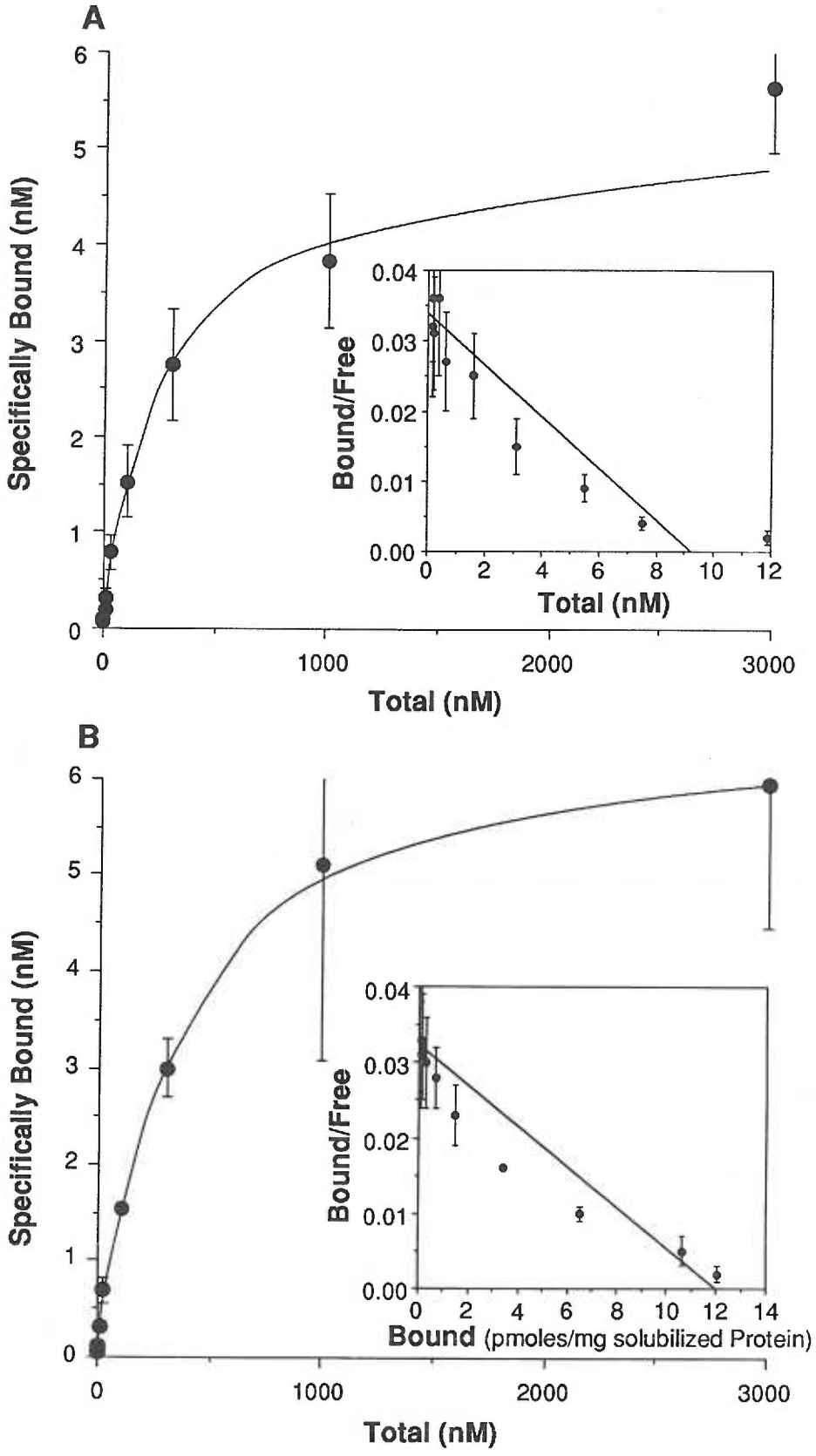

120
100
80
60
40
20
0
% Control Specific Binding
.1
1
10
100
1000
10000
100000
[Drug] nM

Since the pharmacology of [^{3}H]DTG binding is so different in the NCB-20 cells than the guinea pig brain membranes, we compared the association and dissociation rates for [^{3}H]DTG in both tissues (data not shown). Linear transformation of the association and dissociation binding data of [^{3}H]DTG binding in NCB-20 cell membranes gave values of 4.6 x10^6 $M^{-1}min^{-1}$ and 0.08 min^{-1} for the association and dissociation rate constants, respectively. This gives a calculated dissociation constant value (K_D) of 17 nM as compared to the K_D of 62 nM derived from Scatchard analysis (Table 1). The K_D's measured by these two different methods are, therefore, not in complete agreement. Several possible explanations can be suggested for this discrepancy: 1) for the linear transformation analysis of the kinetics of binding data, the calculated concentration of receptor was based on the B_{max} value for [^{3}H]DTG binding equal to 10 pmol/mg membrane protein without taking into account the relatively large error in the estimation of this figure (Table 1); 2) the very fast association and dissociation rates for [^{3}H]DTG in this tissue made it difficult for them to be measured as precisely; 3) the equilibrium saturation binding experiments and kinetic experiments were conducted at different temperatures. Just as for the kinetics of [^{3}H]DTG binding in NCB-20 cell membranes, (+)[^{3}H]3-PPP associates with and dissociates from a single population of sites in guinea pig brain membranes at the rates of 4.1 x 10^5 $M^{-1}min^{-1}$ and 0.02 min^{-1}, respectively. These rates are significantly lower than that seen in cell membranes. The ratio of these rates gives a calculated K_D of 49 nM which compares closely to that obtained from saturation binding analysis (Table 1).

D. Drug Specificity and Equilibrium Saturation Binding Analysis of [^{3}H]DTG and (+)[^{3}H]3-PPP Binding in Solubilized NCB-20 Cell Membranes

Scatchard analysis of [^{3}H]DTG and (+)[^{3}H]3-PPP binding in solubilized NCB-20 cells shows saturable binding to a homogeneous population of sites. There is a relative decrease in affinity for both [^{3}H]DTG and (+)[^{3}H]3-PPP binding in solubilized compared to particulate membranes (table 1, fig. 8). The K_D values increase from 62 nM to 116 nM for [^{3}H]DTG binding and from 66nM to 146nM for (+)3-PPP binding in particulate and solubilized cell

Figure 8. Scatchard analysis (inset) of equilibrium saturation binding curves for [^{3}H]DTG(A) and (+)[^{3}H]3-PPP(B) binding in solubilized NCB-20 cell membranes (values = mean ± s.e.m.; n = 4 - 5).


A
Specifically Bound (nM)
Total (nM)
Bound/Free
Total (nM)
B
Specifically Bound (nM)
Total (nM)
Bound/Free
Bound (pmoles/mg solubilized Protein)

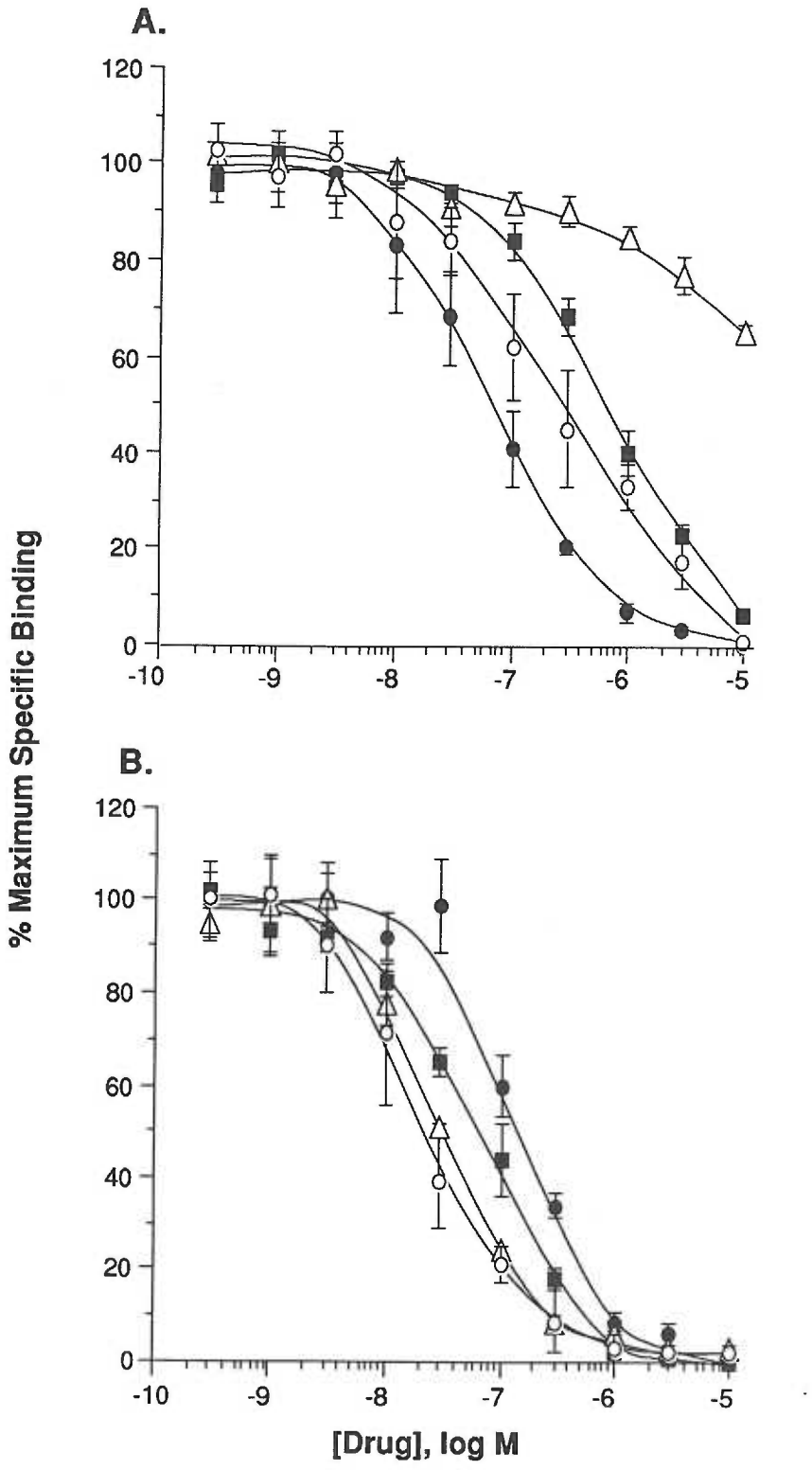
membranes, respectively. This parallel two-fold increase in dissociation constant for both radioligands is most likely attributable to the nonspecific effects of detergent. More remarkable, however, are the equivalent B_{max} values obtained for these radioligands when saturation binding curves are generated in solubilized NCB-20 membranes (B_{max} = 7.04 and 7.9 pmol/mg of solubilized cell membrane protein for [^{3}H]DTG and (+)[^{3}H]3-PPP binding, respectively). Analysis of Scatchard plots using the LIGAND program suggests a one-site model as a best fit for both radioligands.

When NCB-20 cell membranes are first solubilized with sodium cholate and then assayed for [^{3}H]DTG binding, the sigma ligands (+)SKF10,047, haloperidol, and (+)3-PPP become much more potent displacers than when tested in cell membrane suspensions (compare fig 9A and 9C; table 4). However, the significantly lower Hill slope value for haloperidol vs [^{3}H]DTG that is observed in cell membrane suspensions still remains in the solubilized cell preparation. No significant change is seen for the K_i value of DTG against (+)[^{3}H]3-PPP binding when compared to its affinity in particulate cell membrane preparations (compare values between Tables 2 and 4). The inhibition of (+)[^{3}H]3-PPP binding in solubilized cell membranes by the four compounds tested is quite similar to that seen in particulate cell membranes (Table 4; compare fig. 9B and 9D).

E. Photoaffinity Labeling of NCB-20 Cell Membranes with [^{3}H]N_3DTG

The binding subunit of the sigma receptor has been identified in guinea pig brain membranes as a 29 kDa protein with the selective photoaffinity ligand [^{3}H]N_3DTG (Kavanaugh et al, 1988). When NCB-20 cell membranes are photolabeled with [^{3}H]N_3DTG, a radiolabeled protein with electrophoretic mobility equal to a 29 kDa polypeptide is visualized (Fig. 10). In addition, a fainter band corresponding to 20 kDa is seen. There is noticeable labeling of background bands for NCB-20 cell membranes in this particular gel. This is most likely due to over-exposure of the gel in order to adequately show the 29 kDa band in the guinea pig B_o lane (B_o = total binding) and the loading of many more counts for labeled cell

Figure 9. Displacement curves for inhibition of sigma radioligand binding in NCB-20 cell membranes. (A)Inhibition of [^{3}H]DTG binding in particulate cell membranes; (B)Inhibition of (+)[^{3}H]3-PPP binding in particulate cell membranes; (C)Inhibition of [^{3}H]DTG binding in solubilized cell membranes; (D)Inhibition of (+)[^{3}H]3-PPP binding in solubilized cell membranes. Closed circles = DTG; closed squares = (+)3-PPP; open circles = haloperidol; open triangles = (+)SKF-10,047 (values = mean ± s.e.m.; n = 3).


A.
B.
% Maximum Specific Binding
120
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
[Drug], log M

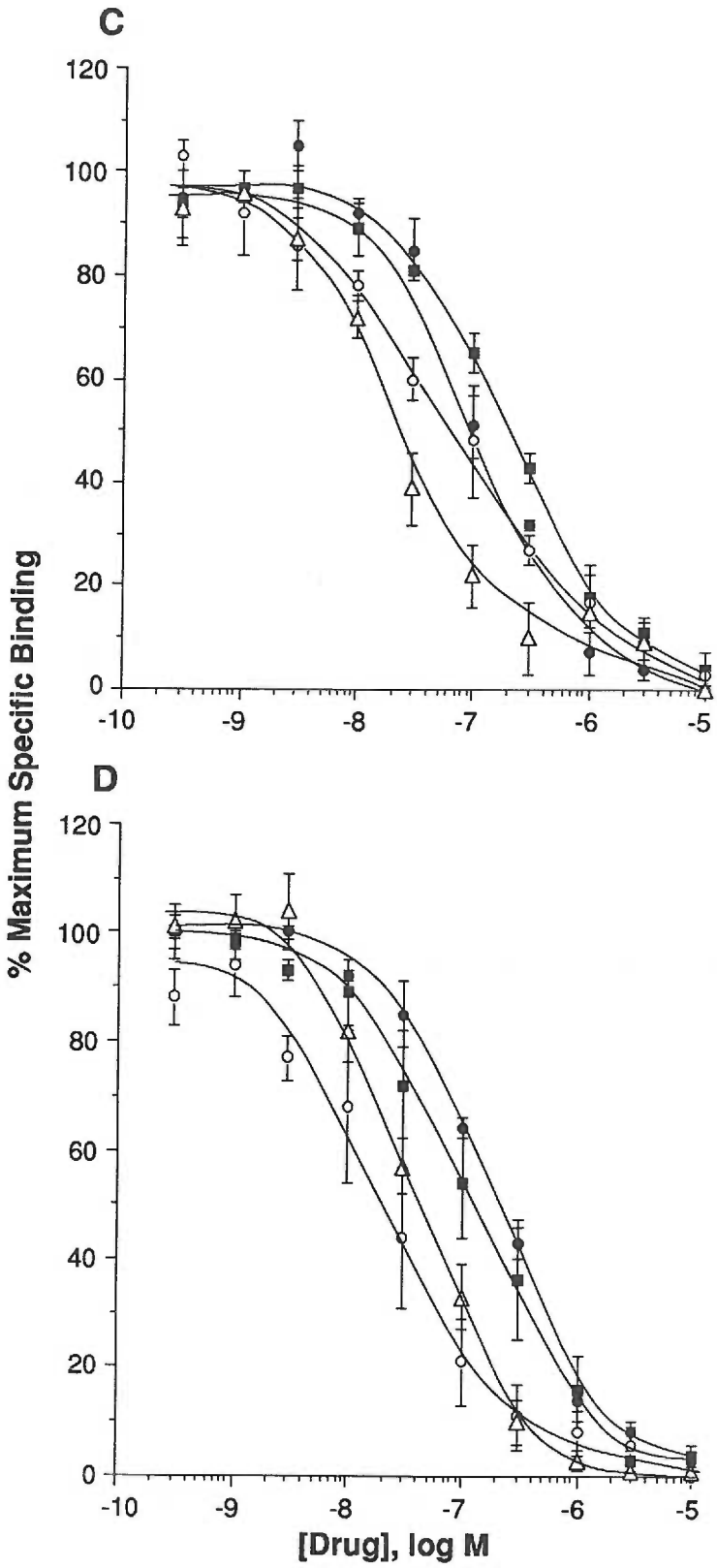

C
D
% Maximum Specific Binding
120
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
[Drug], log M

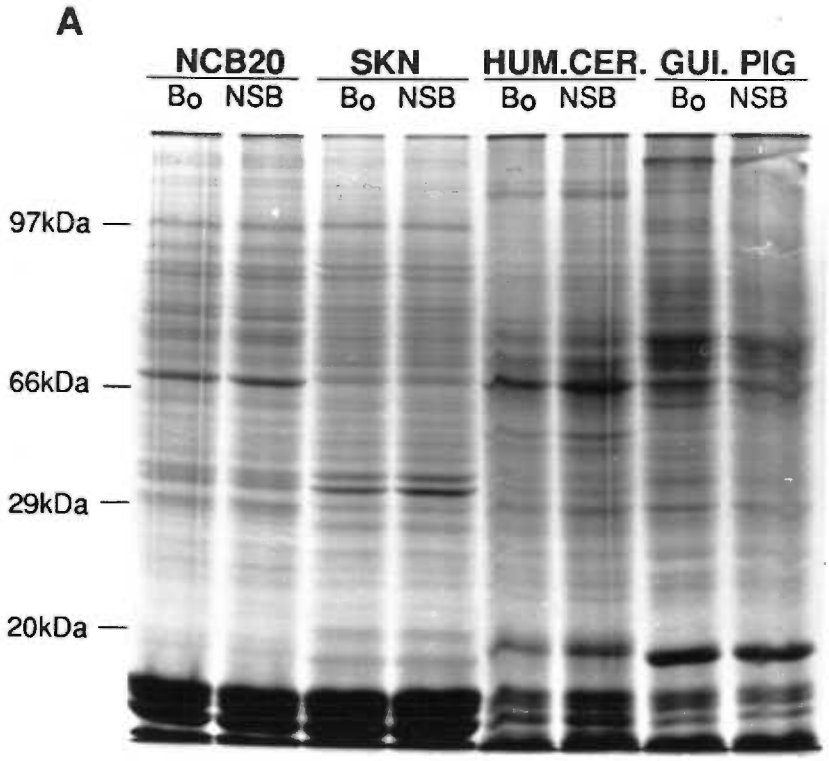
Table 4. Displacement of [^{3}H]DTG and (+)[^{3}H]3-PPP binding in solubilized NCB-20 cell membranes

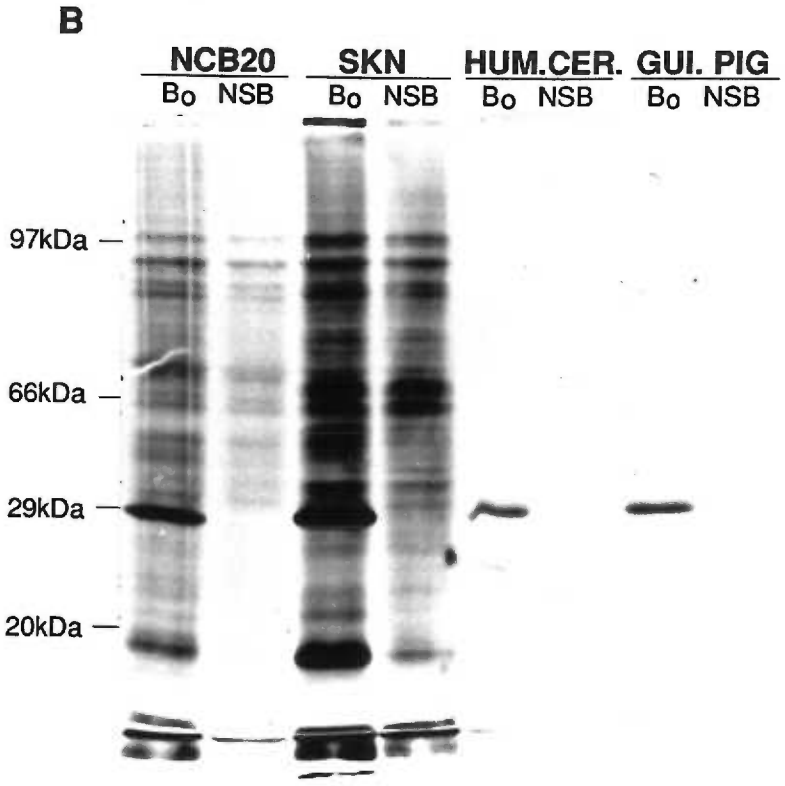
	[^{3}H]DTG		(+)[^{3}H]3-PPP	
Drug	**K_i (nM ± s.e.m.)**	**slope (±s.e.m.)**	**K_i (nM ± s.e.m.)**	**slope (±s.e.m.)**
DTG	116 ± 26[a]	0.83 ± .16	148 ± 43	1.18 ± .02
(+)3-PPP	429 ± 118	0.85 ± .15	146 ± 59[a]	0.70 ± .16
Haloperidol	123 ± 39	0.60 ± .03	28 ± 13	0.79 ± .19
(+)SKF10,047	23 ± 5	0.92 ± .21	29 ± 5	1.14 ± .02

Each experiment was run in triplicate; n = 3-4.

[a] K_D value.

Figure 10. NaDodSO$_4$/PAGE of solubilized NCB-20 cell, SKN-MC cell, human cerebellum, and guinea pig brain membranes following photoaffinity labeling with [^{3}H]N$_3$DTG, as described in Materials and Methods. (A)Coomassie Blue staining of the gel; (B)Fluorograph of gel after treatment with EN^{3}HANCE and exposure to Kodak X-Omat AR film for 41 days. ^{3}H-cpm's loaded per 50 ul sample = 87,500 and 31,760 for NCB-20 cell membranes (B_o and NSB, respectively); 105,325 and 50,475 for SKN-MC membranes; 10,270 and 5,965 for human cerebellum membranes; and 21,845 and 4,070 for guinea pig brain membranes. B_o = total binding; NSB = nonspecific binding (binding in the presence of 10 uM haloperidol). Molecular weight markers (Sigma) are trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), and phosphorylase b (97 kDa).


A
NCB20
SKN
HUM.CER.
GUI. PIG
Bo NSB
Bo NSB
Bo NSB
Bo NSB
97kDa
66kDa
29kDa
20kDa


B
NCB20
SKN
HUM.CER.
GUI. PIG
Bo NSB
Bo NSB
Bo NSB
Bo NSB
97kDa
66kDa
29kDa
20kDa

membranes as compared to human cerebellum or guinea pig brain membranes. For unknown reasons, the nonspecific labeling of NCB-20 membranes is lessened by including haloperidol in the incubation mixture, although the labeling of the 29 kDa band is clearly more greatly reduced.

Human cerebellar membranes exhibit similar characteristics for [^{3}H]DTG binding as seen in NCB-20 particulate cell membranes (Sonders et al, 1987), and preliminary data using membranes prepared from a human neuroblastoma cell line, SKN-MC, also reveal differential binding of [^{3}H]DTG and (+)[^{3}H]3-PPP similar to that described herein for NCB-20 cells (data not shown). When membranes were prepared from each of these tissues and derivatized with the azide compound, specific labeling of a 29 kDa polypeptide was revealed as well (Fig 10). Labeling of this band was prevented by the inclusion of 10 uM haloperidol in the incubation mixture.

Specificity of covalent labeling was verified by addition of various drugs to the incubation mixture prior to light exposure (Fig. 11). In NCB-20 cell membranes, addition of 10 uM haloperidol or (+)3-PPP to the incubation mixture inhibits labeling of the 20 kDa and 29 kDa bands. Addition of 10 uM dopamine, morphine, GABA (not shown), or scopolamine (a muscarinic-cholinergic receptor antagonist; not shown) results in disapearance of the 20 kDa band while leaving labeling of the 29 kDa polypeptide unaffected. As seen in NCB-20 cell membranes, labeling of a 20 kDa band in the B_0 lane of SKN-MC cell membranes also appears to be nonspecific, as labeling of this band is significantly reduced in the presence of non-sigma compounds.

F. Gel Filtration Analysis of Solubilized NCB-20 Cell Membranes

Proteins were solubilized with 20 mM sodium cholate/Tris after photoaffinity labeling and subject to chromatography on a Sepharose 6B column. Under these nondenaturing conditions, the major peak of radioactivity elutes as a 29 kDa species (Fig 12A). While this 29 kDa polypeptide appears to partially elute as part of a 150,000 molecular weight complex, this association is clearly not as prominent as that seen

Figure 11. Fluorograph of $NaDodSO_4$/PAGE of solubilized NCB-20(A) and SKN-MC(B) cell membranes following photoaffinity labeling with [^{3}H]N_3DTG as described in Materials and Methods. Lane 1 = total binding (9,800 cpm's and 39,775 cpm's loaded from NCB-20 and SKN-MC cell membranes, respectively); lane 2 = labeling in the presence of 10 uM dopamine; lane 3 = labeling in the presence of 10 uM morphine; lane 4 = labeling in the presence of 10 uM haloperidol; lane 5 = labeling in the presence of 10 uM (+)3-PPP. Molecular weight markers (Sigma) are trypsin inhibitor (20 kDa), and carbonic anhydrase (29 kDa).

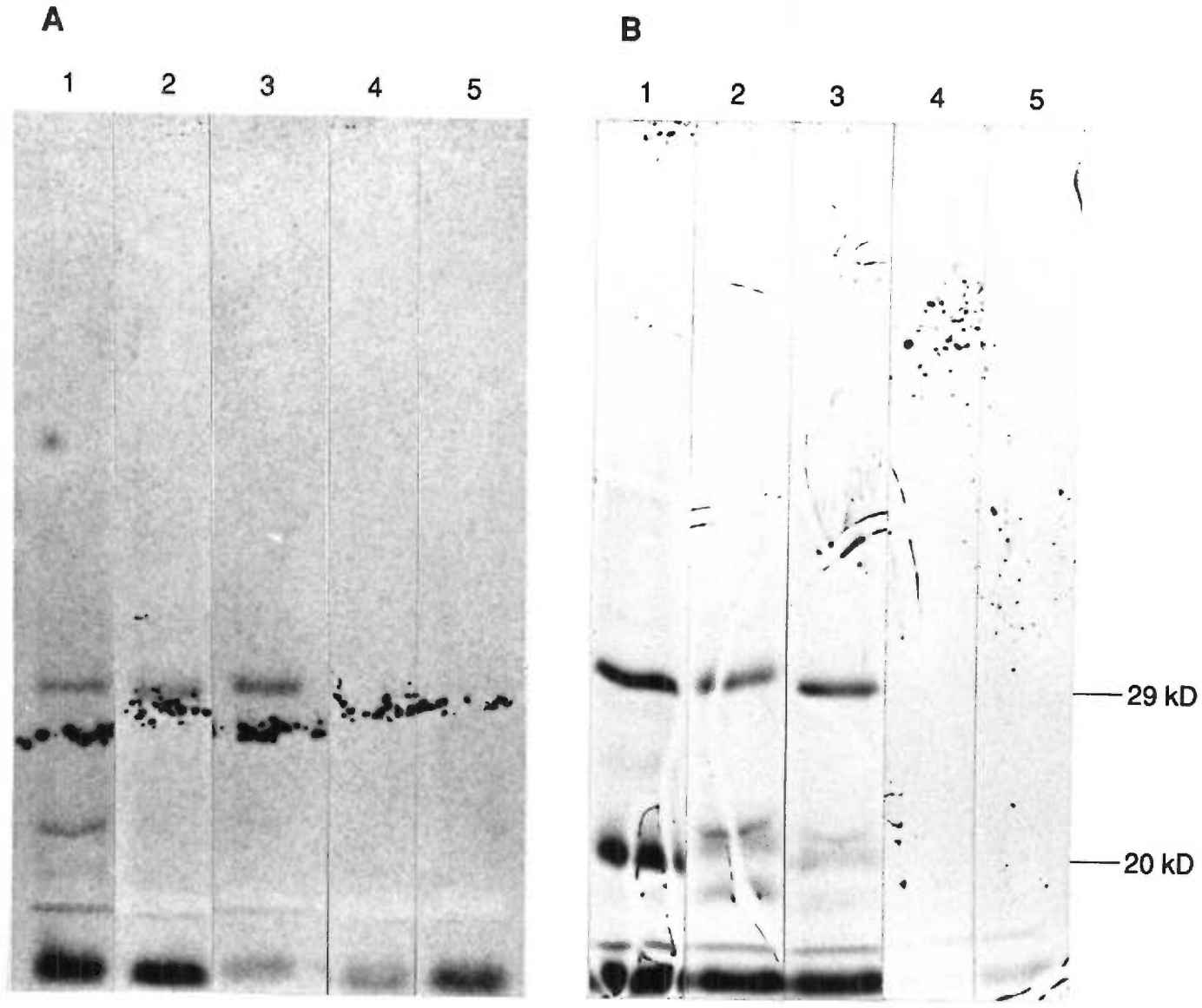
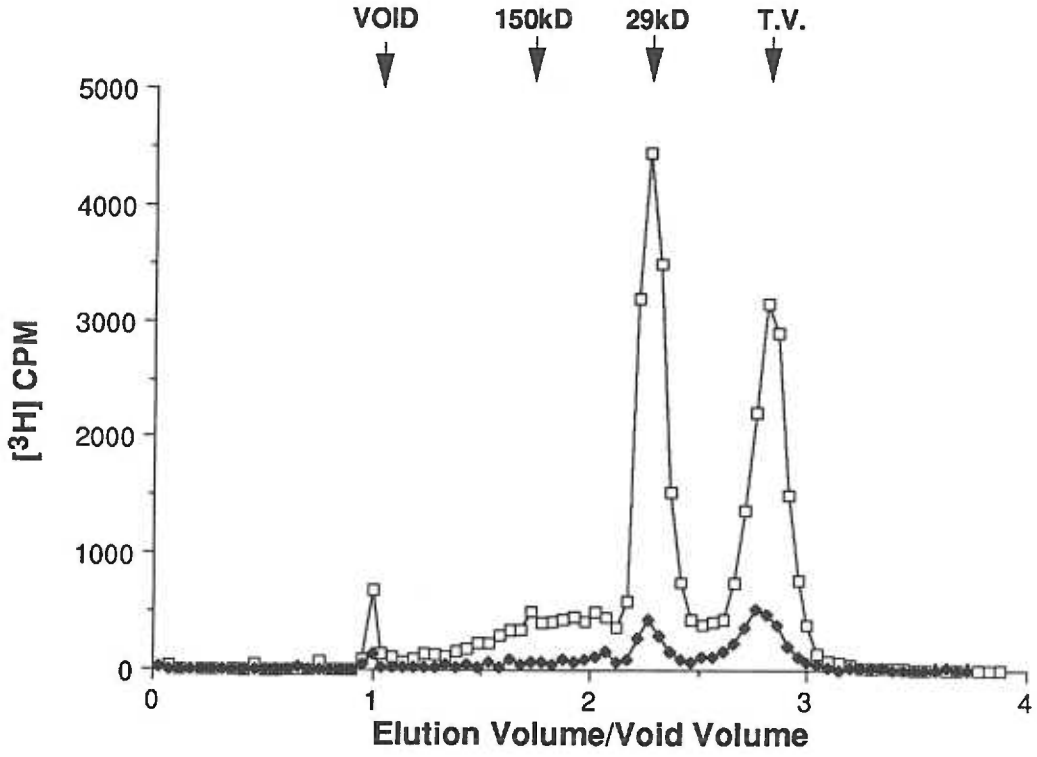
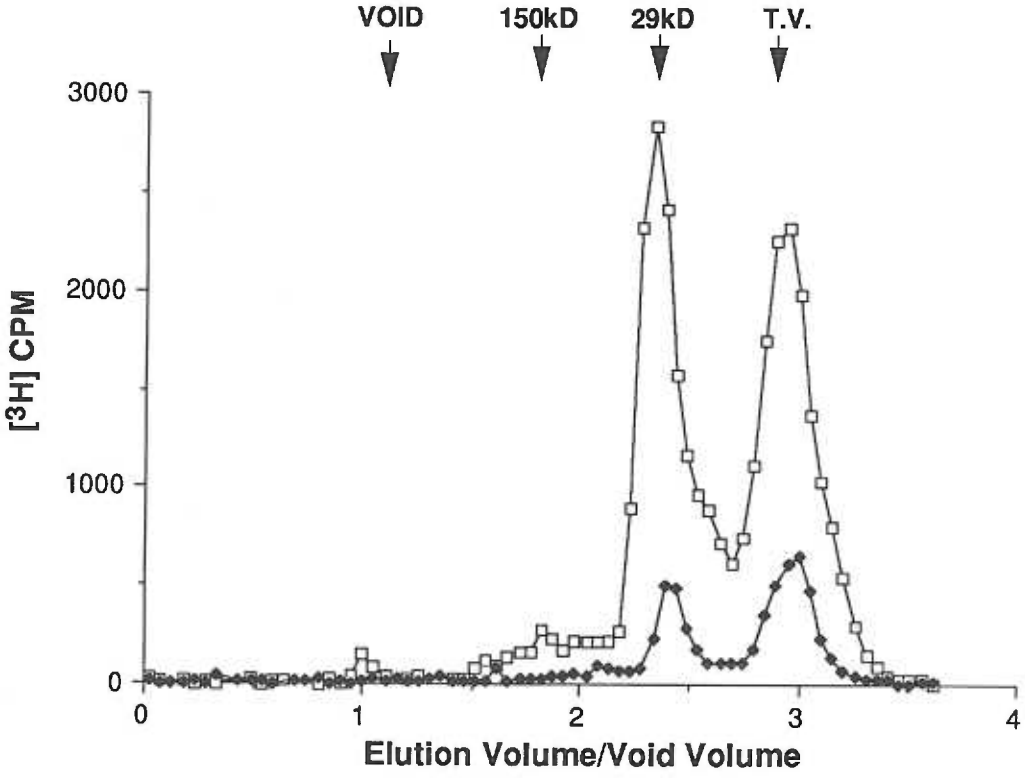

A
1
2
3
4
5
B
1
2
3
4
5
29 kD
20 kD

Figure 12. Sepharose CL-6B chromatography of photoaffinity labeled membrane proteins from NCB-20 cells, SKN-MC cells, human cerebellum, and guinea pig brain, as described in Materials and Methods. The elution profiles show specific labeling of a Mr 150,000 complex and a Mr 29,000 protein in varying proportions between tissues. (T.V. = Total volume)

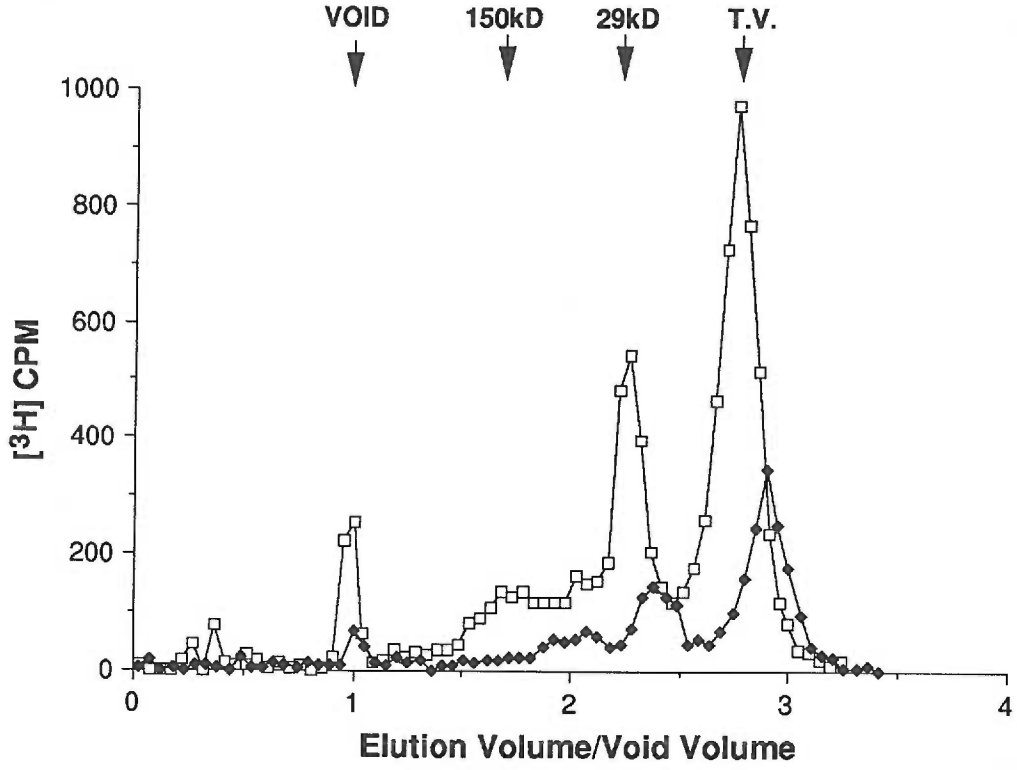
A. NCB-20 Cells



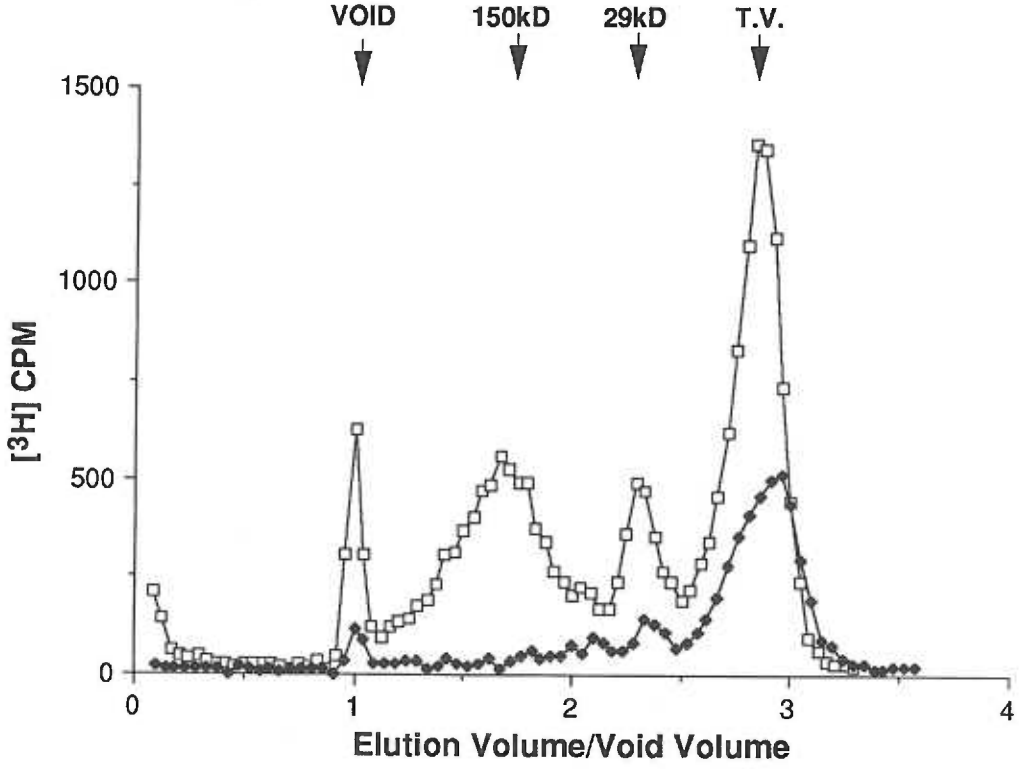
B. SKN-MC Cells



C. Human Cerebellum



D. Guinea Pig Brain



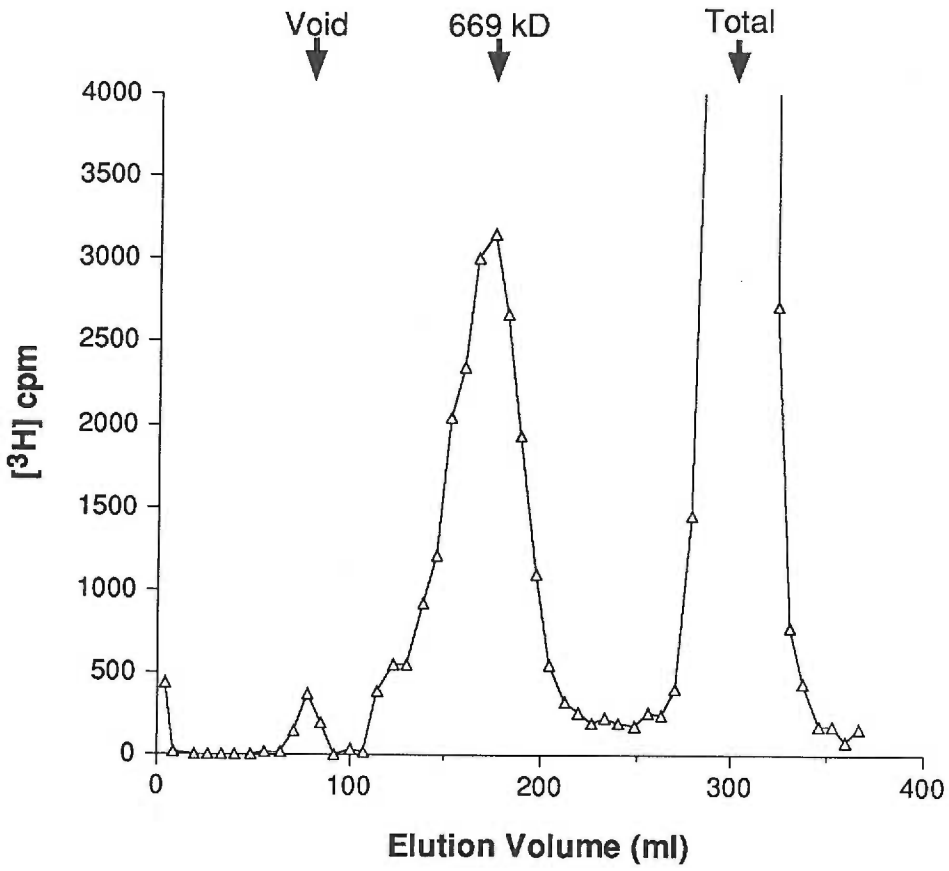
with labeled and solubilized guinea pig brain membranes (Fig 12D; Kavanaugh et al, 1988). Both of these migrating species are haloperidol-sensitive. When membranes from human cerebellum and SKN-MC cells are treated in the same way and applied to the same gel filtration column, an elution profile very similar to that of NCB-20 cells is obtained (fig 12B,C).

Estimation of the size of the native sigma complex was analyzed by gel filtration chromatography of underivatized, solubilized cell membranes labeled reversibly with [^{3}H]DTG. Under these conditions, [^{3}H]DTG dissociates slowly enough to reveal a major peak of radioactivity associated with a complex of molecular weight 669,000 (Stokes radius of 8.7 nM; Fig 13). From this information, it can be inferred that the 29 kDa [^{3}H]DTG binding subunit is part of a much larger complex which may represent the functional, native sigma receptor. This 669 kDa macromolecular complex is the same size as that previously identified in solubilized guinea pig brain membranes (Kavanaugh et al, 1989). It is not clear how the 150 kDa species identified in photoaffinity labeled membranes is related to the 669 kDa complex.

G. Discussion

Sigma receptor binding sites in NCB-20 cells have been characterized biochemically and pharmacologically using the sigma-specific radioligands [^{3}H]DTG and (+)[^{3}H]3-PPP. Unlike results obtained in guinea pig brain membranes (Weber et al), sigma receptor binding assays performed in NCB-20 membrane suspensions reveal a different pharmacological profile for [^{3}HDTG compared to (+)[^{3}H]3-PPP binding. The drug specificity pattern for (+)[^{3}H]3-PPP binding more closely reflects that obtained in guinea pig (Weber et al) and rat brain membranes (Largent et al, 1984), although the (+)[^{3}H]3-PPP site in the cell membranes displays a clear reduction in stereoselectivity for (+) and (-)cyclazocine, (+) and (-)pentazocine, and (+)butaclamol. The pattern of [^{3}H]DTG binding, however, is very different from that seen with this ligand in guinea pig brain membranes. This is evidenced by a significantly decreased potency for displacement of [^{3}H]DTG binding by haloperidol, (+)3-PPP and

Figure 13. Sepharose CL-6B chromatography of cholate-solubilized sigma receptors as described in Materials and Methods. Following incubation of 4 ml of solubilized material with [^{3}H]DTG, the entire sample was loaded on to a pre-equilibrated sepharose CL-6B column, eluted, and fractions were collected. 5% of the recovered counts eluted with the 669 kDa (Stokes radius 8.6 nm) peak. Standards (not all shown) were blue dextran (elutes with the void volume); thyroglobulin (8.6 nm); alcohol dehydrogenase (4.6nm); bovine serum albumin (3.6 nm); carbonic anhydrase (2.0 nm); and tryptophan (elutes with the total column volume).


Void
669 kD
Total
4000
3500
3000
2500
2000
1500
1000
500
0
[3H] cpm
0
100
200
300
400
Elution Volume (ml)

(+)pentazocine, and by very little displacement by (+)SKF-10,047 and (+)cyclazocine even at 10 uM (these findings are corroborated by the results of Friedl and Glaser). Remarkably, the B_{max} values for the two radioligands are dissimilar in cell membrane suspensions with twice as many maximum specific binding sites per mg of membrane protein for [^{3}H]DTG as compared to (+)[^{3}H]3-PPP binding. When assays are performed in solubilized NCB-20 membranes, however, the displacement of [^{3}H]DTG binding by the sigma-specific ligands (+)3-PPP, haloperidol, and (+)SKF-10,047 is more typical of, though not identical to, sigma receptor pharmacology than in particulate cell membranes. Thus, in comparison to binding in brain membranes, haloperidol has a reduced, whereas (+)SKF-10,047 has an increased affinity for the [^{3}H]DTG site in solubilized cell membranes. The [^{3}H]DTG sites in solubilized cell membranes differ then from those in cell and brain membrane suspensions. The K_i values for DTG, (+)3-PPP, haloperidol and (+)SKF-10,047 displacement of (+)[^{3}H]3-PPP binding are quite similar in particulate and solubilized cell membranes. Also, the B_{max} values per mg of solubilized membrane protein for [^{3}H]DTG and (+)[^{3}H]3-PPP binding are equivalent.

It appears that the unusual pharmacological profile for [^{3}H]DTG binding in NCB-20 cell membrane suspensions can not be attributed to differences in molecular properties of the sigma receptor itself as defined by photoaffinity labeling of cell membranes with [^{3}H]N_3DTG or by gel filtration experiments with solubilized cell membranes. The sizes of the binding subunit for [^{3}H]DTG (29 kDa -- Stokes radius of 4.6nm) and the apparent native sigma receptor complex (669 kDa -- Stokes radius of 8.7 nm) correspond to the same molecular weights previously identified in guinea pig brain membranes (Kavanaugh et al, 1988; Kavanaugh et al, 1989). In addition, a 29 kDa [^{3}H]DTG binding subunit was also specifically labeled in human cerebellum and SKN-MC cell membranes, two other tissues which display an atypical, non sigma-like pharmacological profile for [^{3}H]DTG binding. Hence, it is unlikely that the unusual drug specificity pattern of [^{3}H]DTG binding is simply an artifact of cell culture.

It might be interesting to investigate whether [^{3}H]DTG binding assays conducted in solubilized human cerebellum or SKN-MC cell membranes

would reveal a transformation of the [^{3}H]DTG pharmacological profile to one more typical of sigma receptors (as was seen for solubilized NCB-20 cell membranes) It might also be interesting to test the sensitivity of (+)[^{3}H]3-PPP and [^{3}H]DTG binding in NCB-20 cells to GTP or GTP analogs in Mg^{2+}-pretreated cell membranes in light of recent reports proposing the interaction of sigma receptors with G-proteins. Perhaps such a coupling differentially affects [^{3}H]DTG and (+)[^{3}H]3-PPP binding in NCB-20 cells.

Bowen and Hellewell have reported pharmacological differences for sigma receptors on PC-12 cells (a rat pheochromocytoma culture cell line) compared to those characteristic of rat and guinea pig brain membranes. However, unlike the sigma receptors of NCB-20 cells, both (+)[^{3}H]3-PPP and [^{3}H]DTG recognized a single population of sites in PC-12 cells with greatly reduced affinity for (+)benzomorphan compounds (in contrast to sigma receptors in NCB-20 cells which show differential sensitivity of [^{3}H]DTG and (+)[^{3}H]3-PPP sites to displacement by (+) benzomorphan drugs). Hence, whereas [^{3}H]DTG- and (+)[^{3}H]3-PPP-labeled sites appear to coincide in many instances, this appears to be a tissue-dependent occurrence.

It is difficult, at this point, to explain the striking differences in the pharmacological profile between these two sigma ligands. It is helpful to take into account recently published data providing evidence for two populations of haloperidol-sensitive [^{3}H]DTG binding sites based on "binding surface analysis" (Rothman). In this experimental design, accurate parameter estimates are obtained by analyzing binding as a function of the binding of both the radioligand and the inhibitor/displacer simultaneously. Three axes are required to plot the data, generating a three-dimensional surface. A total "binding surface" is generated by constructing several saturation curves in the presence or absence of increasing concentrations of a given displacer, and several displacement curves are constructed as a function of varying concentrations of radioligand. This kind of analysis leads to marked decrease in the standard deviations of binding parameters (i.e. B_{max} and K_i values) and the data may be pooled and fit to one- and two-site models for comparison of goodness-of-fit. In such studies, the investigators have found that

[^{3}H]DTG recognizes two binding sites in guinea pig brain membranes with roughly equivalent affinities (Reid et al). These sites are distinguished by the benzomorphan opiates (+)cyclazocine and (+)pentazocine, each exhibiting approximately 100-fold differences in their affinity for these two sites.

It is curious that while [^{3}H]DTG and (+)[^{3}H]3-PPP have similar B_{max} values in solubilized NCB-20 membranes, significantly different B_{max} values for binding of these two ligands are obtained in cell membrane suspensions. One possible explanation is the existence of two sigma sites or receptor states (Beart et al; Reid et al; Itzhak and Khouri; Itzhak) which bind [^{3}H]DTG equally well but which are differentially affected by solubilization. Perhaps solubilization inactivates a [^{3}H]DTG site (receptor state) not shared with (+)[^{3}H]3-PPP, or converts this unshared receptor site (state) to one they hold in common by functional loss of a regulatory factor; either case would be expected to give the same maximum number of specific binding sites per mg of solubilized membrane protein for these two radioligands.

Inspite of the unresolved questions regarding the cause for the differential binding of [^{3}H]DTG and (+)[^{3}H]3-PPP, it remains clear that NCB-20 cells should serve as a useful model for investigating sigma receptor-mediated cellular responses and possible sigma receptor subtypes. It may be that there is a functional correlate for the differences between sigma ligand displacement of [^{3}H]DTG vs (+)[^{3}H]3-PPP binding in these cells. Studies were therefore initiated to characterize the modulation of agonist-stimulated inositol phosphate accumulation in NCB-20 cells by sigma drugs.

2. CHARACTERIZATION OF THE EFFECT OF SIGMA COMPOUNDS ON AGONIST-STIMULATED PHOSPHOINOSITIDE HYDROLYSIS IN NCB-20 CELLS

A. Brief Introduction

Preliminary findings in rat brain synaptoneurosomes (Bowen et al, 1988a) have suggested a role for sigma receptors in the negative modulation of

carbachol-induced phosphoinositide turnover (carbamoylcholine, or carbachol, is a cholinergic receptor agonist). The evidence presented in support of this notion was that the rank order of potency for the five sigma ligands tested correlated well with their affinity at sigma receptors; compounds tested that do not bind to the sigma receptor also failed to antagonize the induced response.

That actions mediated by the sigma receptor might interface with the phosphoinositide signal transduction system is intriguing considering the important role this system is believed to play in neural tissues (Fisher and Agranoff; Berridge; Majerus et al; Berridge and Irvine). Ligand-induced stimulated turnover of inositol lipids initiates a bifurcating signalling pathway by generating two recognized second messenger molecules, inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DG), serving distinct functions. The initial ligand-stimulated step is the breakdown of phosphotidylinositol-4,5-bisphosphate (PIP_2) by activation of phospholipase C (PL-C), a calcium/phospholipid-dependent phosphoinositidase. This is believed to be mediated by coupling of the receptor to PL-C via a GTP-binding protein. Hydrolysis of PIP_2 results in the concomitant formation of $InsP_3$ and DG leading to the mobilization of intracellular Ca^{2+} stores and the stimulation of a calcium/phospholipid-dependent protein kinase, protein kinase C (PK-C), respectively. Hence, certain calcium- and phosphorylation-sensitive cellular processes can be regulated by the activation of this pathway. Lipid and inositol phosphate cycles then function so as to remove the second messenger molecules and regenerate phosphotidylinositol (PI) which is sequentially phosphorylated to regenerate PIP_2.

We were interested in complementing our biochemical and pharmacological studies of the sigma receptor in NCB-20 cells with the examination of the effects of sigma ligands on the phosphoinositide transduction system. This hybrid neurotumor cell line represents a homogeneous population of cells which is amenable to experimental manipulations and has served as a tool for the study of a variety of nerve cell functions. MacDermott et al used NCB-20 cells to investigate serotonin-receptor mediated cell depolarizaiton and activation of

adenylate cyclase. Berry-Kravis et al (1988) have reported finding that specific proteins in NCB-20 cells are phosphorylated as a result of neurotransmitter-mediated alteration of cAMP levels or TPA-mediated activation of protein kinase C. Since NCB-20 cells are capable of producing functional synapses with smooth muscle when intracellular cAMP levels are elevated for a prolonged period of time, they surmised that NCB-20 cells should provide a useful mammalian system for studying synaptic regulation and its relationship to protein phosphorylation. Berry-Kravis and Dawson have observed the development of serotonin "supersensitivity" induced by prolonged exposure of NCB-20 cells to a delta-opioid receptor agonist and reasoned that studies with NCB-20 cells may help in elucidating the biological mechanism whereby neurotransmitter interactions produce desensitization or facilitation in the intact nervous system. In 1988, Zhu and Chuang published a paper indicating that NCB-20 cells may represent a useful model for studying the regulation of gene expression of delta-opioid, alpha$_2$-adrenergic, and muscarinic cholinergic receptors on the basis of their differential regulation by butyrate and dibutyryl cyclic AMP in these cells.

Of particular relevance to our goals, it has been shown that NCB-20 cells express functional muscarinic cholinergic receptors coupled to PL-C (Chuang). Addition of carbachol in the presence of Li^+ to cells prelabeled with [^{3}H]-*myo*-inositol increases the accumulation of [^{3}H]-Inositol-1-phosphate ([^{3}H]InsP_1) by more than 4-fold over basal levels. In addition, the stimulation of phosphoinositide turnover by the biologically active hormone, bradykinin, has been well characterized in NCB-20 cells (Francel and Dawson; Chuang and Dillon-Carter). Thus, NCB-20 cells appear to be quite suitable for our intended investigations. Our initial studies have focused on characterizing the nature of the inhibition of stimulated [^{3}H]InsP_1 accumulation by sigma ligands in NCB-20 cells. We chose to test a relatively large number of sigma-active drugs belonging to a variety of structural classes to strengthen the validity of our observations and help in our efforts to correlate sigma-binding activity with functional activity. These initial studies were conducted with a view to eventually addressing the more significant question regarding whether the effects of these compounds are mediated by sigma receptor activation.

B. Inhibition of Carbachol-induced [^{3}H]InsP_1 Accumulation in NCB-20 Cells by Sigma Compounds

Figure 14 illustrates a typical elution profile obtained from acid extracts of carbachol-stimulated phosphoinositide turnover in NCB-20 cells. The accumulation of [^{3}H]InsP_1 was dependent on the presence of lithium and reached maximum levels after incubation with agonist for 20 to 30 minutes. No increases in inositol bis- or trisphosphate levels over basal levels were ever detected after stimulation with carbachol. On average, carbachol elicited a 2.5-fold increase in [^{3}H]InsP_1 levels, although stimulations of up to 5-fold over basal response were sometimes observed. Figure 15 demonstrates that the carbachol response is mediated strictly via muscarinic cholinergic receptors as evidenced by its sensitivity to the muscarinic-cholinergic receptor antagonist, atropine, and its insensitivity to the nicotinic-cholinergic receptor antagonist, hexamethonium. In accordance, the nicotinic receptor agonist, dimethylphenylpiperazine (DMPP), failed to raise [^{3}H]InsP_1 levels.

The dose response curves for the drugs tested in the carbachol-stimulated response paradigm are displayed in Figure 16, whereas the corresponding ED_{50} values (the dose required to effect a 50% decrease in carbachol-stimulated [^{3}H]InsP_1 accumulation) are shown in Table 5. Nine of the twelve compounds examined produced a dose-dependent reduction in the levels of [^{3}H]InsP_1 compared to control cells exposed only to 500uM carbachol. (+) and (-) pentazocine, (+)cyclazocine, and NH_2DTG fully blocked the stimulation of phosphoinositide hydrolysis within the range of doses tested. No effect of these compounds on basal levels of [^{3}H]InsP_1 could be detected at the concentrations tested (data not shown). Stimulation with carbachol in the presence of increasing concentrations of DTG also lead to a dose-dependent decrease in the response. However, the maximum reduction effected by 50 uM DTG was 65% of control and was not potentiated by further increases in DTG concentration. DTG was found not to alter basal responses until present at 500 uM concentration; at this concentration, DTG evoked a significant increase (65%) in basal levels of [^{3}H]InsP_1 which may explain the reduced inhibition of the carbachol

Figure 14. Representative profile for step-wise elution of inositol phosphates from acid extracts of control (open squares) or carbachol-stimulated (closed circles) NCB-20 cells. 2-ml fractions were collected and 0.5-ml aliquots were counted for radioactivity.

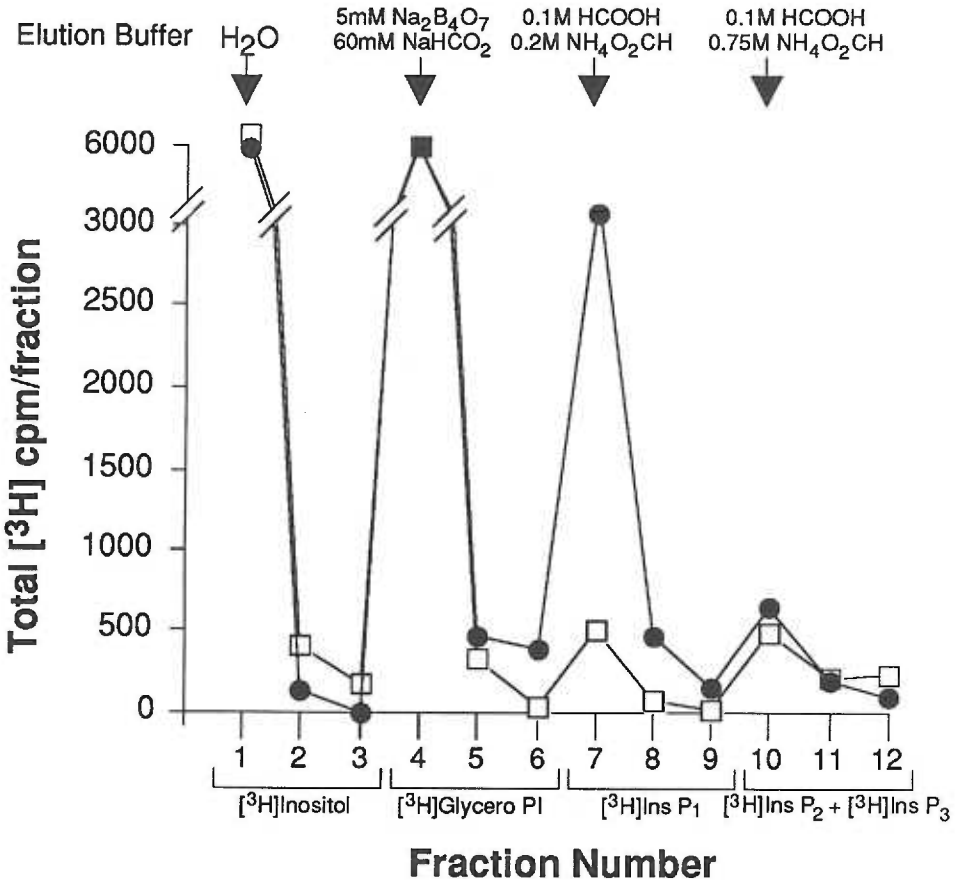

Elution Buffer
H2O
5mM Na2B4O7
60mM NaHCO2
0.1M HCOOH
0.2M NH4O2CH
0.1M HCOOH
0.75M NH4O2CH
Total [3H] cpm/fraction
6000
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12
[3H]Inositol
[3H]Glycero PI
[3H]Ins P1
[3H]Ins P2 + [3H]Ins P3
Fraction Number

Figure 15. Effect of muscarinic- and nicotinic-cholinergic receptor anatagonists (atropine and hexamethonium, respectively) on the carbachol-induced accumulation of $[^3H]InsP_1$ in NCB-20 cells ([DMPP] = 1 mM).

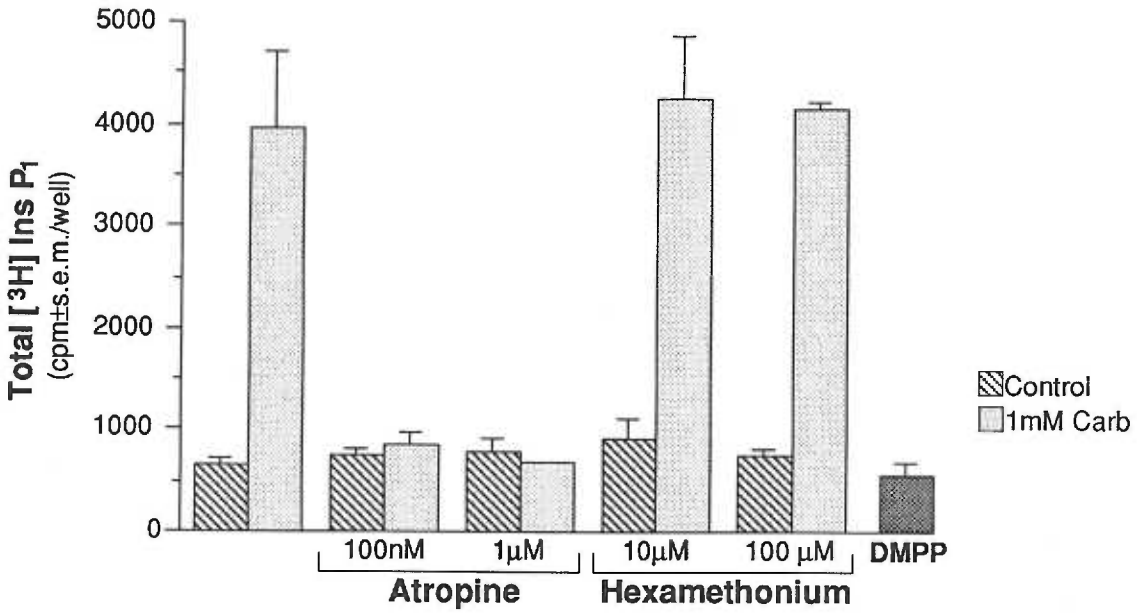
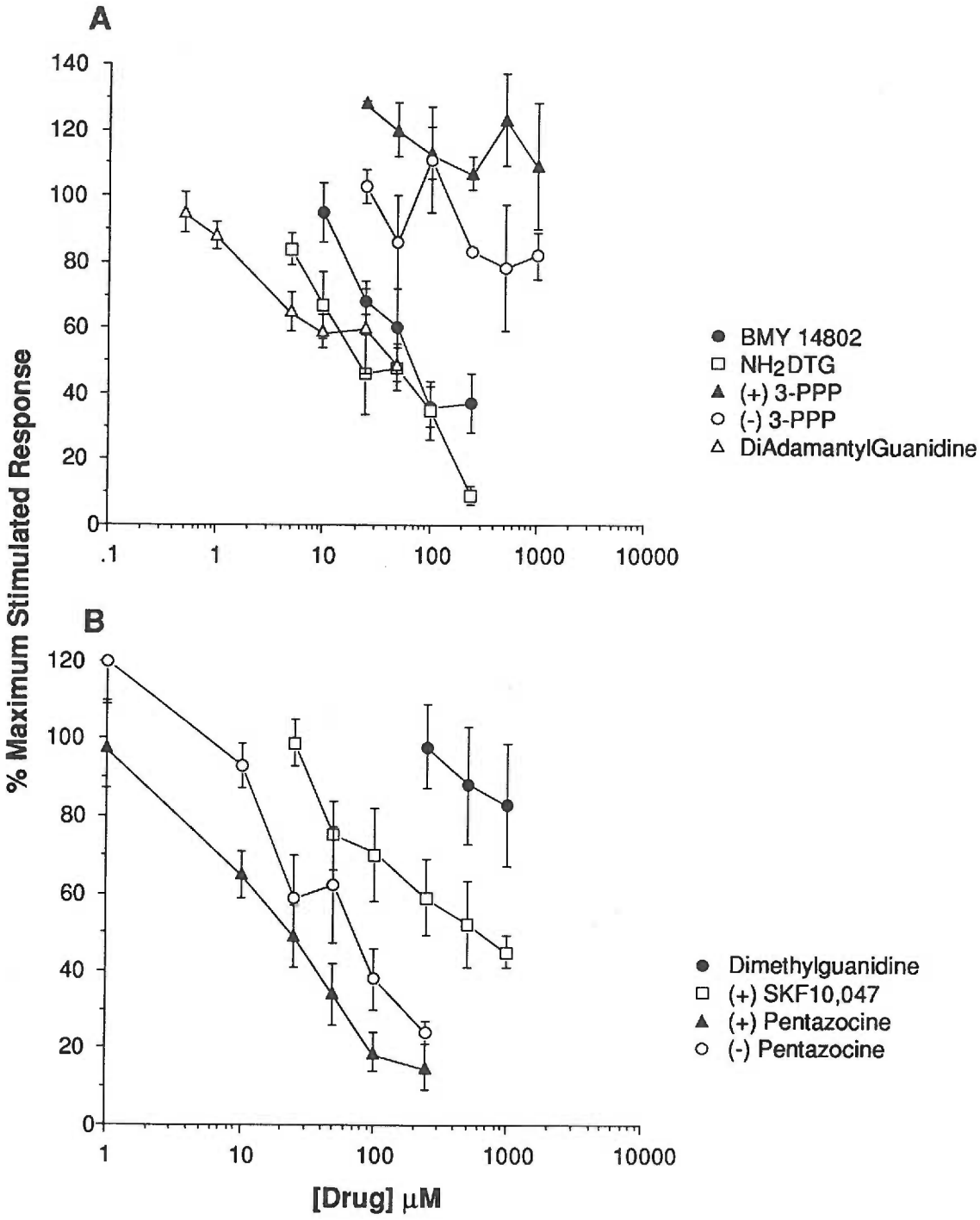

Total [3H] Ins P1
(cpm±s.e.m./well)
5000
4000
3000
2000
1000
0
100nM
1μM
10μM
100 μM
DMPP
Atropine
Hexamethonium
Control
1mM Carb

Figure 16. (A-C)Dose response curves for inhibition of carbachol-stimulated increases in [^{3}H]InsP_1 levels in NCB-20 cells by various drugs (values = mean ± s.e.m.; n = 3-14).


A
140
120
100
80
60
40
20
0
.1
1
10
100
1000
10000
BMY 14802
NH2DTG
(+) 3-PPP
(-) 3-PPP
DiAdamantylGuanidine
B
120
100
80
60
40
20
0
1
10
100
1000
10000
% Maximum Stimulated Response
[Drug] μM
Dimethylguanidine
(+) SKF10,047
(+) Pentazocine
(-) Pentazocine

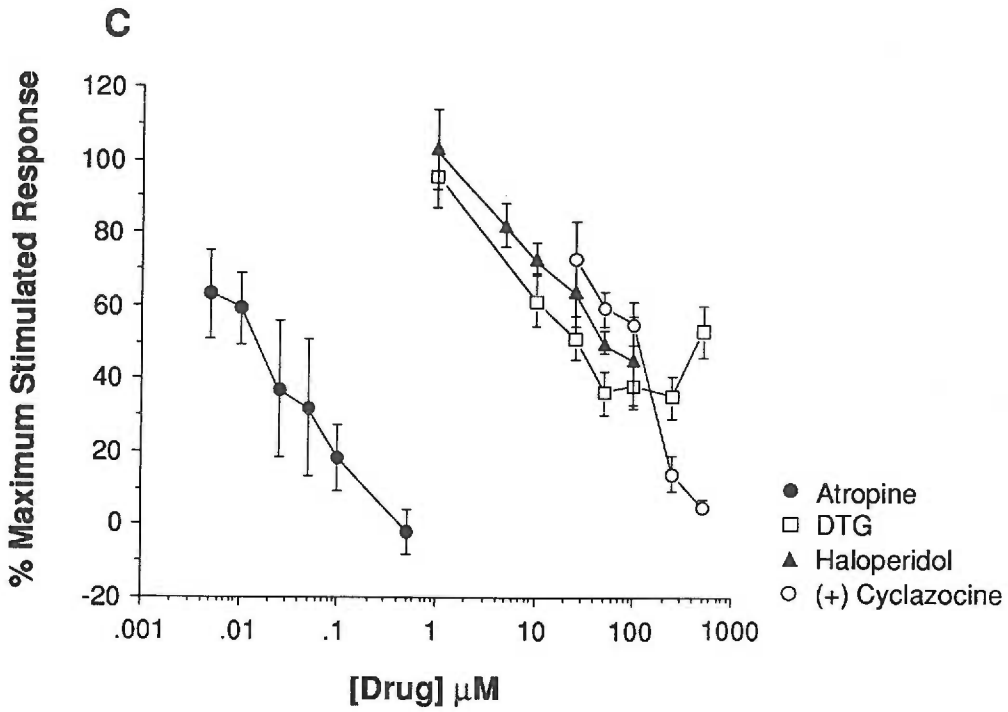

C
% Maximum Stimulated Response
120
100
80
60
40
20
0
-20
.001
.01
.1
1
10
100
1000
[Drug] μM
Atropine
DTG
Haloperidol
(+) Cyclazocine

Table 5. ED_{50} Values for Inhibition of Carbachol-Stimulated [^{3}H]$InsP_1$ Accumulation in NCB-20 Cells

Drug	ED_{50} (μM ± s.e.m.)
Atropine	.025 ± .018
(+) Pentazocine	28 ± 5
Diadamantylguanidine	31 ± 5
NH_2DTG	47 ± 9
DTG	49 ± 13
Haloperidol	61 ± 17
(-) Pentazocine	65 ± 22
BMY 14802	73 ± 16
(+) Cyclazocine	83 ± 17
(+) SKF-10,047	492 ± 162
Dimethylguanidine [1]	> 1mM
(+) 3-PPP [2]	> 1mM
(-) 3-PPP [3]	> 1mM

Experiments were conducted in duplicate or triplicate; n=3-14

[1] ED_{20} for dimethylguanide=1mM
[2] No significant inbibition of stimulated response with 1mM (+)3-PPP
[3] ED_{20} for (-)3-PPP=1mM

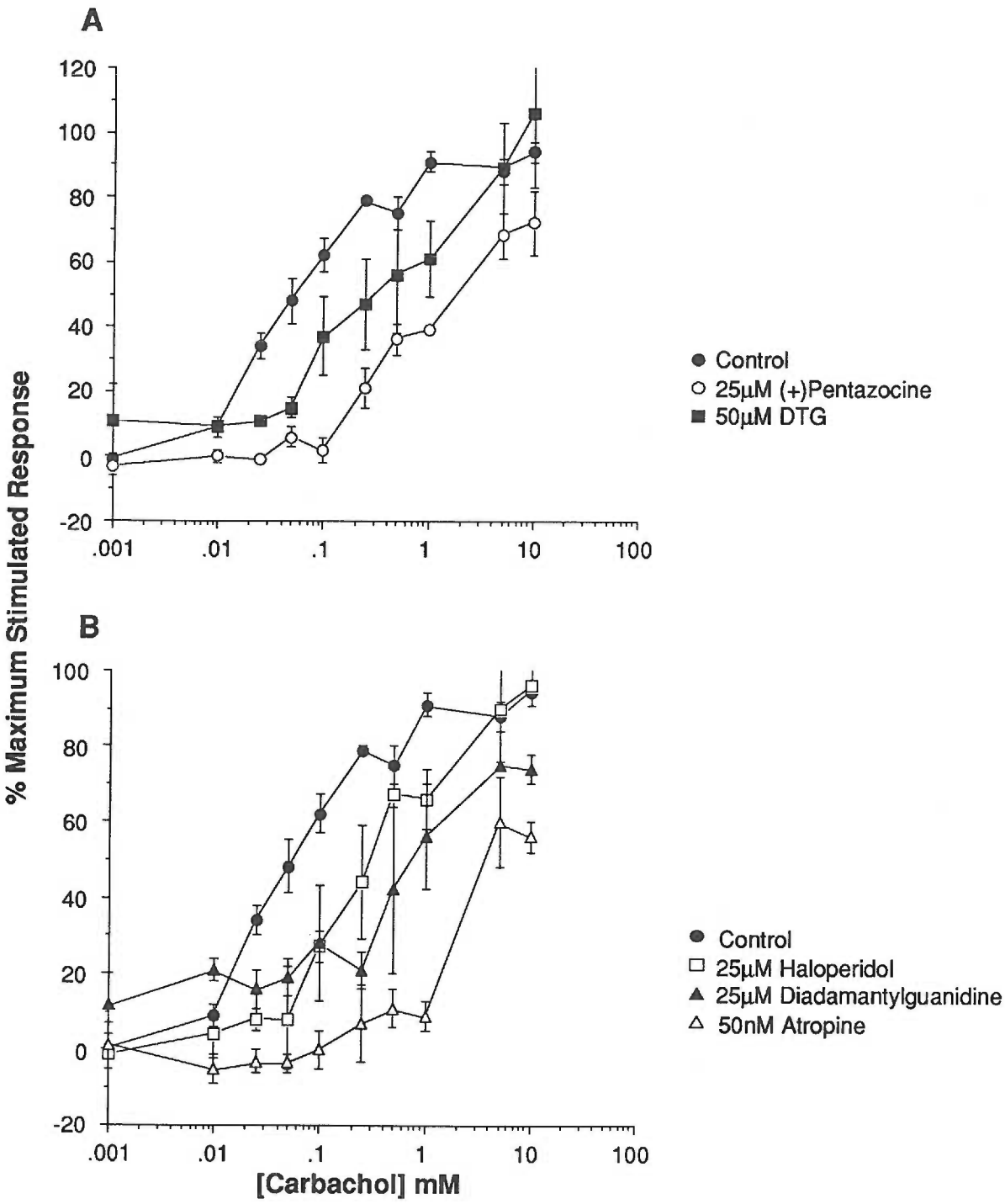
response at that dose of DTG. The antipsychotic drug, haloperidol, and the DTG congener, diadamantylguanidine, antagonized the response, although 100% inhibition was not reached at the highest concentrations tested; the high percentage of methanol required to properly dissolve these compounds prevented them from being tested at higher concentrations without inducing methanol-related changes in inositol phosphate levels. The potential therapeutic compound, BMY 14802, elicited up to a 65% decrease in stimulated [^{3}H]InsP$_1$ levels at the highest dose tested. Higher concentrations of BMY 14802 produced a significant increase in basal levels of [^{3}H]InsP$_1$ (an approximate 75% increase) and therefore were not included in the dose response analysis. The sigma ligand (+)SKF-10,047 was observed to produce a 55% decrease in the carbachol response at the highest dose applied.

Both stereoisomers of 3-PPP failed to inhibit carbachol-stimulated [^{3}H]InsP$_1$ accumulation by 50% at 1 mM concentration. (+)3-PPP appeared not to antagonize the response to any significant extent. Antagonism by (-)3-PPP reached a maximum of 20% at 1 mM with a high degree of variability. The compound, dimethylguanidine, which fails to bind the sigma receptor in radioligand binding assays also failed to produce substantial inhibition of the stimulated response at 1mM concentration. Neither (+)3-PPP, (-)3-PPP, nor dimethylguanidine had any effect on basal levels of [^{3}H]InsP$_1$.

C. The Nature of the Antagonism of the Carbachol Response by Sigma Ligands

Carbachol dose response curves were constructed in the presence and absence of several sigma ligands in an effort to determine the nature of the inhibitory effect of these drugs. Figure 17 illustrates the rightward shift of the carbachol dose response curve that results from the inclusion of (+)pentazocine, DTG, haloperidol, or diadamantylguanidine in the assay at concentrations equal to or below their respective ED_{50} values. It can be seen that the antagonism by these four sigma compounds can be overcome by increasing concentrations of agonist, indicative of an inhibition that is competitive in nature. The drugs differ, though, in their efficacy. For

Figure 17. Carbachol dose response curves for the elevation of [^{3}H]InsP_1 levels in NCB-20 cells in the absence or presence of sigma compounds or the muscarinic-receptor antagonist, atropine (values = mean ± s.e.m.; n = 3 - 7).


A
120
100
80
60
40
20
0
-20
.001
.01
.1
1
10
100
Control
25µM (+)Pentazocine
50µM DTG
B
100
80
60
40
20
0
-20
.001
.01
.1
1
10
100
% Maximum Stimulated Response
[Carbachol] mM
Control
25µM Haloperidol
25µM Diadamantylguanidine
50nM Atropine

example, whereas 25 uM (+)pentazocine is effective in producing a 40-fold increase in the IC_{50} for carbachol (from 50 uM to 2 mM), 50 uM DTG increases the IC_{50} value for carbachol only 5-fold (from 50 uM to 250 uM). It is evident that higher concentrations of agonist are needed to overcome the inhibition of 50 nM atropine, a competitive muscarinic cholinergic receptor antagonist.

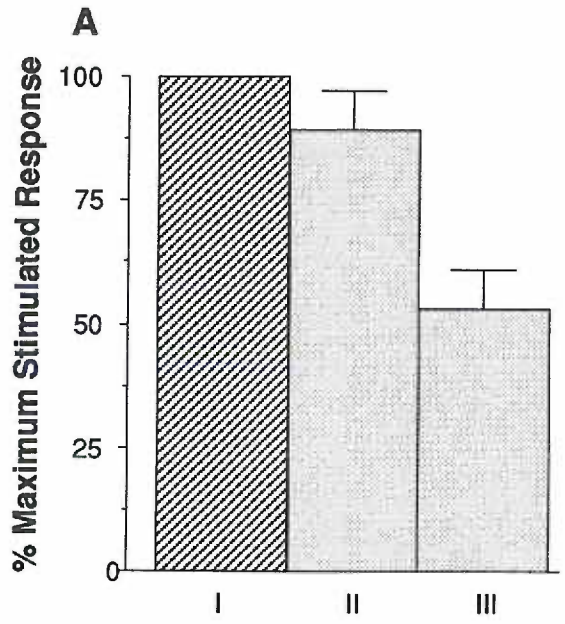
A series of experiments was conducted to investigate the reversibility of the effects of DTG, the results of which are shown in Figure 18. The experimental design consisted of stimulating the cells with carbachol (or vehicle) under three separate conditions: i) cells were exposed to vehicle (physiological salt solution) for 5 min, the medium was then removed and cells rinsed before replacement with fresh Li^+-containing medium and stimulation with 500 uM carbachol or vehicle; ii) cells were pretreated with 50 uM DTG for 5 min, the medium was then removed and cells rinsed before replacement with fresh Li^+-containing medium and stimulation with 500 uM carbachol or vehicle; iii) cells were exposed to vehicle for 5 min, the medium was then removed and cells rinsed before replacement with fresh Li^+-containing medium followed by stimulation with carbachol (or vehicle) in the presence of 50 uM DTG. The effectiveness of the rinses was measured by monitoring the amount of [^{3}H]DTG remaining in the medium in parallel plates. It was found that following the rinsing protocol, 3% of the added counts were left in the medium and 0.5% more counts were extractable from the cells with 0.2N NaOH.

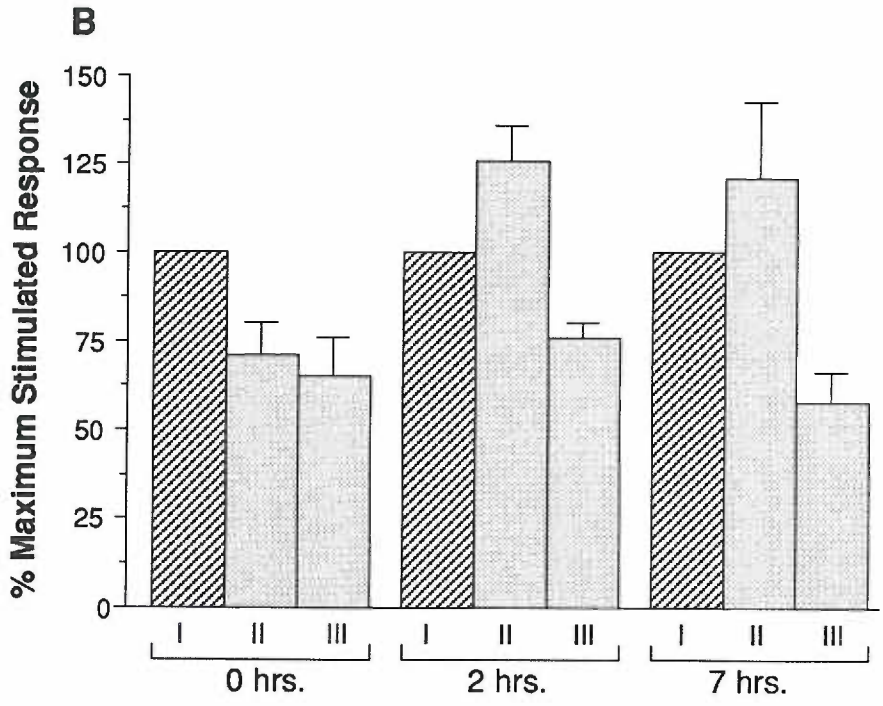
It can be seen (Fig 18A) that when cells are rinsed after treatment with 50 uM DTG, the response to carbachol is nearly fully recovered. The rinsing treatment itself does not alter the inhibitory effects of DTG (examined under protocol iii). When a two- or seven-hour period is allowed to elapse between the time the cells are rinsed and the moment they are exposed to agonist, the cells pretreated with 50 uM DTG accumulate as much, if not more, [^{3}H]$InsP_1$ as in the control condition (Fig 18B). The carbachol response can still be antagonized, however, when DTG and carbachol are added concomitantly at the respective time points. The time course studies of the reversibility of DTG's actions in NCB-20 cells were initiated in light of the results of Bowen et al (1989) indicating that

Figure 18. Reversibility of the inhibition by 50 uM DTG of carbachol-stimulated phosphoinositide hydrolysis.
(A)Experimental conditions:

	Pretreatment (5 min)	Wash	Stimulating agent
I)	PSS	3X rinse	500 uM carbachol (or PSS)
II)	50 uM DTG	3X rinse	500 uM carbachol (or PSS)
III)	PSS	3X rinse	500 uM carbachol (or PSS) in the presence of 50 uM DTG.

(B)Experimental conditions as for (A). In addition, the time elapsed between the rinses and the beginning of the stimulation period is varied from 0, to 2, or 7 hr.


A
% Maximum Stimulated Response
100
75
50
25
0
I
II
III


B
% Maximum Stimulated Response
150
125
100
75
50
25
0
I
II
III
I
II
III
I
II
III
0 hrs.
2 hrs.
7 hrs.

the effects of (+)pentazocine (50 uM) were not reversible in rat brain synaptoneurosomes. We deduced that this may be a time-dependent phenomenon given that our protocols differed with respect to the time elapsed between removal of the unbound sigma ligand and subsequent stimulation with muscarinic agonist. Whereas we initially included a seven hour waiting period for cells to return to their original, pre-wash condition (morphologically), our fellow investigators added agonist as soon as the synaptoneurosomes were resuspended after removal of (+)pentazocine. As our results indicate, however, the reversibility of DTG's effects are observed at every time point.

The antagonism by DTG of the carbachol response is not a function of its preincubation time prior to the addition of agonist; the extent of inhibition is equivalent whether DTG is added 30 minutes prior to or concomitant with the addition of agonist (not shown). When the time course for the accumulation of $[^3H]InsP_1$ by carbachol is plotted in the presence of 50 uM DTG, the levels of tritiated inositol monophosphates are reduced at every time point compared to control (not shown). Thus, there is no delay in the development of the inhibitory effect of DTG.

The correlation between K_i values for displacement of $[^3H]$DTG binding and ED_{50} values in the antagonism of the carbachol response is not very strong (Fig 19A). When all twelve compounds are included in the analysis, the correlation coefficient equals 0.56 ($P<0.1$) when a plot of K_i vs ED_{50} values is analyzed. When.rank orders of potency are compared, $r = 0.47$ ($P>0.1$) (not shown). The degree of correlation is slightly decreased when (+) and (-)3-PPP, and dimethylguanidine are excluded from the regression analysis ($r = 0.52$ ($P>0.1$) and $r = 0.39$ ($P>0.1$) for correlation of Ki vs ED_{50} in terms of values or rank orders of potency, respectively).

The correlation between K_i values for displacement of (+)$[^3H]$3-PPP binding and ED_{50} values for inhibition of the carbachol-induced response is even less than that described above for $[^3H]$DTG binding. While the correlation coefficient is 0.27 ($P>0.1$) when K_i and ED_{50} values are compared (fig 19B), this value approaches zero when the rank orders of

Figure 19. Correlation plot of ED_{50} values against the carbachol response vs K_i values for inhibition of [^{3}H]DTG(A) or (+)[^{3}H]3-PPP(B) binding by various drugs in NCB-20 cells.

(A) r = 0.56($P>0.1$) when the ED_{50} values for dimethylguandine, (+)3-PPP, and (-)3-PPP are set at 1 mM and the K_i values for (+)cyclazocine and (+)SKF-10,047 are each set at 10 uM.

r = 0.67($P<0.05$) when (+)3-PPP is excluded.

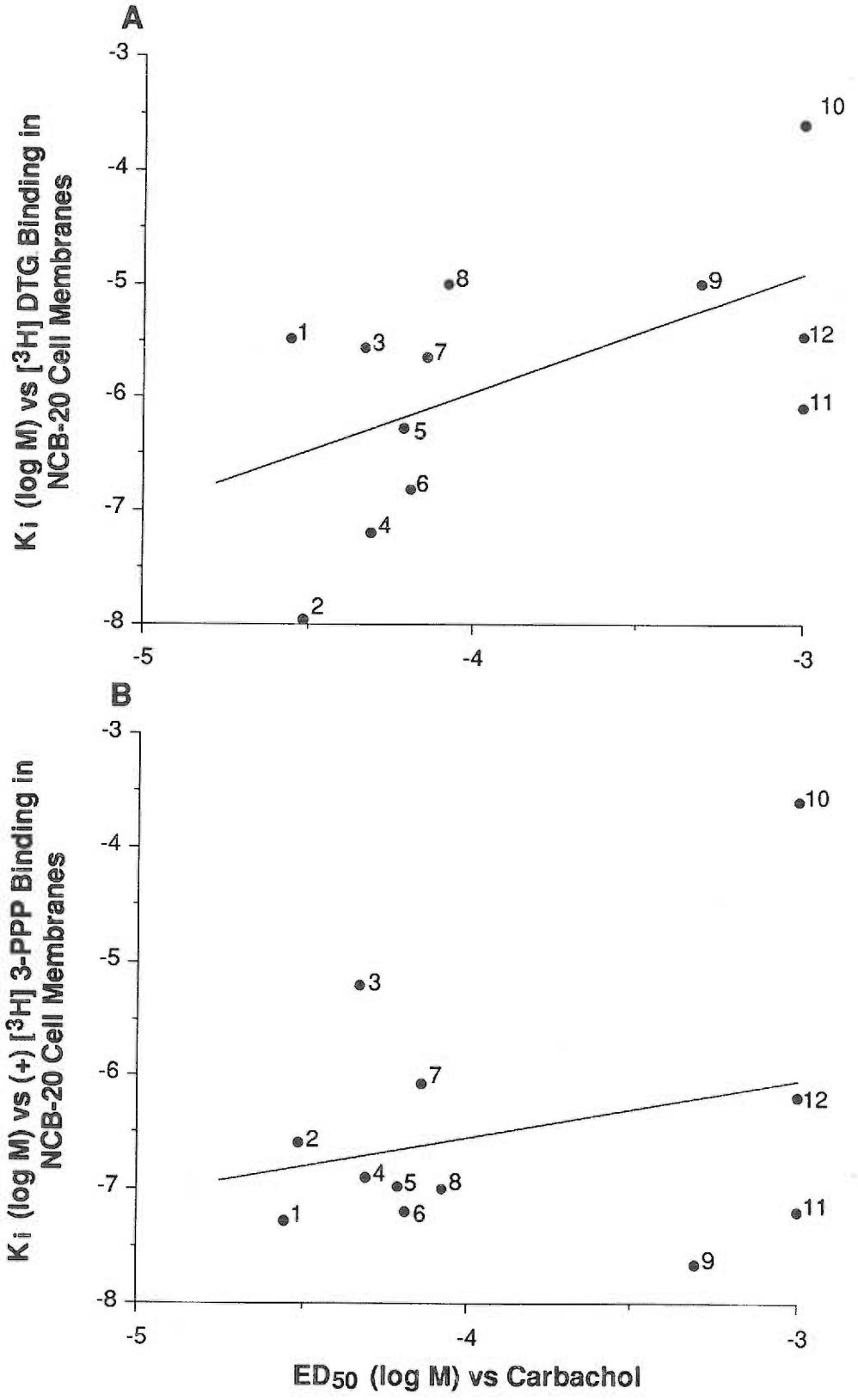
r = 0.52($P>0.1$) when dimethylguanidine, (+)cyclazocine, and (+)SKF-10,047 are excluded.

(B) r = 0.27($P>0.1$) when the ED_{50} values for dimethylguanidine, (+)3-PPP, and (-)3-PPP are set at 1 mM.

r = 0.41($P>0.1$) when (+)3-PPP is excluded.

r = -0.40($P>0.1$) when dimethylguanidine, (+)3-PPP, and (-)3-PPP are excluded.

1. (+)pentazocine; 2. diadamantylguanidine; 3. NH2DTG; 4. DTG; 5. haloperidol; 6. (-)pentazocine; 7. BMY 14802; 8. (+)cyclazocine; 9. (+)SKF-10,047; 10. dimethylguanidine; 11. (+)3-PPP; 12. (-)3-PPP.


A
B
Ki (log M) vs [3H] DTG Binding in NCB-20 Cell Membranes
Ki (log M) vs (+) [3H] 3-PPP Binding in NCB-20 Cell Membranes
ED50 (log M) vs Carbachol
-3
-4
-5
-6
-7
-8
1
2
3
4
5
6
7
8
9
10
11
12

potency for the two parameters are compared instead ($r = 0.03$ ($P>0.1$).

D. The Effect of Sigma Compounds on Bradykinin-stimulated [^{3}H]InsP_1 Accumulation

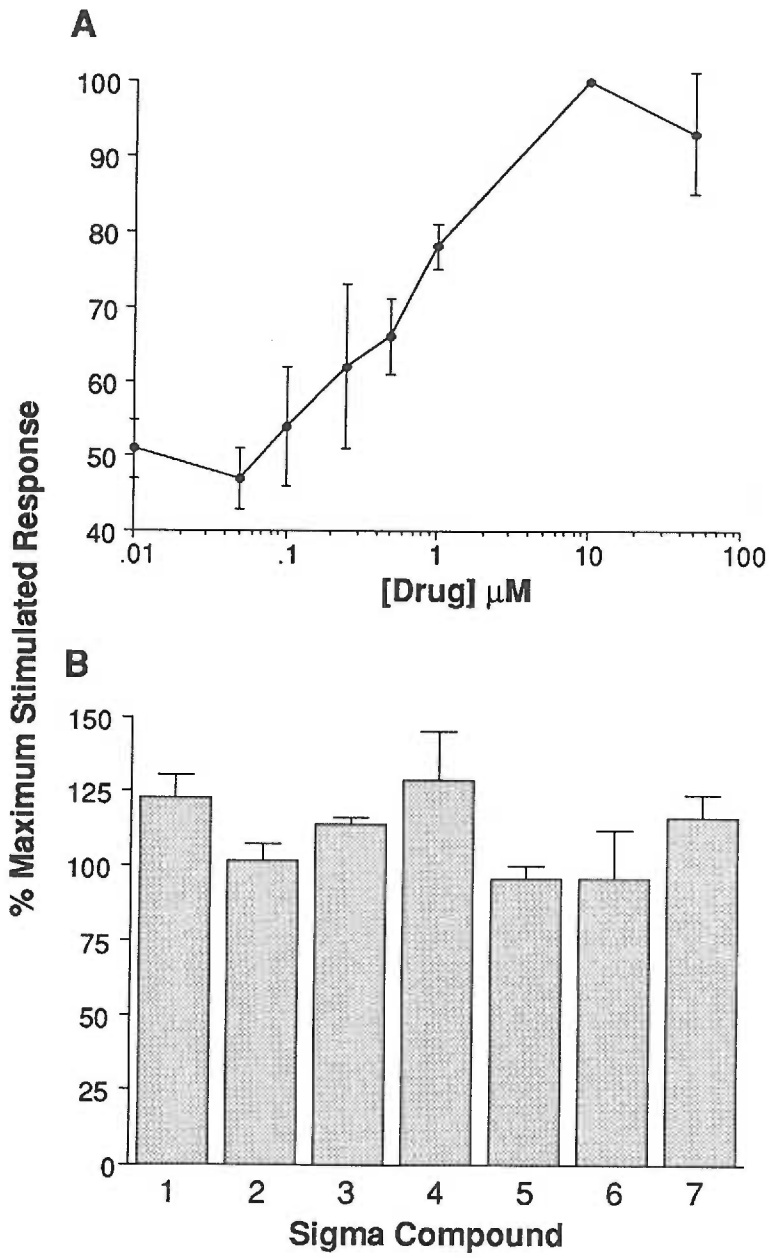
NCB-20 cells also express functional bradykinin receptors capable of eliciting a robust accumulation of inositol phosphates and with greater potency than carbachol (Chuang and Dillon-Carter). The dose response curve for bradykinin is shown in Fig 20A and indicates an ED_{50} value of 100 nM. On average, exposure of cells to 1 uM bradykinin lead to a 5-fold increase in stimulated [^{3}H]InsP_1 accumulation. In contrast to that observed in the case of carbachol stimulation, sigma ligands were ineffective in diminishing the bradykinin response at the highest dose tested (Fig 20B).

E. Discussion

The carbachol response in NCB-20 cells is mediated via muscarinic cholinergic receptors and characterized by a 2.5- to 5-fold increase in the stimulated accumulation of [^{3}H]InsP_1. As previously reported (Chuang), this response can not be measured in the abscence of Li^+. Stimulated levels of [^{3}H]inositol polyphosphates were not detectable under any conditions.

The effects of a series of compounds on agonist-stimulated PI hydrolysis were characterized and the results indicated that all drugs tested known to bind sigma receptors in radioligand binding assays, except for the stereoisomers of 3-PPP, were seen to modulate the carbachol response in an antagonistic fashion. The sigma inactive guanidine congener, dimethylguanidine, failed to inhibit the accumulation of [^{3}H]InsP_1 to a significant extent at 1mM concentration. In general, it is apparent that the doses required for the sigma ligands to exert their antagonistic effects far exceed their respective K_i values against (+)[^{3}H]3-PPP and [^{3}H]DTG binding in cell membrane suspensions (compare Tables 1 and 5; for example, the ED_{50}/K_i vs (+)[^{3}H]3-PPP ratios for the drugs tested range from approximately 8 for NH_2-DTG to 528 for (+)pentazocine, and >22,000

Figure 20. (A)Bradykinin dose response curve for the accumulation of [^{3}H]InsP1 in NCB-20 cells. (B)The effect of various sigma compounds on the response elicited by 1 uM bradykinin (values = mean ± s.e.m.). 1. 500 uM (+)cyclazocine; 2. 50 uM diadamantylguanidine;3. 500 uM DTG; 4. 100 uM haloperidol; 5. 500 uM NH_2DTG; 6. 250 uM (+)pentazocine; 7. 250 uM (-)pentazocine.


A
100
90
80
70
60
50
40
.01
.1
1
10
100
[Drug] μM
% Maximum Stimulated Response
B
150
125
100
75
50
25
0
1
2
3
4
5
6
7
Sigma Compound

for (+)SKF; the ED_{50}/K_i vs [^{3}H]DTG ratios vary from 8 for (+)pentazocine, to 424 for (-)pentazocine, and 1,000 for DTG) It should be noted that the ED_{50} values reported here for the antagonism of stimulated phosphoinositide hydrolysis by sigma ligands are on par with those reported for sigma receptor regulation of contractile responses of the guinea pig ileum longitudinal muscle/myenteric plexus (LMMP) preparation and phosphoinositide-linked receptor systems in rat brain neurosynaptosomes. Both of these *in vitro* bioassay systems implicate a sigma receptor-mediated mechanism for the actions of the sigma ligands tested (Campbell et al, 1989; Campbell et al, submitted; Bowen et al, in press). A dose of carbachol equivalent to 50 times its K_i value vs (-)[^{3}H]QNB (a nonselective muscarinic antagonist) binding is necessary to elicit 50% of its maximum response in NCB-20 cells (K_i = 1 uM, ED_{50} = 50 uM; tables 7 and 5, respectively). Likewise, whereas the K_D for [^{3}H]oxotremorine-M (a muscarinic agonist) in synaptoneurosomes is 39 nM, its ED_{50} value for stimulation of phosphoinositide hydrolysis is approximately 10uM (Bowen et al, in press). It is therefore not feasible to rule out an indirect action of sigma drugs on the muscarinic receptor-linked PI system via sigma receptor activation based solely on the comparatively high doses of sigma drug required for antagonism of the response.

There is apparent specificity of this inhibitory effect of sigma compounds as bradykinin signalling in NCB-20 cells is not disturbed by incubation with high concentrations of sigma drugs.

The abherent behavior of (+)3-PPP in the NCB-20 cell PI bioassay is not unique. The data of Bowen et al (in press) indicate that whereas (+)3-PPP does produce a concentration-dependent attenuation of the carbachol effect, a plateau is reached with 100 uM (+)3-PPP at about 40% inhibition of the response, and maintained even at a drug concentration of 1mM. (+) and (-)3-PPP were also found to correlate poorly in the guinea pig LMMP preparation, both displaying lower potency in the bioassay compared with their binding affinities in [^{3}H]DTG binding assays (Campbell et al, 1989).

The inhibitory effects of sigma drugs appear to be reversible and competitive in nature, as inferred by experiments conducted on a more limited number of sigma ligands. It is not possible to ascertain from these data alone whether the competitive inhibition is the result of direct, anticholinergic effects of these compounds or the result of allosteric interactions with a distinct site, activation of which can modulate the affinity of muscarinic agonists. However, the poor correlation between the potency of sigma ligands in the PI assay and their binding affinity in sigma radioligand binding assays (Fig 19) argues against the notion of sigma receptor involvement. Two other observations argue the same point. For one, the sigma-specific ligand (+)3-PPP (100 uM) was unable to reverse the inhibitory effects of DTG (100 uM) when added simultaneously (not shown). Since (+)3-PPP has no effect on its own, it would seem reasonable to expect it to directly compete with DTG at the sigma site thereby attenuating its effects. In this context, (+)3-PPP would behave as an antagonist and DTG an agonist at the sigma site. However, this was not the case. Another important observation is that, when present, the effects of the sigma drugs tested were all of an inhibitory mode - - no clear "agonists" or "antagonists" were identified. Thus, the neuroleptic, haloperidol, and the psychotomimetic, (+)SKF-10,047, both reduced the magnitude of the stimulated response. These findings prompted us to investigate the possibility that sigma drugs exert their inhibitory actions by competing directly with carbachol for binding at the muscarinic receptor ligand binding site, effectively acting as antimuscarinic agents.

3. THE EFFECT OF SIGMA COMPOUNDS ON THE SPECIFIC BINDING OF (-)[^{3}H]QNB, A HIGH AFFINITY, NONSELECTIVE MUSCARINIC-CHOLINERGIC RECEPTOR ANTAGONIST

A. Brief Introduction

Receptor classes capable of inhibiting stimulated phosphoinositide breakdown have recently been identified (Linden and Delahunty). Hence, it appears that, just as for adenylate cyclase activity, the activity of phospholipase C can be modulated in a stimulatory and inhibitory fashion.

The receptor-mediated regulation of PL-C activity can be attained by direct or indirect mechanisms. For example, in GH_3 cells, adenosine analogs acting at A_1 receptors seem to decrease thyrotropin releasing hormone(TRH)-stimulated phosphoinositide breakdown by directly inhibiting PL-C via coupling with a GTP-binding protein. On the other hand, dopamine receptor-mediated inhibition of TRH response in pituitary lactotroph cells (Enjalbert et al) appears to be secondary to a reduction by dopamine of intracellular calcium levels (Vallar et al). Similarly, activation of the NMDA-subtype of excitatory amino acid receptors strongly inhibits the stimulation of PI hydrolysis by certain agonists; these affects appear to be secondary to Na^+ influx through the NMDA-associated cation channel (Baudry et al; Morrisett et al; Gonzales and Moerschbaecher).

While receptor-mediated mechanisms for negative coupling to PL-C activity are being discovered, the data we have so far accumulated regarding the inhibitory effects of sigma drugs on carbachol-stimulated PI turnover in NCB-20 cells suggest that one of the more likely mechanisms of action for these drugs is direct competition at the muscarinic binding site. The strongest piece of evidence for this notion is the relatively poor correlation between the potency of sigma ligands in the PI assay and their binding affinity at the sigma site ($r = 0.56$ and 0.27 for ED_{50} vs [^{3}H]DTG binding and ED_{50} vs (+)[^{3}H]3-PPP, respectively (fig 19A,B); $r = 0.47$ and 0.03 for rank orders of potency in the bioassay vs [^{3}H]DTG binding assay and rank orders of potency in the bioassay vs (+)[^{3}H]3-PPP binding assay, respectively). Such a direct interaction might also account for the apparent specificity of these effects as indicated by the lack of effect of sigma compounds on bradykinin-induced responses (Fig. 20B). In order to test this hypothesis, studies were conducted to investigate the nature and degree of sigma drug interference with binding of tritiated (-)quinuclidinyl benzilate ((-)[^{3}H]QNB), a high affinity muscarinic antagonist (Nathanson). The resulting pharmacological profile for sigma drug inhibition of (-)[^{3}H]QNB binding would then permit us to establish the correlation between the potency of inhibition of carbachol responses by sigma ligands and their potency as displacers of radioligand

specific binding at muscarinic receptors and thereby ascertain the validity of this proposal.

B. Binding Characteristics of (-)[^{3}H]QNB in NCB-20 Cell Membrane Suspensions.

Equilibrium saturation binding analysis of (-)[^{3}H]QNB binding in NCB-20 cell membrane suspensions reveals saturable, high affinity binding to a homogenous population of sites (Fig. 21A). The K_D and B_{max} values obtained were 129 pM and 66 fmol/mg membrane protein, respectively (Table 6).

C. Displacement of (-)[^{3}H]QNB Binding by Sigma Ligands in NCB-20 Cell Membrane Suspensions

The K_i values for binding of sigma compounds at muscarinic sites fall in the low micromolar range, except for diadamantylguanidine, whose affinity is the greatest (K_i of 77 nM; table 7 and fig. 22). The range of Ki values for sigma drugs against (-)[^{3}H]QNB binding is much less than that characteristic of displacement of sigma-specific radioligand binding (compare Tables 1 and 7). The pharmacological profile shows only negligeable stereoselectivity for benzomorphan enantiomers. The displacement curves are monophasic, and blockade of (-)[^{3}H]QNB binding is complete within the range of doses tested for all ligands examined, except dimethylguanidine and (-)3-PPP which display 70% and 75% inhibition of (-)[^{3}H]QNB binding at 1mM concentration, respectively (Fig.22).

Scatchard analysis of (-)[^{3}H]QNB binding in the presence of 5 uM (+)pentazocine or 10 uM DTG results in significant increases in its K_D value, lowering its affinity from 129 pM to 288 pm and 549 pM, respectively. The decrease in (-)[^{3}H]QNB binding is not accompanied by a significant change in the maximum number of binding sites (Table 6; fig 21B). These results indicate that (+)pentazocine and DTG competitively inhibit the binding of the labeled muscarinic antagonist.

Figure 21. (A)Equilibrium saturation binding curve and Scatchard analysis (inset) of (-)[^{3}H]QNB binding in NCB-20 membranes. (B)Scatchard analysis of equilibrium saturation binding data for (-)[^{3}H]QNB in the absence (closed circles) or presence of 10 uM DTG (closed boxes) or 5 uM (+)pentazocine (open circles) in NCB-20 membranes (values = mean ± s.e.m.; n = 4 - 5).

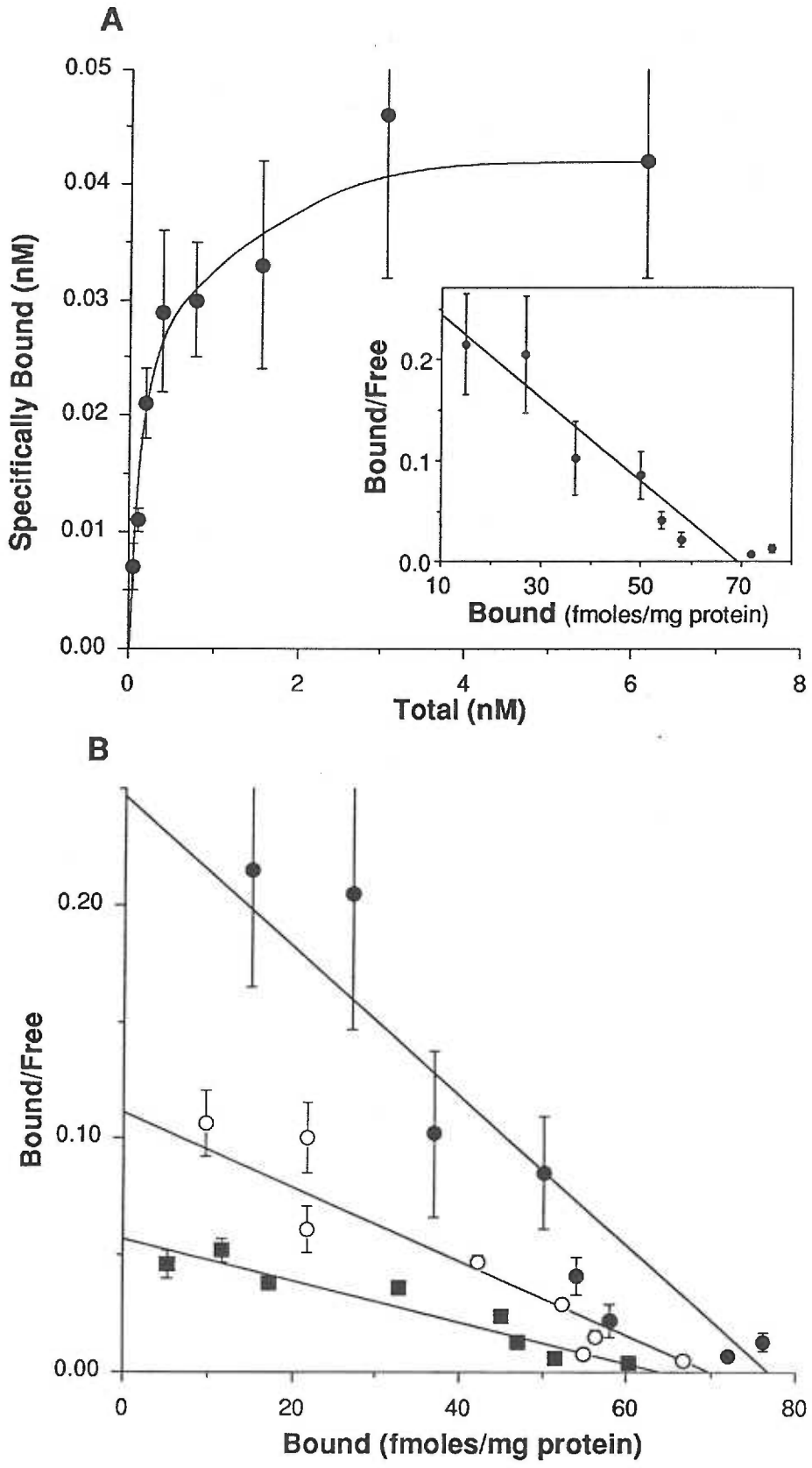

A
Specifically Bound (nM)
0.05
0.04
0.03
0.02
0.01
0.00
0
2
4
6
8
Total (nM)
Bound/Free
0.2
0.1
0.0
10
30
50
70
Bound (fmoles/mg protein)
B
Bound/Free
0.20
0.10
0.00
0
20
40
60
80
Bound (fmoles/mg protein)

Table 6. Equilibrium Saturation Binding Analysis of (-)[^{3}H]QNB Binding in NCB-20 Cell Membranes

	(-)[^{3}H]QNB	
Condition	K_D (pM ± s.e.m.)	B_{max}*
Control	129 ± 15	66 ± 9
in the presence of 5μM (+)pentazocine	288 ± 29	58 ± 5
in the presence of 10μM DTG	549 ± 163	59 ± 9

* (fmoles/mg protein ± s.e.m.)

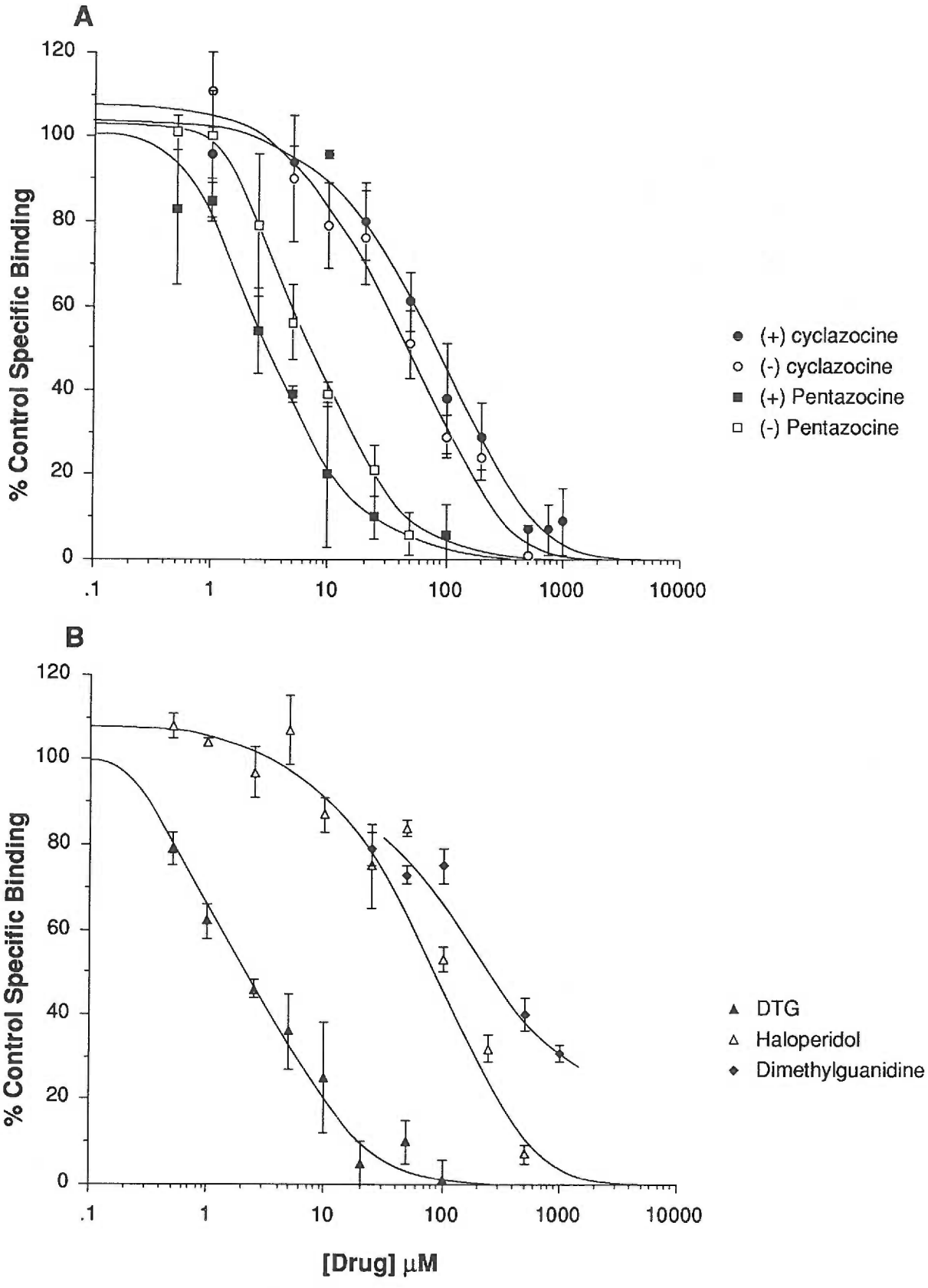
Experiments were run in duplicate or triplicate; n=4-5

Table 7. Displacement of (-)[^{3}H]QNB Binding in NCB-20 Cell Membranes by Various Drugs

Drug	Ki (μM ± s.e.m.)
Atropine	0.009 ± 0.001
Diadamantylguanidine	0.077 ± 0.020
Carbachol	1 ± 1
DTG	2 ± 1
(+) Pentazocine	2 ± 1
(-) Pentazocine	3 ± 1
BMY 14802	11 ± 5
(-) Cyclazocine	21 ± 14
NH_2DTG	35 ± 16
(-) SKF-10,047	43 ± 21
(+) Cyclazocine	45 ± 16
Haloperidol	60 ± 2
(+) 3-PPP	62 ± 10
(+) SKF-10,047	72 ± 24
Dimethylguanidine	188 ± 15
(-) 3-PPP	241 ± 21

Each experiment was run in duplicate; n=2-4

Figure 22. (A - E)Binding curves for displacement for specific (-)[^{3}H]QNB binding in NCB-20 cell membrane suspensions by various compounds (values = mean ± s.e.m.; n = 2 - 3).


A
120
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80
60
40
20
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% Control Specific Binding
.1
1
10
100
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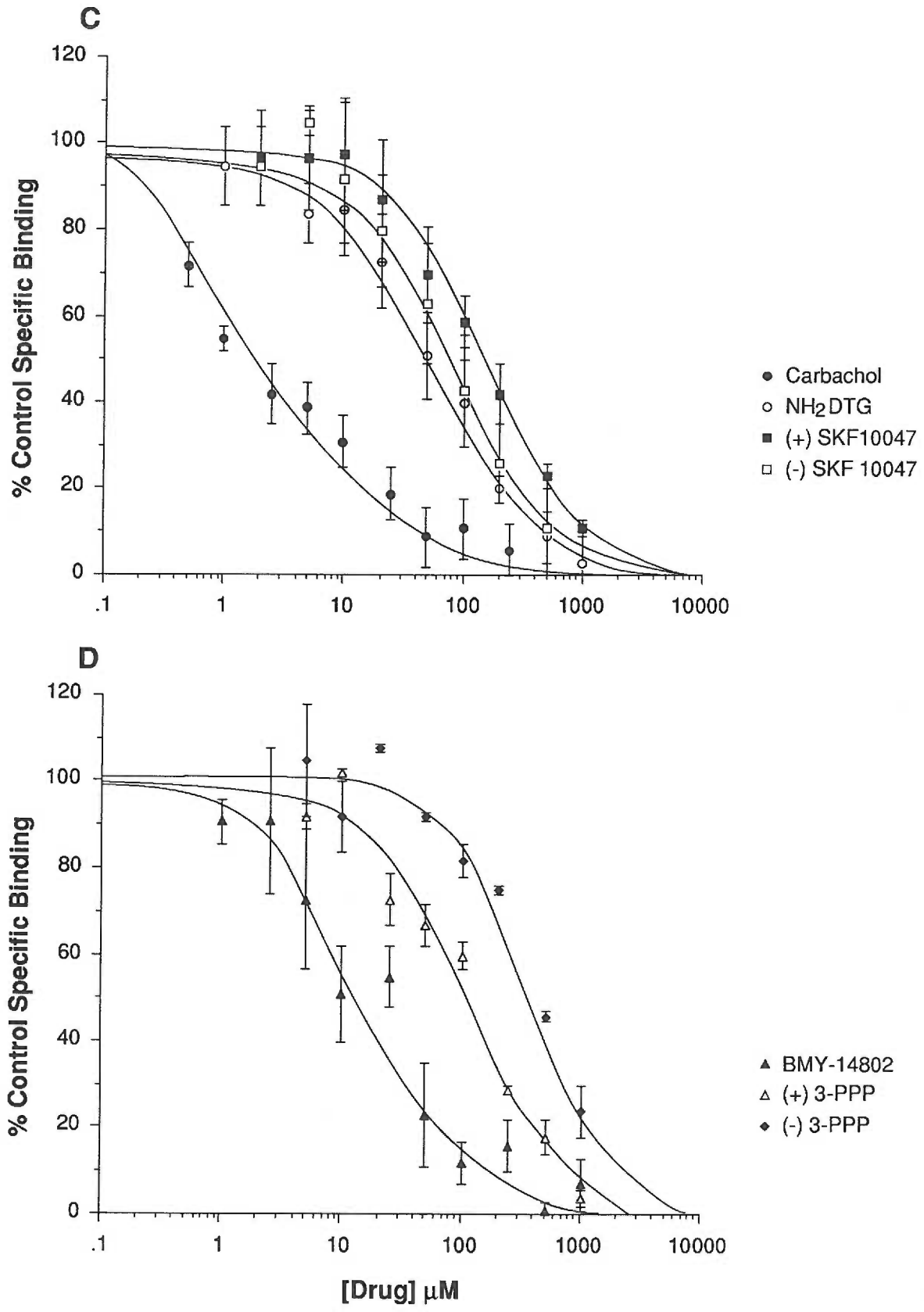
(+) cyclazocine
(-) cyclazocine
(+) Pentazocine
(-) Pentazocine
B
120
100
80
60
40
20
0
% Control Specific Binding
.1
1
10
100
1000
10000
[Drug] μM
DTG
Haloperidol
Dimethylguanidine


C
120
100
80
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0
% Control Specific Binding
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1
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10000
Carbachol
NH2DTG
(+) SKF10047
(-) SKF 10047
D
120
100
80
60
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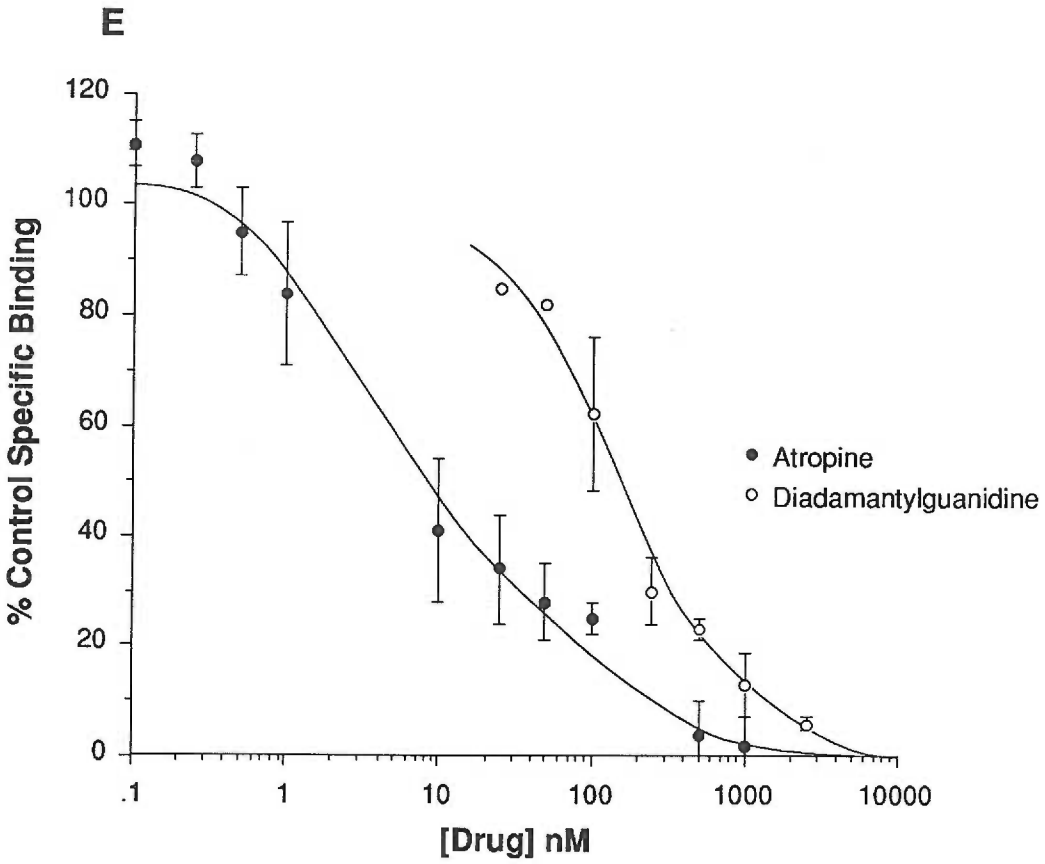
% Control Specific Binding
.1
1
10
100
1000
10000
[Drug] µM
BMY-14802
(+) 3-PPP
(-) 3-PPP


E
120
100
80
60
40
20
0
% Control Specific Binding
.1
1
10
100
1000
10000
[Drug] nM
Atropine
Diadamantylguanidine

The correlation between K_i's vs (-)[^{3}H]QNB binding and ED_{50} values for inhibition of the carbachol response is 0.74 ($P<0.01$) (fig. 23) and increases to 0.82 ($P=0.001$) when assessed in terms of rank orders of potency (not shown). We've previously seen that the correlation between bioactivity and binding vs [^{3}H]DTG or (+)[^{3}H]3-PPP equals 0.56 and 0.27, respectively (Fig. 19A,B).Thus, the biological potency of the ligands tested correlates better with their binding affinities at muscarinic-cholinergic rather than sigma receptor sites.

The stronger correlation coefficient for bioactivity and binding vs [^{3}H]DTG ($r = 0.56$) than for bioactivity and binding vs (+)[^{3}H]3-PPP ($r = 0.27$) is likely a consequence of the fact that the pharmacological profile for drug displacement of (-)[^{3}H]QNB binding is also more closely related to that for displacement of [^{3}H]DTG than (+)[^{3}H]3-PPP binding in NCB-20 cells ($r = 0.78$ ($P<0.001$) and 0.33 ($P>0.1$). Fig. 24A,B), respectively. The explanation for this is not clear. Perhaps there are some shared topographical features between muscarinic receptors and sites labeled by [^{3}H]DTG in NCB-20 cells. Alternatively, the relationship may just be coincidental. It may be important to note that the degree of correlation varies considerably as a function of the particular drugs included in the analysis. For example, if the three guanidine congeners, diadamantylguanidine, NH_2-DTG, and dimethylguanidine, and the sigma-specific drug, BMY 14802, are excluded from the analysis, the r value changes from 0.78 ($P<0.001$) to 0.56 ($P<0.1$) for K_i's against (-)[^{3}H]QNB vs [^{3}H]DTG. Evaluation of an analogous drug profile comparing binding affinities against (-)[^{3}H]QNB vs (+)[^{3}H]3-PPP only changes the r value from 0.33 ($P>0.1$) to 0.30 ($P>0.1$).

D. Discussion

The evidence gathered suggests that direct interaction of sigma ligands at muscarinic receptors is the likeliest explanation for the antagonism by sigma drugs of the carbachol response in NCB-20 cells. For one, the demonstrated competitive inhibition of the carbachol response by sigma compounds parallels the competitive effects of DTG and (+)pentazocine on (-)[^{3}H]QNB binding in NCB-20 cell membranes (DTG and (+)pentazocine decrease the affinity of (-)[^{3}H]QNB for the muscarinic receptor without

Figure 23. Correlation plot of ED_{50} values vs K_i values for inhibition of (-)[^{3}H]QNB binding in NCB-20 cell membranes.

r = 0.74($P<0.05$) when the ED_{50} values for (+)3-PPP, (-)3-PPP, and dimethylguanidine are set at 1 mM.

r = 0.75($P<0.05$) when (+)3-PPP is excluded from the analysis.

r = 0.62($P>0.1$) when (+)3-PPP, (-)3-PPP, and dimethylguanidine are excluded.

1. (+)pentazocine; 2. diadamantylguanidine; 3. NH_2DTG; 4. DTG; 5. haloperidol; 6. (-)pentazocine; 7. BMY 14802; 8. (+)cyclazocine; 9. (+)SKF-10,047; 10. dimethylguanidine; 11. (+)3-PPP; 12. (-)3-PPP.

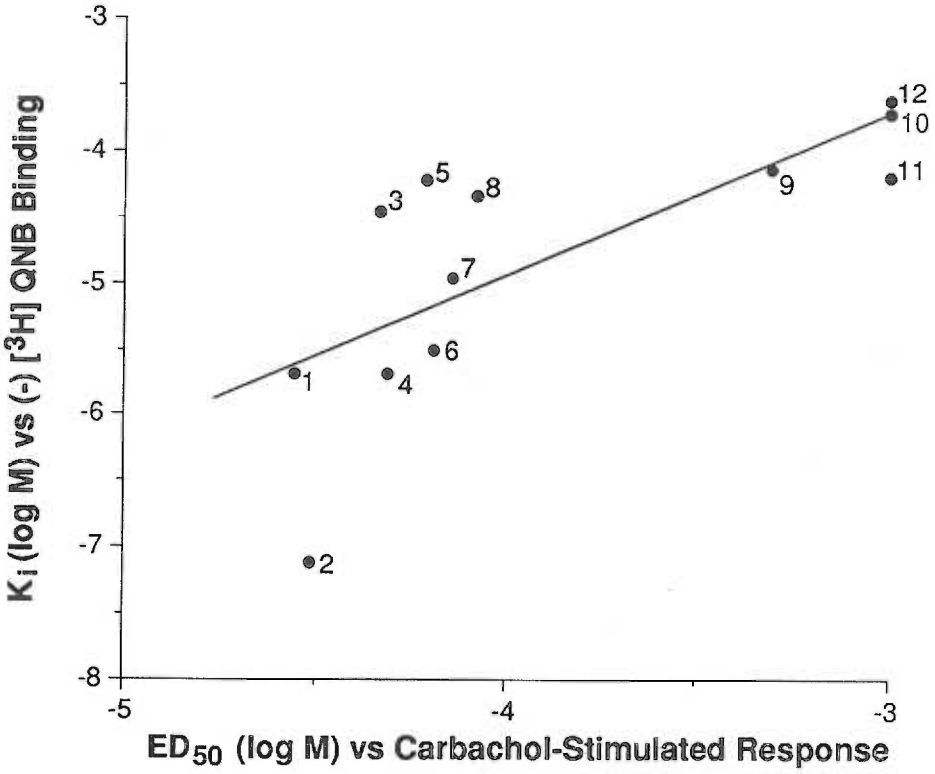

-3
-4
-5
-6
-7
-8
-5
-4
-3
K_i (log M) vs (-) [^{3}H] QNB Binding
ED_{50} (log M) vs Carbachol-Stimulated Response
1
2
3
4
5
6
7
8
9
10
11
12

Figure 24. Correlation plot of K_i's against [^{3}H]QNB binding vs K_i's against [^{3}H]DTG binding(A) or (+)[^{3}H]3-PPP binding(B) in NCB-20 cell membranes.

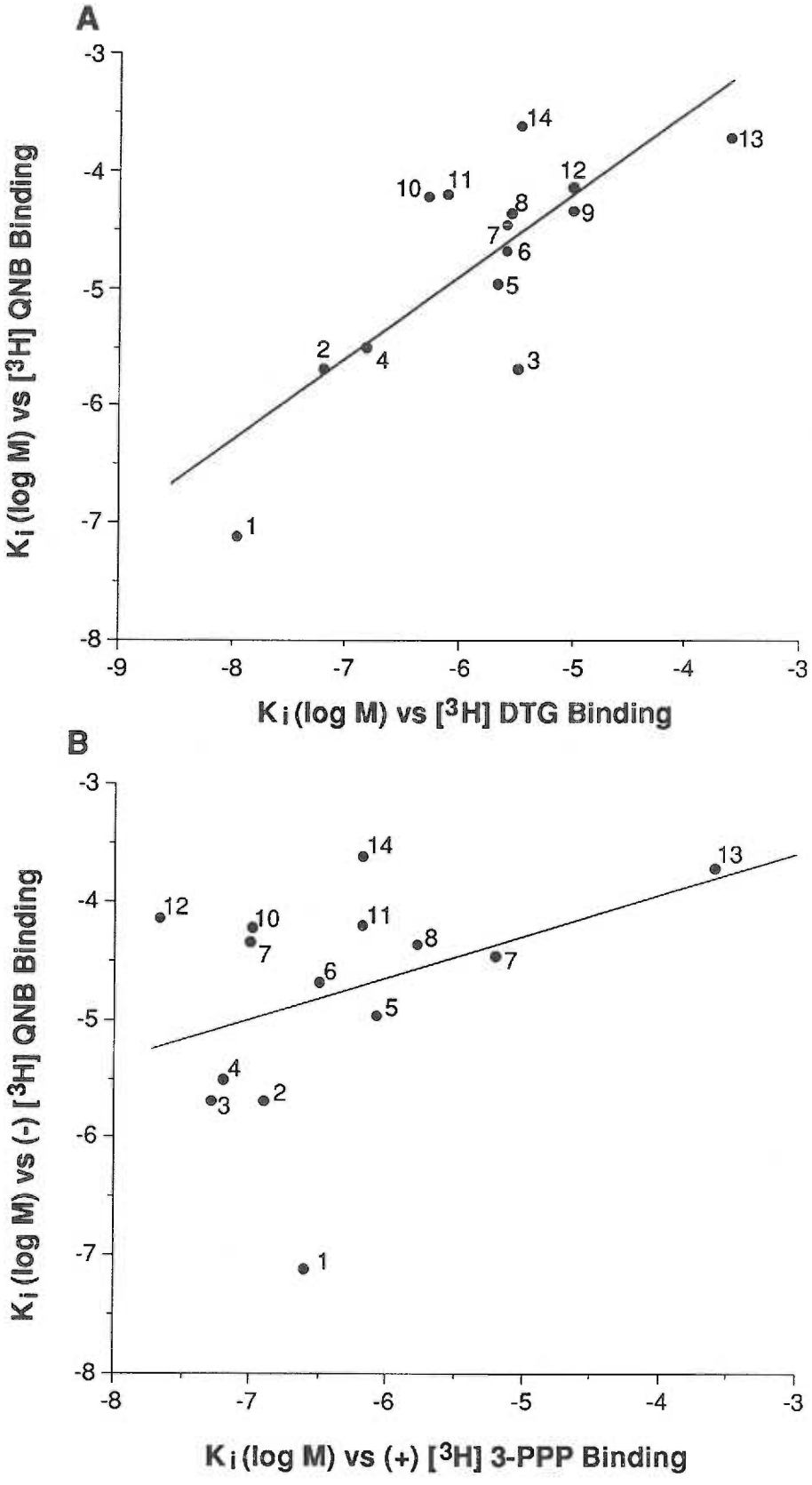
(A) r = 0.78($P<0.001$) when K_i values for displacement of [^{3}H]DTG binding by dimethylguanidine, (+)cyclazocine, and (+)SKF-10,047 are set at 250 uM, 10 uM, and 10 uM, respectively.

r = 0.77($P<0.01$) when dimethylguanidine is excluded.

r = 0.76($P<0.01$) when dimethylguanidine, (+)cyclazocine, and (+)SKF-10,047 are excluded.

(B) r = 0.33($P>0.1$) when the K_i for displacement of (+)[^{3}H]3-PPP binding by dimethylguanidine is set at 250 uM.

r = 0.14($P>0.1$) when dimethylguanidine is excluded from the analysis.


A
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-6
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-7
-6
-5
-4
-3
14
13
12
10
11
8
9
7
6
5
2
4
3
1
Ki (log M) vs [3H] QNB Binding
Ki (log M) vs [3H] DTG Binding
B
-3
-4
-5
-6
-7
-8
-8
-7
-6
-5
-4
-3
14
13
12
10
11
8
7
7
6
5
4
2
3
1
Ki (log M) vs (-) [3H] QNB Binding
Ki (log M) vs (+) [3H] 3-PPP Binding

affecting the maximum number of binding sites). Similar effects have recently been reported by Candura et al in their study of the inhibition of carbachol-induced PI metabolism by sigma compounds in rat brain cerebral cortex slices. Secondly, the potency of sigma drugs in the PI bioassay correlates best with their ability to displace (-)[^{3}H]QNB rather than [^{3}H]DTG or (+)[^{3}H]3-PPP binding.in NCB-20 cells. In general, the concentrations of sigma drugs required to produce a 50% reduction in the response to carbachol is similar to that responsible for displacement of 50% of specific (-)[^{3}H]QNB binding. This is in line with the effects of atropine, a known muscarinic antagonist. Thus, the ED_{50} value for antagonism of the carbachol response by atropine is 25 nM, while the K_i for atropine vs (-)[^{3}H]QNB binding is 9 nM. In contrast, the ED_{50} value for the muscarinic agonist, carbachol, is 50 times greater than its inhibition constant (K_i) against (-)[^{3}H]QNB binding.

Results indicated that the K_i's for sigma drugs against (-)[^{3}H]QNB binding correlated better with their ability to displace [^{3}H]DTG than (+)[^{3}H]3-PPP binding in NCB-20 cells. As previously mentioned, the drug specificity profile for (+)[^{3}H]3-PPP binding in NCB-20 cells resembles the prototypic profile for the sigma receptor as established in other tissues, whereas that of [^{3}H]DTG binding in NCB-20 cell membranes is unusual. The question may then arise as to the relationship between the muscarinic site and the "second DTG site". That these two sites may be one and the same is ruled out by two strong pieces of evidence: 1) 250uM atropine shows no significant displacement of specific [^{3}H]DTG binding (nor does it affect (+)[^{3}H]3-PPP binding -- data not shown) and, 2) the difference in B_{max} values between (+)[^{3}H]3-PPP- and [^{3}H]DTG-labeled sites can not be accounted for by the number of (-)[^{3}H]QNB-labeled sites (there appears to be approximately 6 pmol/mg membrane protein more of [^{3}H]DTG than (+)[^{3}H]3-PPP sites, and only 66 fmol/mg membrane protein of total (-)[^{3}H]QNB sites).

Assuming a model of direct interaction of sigma ligands with muscarinic receptors, however, makes it difficult to explain a few discrepancies. For example, diadamantylguanidine would have been expected to be a more potent antagonist than that observed based on its high affinity for sites

labeled by (-)[^{3}H]QNB (K_i = 77 nM, whereas ED_{50} = 31 uM). The observed lack of antagonism by (+)3-PPP is also difficult to explain light of its ability to displace specific (-)[^{3}H]QNB binding at low micromolar concentrations. Whereas dimethylguanidine and (-)3-PPP appear to produce only a 20% inhibition of the carbachol response at 1 mM concentration, this may be related to the fact that they are also the least potent displacers of (-)[^{3}H]QNB binding of the drugs screened. These discrepancies may be attributable to the difference in the agent used for stimulation of the PI response (i.e. carbachol) versus that employed in the muscarinic receptor radioligand binding assays (i.e. (-)[^{3}H]QNB). In addition, the measurement of bioactivity and binding affinity were conducted in different preparations; the bioactivity was measured using whole cells whereas the binding assays were conducted on cell membrane preparations. It may also simply be that higher doses of (+)3-PPP are needed to detect its effects (the observed weakness in the bioactivity of (+)3-PPP relative to its binding affinity has been reported for the guinea pig LMMP and rat brain synaptoneurosomes as well (Campbell et al, 1989, and Bowen et al, in press, respectively).

The possibility that sigma drugs affect muscarinic responses by acting at an allosterically coupled, non sigma-like site, can not be definitively ruled out. The observations that sigma compounds completely inhibit (-)[^{3}H]QNB binding over a reasonable range of drug concentrations and change the K_D value and not the B_{max} value for (-)[^{3}H]QNB binding are indicative of a direct interaction but not sufficient to rule out an allosteric one. A more conclusive means of investigating this question would be to investigate the effects of sigma compounds at appropriate concentrations on the kinetic parameters of specific binding of muscarinic receptor radioligand. Such studies have recently been conducted to delineate the mechanism(s) by which gallamine (a nicotinic neuromuscular blocker) (Ellis et al) and progesterone (Klangkalya and Chan) decrease the binding of (-)[^{3}H]QNB. If sigma drugs do indeed directly compete for the occupancy of muscarinic receptors, then they would not be expected to alter the rate of dissociation of (-)[^{3}H]QNB.

Interestingly, it has recently been shown that DTG, in micromolar quantities, is able to block nicotinic cholinergic receptors recorded in single guinea pig myenteric neurons (Galligan et al). This inhibition is due to blockade of nicotinic-ion channels. Correspondingly, the researchers were able to demonstrate that, unlike the inhibition of electrically- or 5-HT-evoked contractions of the longitudinal muscle by sigma drugs, the inhibition of contractions evoked by the nicotinic agonist, DMPP, are not related to actions at sigma receptors.

Our data regarding sigma drug effects on carbachol-stimulated PI hydrolysis in NCB-20 cells are at odds on several fronts with those reported by the laboratory of Wayne Bowen (Tolentino and Bowen; Bowen et al, in press) working with rat brain synaptoneurosomes. These investigators have found that the correlation between sigma binding affinities (K_i) and ED_{50} values for blockade of carbachol- or oxotremorine-M-stimulated PI turnover is very strong ($r = 0.81$ and 0.92, respectively). Dose response curves for the muscarinic agonist, oxotremorine-M, established in the presence of 10 uM (+)pentazocine, displayed very little change in the concentration of agonist required to produce half-maximal stimulatory effects but caused a significant reduction in the maximum stimulatory response itself. Hence, they observed a <u>noncompetitive</u> inhibition of the stimulated response by sigma ligands. When intact synaptoneurosomes were pretreated with 50 uM (+)pentazocine, followed by removal of free ligand, the subsequent oxotremorine-M-induced response was still inhibited by greater than 50% of control. This irreversibility of the effects of (+)pentazocine is in contrast to the reversibility of DTG's effects on carbachol response that we observe in NCB-20 cells. Scatchard analysis of synaptoneurosomes pretreated with 50 uM (+)pentazocine followed by washing shows that subsequent [^{3}H]oxo-M binding undergoes a 50% reduction in B_{max} with no effect on K_D -- hence, a <u>non-competitive</u> effect. Other experiments support the notion that this reduction in Bmax is a consequence of the occupancy of sigma receptors, and not muscarinic receptors. Thus, the researchers propose that occupation of sigma receptors causes a down-regulation of cholinergic receptors which is responsible for the observed negative modulation of muscarinic agonist-stimulated phosphoinositide metabolism by sigma-

specific ligands. It would be interesting to know how the time-dependence for antagonism of the oxo-M response by sigma ligands in rat brain synaptoneurosomes correlates with the time-dependence for disapearance (down regulation) of (-)[^{3}H]QNB binding sites.

At this point, it is difficult to clearly reconcile our contrasting results, which are likely a function of several factors. Perhaps there are inherent differences between the sigma receptors of NCB-20 cells and those found in rat brain synaptoneurosomes. Since the molecular and biochemical correlates of sigma receptor function remain largely unknown, there are no tools available for directly comparing the functional integrity of these receptors in these two preparations. While our conclusions differ with respect to the involvement of sigma receptors in the modulation of receptor-mediated PI hydrolysis, our respective studies appear to be internally consistent.

Perhaps our own data would yield stronger correlations if we were to use the same muscarinic agonist to stimulate PI hydrolysis in our cells as to label muscarinic sites in cell membranes (i.e. use oxotremorine-M and [^{3}H]oxo-M, respectively). We have observed that 5 uM (+)pentazocine causes a doubling of the K_D of (-)[^{3}H]QNB and that 25 uM (+)pentazocine causes a 40-fold increase in the dose of carbachol required to effect 50% of its maximum response (ED_{50}). Is the 40-fold increase in carbachol's ED_{50} value completely accounted for by a decrease in its affinity for the muscarinic site? If sigma drugs are acting as direct antagonists, then why do they exhibit such different efficacies (the degree of rightward shift of the carbachol dose response curve is dependent on the sigma ligand used)? Perhaps it would be more appropriate to employ the same muscarinic ligand in the bioassay as the binding assay, thereby rendering a direct comparison even more valid.

It is also important not to overlook the fact that (-)[^{3}H]QNB binding assays were not conducted on whole NCB-20 cells, but rather on cell membrane suspensions and in a different buffer than that used in the bioassay. We reasoned it was important to conduct our original sigma-specific binding assays in particulate cell membranes to enable us to directly compare our

results with those of studies conducted in other tissues (i.e. guinea pig and rat brain membranes). This turned out to be especially important given the unusual pharmacological profile we obtained for [^{3}H]DTG binding in these cells. Because we were interested in comparing the affinities of sigma drugs for displacement of [^{3}H]DTG and (+)[^{3}H]3-PPP binding with those against a muscarinic receptor-specific radioligand, (-)[^{3}H]QNB binding assays were also conducted in cell membranes. However, it may be helpful at this point to develop an assay for sigma and muscarinic receptor-specific radioligand binding in intact NCB-20 cells.

Whereas these changes in experimental design may strengthen the validity of our results, they would not be expected to necessarily rectify the nature of the differences reported for sigma drug action on NCB-20 cells vs rat brain synaptoneurosomes. For example, the competitive effects of (+)pentazocine on the carbachol dose response curve in intact NCB-20 cells would remain in contrast to its noncompetitive effects in analogous experiments with synaptoneurosomes. Likewise, the reversibility of DTG's effects on the agonist-stimulated response in intact NCB-20 cells would still be opposed to the observed irreversibility of (+)pentazocine's effects in synaptoneurosomes.

As previously mentioned, sigma receptors in PC-12 cell membranes appear pharmacologically distinct from those of rat and guinea pig brain membranes. These observations sparked the interest of Dr. Bowen's group in studying the effects of sigma drugs on agonist-stimulated phosphoinositide hydrolysis in PC-12 cells to complement their similar work conducted in rat brain synaptoneurosomes. Unfortunately, they were unsuccessful in establishing a reliable bioassay for measurement of PI metabolism in PC-12 cells (personal communication).

Thus, the data we have gathered support the notion that sigma ligands interact with muscarinic receptors in NCB-20 cells (at micromolar concentrations), enabling them to antagonize carbachol-stimulated phophoinositide hydrolysis. This model can be easily tested. One would predict, for example, that the antagonistic effects of atropine and sigma ligands on the carbachol- or oxotremorine-M-induced responses would be

additive (at submaximal doses) if both classes of antagonists were competing for binding at the same site. According to our model, (-)[^{3}H]QNB or [^{3}H]Oxo-M binding characteristics in cell membranes or whole cells would not be altered as a result of pretreating cells with a sigma ligand followed by removal of free ligand prior to Scatchard analysis of radioligand binding. Finally, the antagonism by sigma drugs (or atropine) of the stimulated response would not be expected to be dependent on the assay being performed on intact cells.

It is understood that the conclusions drawn from experiments conducted *in vitro* do not necessarily apply directly to the counterpart *in vivo* experiments. As an example of particular relevance to the work presented herein, PCP has been to enhance (-)[^{3}H]QNB binding *in vivo* (Boggan et al), and yet inhibit (-)[^{3}H]QNB binding *in vitro* (Aronstam et al). It is undeniable, however, that much progress is likely to be made in our understanding of the biological relevance of the complex interactions characteristic of the central nervous system. Once the molecular and biochemical features of the sigma receptor(s) have been clarified, it should be possible to more accurately predict receptor function in complex systems.

IV. SUMMARY

The sigma-specific radioligands, [^{3}H]DTG and (+)[^{3}H]3-PPP, each bind saturably and with high affinity to an apparent homogeneous population of sites in NCB-20 membrane suspensions. There appears to be about twice as many [^{3}H]DTG as (+)[^{3}H]3-PPP sites in cell membranes. The pharmacological profiles for displacement of specific [^{3}H]DTG or (+)[^{3}H]3-PPP binding in cell membranes are dissimilar; most notably, the benzomorphan opiates and the antipsychotic drug, haloperidol, are much weaker displacers of [^{3}H]DTG than (+)[^{3}H]3-PPP specific binding.

Binding assays conducted in cholate-solubilized cell membranes reveal linear Scatchard plots for [^{3}H]DTG and (+)[^{3}H]3-PPP binding, fitting best to a one-site model when tested by computer analysis. [^{3}H]DTG and (+)[^{3}H]3-PPP share the same maximum number of binding sites per mg of

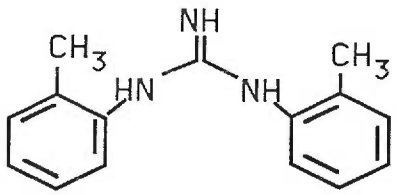
solubilized cell membrane protein. In contrast to that observed in particulate cell membranes, the sigma compounds (+)SKF-10,047, haloperidol, DTG, and (+)3-PPP displace specific [^{3}H]DTG and (+)[^{3}H]3-PPP binding with comparable potencies in solubilized cell membranes.

The binding subunit of the sigma receptor in NCB-20 cells corresponds to a 29 kDa protein, as revealed by specific labeling with the photoaffinity probe, [^{3}H]N_3DTG. This corresponds to the same size as the protein specifically labeled by [^{3}H]N_3DTG in guinea pig brain membranes (Kavanaugh et al, 1988). Sepharose CL-6B chromatography of solubilized sigma receptors pre-incubated with [^{3}H]DTG reveals labeling associated with a single peak, corresponding to a complex of Mr 669,000. This is in agreement with the identical experiments conducted in solubilized guinea pig brain membranes (Kavanaugh et al, 1989).

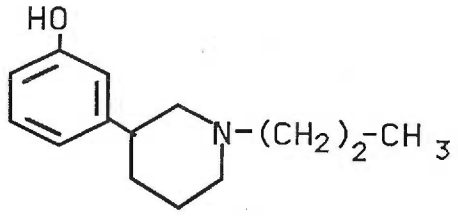
Sigma compounds inhibit carbachol-stimulated PI hydrolysis in NCB-20 cells in a competitive and reversible manner. These drugs have no significant effects on bradykinin-elicited responses. Our data suggest that sigma drugs most likely inhibit the carbachol response by interacting directly with muscarinic receptors present in NCB-20 cells.

V. APPENDIX

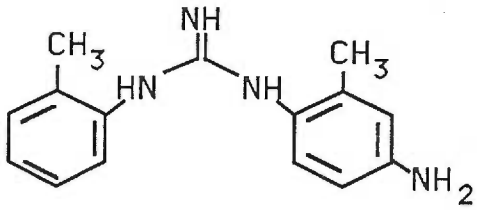
Figure i. Structures of various sigma- and PCP-receptor ligands.



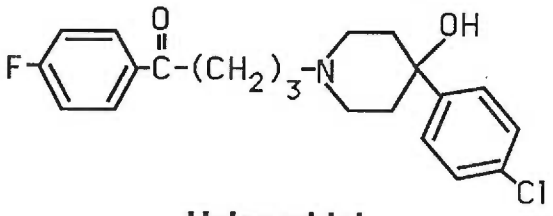
DTG



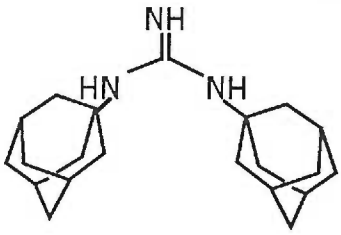
(±)3-PPP



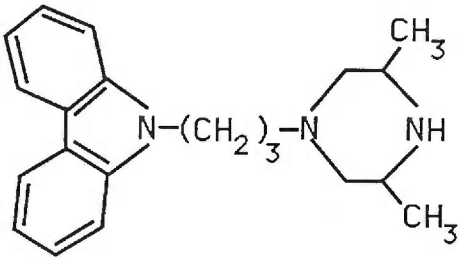
NH_2DTG



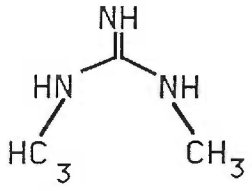
Haloperidol



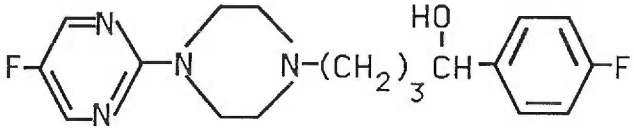
Diadamantylguanidine



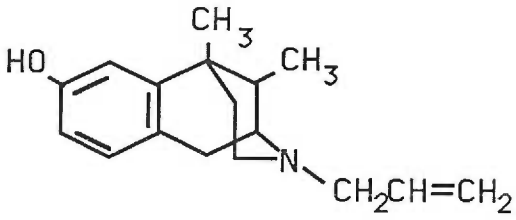
BW234U
(Rimcazole)



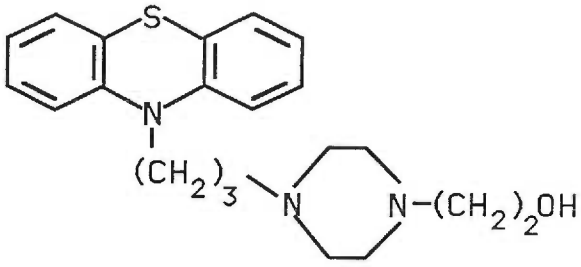
Dimethylguanidine



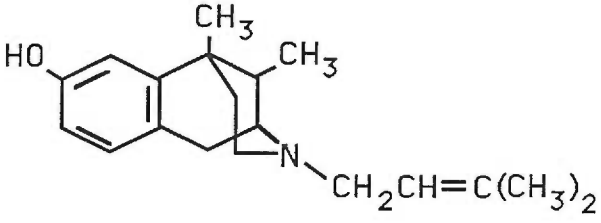
BMY 14802



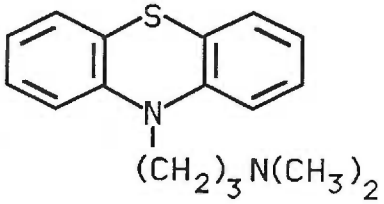
(±)SKF-10,047



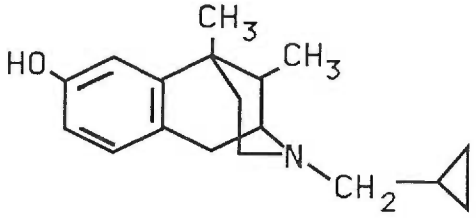
Perphenazine



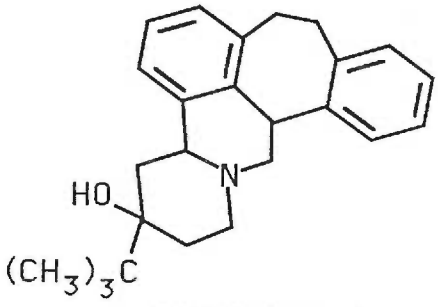
(±)Pentazocine



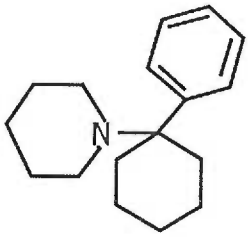
Chlorpromazine



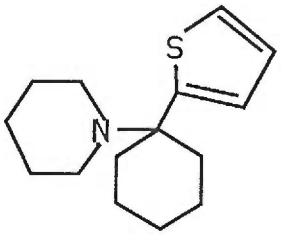
(±)Cyclazocine



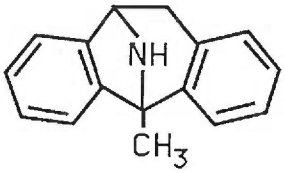
(±)Butaclamol



PCP
(Phencyclidine)



TCP



MK-801

Figure ii. Ability of various drugs to displace [^{3}H]DTG and (+)[^{3}H]3-PPP from guinea pig brain membranes. The table indicates IC_{50} values (mean ± s.e.m.; n=2-4) and the correlation coefficient is 0.95($P<0.00001$) for the plot shown (below). 1. haloperidol; 2. DTG; 3. perphenazine; 4. (+)pentazocine; 5. (+)3-PPP; 6. (-)pentazocine; 7. (-)3-PPP; 8. (+)cyclazocine; 9. (-)butaclamol; 10. (+)SKF-10,047; 11. PCP; 12. (+)butaclamol; 13. (-)cyclazocine; 14. (-)SKF-10,047.

(These data were reproduced from Weber et al).

Drug	IC_{50}, nM Against [^{3}H]Tol_2Gdn	Against (+)-[^{3}H]3-PPP
Haloperidol	5.0 ± 0.3	17 ± 1
Tol_2Gdn	28 ± 1	53 ± 9
Perphenazine	42 ± 10	21 ± 3
(+)-Pentazocine	43 ± 2	8 ± 3
(−)-Pentazocine	135 ± 3	81 ± 1
(±) Pentazocine	69 ± 1	ND
(+)-3-PPP	76 ± 4	33 ± 12
(−)-3-PPP	280 ± 21	235 ± 60
(+)-Cyclazocine	365 ± 25	47 ± 12
(−)-Cyclazocine	2600 ± 210	1000 ± 0
Spiperone	690 ± 21	ND
(−)-Butaclamol	530 ± 49	183 ± 5
(+)-Butaclamol	2150 ± 250	2100 ± 71
(+)-SKF 10,047	625 ± 88	93 ± 5
(−)-SKF 10,047	4000 ± 566	2850 ± 390
PCP	1050 ± 106	1000 ± 71
U50,488H	1350 ± 106	ND
Trifluoperazine	345 ± 4	ND
Triflupromazine	605 ± 67	ND
Chlorpromazine	1475 ± 265	ND
Amitriptyline	300 ± 7	ND
Imipramine	520 ± 14	ND
Desipramine	4000 ± 212	ND
Nortriptyline	2000 ± 640	ND
Guanabenz	4600 ± 283	ND
Clonidine	>10,000	ND
Cocaine	>10,000	ND

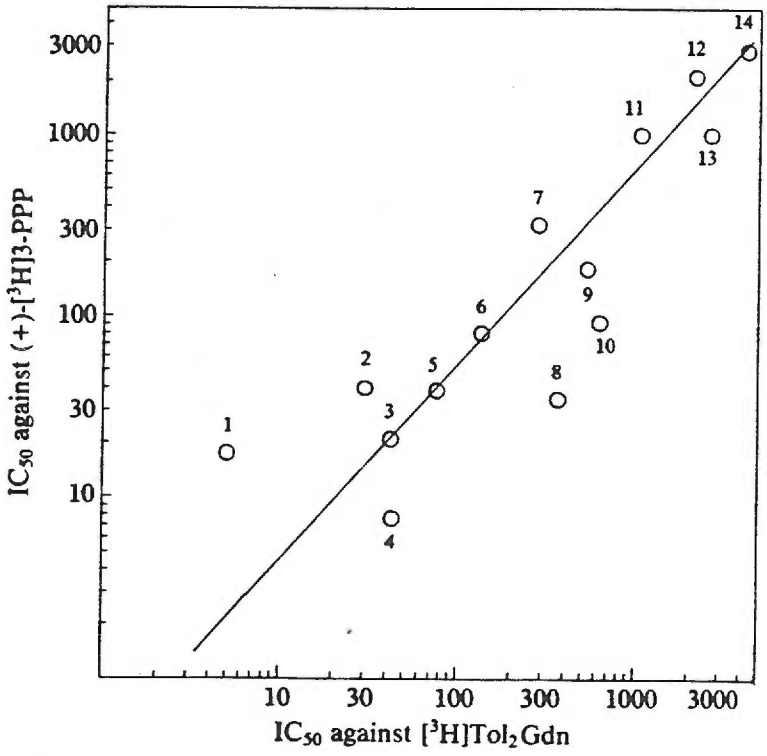
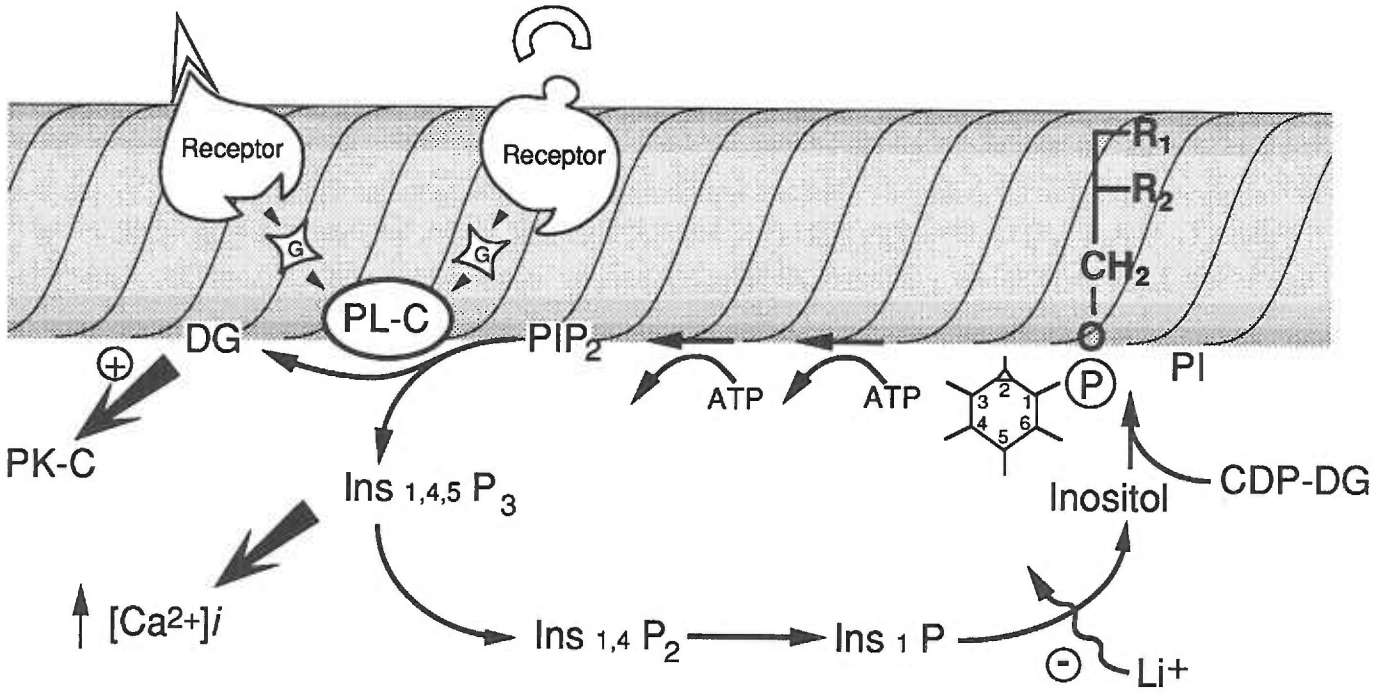


Figure iii. Schematic illustration of receptor-mediated stimulation of phosphoinositide hydrolysis. The initial ligand-stimulated step is the breakdown of phosphotidylinositol-bisphosphate (PIP_2) by activation of phospholipase C (PL-C) via coupling of the receptor to a GTP-binding protein. Hydrolysis of PIP_2 results in the concomittant formation of the second messenger molecules inositol-1,4,5-trisphosphate ($InsP_3$), which leads to mobilization of intracellular Ca^{2+} stores, and diacylglycerol (DG), which stimulates protein kinase C (PK-C). Lipid and inositol phosphate cycles are responsible for removal of the messenger molecules and regeneration of membrane-bound phosphoinositides.


Receptor
Receptor
G
G
PL-C
DG
PIP2
ATP
ATP
R1
R2
CH2
O
PI
P
PK-C
Ins 1,4,5 P3
Inositol
CDP-DG
[Ca2+]i
Ins 1,4 P2
Ins 1 P
Li+

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