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On the Neuropharmacology of Harmane and Other β-Carbolines

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MÜLLER, W. E., K. J. FEHSKE, H. O. BORBE, U. WOLLERT, C. NANZ AND H. ROMMELSPACHER. *On the neuropharmacology of harmane and other β-carbolines.* PHARMAC. BIOCHEM. BEHAV. **14**(5) 693–699, 1981.—β-Carbolines have been recently proposed as candidates for the unknown endogenous ligand of the benzodiazepine receptor. Out of the β-carboline derivatives already found in the mammalian CNS, harmane is clearly the most potent inhibitor of benzodiazepine receptor binding. Therefore, it has been considered as possible endogenous ligand for this new receptor system. However, a certain degree of specificity might be a basic condition to accept the hypothesis of harmane as the endogenous ligand. Thus, the effects of harmane as well as other β-carbolines on several neurotransmitter receptor binding systems *in vitro* and on some neuropharmacological tests *in vivo* were investigated. Harmane developed the highest affinity towards the benzodiazepine binding site among all systems investigated. Its IC_{50}-values for inhibiting opiate and muscarinic cholinergic receptor binding were about four times lower than those for dopamine and serotonin receptor binding but were about four times higher than that found for the benzodiazepine receptor binding. Norharmane exerted a remarkable displacing activity only at the benzodiazepine binding site. Harmine affected mainly the opiate and cholinergic muscarinic system, whereas tetrahydronorharmane turned out to be a potent inhibitor of serotonin and dopamine receptor binding. Doses of harmane needed to produce convulsions as indication of its possible benzodiazepine receptor agonistic properties are also sufficient to diminish nociception and decrease body temperature whereas the apomorphine-induced licking rate was affected at higher doses. The data demonstrate that harmane affects not only the benzodiazepine binding site but also other neuronal mechanisms. Furthermore, only minor changes of the β-carboline structure lead to substantially different effects. Therefore, the search for other β-carbolines with higher affinity for the benzodiazepine binding site as harmane seems to be promising.

Harmane β-Carbolines Benzodiazepine receptor Endogenous ligand Specificity Muscarinic Serotoninergic Dopaminergic mechanisms

THE PHYSIOLOGICAL function of the benzodiazepine receptor and the nature of its endogenous ligand are still not yet known [2, 18, 33]. Similar to the opiate receptors, none of the already known CNS neurotransmitters fulfilled the criteria expected from an endogenous ligand of this new receptor system [4, 16, 32]. However, in contrast to the opiate receptor where the search for the unknown ligand had finally led to a new class of neuroactive compounds, the endorphins, the thorough search for the endogenous ligand of the benzodiazepine receptor has not yet been successful. Until now a large variety of quite different compounds has been considered as putative endogenous ligands, including peptides, purine derivatives, nicotinamide, and very recently β-carboline derivatives [3, 15, 18, 25].

With respect to the β-carbolines as possible endogenous ligands of the benzodiazepine receptor Braestrup *et al.* [3] identified a high-affinity inhibitor of benzodiazepine receptor binding in human urine [20] as norharmane (β-carboline)-3-carboxylic acid ethyl ester, with an IC_{50} in inhibiting specific [^{3}H]flunitrazepam binding of 7 nmol/l. However, since the ester formation was obviously an artefact of the isolation procedure, the authors concluded that this compound does not represent the endogenous ligand of the benzodiazepine receptor. The free acid (IC_{50}=31 μmol/l) [3] has not yet been found *in vivo*.

Independent from these authors we investigated the affinity of several β-carboline derivatives for the benzodiazepine receptor [25]. The compound with the highest

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affinity we found (harmane, IC_{50}=7 μmol/l) has been currently identified in urine and brain of rats as well as in the urine and platelets of humans [1, 27, 29]. Thus, harmane is the most potent endogenously occurring inhibitor of benzodiazepine receptor binding known so far and we suggested that harmane or another related β-carboline derivative could function as the endogenous ligand of the benzodiazepine receptor [25].

Harmane and other related β-carbolines have rather benzodiazepine antagonistic than benzodiazepine-like effects *in vivo*, e.g., increased anxiety, CNS stimulation, and convulsions have been reported [8, 9, 30]. This had led us to the conclusion that harmane or other related β-carbolines might act as agonists at the benzodiazepine receptor, where the benzodiazepine themselves act as antagonists [25]. This assumption has been supported by recent findings about a competitive antagonism by diazepam of harmane-induced convulsions *in vivo* and about a competitive inhibition by harmane of benzodiazepine receptor binding *in vitro* [26].

On the other hand, several other symptoms of CNS activities of harmane or other β-carbolines have been reported, which possibly can not be explained by benzodiazepine receptor mediated mechanisms, but very likely indicate effects on catecholaminergic, cholinergic, and serotoninergic transmission, e.g., psychotomimetic, tremorogenic, and antipsychotic effects [8, 10, 19, 31]. However a certain degree of specificity would be a further important criterion to accept the hypothesis of an endogenous function of harmane. Therefore, we examined the specificity of harmane as an inhibitor of benzodiazepine receptor binding by investigating its affinities for several other neurotransmitter receptor systems in rat or calf brain homogenates. The experiments were extended to some other related β-carbolines. Furthermore, we studied the actions of harmane in several behavioural test situations typical for CNS activities not related to the benzodiazepines.

METHOD

In vitro *Experiments*

Receptor binding experiments were performed according to procedures already published using rat brain homogenates (male Sprague-Dawley rats, 150–200 g body weight) or calf brain homogenates (male calfs, obtained freshly from a local slaughterhouse).

Muscarinic cholinergic receptor. One ml aliquots of a whole rat brain homogenate (one rat brain in 300 ml 67 mmol/l phosphate buffer pH 7.4) were incubated for 1 hr at 20°C together with 1 nmol/l [^{3}H]QNB and different concentrations of the displacers, according to [35]. Unspecific binding was obtained from experiments performed in the presence of atropine 10 μmol/l. The incubation was stopped by rapid filtration through Whatman GF/B filters followed by three washing steps with 3 ml ice cold buffer each. After extraction into the scintillation liquid (Quickszint 402, Zinsser, Frankfurt, FRG) the radioactivity was determined by liquid scintillation spectrometry (Packard, model 3255).

Opiate receptor. One ml aliquots of a whole rat brain homogenate (one rat brain in 200 ml 50 mmol/l Tris-HCl buffer, pH 7.4) were incubated for 15 min at 37°C together with 6 nmol/l [^{3}H]naloxone and different concentrations of the displacers [23]. Unspecific binding was obtained from experiments performed in the presence of cold naloxone 3 μmol/l. The incubation tubes were put in ice for further 5 min and filtered through Whatman GF/B filters followed by three washing steps with three ml ice-cold buffer each. The determination of the radioactivity bound on the filters has been described above.

Dopamine receptor. Calf striata were homogenized in 40 volumes 50 mmol/l Tris-HCl buffer pH 7.7 and centrifuged two times at 48,000×G. The final pellet was resuspended in 90 volumes of 50 mmol/l Tris-HCl buffer pH 7.1 containing 10 μmol/l pargyline, 4 mmol/l $CaCl_2$, 120 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgCl_2$, and 0.1% ascorbic acid.

After a preincubation for 5 min at 37°C, 2 ml aliquots of the homogenate were incubated for 10 min at 37°C together with 1.2 nmol/l [^{3}H]spiroperidol and different concentrations of the displacers [5,14]. Unspecific binding was obtained from experiments performed in the presence of dopamine 1 mmol/l. After rapid filtration through Whatman GF/B filters and three washing steps with three ml ice cold buffer each bound radioactivity was determined as described above.

Serotonin receptor. Frontal cortex of calf brain was homogenized in 10 volumes of 0.32 mol/l saccharose and centrifuged at 700×G for 10 min. The supernatant was centrifuged again at 48,000×G for 10 min. The pellet was resuspended in 50 mmol/l Tris-HCl buffer pH 7.5 and centrifuged again at 48,000×G for 10 min. The final pellet was resuspended in 100 volumes 50 mmol/l Tris-HCl buffer pH 7.7 containing 4 mmol/l $CaCl_2$, 20 μmol/l pargyline, and 0.1% ascorbic acid.

After a preincubation at 37°C for 15 min, one ml aliquots of the membrane suspension were incubated for 10 min at 37°C together with 6 nmol/l [^{3}H]serotonin and different concentrations of the displacers [22]. Unspecific binding was obtained from experiments performed in the presence of 30 μmol/l cold serotonin. The incubation was stopped by rapid filtration through Whatman GF/B filters followed by three washing steps with three ml ice cold buffer each. Bound radioactivity was determined as described above.

In vivo *Experiments*

Female Wistar rats (170–210 g) were kept under conventional conditions and had free access to dry food (standard chow, Altromin, Lage, FRG) and tap water. The animals were used for only one experiment. All behavioural experiments were performed during the afternoon. The observations were carried out by only one experimenter, who was not informed about the drug and the dose injected. All drugs were dissolved in 0.9% sodium chloride. All drug weights indicated refer to the free bases.

Apomorphine antagonism. After injection of 1.0 mg/kg apomorphine subcutaneously (SC), the rats were placed individually into a plastic tube with an inner diameter of 6 cm. Five min later (if not stated otherwise) harmane was injected into the tail vein (intravenously, IV). The compulsive licking movements were counted every 10 min for 30 sec. The observation period lasted 90 min.

Body temperature. Rats were placed into plastic tubes as described above. They were allowed to adapt for 15 min. Thereafter, the body temperature was measured with a thermistor probe (type TE3, Ellab Copenhagen, Denmark), which was inserted 3 cm deep into the rectum. Then, harmane was injected IV and the controls received vehicle.

Nociception. Rats were placed gently on a copper plate (15.5×31 cm) which was kept at a constant temperature of 58°C by a thermostat. The reaction time until the animal blew or licked one of the paws or jumped was measured by means

of a stopwatch (hot plate test as elaborated by Eddy and Leimbach [6]. Then, the rats were placed into a plastic tube and adapted for 15 min. Thereafter, harmane or vehicle were injected IV. The animals were removed from the tube and the test was repeated after various time intervals.

Statistics. The dose-response curves were approximated by orthogonal polynomials. The polynomial degree was estimated up to a quaternary trend component. In all samples a parabolic trend described the curves sufficiently well. Then, the orthogonal polynomial coefficients were calculated. To assess differences in the level of the curves the exact Fisher-Yates test was applied.

The shape of the curves was calculated following the Freeman-Halton test [7] with four different sign patterns (+−−−; ++−+; ++−−; +−−+). The Lehmacher-Wall test [13] was used for ordinal scale measurements of the response curves.

Materials

[^{3}H]Quinuclidinylbenzylate (QNB) (specific activity 29 Ci/mmole), [^{3}H]naloxone (specific activity 50 Ci/mmole), [^{3}H]spiroperidol (specific activity 25 Ci/mmole), and [^{3}H]serotonin creatinine sulfate (specific activity 29 Ci/mmole) were obtained from New England Nuclear (Dreieich, FRG).

All drugs were gifts from the German manufacturers. All β-carbolines were obtained from Sigma (Munich, FRG) except tetrahydronorharmane which was synthesized as described by Vejdelek *et al.* [34]. All other chemicals were obtained from commercial suppliers.

RESULTS

In order to investigate the specificity of the interaction of harmane with the benzodiazepine receptor we studied its relative potencies as inhibitor of several radioligand receptor binding systems. Each of them has been demonstrated to specifically label a definite neurotransmitter receptor, e.g., specific [^{3}H]QNB binding the muscarinic cholinergic receptor [35], specific [^{3}H]naloxone binding the opiate receptor [23], specific [^{3}H]spiroperidol binding the dopamine receptor [14], and specific [^{3}H]serotonin binding the serotonin receptor [22]. In each case, we checked the specificity of our radioligand binding systems by determing IC_{50} values (concentrations which inhibit specific radioligand binding by 50%) of typical receptor ligands of known potency, as given in Tables 1, 2, and 3.

In the case of harmane, a three times lower affinity for the muscarinic cholinergic and an about five times lower affinity for the opiate receptors were found compared with that for the benzodiazepine receptor (Tables 1 and 2). Harmane is clearly less active at the dopamine and serotonin receptors, with at least ten times higher IC_{50} values than for the benzodiazepine receptor (Table 3). Norharmane exhibits a higher degree of specificity as harmane, since its affinity for all four other receptor systems is at least ten times lower than that for the benzodiazepine receptor, as indicated by its IC_{50} values in Tables 1, 2, and 3.

In contrast to harmane and norharmane, tetrahydronorharmane is most potent in inhibiting specific [^{3}H]spiroperidol binding and specific [^{3}H]serotonin binding but is considerably less potent at the benzodiazepine, muscarinic cholinergic, and opiate receptors (Tables 1, 2, and 3).

A third specificity profile seems to be present in the case of the 7-methoxy-derivatives of harmane and dihydro-

TABLE 1

INHIBITION OF BENZODIAZEPINE AND MUSCARINIC CHOLINERGIC RECEPTOR BINDING BY SEVERAL β-CAROLINES

	IC_{50} [^{3}H]-flunitrazepam	IC_{50} [^{3}H]QNB
Harmane	7 ± 1	24 ± 5
Norharmane	8 ± 1	133 ± 33
Harmine	134 ± 9	5 ± 1
Harmaline	390 ± 42	52 ± 14
Tetrahydronorharmane	920 ± 95	638 ± 128
Clonazepam	0.002	—
Chlordiazepoxid	0.8	—
Atropine	—	0.004
Pilocarpine	—	6.2

The concentration of the displacing agents which inhibits specific receptor binding by 50% (IC_{50}) was determined by log-probit analysis, using 4–6 different concentrations of the displacers. The data are means ± S.E.M. of 3–6 individual determinations.

TABLE 2

INHIBITION OF OPIATE RECEPTOR BINDING BY SEVERAL β-CARBOLINES

	IC_{50} [μM]		IC_{50} 150 mM Na^+ / IC_{50} 0 mN Na^+
	0 mM Na^+	150 mM Na^+	
Harmane	2.8 ± 4	42 ± 1	1.5
Norharmane	118 ± 4	345 ± 74	2.9
Harmine	7 ± 1	20 ± 1	2.9
Harmaline	13 ± 3	60 ± 4	4.6
Tetrahydronor-harmane	353 ± 70	1050 ± 50	3.0
Naloxone	0.0025	0.0029	1.1
Morphine	0.024	0.47	19.6

IC_{50} values were determined as described under Table 1.

TABLE 3

INHIBITION OF DOPAMINE AND SEROTONIN RECEPTOR BINDING BY SEVERAL β-CAROLINES

	IC_{50} [μM]	
	[^{3}H]spiroperidol	[^{3}H]serotonin
Harmane	163 ± 22	101 ± 8
Norharmane	317 ± 33	180 ± 29
Harmine	69 ± 6	128 ± 18
Harmaline	207 ± 15	115 ± 26
Tetrahydronorharmane	26 ± 3	4 ± 1
Chlorpromazine	0.06	—
Dopamine	5.1	7.6
Cyproheptadine	—	5.0
Serotonin	—	0.01

IC_{50} values were determined as described under Table 1.

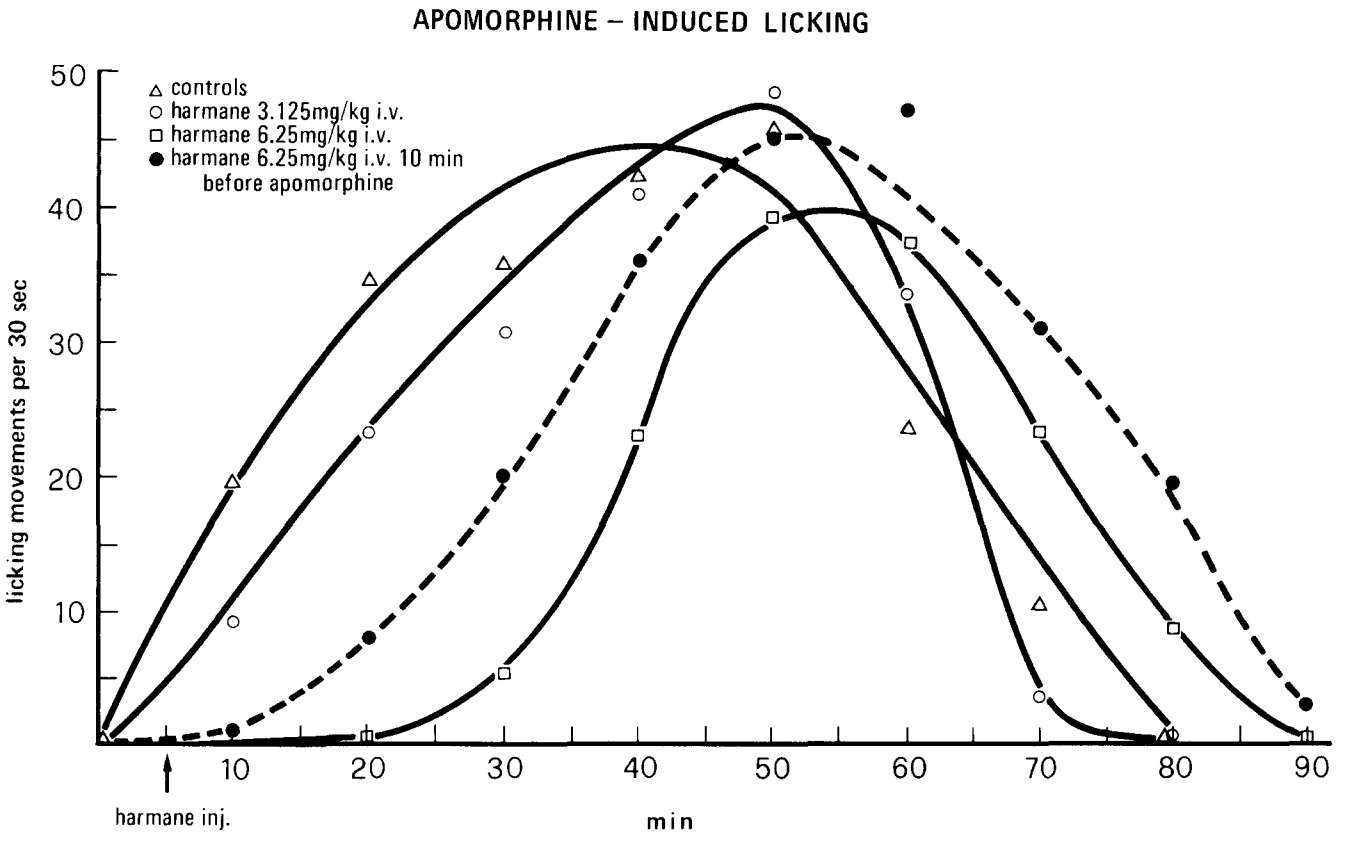

FIG. 1. Effect of harmane on the apomorphine-induced licking rate. Following IC injection of apomorphine (1 mg/kg) the rats were placed into a plastic tube. Five min later vehicle and various doses of harmane respectively were injected IV. In another experiment, harmane was injected 10 min before apomorphine (dotted line). Values represent the means from ten rats.

harmane (harmine and harmaline) which are most potent at the [^{3}H]naloxone or [^{3}H]QNB binding sites but which are substantially less active at all other receptor systems investigated (Tables 1, 2, and 3).

In respect to the essential problem of the specificity of harmane as a possible benzodiazepine receptor agonist [25,26] it is now of major importance if neuropharmacological actions *in vivo* can be demonstrated which correlate to the effects of harmane on the receptor systems investigated in this study. As already mentioned, the convulsive activity of harmane (ED_{50} in rats=3.6 mg/kg IV) has been correlated with its possibly agonistic properties at the benzodiazepine receptor [26]. It seems now very important to know if doses of harmane similar to its ED_{50} as a convulsant are also sufficient to produce symptoms of CNS activities not correlated with the benzodiazepine receptor. Since dopamine antagonistic [10, 11, 24] and serotoningergic [11, 12, 24] properties of β-carbolines have already been suggested we tested convulsive doses of harmane in two animal models which can indicate antidopaminergic and sertoninergic activity. Furthermore, using the hot-plate test, we checked whether for harmane as for tetrahydronorharmane [24] analgesic properties can be demonstrated.

Apomorphine-Induced Licking Movements

Treatment with 6.25 mg/kg harmane causes a delay of the apomorphine-induced licking movements (Fig. 1). This effect lasts roughly 20 min since the difference between the two curves is neutralized if a timelag of 20 min is assumed (α(minimum)=8.7%, adjusted), calculated by comparing the response rate at time 10 with that at time 30, time 20 with that at time 40, etc. This delay is not detectable after injection of 3.125 mg/kg harmane. The shift to the right of the increasing side of the time course of apomorphine is diminished when 6.25 mg/kg harmane is administered 10 min before apomorphine. However, the difference is suspended if a timelag of 10 min is assumed between the two regimens (α (minimum)=53%, adjusted). The maximal licking rate (approximately 60 min after apomorphine) and the decreasing side of the curves are not affected, which speaks for a short-lasting action of harmane (Fig. 1).

Body Temperature

The body temperature of vehicle-treated rats increases after the injection up to a maximum at about 25 min after injection (Fig. 2). Administration of only 1.56 mg/kg harmane significantly alters the shape of the time course curve when compared with controls by the method of orthogonal polynomes. 3.125 mg/kg of harmane or more cause a hypothermia in a dose-dependent manner (Fig. 2). One hundred min after injection of 3.125 or 6.25 mg/kg harmane the body temperature was back at the control levels (Fig. 2).

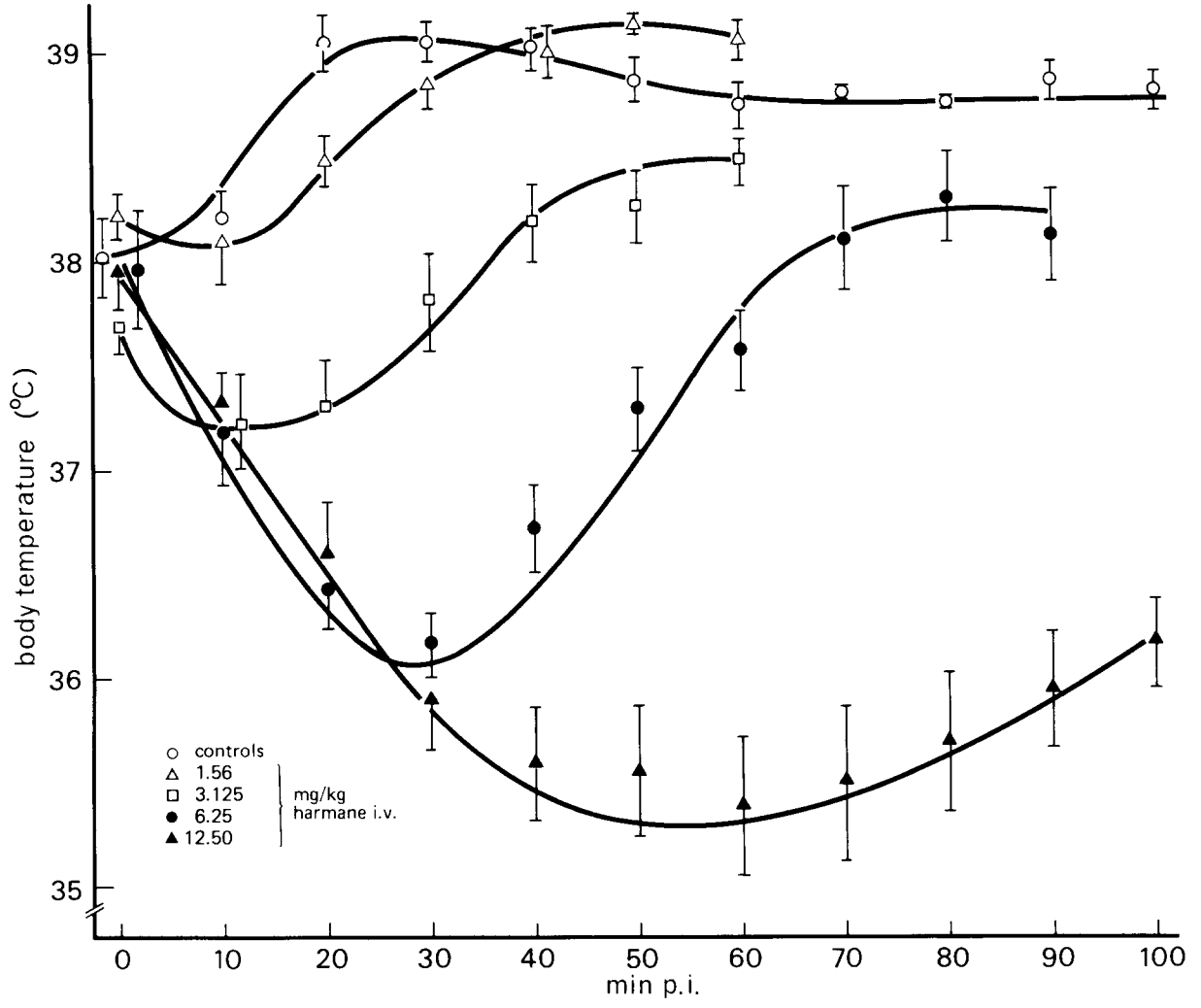

FIG. 2. Time course of the effect of harmane on the body temperature. After placing rats into a plastic tube, vehicle and various doses of harmane respectively were injected IV. Values represent the means±S.E.M. from ten rats.

Hot-Plate Test

The effect of harmane on the nociception of rats was investigated using the hot plate test. Vehicle-treated animals showed no changes of the reaction time during the observation period while treatment with harmane caused a prolongation of the reaction time (Fig. 3). This effect was detectable 20 min after injection of only 3.125 mg/kg harmane (Fig. 3).

DISCUSSION

The data reported in this communication clearly indicate that harmane and some other related β-carboline derivatives interact not only with benzodiazepine receptor binding but also with several other neurotransmitter receptors of the mammalian CNS with relative high affinity. Minor differences in the structural parameters lead to large differences in the affinities for the different receptors. Examples are the high affinity of tetrahydronorharmane for the dopamine and serotonin receptors and the relative high affinities of harmane, harmine, and harmaline for the muscarinic cholinergic and the opiate binding sites which are not found in the case of norharmane. It is now of major importance to demonstrate neuropharmacological activities of harmane *in vivo* possibly related to its activity at the receptor systems investigated.

As already mentioned, harmane is a fairly potent inhibitor of specific [^{3}H]QNB binding *in vitro*. However, whether it exhibits cholinergic or anticholinergic activities *in vivo* is controversial. A few observations might indicate weak cholinergic activity [28] while atropine-like effects were in general not observed for harmane or other β-carbolines [9,28]. In general, the effects of harmane or other β-carbolines on the muscarinic cholinergic receptor *in vivo* might be weak if present at all, which largely contrasts to the relative high potency at specific [^{3}H]QNB binding *in vitro*.

As found for specific [^{3}H]QNB binding, harmane, har-

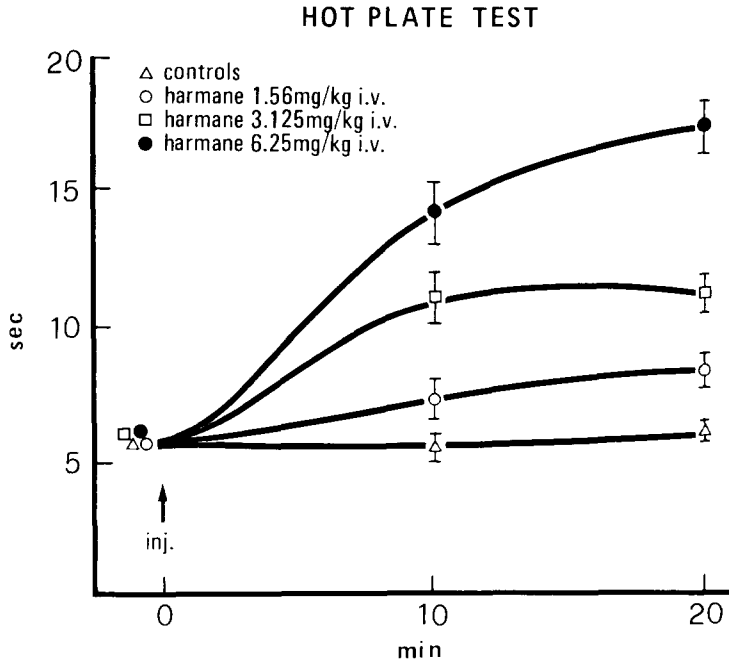

FIG. 3. Time course of the effect of harmane on the nociception using the hot plate test. Values represent the means±S.E.M. from ten rats.

mine, and harmaline are fairly potent inhibitors of specific [^{3}H]naloxone binding. Using the sodium shift (the ratio of the IC_{50}'s in the presence and without 150 mmol/l NaCl) as an indicator of opiate agonistic properties [23] harmane must be classified nearly as a pure opiate antagonist, with a sodium shift only slightly higher than that of the pure antagonist naloxone (Table 2). The sodium shifts for all other β-carboline derivatives investigated are definitely higher than that of harmane and are in the range of that of the mixed agonist pentazocine (sodium shift=3.3) [23]. These data make it very unlikely that the effects of harmane on the nociception of rats are mediated by opiate agonist properties. Thus, the relative high potency of harmane in the hot-plate test might indicate other analgesic mechanisms. A point in case could be a serotonin-agonistic activity, as it has been discussed for the analgesic effects of tetrahydronorharmane which could be inhibited by a serotonin receptor antagonist [24]. With respect to the doses effective in the hot-plate test and those producing convulsions it may be noticed that preceding convulsions e.g., induced by bicuculline elicit higher activity of serotonin-mediated mechanisms [21]. This observation may explain the discrepancy between the low affinity of harmane to the serotonin binding site compared with that to the benzodiazepine binding site and the similar potency of the compound in the *in vivo* experiments. In this connection it is noteworthy that the harmane-induced convulsions lasted less than 60 sec.

The data of this communication give strong evidence that β-carbolines can also affect the dopamine receptors. In respect to dopaminergic transmission, a dopamine receptor antagonism may explain that β-carbolines exhibit antidopaminergic properties [10,24] and harmane inhibits the apomorphine induced licking movements (Fig. 2).

In summary, the results of the present communication have clearly shown that besides some already known effects on the benzodiazepine receptor, harmane can also influence some other neurotransmitter receptors with quite high potencies. More important, harmane doses in the range of its ED_{50} as convulsant are sufficient to produce some behavioral changes *in vivo* very likely mediated by its interaction with the dopaminergic and serotoninergic systems. Both neuronal systems might be activated by the preceding convulsions. Since the convulsant activity of harmane has been correlated to its possible agonistic properties at the benzodiazepine receptor the data reported clearly indicate that the interaction of harmane might produce some consequent actions of other neuronal mechanisms.

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