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[^{3}H]Noscapine Binding Sites in Brain: Relationship to Indoleamines and the Phosphoinositide and Adenylyl Cyclase Messenger Systems

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SUMMARY

High affinity [^{3}H]noscapine binding sites are brain specific, ion insensitive, and present in a variety of species and show strict structure-activity requirements. Among neurotransmitter-related structures, indoleamines and β-carbolines display highest affinity for [^{3}H]noscapine sites. Noscapine inhibits carbachol-stimulated phosphoinositide turnover in guinea pig and rat brain slices, with structural analogs possessing similar relative potencies for binding to [^{3}H]noscapine binding sites and inhibiting phosphoinositide turnover. Noscapine and its derivatives also markedly enhance the ability of forskolin to augment cAMP levels in brain slices, with relative potencies paralleling affinities for noscapine binding sites.

Noscapine, a phthaleideisoquinoline alkaloid constituting 1–10% of the alkaloid content of opium, has been used clinically as an antitussive agent (1). Mechanisms for its antitussive action are unknown, although animal studies have suggested a central nervous system site of action (2). Noscapine is of the same order of potency as codeine in blocking cough (3, 4). Unlike codeine and other narcotic antitussive agents, noscapine lacks addictive, analgesic, respiratory depressant, and sedative properties (1), indicating a specific receptor distinct from opiate receptors.

Karlsson *et al.* (5) described high affinity, saturable, stereospecific binding sites for noscapine in guinea pig brain homogenates. They also demonstrated that [^{3}H]noscapine binding was enriched in the synaptosomal subcellular fraction, that several known neurotransmitter receptor ligands failed to displace [^{3}H]noscapine binding, and that noscapine binding sites probably differed from other previously described antitussive binding sites.

To explore the physiological role of noscapine binding sites, we have conducted detailed structure-activity analyses and examined the influence of noscapine on second messenger systems. We now report selective interactions of indoleamines with [^{3}H]noscapine binding sites. We also report the regulation by noscapine and related alkaloids of PI and adenylyl cyclase messenger systems, with the relative potencies of the alkaloids paralleling their affinities for noscapine binding sites.

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Materials and Methods

Chemicals and drugs. [^{3}H]Noscapine (13 Ci/mmol), 2-^{125}I-melatonin (2200 Ci/mmol), [*methyl*-^{3}H]SAM (11 Ci/mmol), and [5-^{3}H]cytidine (27.8 Ci/mmol) were obtained from DuPont/NEN (Boston, MA). ^{125}I-cAMP radioimmunoassay kits were from Amersham Corporation (Arlington Heights, IL). Quinazodine was generously provided by Bristol-Myers Squibb (Wallingford, CT) and narceine was from K & K Laboratories (Cleveland, OH). Neurotransmitter receptor agonists and antagonists and analogs of noscapine, serotonin, melatonin, tryptamine, and β-carboline were obtained from either Aldrich Chemical Co. (Milwaukee, WI), Research Biochemicals (Natick, MA), or Sigma Chemical Co. (St. Louis, MO). Protein concentration was determined using Coomassie protein assay reagent from Pierce (Rockford, IL). All other drugs and chemicals were from Sigma.

Crude membrane preparation. Tissues from adult male Hartley short-hair guinea pigs, Sprague-Dawley rats, BALB/c mice, and Syrian

ABBREVIATIONS: PI, phosphoinositide; PDE, phosphodiesterase; HIOMT, hydroxyindole-*O*-methyl transferase; CDP-DAG, cytidine diphosphate-diacylglycerol; IBMX, 3-isobutyl-1-methylxanthine; PAPP, *p*-aminophenylethyl-*m*-trifluoromethylphenyl piperazine; 8-hydroxy-DPAT, (±)-8-hydroxy-dipropylaminotetralin hydrobromide; TFMPP, *m*-trifluoromethylphenylpiperazinehydrochloride; (±)-DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride; SAM, *S*-adenosylmethionine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; G protein, guanine nucleotide-binding protein; GABA, γ-aminobutyric acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; GDPβS, guanosine-5′-*O*-(2-thio)diphosphate; ATPγS, adenosine-5′-*O*-(3-thio)triphosphate; ADPγS, adenosine-5′-*O*-(2-thio)diphosphate; 5HT, 5-hydroxytryptamine; 3-PPP, 3-(3-hydroxyphenyl)-*N*-(1-propyl)p:peridine.

hamsters were prepared as follows. Freshly dissected tissue was used due to the instability of [^{3}H]noscapine binding sites during freeze-thawing. Brain and other tissues were homogenized (100 mg of wet weight/ml of buffer) with a Brinkmann Polytron in 50 mM Tris, pH 7.4 (at 0°), buffer. Homogenates were centrifuged (15 min, 30000 × g), the supernatants were discarded, and the pellets were resuspended in the same volume of buffer. Homogenization, centrifugation, and resuspension of the membrane pellets were repeated twice to prepare washed membranes.

[^{3}H]Noscapine binding to membranes. The binding of [^{3}H]noscapine to washed membranes was as described by Karlsson *et al.* (5), with several modifications. Membranes (150 μl, 200–400 mg of protein) were incubated with 5 nM [^{3}H]noscapine in a total volume of 0.5 ml of 50 mM Tris, pH 7.4, buffer for 30 min at 0°. The membranes were then filtered over 0.3% polyethylenimine-soaked filters using a Brandel cell harvester and were washed twice with 4 ml of ice-cold wash buffer (50 mM Tris, pH 7.4, 100 mM choline chloride, 0.01% Triton X-100), and the filters were quantitated by liquid scintillation counting. Nonspecific binding was measured in the presence of 10 μM unlabeled noscapine. Specific [^{3}H]noscapine binding represented <10% of added radioactivity. K_d and B_{max} values were calculated using the EBDA/SCAFIT equilibrium binding data analysis program (6). For drug competition experiments, drugs were dissolved in either water, dimethylsulfoxide, or equimolar HCl or NaOH. Drugs were diluted into the assay such that the solvent concentrations were <0.1% and had no inhibitory effect.

Measurement of endogenous *N*-acetylserotonin levels. The detection of *N*-acetylserotonin was based on the radioenzymatic assay described by Hussain and Sole (7), which utilizes HIOMT to transfer a methyl group from [^{3}H]SAM to *N*-acetylserotonin, forming [^{3}H] melatonin. HIOMT was purified according to the method of Axelrod and Weissbach (8). Guinea pig brain homogenate supernatants, prepared as described above, were rapidly frozen with dry ice to be assayed as follows. Reaction mixtures consisting of 40 μl of 0.5 M sodium phosphate buffer, pH 7.95, 10 μl of [^{3}H]SAM (1 μCi), 25 μl of HIOMT (4 units), and 25 μl of brain homogenate supernatants were incubated for 30 min at 37°. The reaction was terminated by the addition of 25 μl of melatonin (1 mM stock) and 200 μl of 0.5 M borate buffer, prepared as described (7), and was then extracted with 1 ml of toluene/isoamyl alcohol (7:3). The organic phase (800 μl) was removed and extracted again with 0.5 M borate buffer, and 600 μl of the organic phase were transferred to Eppendorf tubes for evaporation and determination of radioactivity by liquid scintillation counting. The assay was linear for *N*-acetylserotonin levels in the 10–10,000 pg range.

Measurement of carbachol-stimulated PI turnover. Carbachol-stimulated PI turnover was assayed as described (9), with the following modifications. Guinea pig brain slices (300 μm × 300 μm) were prepared, allowed to recover as described (10), and then incubated for 1 hr in Krebs/HEPES buffer containing 0.4 μCi/ml [2-^{3}H]cytidine. Lithium chloride (10 mM final concentration) was then added, followed by carbachol (1 mM) 10 min later. Noscapine and other drugs (25 μl) were added 20 min before the addition of $LiCl_2$. The reactions were terminated after 1 hr of carbachol exposure, [^{3}H]CDP-DAG was extracted, and radioactivity was counted (9). Typical ratios of carbachol-stimulated to basal [^{3}H]CDP-DAG were 10:1.

Measurement of forskolin-stimulated cAMP levels. Guinea pig striatal sections (300 μm × 300 μm) were prepared and allowed to recover for 30 min, as described (10). Gravity-settled slices (50 μl) were transferred to tubes containing 250 μl of oxygenated (95:5% O_2/CO_2) Krebs/HEPES buffer (at 37°) with noscapine, forskolin, or a mixture of both. After a 20-min incubation at 37°, which we determined to be the time for maximal cAMP production (data not shown), the reaction was terminated and cAMP was extracted by the addition of ice-cold 15% trichloroacetic acid (400 μl), followed by sonication. The samples were centrifuged (10 min, 12,000 × g), and 600 μl were removed and extracted with 3 × 3 ml of water-saturated diethyl ether. Aliquots (500 μl) were lyophilized and resuspended in 100 μl of sodium bicarbonate buffer (10%). cAMP levels were measured using 1/200 and 1/400 sample dilutions, with a ^{125}I-cAMP radioimmunoassay kit (Amersham). cAMP standards were linear in a 10–1000 fmol of cAMP/tube range, and typical basal cAMP levels in 50 μl of striatal slices were approximately 10 pmol.

Results

Properties of [^{3}H]noscapine binding sites. Confirming the report of Karlsson *et al.* (5), we observe reversible saturable binding of [^{3}H]noscapine to guinea pig whole-brain (minus cerebellum) membranes. Total binding of [^{3}H]noscapine in typical experiments is approximately 5–6 times nonspecific binding levels in the presence of 10 μM unlabeled noscapine. Scatchard analysis of [^{3}H]noscapine binding using increasing concentrations of both labeled and unlabeled noscapine reveals a single population of binding sites, with a K_d of 8–10 nM and a B_{max} of approximately 1 pmol/mg of protein, similar to results of Karlsson *et al.* (5) (data not shown).

We also detect saturable [^{3}H]noscapine binding in rat, mouse, and hamster brain. [^{3}H]Noscapine binding in rat and mouse brain (four experiments) displays K_d values of 8.8 ± 2.7 nM (mean ± standard error) and 12.7 ± 3.6 nM, respectively, which are similar to that of guinea pig brain (four experiments) (K_d = 10.8 ± 3.1 nM), whereas hamster brain (four experiments) has approximately one fourth the affinity for [^{3}H]noscapine (K_d = 40.2 ± 6.4 nM). Similar B_{max} values in guinea pig and hamster brain are observed, with respective values of 1086 ± 110 and 1101 ± 201 fmol/mg of protein. In mouse and rat brain, however, respective B_{max} values of 315 ± 60 and 333 ± 27 fmol/mg of protein are about one third the levels in guinea pig brain.

To determine whether noscapine binding sites are selective for brain tissue, we evaluated a range of guinea pig peripheral tissues. No saturable binding is demonstrable in spleen, lung, large and small intestine, testis, trachea, pancreas, and stomach (data not shown). Liver and kidney display saturable binding with respective K_d values of 196 and 125 nM and B_{max} values of 9.54 and 7.6 pmol/mg of protein. The drug specificity of liver and kidney binding sites differs markedly from that in the brain (Table 1). All drugs examined in liver inhibit <50% of [^{3}H] noscapine binding at 10 μM. Greater drug potency is observed in kidney, but with markedly different structure-binding relationships than in the brain. For instance, papaverine is at least 1000 times more potent than laudanosine in brain membranes,

TABLE 1

Pharmacology of central and peripheral [^{3}H]noscapine binding sites

[^{3}H]Noscapine binding in guinea pig brain, liver, and kidney was measured as described in Materials and Methods. IC_{50} values (concentration that displaces 50% of specific binding) were calculated from [^{3}H]noscapine displacement curves using five to 10 different drug concentrations. The maximum drug concentration examined was 10 μM. Data shown are mean values of experiments conducted three times in duplicate, with values varying <20%.

Drug	IC_{50}		
	Brain	Liver	Kidney
		nM	
Papaverine	13	>10,000	545
Hydrastine	32	>10,000	1,000
Harmol	75	>10,000	3,000
Harmine	460	>10,000	156
Quinazodine	820	>10,000	640
Prazosin	1,300	>10,000	1,720
Laudanosine	>10,000	>10,000	173

whereas in kidney laudanosine is severalfold more potent than papaverine.

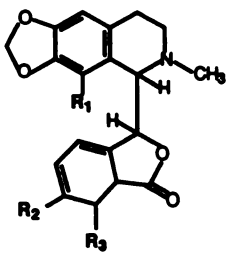
Karlsson *et al.* (5) reported the failure of cations to influence [^{3}H]noscapine binding. We confirm their results, demonstrating that the chloride salts of Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, K^+, Na^+, and Li^+ are all inactive up to 50 mM (data not shown). In addition, the sodium salts of the following anions fail to influence [^{3}H]noscapine binding: 50 mM PO_4^{2-}, SO_4^{2-}, SCN^-, Cl^-, I^-, Br^-, NO_3^-, and F^- (data not shown).

We are unable to demonstrate a G protein linkage to the noscapine binding site. The nonhydrolyzable GTP analog GTPγS fails to shift the affinity of [^{3}H]noscapine binding (data not shown). The affinity of noscapine, hydrastine, primaquine, narceine, and harmol for [^{3}H]noscapine binding is unaltered by the guanine and adenine nucleotides (up to 100 μM) GTP, GDP, GTPγS, GDPβS, ATP, ADP, ATPγS, and ADPβS (data not shown).

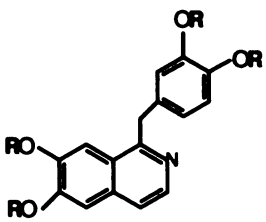
Structure-activity relationships. As observed previously (5), strict structure-activity requirements exist for phthaleideisoquinoline interactions with the noscapine binding site (Fig. 1). Most striking is the virtual abolition of affinity for the noscapine receptor resulting from the transformation of the two methoxy groups of noscapine, R_2 and R_3 (Fig. 1), into the methylenedioxy bridge of bicuculline. The role of sterochemistry in the inactivity of (+)-bicuculline is not readily assessed, because (−)-bicuculline is not available and (−)-bicuculline methochloride is derived from (+)-bicuculline. Removal of the methoxy group from position R_1 in the transformation of noscapine to hydrastine results in only a minor 4-fold decrease in potency, which differs from the <2-fold decrease observed by Karlsson *et al.* (5). Interestingly, the isoquinoline smooth muscle relaxant papaverine differs substantially in structure from noscapine but is approximately as potent as noscapine for the binding site. In contrast, laudanosine, in which the heterocylic ring of papaverine is reduced and a *N*-methyl group is added, lacks affinity for [^{3}H]noscapine binding sites.

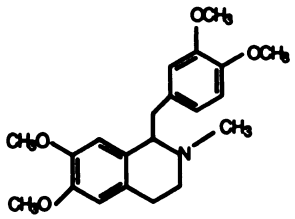
Like Karlsson *et al.* (5), we find that most agents affecting adrenergic, cholinergic, dopaminergic, GABAergic, glutamatergic, and histaminergic neurotransmitter systems lack detectable affinity for noscapine binding sites (Fig. 2 legend), whereas serotonin has a K_i of 10 μM (Fig. 2). Indoleamines manifest notable structure-activity relationships for [^{3}H]noscapine binding sites (Fig. 2). The primary amine structure is not required, inasmuch as its transformation into an alcohol, in 5-hydroxytryptophol, produces a 10-fold increase in affinity. Increasing the lipophilicity of the amine group by *N*-acetylation provides yet higher affinity, 16-fold greater than that of serotonin. Conversion of the 5-hydroxy group into a methoxy does not markedly alter affinity. Thus, 5-methoxytryptamine has potency similar to that of serotonin, and melatonin has affinity similar to that of *N*-acetylserotonin. Substitutions at the 2-position of the indole ring enhance affinity. Thus, 2-methylserotonin is approximately 3 times more potent than serotonin, and 2-iodomelatonin is 15 times more potent than melatonin. A substituent at the 5-position is important for binding, inasmuch as tryptamine is less than one tenth as potent as serotonin. Noscapine binding sites do not involve any conventional serotonin receptors, inasmuch as a variety of serotonin receptor subtype-selective drugs and serotonin uptake blockers are inactive (Fig. 2 legend).

A number of β-carbolines have been proposed as endogenous



	K_i (nM)	R_1	R_2	R_3
(-) Noscapine	8	OCH_3	OCH_3	OCH_3
(-) Hydrastine	32	H	OCH_3	OCH_3
(+) Bicuculline	>10,000	OCH_3	$O\text{-}CH_2\text{-}O$	
(-) Bicuculline methchloride	>10,000	OCH_3	$O\text{-}CH_2\text{-}O$	





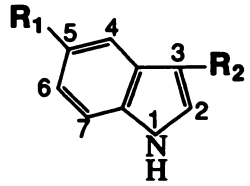
Laudanosine, K_i= >50 μM

	K_i (nM)	R
Papaverine	13	CH_3
Ethaverine	29	CH_2CH_3

Fig. 1. Structure-activity relationships of noscapine analogs. The affinities of noscapine and structurally related compounds for [^{3}H]noscapine binding sites and the inhibitory constant values (K_i) were determined as described in Materials and Methods. K_i data are mean values of three experiments with five to 10 different drug concentrations assayed in duplicate, with standard errors of <10%. Structures having affinity for [^{3}H]noscapine binding sites are (K_i) the quinolines primaquine (200 nM) and chloroquine (1 μM) and the phenylethylamine narceine (393 nM).

neuromodulators metabolically derived from serotonin (11, 12). Some of these display substantial affinity for noscapine binding sites (Fig. 3). Harman and harmol are approximately twice as potent as iodomelatonin. The methyl group at position R_1 (Fig. 3) is of apparent importance for activity, because its removal in the conversion of harman to norharman results in a 10-fold decrease in potency. Reduction of the pyridine moiety, generating dihydro-β-carbolines, reduces affinity, whereas further reduction to tetrahydro-β-carbolines generates compounds that are all inactive at 10 μM. Because β-carbolines affect GABA-benzodiazepine receptors (13), we evaluated a variety of benzodiazepines agonists and antagonists, which all lack affinity for the [^{3}H]noscapine binding site (Fig. 2 legend).

High affinity binding sites for ^{125}I-melatonin have been described (14, 15). To ascertain whether noscapine binding sites are related to these putative melatonin receptors, we monitored ^{125}I-melatonin binding to guinea pig brain membranes (data not shown). We confirm the high affinity of ^{125}I-melatonin, with a K_d of approximately 0.6 nM. Noscapine (10 μM), however, inhibits only 30% of ^{125}I-melatonin binding, indicating that

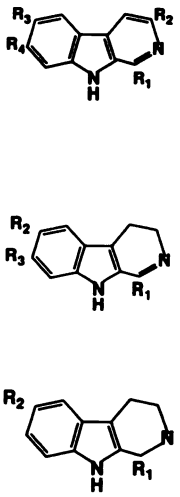


	R_1	R_2	K_i (μM)
Serotonin and analogs	OH	$CH_2CH_2NH_2$	10.9
	OH	CH_2CH_2OH	1.0
	OH	CH_2COOH	9.5
	OH	$CH_2CH(COOH)NH_2$	>100.0
	OH	$CH_2CH_2NHCH_3$	14.0
	OH	$CH_2CH_2NHCOCH_3$	0.7
	2-Methylserotonin		4.6
Melatonin and analogs	CH_3O	$CH_2CH_2NHCOCH_3$	1.2
	CH_3O	$CH_2CH_2NH_2$	12.1
	CH_3O	$CH_2CH_2N(CH_3)_2$	17.2
	CH_3O	CH_2CH_2OH	2.9
	CH_3O	$COCH_3$	>100.0
	CH_3O	H	5.5
	2-Iodomelatonin		0.1
	6-Hydroxymelatonin		2.3
Tryptamine and analogs	H	$CH_2CH_2NH_2$	>100.0
	H	$CH_2CH_2NHCH_3$	68.0
	H	$CH_2N(CH_3)_2$	50.0
	H	$CH_2CH(CH_3)NH_2$	51.7
	H	CH_2CH_2OH	13.0
	CH_3	$CH_2CH_2NH_2$	78.0
	F	$CH_2CH_2NH_2$	>100.0

Fig. 2. Structure-activity relationships of serotonin (5HT) and other indoleamines at [^{3}H]noscapine binding sites. The affinities (K_i) of 5HT and other related indoleamines for displacing [^{3}H]noscapine from its binding site were determined as described for Fig. 1. Serotonin subtype-specific drugs inactive (K_i > 10 μM) at competing for [^{3}H]noscapine binding were $5HT_{1a}$ (PAPP and 8-hydroxy-DPAT), $5HT_{1b}$ (CGS-12066B and TFMPP), $5HT_2$ [(±)-DOI, ketanserin, pirenperone, and spiperone], and $5HT_3$ (MDL 7222, quipazine, and metoclopramide), as well as 5HT uptake blockers (clomipramine and fenfluramine). Other inactive neurotransmitter class drugs tested were of the following types: adrenergic (norepinephrine, clonidine, propranalol, ephedrine, and yohimbine), antitussive (carbetapentane, chlophendianol, oxeladin, and dextromethorphan), benzodiazepine (diazepam, clonazepam, PK 11195, and Ro 15–1788), dopaminergic (chlorpromazine, bromocryptine, haloperidol, and bupropion), GABAergic [(±)-bicuculline, baclofen, and muscimol], histaminergic (histamine, cimetidine, cyproheptadine, and diphenhydramine), muscarinic (atropine, carbachol, pirenzapine, and metoclopramide), opiate (codeine, morphine, and naloxone), phencyclidine/σ [(+)-3PPP and pentazocine], and purinergic (ATP, caffeine, theophylline, cyclohexyl adenosine, and 1,3-dipropyl-8-phenylxanthine).

[^{3}H]noscapine binding sites are not related to ^{125}I-melatonin sites.

The substantial affinity of *N*-acetylserotonin for noscapine binding sites, comparable to the affinity of serotonin itself for various serotonin receptor subtypes (16, 17), raises the possibility that noscapine binding sites might represent *N*-acetylserotonin receptors. Endogenous *N*-acetylserotonin has been detected in mammalian brain by immunohistochemistry (18). Utilizing an enzymatic-isotopic assay (7), we measured *N*-acetylserotonin levels in various regions of guinea pig brain



β-Carbolines	R_1	R_2	R_3	R_4	K_i (nM)
Norharmane	H	H	H	H	329
β-CCM[a]	H	HCO_2CH_3	H	H	348
3-HMC[b]	H	CH_2OH	H	H	262
FG-7142[c]	H	$CONHCH_3$	H	H	837
Harmane	CH_3	H	H	H	37
Harmol	CH_3	H	H	OH	46
Harmine	CH_3	H	H	CH_3	222

Dihydro-β-Carbolines	R_1	R_2	R_3	K_i (nM)
6-Methoxyharmalan	CH_3	OCH_3	H	2197
Harmalol	CH_3	H	OH	412
Harmaline	CH_3	H	OCH_3	1797

Tetrahydro-β-Carbolines	R_1	R_2	KI (nM)
Tetrahydro-β-carboline	H	H	>10,000
1-Methyl-tetrahydro-β-carboline	CH_3	H	>10,000
6-Methoxy-tetrahydro-β-carboline	H	OCH_3	>10,000

Fig. 3. Structure-activity relationships of β-carbolines at [^{3}H]noscapine binding sites. Procedures used to generate K_i values for β-carbolines at the [^{3}H]noscapine binding site were as described for Fig. 1. [a]Methyl β-carboline-3-carboxylate; [b]3-hydroxymethyl-β-carboline; [c]*N*-methyl-β-carboline-3-carboxamide.

TABLE 2

_N_-Acetylserotonin levels and [^{3}H]noscapine binding levels in guinea pig brain

N-Acetylserotonin levels and [^{3}H]noscapine binding levels (B_{max}) were measured as described in Materials and Methods. *N*-Acetylserotonin levels and [^{3}H]noscapine binding levels are mean ± standard error values (three determinations) of experiments conducted twice and three times, respectively.

Brain region	*N*-Acetylserotonin level	[^{3}H]Noscapine binding level
	ng/g	*pmol/mg*
Striatum	34.0 ± 0.35	1.23 ± 0.127
Hypothalamus	32.5 ± 1.90	1.34 ± 0.175
Cortex	23.2 ± 0.10	1.00 ± 0.115
Brainstem	18.6 ± 0.63	1.10 ± 0.062
Colliculi	15.6 ± 0.54	0.95 ± 0.105
Midbrain	14.8 ± 1.38	1.62 ± 0.227
Cerebellum	14.0 ± 1.42	0.65 ± 0.099
Hippocampus	13.5 ± 0.68	1.30 ± 0.068

(Table 2). The highest levels in striatum and hypothalamus are triple the lowest values in areas such as midbrain, cerebellum, and hippocampus. [^{3}H]Noscapine binding levels differ <3-fold between the highest values in the midbrain and the lowest values in the cerebellum and do not parallel relative levels of *N*-acetylserotonin.

Influence of noscapine upon second messenger systems. PI turnover in guinea pig corpus striatum and cerebral cortex was monitored by measuring the incorporation of [^{3}H] cytidine into [^{3}H]CDP-DAG (9). Noscapine (1 mM) does not affect basal PI turnover but markedly inhibits carbachol-stimulated PI turnover (EC_{50}, 10 μM) (Fig. 4). Relative potencies of noscapine derivatives in influencing PI turnover correspond closely to their relative affinities for [^{3}H]noscapine binding sites. Thus, noscapine and papaverine are most potent, hydrastine is 5–10-fold weaker, and bicuculline and laudanosine are virtually inactive.

Several other drugs having affinity for [^{3}H]noscapine binding sites fail to stimulate PI turnover but inhibit carbachol-stimulated PI turnover weakly (Fig. 4 legend). No inhibition of carbachol-stimulated PI turnover by 5-methoxytryptophol, melatonin, or serotonin is detected. We also evaluated the effect

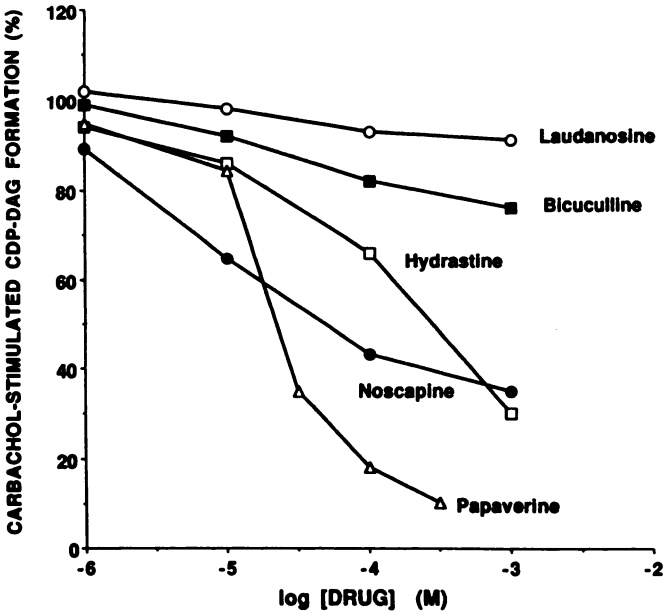


Fig. 4. Inhibition of carbachol-stimulated PI turnover by noscapine. The inhibition of carbachol-stimulated CDP-DAG formation in guinea pig striatum by noscapine and related compounds was examined as described in Materials and Methods. Other drugs tested (at 100 μM) inhibit carbachol-stimulated CDP-DAG formation minimally (percentage inhibition), i.e., harmol (30%), 2-iodomelatonin (23%), *N*-acetylserotonin (9%), 5-methoxytryptophol (0%), melatonin (0%), and serotonin (0%). None of the drugs tested significantly alter basal PI turnover rates. Results, expressed as percentages of maximal stimulation with 1 mM carbachol, are from a typical experiment repeated four times in triplicate.

TABLE 3

Effect of noscapine on carbachol-stimulated PI turnover in rat and guinea pig brain regions

Carbachol-stimulated PI turnover in rat and guinea pig brain was measured as described in Materials and Methods. This experiment was conducted twice in triplicate and data shown are mean values, which varied <10%.

Brain region	PI turnover			
	Guinea pig		Rat	
	Stimulation	Inhibition	Stimulation	Inhibition
	fold	%	*fold*	%
Hippocampus	9.62	73.1	8.92	69.4
Striatum	14.30	54.8	11.20	54.3
Cortex	6.64	70.1	2.45	72.5
Hypothalamus	7.40	77.7	5.12	64.4
Cerebellum	0		0	
Thalamus	5.04	69.8	3.93	57.2
Colliculi	3.95	70.4	1.88	69.0

of noscapine on inhibiting carbachol-stimulated PI turnover, using the [³H]inositol turnover assay (19). Noscapine inhibits carbachol-stimulated PI turnover with similar potency (data not shown) while having no stimulatory effect on PI turnover by itself, thus confirming the effect of noscapine by two independent measures of PI turnover.

The influence of noscapine on carbachol-stimulated PI turnover in various regions of guinea pig and rat brain was compared to determine whether the effects of noscapine were region or species specific (Table 3). Relative stimulation of PI turnover in various brain regions is similar in both rat and guinea pig. Moreover, the relative inhibition of PI turnover by noscapine is similar in various regions.

The effect of noscapine on PI turnover is not restricted to muscarinic cholinergic actions. Serotonin and the α_1-adrenoceptor agonist methoxamine provide 3-fold and 10-fold stimulation of PI turnover, respectively, in guinea pig cerebral cortex (data not shown). Noscapine inhibits serotonin- and methoxamine-stimulated PI turnover with an EC_{50} similar to the values for inhibition of carbachol-stimulated PI turnover (data not shown).

We also evaluated the effect of noscapine on cAMP levels in guinea pig striatal slices (Fig. 5). Noscapine itself, up to 1 mM, does not affect cAMP levels. However, noscapine does augment the stimulation by forskolin of cAMP levels (Fig. 5). Forskolin (1 μM) increases cAMP levels 5-fold over basal concentrations, whereas the combination of 1 mM noscapine and 1 μM forskolin

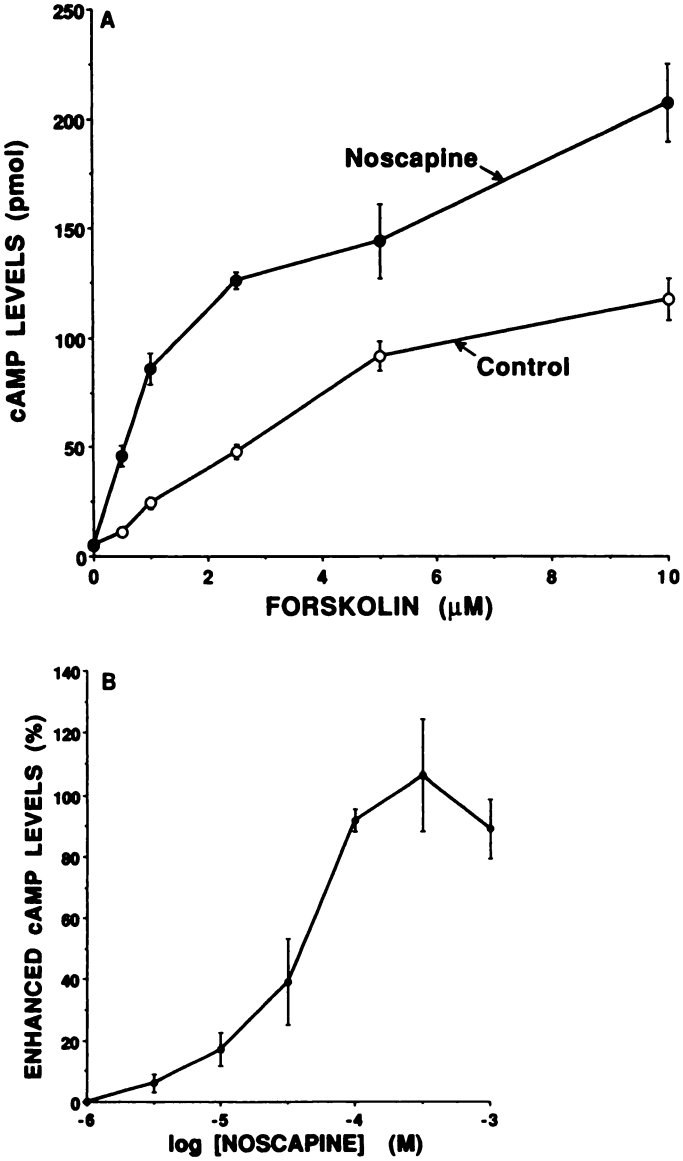


Fig. 5. Enhancement of forskolin-stimulated cAMP levels by noscapine in guinea pig striatum. A, Dose-response curve of forskolin stimulating cAMP levels in the absence (*Control*) or presence of 1 mM noscapine. cAMP levels are expressed as pmol/slice preparation (50 μl). B, Dose-response curve of noscapine enhancing forskolin-stimulated cAMP levels, expressed as percentages of enhanced cAMP levels generated with 2 μM forskolin. The experiment was conducted twice, as described in Materials and Methods, and data are mean ± standard error values (six experiments).

provides a 17-fold increase. Noscapine enhances cAMP levels at all forskolin concentrations explored. Noscapine is one fifth as potent in augmenting forskolin-stimulated cAMP levels (EC_{50}, 50 μM) as in inhibiting carbachol-stimulated PI turnover. The percentage increase of cAMP levels by noscapine is the same in the presence or absence of the PDE inhibitor IBMX (0.5 mM). In the presence of IBMX, the absolute levels of cAMP are approximately 2-fold greater (data not shown).

Relative potencies of noscapine derivatives in enhancing cAMP levels (Fig. 6), in most cases, parallel their effects on PI turnover. Papaverine is more potent than noscapine, whereas bicuculline and laudanosine are virtually inactive and hydrastine and *N*-acetylserotonin are less potent than noscapine. Harmol, iodomelatonin and 5-methoxytryptophol increase cAMP levels less than does noscapine at 100 μM harmol, whereas at 1 mM greater enhancement than for noscapine is observed with these drugs. Among serotonin derivatives, the lesser effect of *N*-acetylserotonin than of iodomelatonin and 5-methoxytryptophol parallels the relative potencies of these agents at [^{3}H]noscapine binding sites. The substantial enhancement of cAMP levels by papaverine presumably reflects its inhibition of PDE (20). Noscapine, hydrastine, harmol, iodomelatonin, and 5-methoxytryptophol were, therefore, examined as potential inhibitors of Ca^{2+}/calmodulin-dependent and -independent PDE. Neither cytosolic nor membrane PDE activity from guinea pig brain is inhibited by noscapine or any other drug tested, at concentrations up to 1 mM (data not shown). Papaverine, as expected, inhibits PDE activity with an IC_{50} of 10 μM.

The enhancement of cAMP levels by certain drugs might reflect a direct, forskolin-like stimulation of adenylyl cyclase.

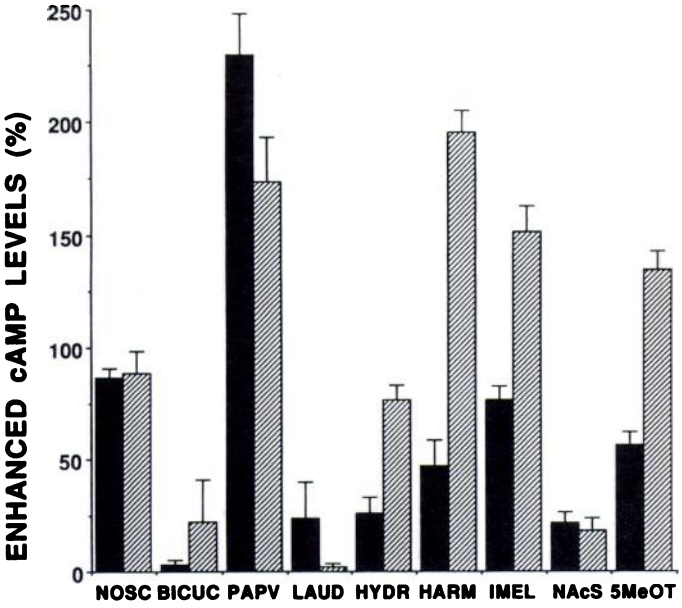


Fig. 6. Pharmacology of enhanced forskolin-stimulated cAMP levels. The effect of noscapine and related drugs on forskolin-stimulated cAMP levels in guinea pig striatal slices was examined as described in Materials and Methods. The experiment was repeated twice and data are mean ± standard error values (six experiments). Results are expressed as percentages of enhanced cAMP levels generated with 1 μM forskolin. Drugs tested at 100 μM (■) and 1 mM (▨) were noscapine (*NOSC*), (−)-bicuculline (*BICUC*), papaverine (*PAPV*), laudanosine (*LAUD*), hydrastine (*HYDR*), harmol (*HARM*), 2-iodomelatonin (*IMEL*), *N*-acetylserotonin (*NAcS*), and 5-methoxytryptophol (*5MeOT*).

We, therefore, examined the effect on cAMP levels of drugs alone or in the presence of noscapine (Table 4). Serotonin, 2-iodomelatonin, and melatonin elevate cAMP levels 50–70%, with no further change in the presence of noscapine. Whether this reflects actions on an indoleamine-stimulated adenylyl cyclase is unclear. The effects are probably not a forskolin-like stimulation of cyclase, because they are not augmented further by noscapine.

Discussion

In the present study we have characterized noscapine binding sites in an effort to ascertain possible endogenous ligands. [^{3}H]Noscapine binding sites are brain specific. Lower affinity, high capacity noscapine binding sites are observed in kidney and liver; they differ dramatically in their drug specificity, compared with sites in brain. After the injection of [^{3}H]noscapine into mice, noscapine is rapidly accumulated to high levels in the liver and kidney (21). Conceivably, noscapine binding in these tissues may involve such sites.

[^{3}H]Noscapine binding sites do not appear to reflect any known receptors. Papaverine, a smooth muscle relaxant, has affinity similar to that of noscapine at [^{3}H]noscapine binding sites. Binding sites for papaverine in peripheral smooth muscle tissues, with reported K_d values of 100 nM, have not been detected in brain (22, 23).

Micromolar concentrations of noscapine allosterically enhance the affinity of dextromethorphan for its binding site (24), which may be associated with σ receptors (25). It is unlikely that noscapine binding sites represent dextromethorphan receptors, because concentrations of dextromethorphan up to 10 μM do not influence [^{3}H]noscapine binding.

Several β-carbolines are potent inhibitors of [^{3}H]noscapine binding. β-Carbolines interact with benzodiazepine receptors (13), although benzodiazepine agonists and antagonists fail to have any affinity for [^{3}H]noscapine binding sites. The β-carboline harman binds to monoamine oxidase A in brain mitochondrial fractions, with harmine, harmaline, and 1-methyltetrahydro-β-carboline being the most potent β-carboline analogs (26). This site appears unrelated to noscapine binding sites, which are localized to synaptosomes and have very different relative affinities for β-carbolines.

TABLE 4

Effect of drugs on cAMP levels in guinea pig striatal slices

cAMP levels in guinea pig striatal slices were examined as described in Materials and Methods. Drugs were tested in the absence (control) or presence of 1 mM noscapine. The experiment was repeated twice and data are mean ± standard error values (six experiments), expressed as pmol of cAMP/slice preparation (50 μl).

Drug (100 μM)	cAMP levels	
	Control	Noscapine (1 mM)
	pmol	
Basal	10.0 ± 1.75	ND[a]
Noscapine	10.1 ± 0.98	ND
Harmol	14.4 ± 1.60	ND
5-Methoxytryptophol	12.6 ± 0.80	ND
N-Acetylserotonin	10.7 ± 0.91	ND
Serotonin	17.6 ± 0.63[b]	15.8 ± 0.84
2-Iodomelatonin	16.6 ± 0.55[b]	18.8 ± 1.88
Melatonin	18.0 ± 1.38[b]	20.3 ± 0.53
Forskolin (2 μM)	66.1 ± 2.37[c]	115 ± 4.21

[a] ND, not determined.
[b] Value differs from control, $p < 0.05$.
[c] $p < 0.001$.

Whereas melatonin displays high affinity for noscapine binding sites, receptors for ^{125}I-melatonin display much higher affinity for melatonin (K_i = 0.6 μM) than noscapine (K_i > 10 μM). Several compounds displaying affinity for noscapine binding (Fig. 2) also influence ^{125}I-melatonin binding sites (15). However, potencies are 2–3 orders of magnitude less at melatonin than at noscapine sites. Although ^{125}I-melatonin may to some extent label the noscapine site, the differences in pharmacological properties indicate that noscapine binding sites do not represent the major physiological melatonin receptors.

Noscapine binding sites do not correspond to any known serotonin receptors. Because *N*-acetylserotonin is about 15-fold more potent than serotonin at inhibiting [^{3}H]noscapine binding, it is possibly an endogenous ligand at these binding sites. The K_i value of *N*-acetylserotonin for noscapine binding sites, about 0.7 μM, is similar to the potencies of most neurotransmitters at their receptor binding sites (27). There is only one report of binding sites for *N*-[^{3}H]acetylserotonin (28). The properties of these binding sites appear to reflect 5HT$_1$ serotonin receptors. Indeed, the incubations at 37° for 30 min may have resulted in conversion of *N*-[^{3}H]acetylserotonin into [^{3}H] serotonin. Regional distributions of endogenous *N*-acetylserotonin and noscapine binding sites do differ, but many neurotransmitters show poor correlations between regional variations in transmitter levels and numbers of receptor binding sites (29). Both *N*-acetylserotonin and its precursor, 5-methoxytryptamine, fail to stimulate PI turnover or affect cAMP levels, whereas only *N*-acetylserotonin weakly inhibits carbachol-stimulated PI turnover in brain slices.

In this study, we assessed PI turnover by monitoring the formation of [^{3}H]CDP-DAG, a procedure whose reliability has been well established (9, 30, 31). This assay is more rapid and sensitive than techniques measuring [^{3}H]inositol incorporation into inositol phosphates (9, 10). Several steps are involved in both assays of PI turnover. In one case, [^{3}H]cytidine is converted to CDP, which is linked to DAG (9, 30, 31). In the other assay, [^{3}H]inositol is accumulated by cells, linked to phosphatidic acid, and subjected to two phosphorylations followed by inositol trisphosphate generation. Inositol trisphosphate is converted to other inositol phosphates, whose total content is measured as a reflection of PI turnover. Because noscapine affects carbachol-stimulated PI turnover with the same potency in both types of assay, it is likely that the observed drug effects involve physiologic PI turnover.

Influences of noscapine on PI turnover may reflect a second messenger system associated with the noscapine binding site. This possibility is supported by the close similarity of relative effects of various agents on PI turnover and [^{3}H]noscapine binding. Thus, bicuculline and laudanosine, structurally very similar to noscapine and papaverine, respectively, lack effects on either [^{3}H]noscapine binding or carbachol-stimulated PI turnover. The concentration-response curve for noscapine inhibiting [^{3}H]CDP-DAG formation is shallower than for papaverine, suggesting that noscapine affects more than one site, although our binding data and those of Karlsson *et al.* (5) indicate one binding site (Hill coefficient = 1).

Noscapine is substantially weaker in influencing PI turnover than in competing for binding sites, probably due to limited bioavailability of drugs in brain slices; bioavailability is much less than that in finely disrupted brain membranes. For most drugs studied, potencies at influencing PI turnover in brain slices tend to be 2–3 orders of magnitude lower than potencies at influencing receptor binding sites (32–34). Therefore, drugs that compete at low micromolar affinities at the noscapine site may not affect PI turnover to detectable levels, due to limits of drug solubility at the millimolar concentrations required. The mechanism by which noscapine inhibits PI turnover is unclear. Numerous agents inhibit carbachol- or other agonist-stimulated PI turnover (for reviews, see Refs. 34 and 35). In some instances these are direct effects involving inhibition of phospholipase C via interactions with G proteins (35). In some cases more indirect actions take place (35, 36). We are unable, however, to show any G protein linkage to the noscapine binding site.

Noscapine markedly enhances the ability of forskolin to augment cAMP levels in brain slices. The relative potencies of noscapine and its derivatives are similar to their relative effects at [^{3}H]noscapine binding sites. Potencies of noscapine and related agents in influencing cAMP levels, however, are lower than their potencies in inhibiting PI turnover. This suggests that effects on cAMP may be indirect. The enhancement of cAMP levels by noscapine and its derivatives (other than papaverine) is not the result of direct inhibition of PDE activity, inasmuch as these compounds fail to inhibit calcium-independent and calcium/calmodulin-dependent PDE activity.

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