Anesthesiology
64:248–252, 1986

Mechanism of Antagonism by Physostigmine of Acute Flunitrazepam Intoxication

E. Vatashsky, M.D., B. Beilin, M.D.,† Michal Razin, B.Sc.,‡ Marta Weinstock, Ph.D.§*

The effect of physostigmine on the loss of consciousness and respiratory depression induced in rabbits by flunitrazepam, 1 mg/kg, was studied to demonstrate whether the restoration of consciousness and respiration rate results from an increase in central cholinergic activity or from an interference by physostigmine with specific binding of flunitrazepam to its receptors. Physostigmine, 0.1–0.4 mg/kg iv, caused a dose-related reversal of consciousness and respiration rate within 15 min of its injection, which lasted 15–30 min depending on the dose. This was associated with peak inhibition of acetylcholinesterase (AChE) in the frontal cortex and medulla, at 15 min, ranging from 35–51%. The analeptic effect of physostigmine in flunitrazepam-treated rabbits was prevented by pretreatment with scopolamine, 1 mg/kg. The effective dose range for physostigmine, 3–12 μmol/kg, is close to concentrations of this agent that inhibit activity in solubilized preparations of AChE from rabbit cortex, 1–3 × 10^{-8} M. However, physostigmine, 10^{-9}–10^{-4} M, failed to displace ^{3}H flunitrazepam from specific binding sites on membranes prepared from rabbit cerebral cortex. It is concluded that physostigmine antagonizes the somnolence and respiratory depression induced by benzodiazepines by restoring cholinergic transmission to normal levels. The effective dose range of physostigmine is small, and serious side effects from overdose can occur as a result of excess cholinergic activity at neuromuscular synapses. (Key words: Anesthetics, intravenous: flunitrazepam. Brain: unconsciousness, respiratory depression. Enzymes: cholinesterase. Neurotransmitters: acetylcholine. Receptors: benzodiazepinese.)

DURING THE PAST few years, many reports have appeared that physostigmine can reverse the loss of consciousness and respiratory depression induced by various benzodiazepine derivatives.[1–4] However, other studies have failed to support these observations.[5]

Physostigmine, an anticholinesterase that readily enters the brain, has been shown to reverse the effects of various drug classes that act in different ways to reduce cholinergic transmission. These classes include tricyclic antidepressants,[6] ketamine,[7] anticholinergics,[8] and narcotic analgesic drugs.[9–10]

Benzodiazepines may produce their characteristic effects by interfering with cholinergic transmission in certain areas of the CNS. Thus, diazepam has been shown to potentiate the depressant actions of adenosine on cortical neurones[11] and, thereby, reduce the release of acetylcholine.[12] Benzodiazepines also have been shown to increase acetylcholine levels in rat and mouse brains, most likely by inhibiting its release.[13] Therefore, one may expect that if benzodiazepines depress consciousness and respiration by reducing acetylcholine release, their effects should be reversed by drugs that increase cholinergic transmission, such as physostigmine.

The reason for the lack of reversal by physostigmine in some clinical studies is not clear. It may be due to the fact that the subjects received too much atropine relative to the dose of physostigmine,[5] or that the dose of physostigmine alone was inadequate. Alternatively, the antagonism of benzodiazepine narcosis by physostigmine may not result from the anticholinesterase effect of the latter, but from an interaction at the benzodiazepine receptor site. Thus, Speeg *et al.*, in 1979,[14] reported that physostigmine inhibits the binding of both ^{3}H-diazepam and ^{3}H-flunitrazepam to human and rat brain membranes, in a dose-dependent manner. This may explain why Nagy and Desci, in 1978,[15] required doses of physostigmine ranging from 0.4 to 1.5 mg/kg iv to reverse diazepam-induced coma. These doses are much greater than those that antagonize the effects of other drugs, such as opiates[10] or ketamine,[16] in laboratory animals.

The present study in rabbits was undertaken to determine under what conditions physostigmine can antagonize the depressant effects of flunitrazepam, and whether its action results from an anticholinesterase effect or from an interaction with the flunitrazepam binding sites.

Materials and Methods

Effect of Physostigmine on Flunitrazepam-Induced Unconsciousness

Male rabbits (mixed strain), weighing 2.5–3 kg, were placed in a restraining hammock to facilitate cannulation.[10] Respiratory rate was counted visually. Drugs were injected through a butterfly needle placed into the marginal ear vein. Flunitrazepam was diluted 3-fold with saline to avoid venous irritation, and injected at a dose of 1.0 mg/kg during a period of 2 min in 36 rabbits. This dose previously had been found to produce a loss of righting reflex and response to external stimuli (touch, noise, etc.) in our strain of rabbits, within 15 min of injection. Once this occurred, the animals were removed from the hammock and placed on their side. The rabbits were divided

* Lecturer in Anesthesiology.
† Instructor in Anesthesiology.
‡ Research Assistant in Pharmacology.
§ Professor of Pharmacology.

Received from the Departments of Anesthesiology and Pharmacology, Hadassah Hospital Hebrew University Medical School, P. O. Box 1172, Jerusalem, Israel. Accepted for publication September 17, 1985.

Address reprint requests to Professor M. Weinstock.

TABLE 1. The effect of Flunitrazepam and Physostigmine on Consciousness

Treatment Group (mg/kg)	n	Somnolence Score ± SEM					
		0	15 min*	30 min	45 min	60 min	90 min
I: flunitrazepam 1.0	6	1 ± 0	3.83 ± 0.17	4 ± 0	3.83 ± 0.21	3.67 ± 0.21	3.67 ± 0.21
II: flunitrazepam 1.0 + physostigmine 0.1	6	1 ± 0	3.67 ± 0.21	4 ± 0	2.67 ± 0.21†	3.50 ± 0.22	4 ± 0
III: flunitrazepam 1.0 + physostigmine 0.2	6	1 ± 0	4 ± 0	3.67 ± 0.21	2.33 ± 0.21†	3.5 ± 0.22	4 ± 0
IV: flunitrazepam 1.0 + physostigmine 0.4	6	1 ± 0	3.83 ± 0.17	4 ± 0	1.67 ± 0.21†	2.0 ± 0.26†	3.3 ± 0.21
V: flunitrazepam 1.0 + scopolamine 1.0 + physostigmine 0.2	6	1 ± 0	4 ± 0	3.83 ± 0.17	3.83 ± 0.17‡	3.83 ± 0.17	3.83 ± 0.17
VI: Flunitrazepam§ 1.0	6	1 ± 0	3.5 ± 0.22	4 ± 0	4 ± 0	3.67 ± 0.21	3.5 ± 0.22

Degree of consciousness scoring was based on the following: 1 = fully awake, able to stand, and reactive to external stimuli; 2 = sleepy but able to stand if assisted, reactive to external stimuli; 3 = sleepy and unable to stand, but reactive to external stimuli; 4 = unable to stand or right itself if placed on back, unreactive to external stimuli.

* Time after injection of flunitrazepam; †$P < 0.01$ cf Gp I; ‡$P < 0.01$ cf Gp III. §Showed no reduction in respiration rate (table 2).

into five groups and given one of each of the following treatments, iv.

Group 1. Saline, 0.1 ml/kg, 30 min after flunitrazepam.
Group 2. Physostigmine, 0.1 mg/kg, 30 min after flunitrazepam.
Group 3. Physostigmine, 0.2 mg/kg, 30 min after flunitrazepam.
Group 4. Physostigmine, 0.4 mg/kg, 30 min after flunitrazepam.
Group 5. Scopolamine, 1 mg/kg, 15 min after flunitrazepam, followed 15 min later, by physostigmine, 0.2 mg/kg. Scopolamine was given to see whether the influence of physostigmine on the hypnotic effect of flunitrazepam was associated with an increase in central cholinergic activity. This agent was chosen in preference to atropine due to its higher degree of penetrability into the central nervous system.[17]

The degree of consciousness (scored as shown in table 1) and respiration rate were assessed every 5 min after injection of flunitrazepam, and thereafter at 15-min intervals for 3 h (table 2).

MEASUREMENT OF BRAIN CHOLINESTERASE INHIBITION

Acetylcholinesterase (AChE) activity was measured in the frontal cortex and medulla oblongata of rabbits given flunitrazepam or saline alone, 15 or 60 min after physostigmine, 0.2 mg/kg, and 15 min after physostigmine, 0.1 or 0.4 mg/kg. Animals were killed by air embolism, and the two brain areas were rapidly dissected out. They were washed in 1 ml 0.1 M phosphate buffer, *p*H 8.0, blotted with filter paper, weighed, homogenized in 10 volumes of 1% Triton® in phosphate buffer, and centrifuged at 1000 g for 1 min. Cholinesterase activity was measured at room temperature in supernatants (50 μl) by the method of Ellman *et al.*[18]

In order to remove the enzyme inhibition by physostigmine, 50 μl aliquots of solubilized enzyme, taken from both physostigmine- and saline-treated rabbits, were incubated in 3 ml phosphate buffer, *p*H 8.0, at 37° C for 30 min. These samples were cooled to room temperature (20–22° C) so that enzyme activity could be measured under the same conditions as before. This was necessary because AChE activity was greater at 37° C than at room temperature (20–22° C) (table 3). This treatment enabled the use of each animal as its own control for the estimation of the degree of enzyme inhibition induced by physostigmine (table 4).

TABLE 2. The Effect of Flunitrazepam and Physostigmine on Respiration Rate

Group*	Respiration Rate ± SEM Breaths/Min					
	0	15 min	30 min	45 min	60 min	90 min
I	112 ± 12†	62 ± 6	65 ± 2	68 ± 4	79 ± 3	88 ± 5‡
II	122 ± 8‡	78 ± 9	72 ± 7§	82 ± 5	91 ± 10	95 ± 10§
III	136 ± 12‡	75 ± 6	72 ± 7†	94 ± 4	85 ± 8	92 ± 8
IV	112 ± 6	68 ± 4	70 ± 7†	99 ± 6	95 ± 7	99 ± 7
V	145 ± 14‡	64 ± 4	76 ± 4	82 ± 5	85 ± 6	88 ± 5
VI	105 ± 8	99 ± 10	112 ± 9	119 ± 13	123 ± 12	137 ± 19

* Treatments are shown in Table 1.

Significantly different from reading at 45 min: †$P < 0.001$; ‡$P < 0.01$; §$P < 0.05$.

TABLE 3. Acetylcholinesterase Activity in Rabbit Brain

Treatment (mg/kg)	n	Activity ($\mu mol/min^{-1} \cdot g^{-1}$)					
		Frontal Cortex			Medulla		
		22°C	37°C	22°C	22°C	37°C	22°C
Saline	6	15.6 ± 1.1	19.1 ± 1.6†	15.7 ± 1.2	33.1 ± 2.2	40.2 ± 2.7†	32.7 ± 1.9
Flunitrazepam 1.0	3	15.7 ± 1.2	—	14.3 ± 1.5	35.0 ± 2.4	—	33.4 ± 2.4
Physostigmine 0.2	6	9.9 ± 0.4*	—	14.7 ± 0.6	21.6 ± 2.2*	—	31.7 ± 1.1

Significantly different from saline and flunitrazepam, * $P < 0.01$. Significantly different from value at 22 C, † $P < 0.05$.

In a further series of experiments, a solubilized preparation of AChE was prepared from the frontal cortex of an untreated rabbit, as previously described. Aliquots (50 μl) of the enzyme preparation were incubated in 3 ml of 0.1 M phosphate buffer for 20 min at 37° C, with four different concentrations of physostigmine. Enzyme activity was measured and the concentration of physostigmine that inhibited the activity by 50% (IC_{50}) was estimated. Each concentration was measured in triplicate.

BENZODIAZEPINE-RECEPTOR BINDING STUDIES

The frontal cortex was removed from three untreated rabbits after decapitation and used for these studies. The cortex was weighed and homogenized in 50 volumes of 50 mM cold Tris® buffer, *p*H 7.4, and spun for 15 min at 49000 g. The pellet was homogenized in 200 volumes of the same buffer and used for binding assay. Incubation media contained 0.7 ml of rabbit cortex homogenate, 0.1 ml of ^{3}H-flunitrazepam (final concentration 2 nM), and 0.1 ml of different concentrations of unlabeled flunitrazepam or physostigmine dissolved in 0.1% ascorbic acid. To determine nonspecific binding, assays were conducted in the presence of 5 μM clonazepam. After incubation for 90 min at 4° C, samples were filtered under vacuum over Whatman® fiberglass filters, washed three times with 4 ml Tris® buffer, and counted for radioactivity.[19]

Drugs used were flunitrazepam (Hoffmann-La Roche, Inc.) and physostigmine salicylate (prepared with 0.1% sodium metabisulfite as antioxidant) (−) scopolamine hydrobromide (Sigma Ltd.).

All drugs were freshly prepared and diluted in sterile saline immediately before use. Doses are expressed throughout in mg/kg body weight of the salt. All data represent the mean value ± SEM.

STATISTICAL ANALYSES

Scored data were analyzed by the Mann-Whitney test. Other data were analyzed by 2-way analysis of variance with one repeated measure (time). Individual means were tested for significant differences with *post-hoc* group comparisons (Winer).[20]

TABLE 4. Inhibition of Acetylcholinesterase (AChE) in Rabbit Brain by Physostigmine

Dose of Physostigmine (mg/kg)	Time after Injection (min)	n	Per cent Inhibition of AChE	
			Frontal Cortex	Medulla
0.1	15	6	35.4 ± 1.9*	34.9 ± 2.3*
0.2	15	6	44.3 ± 1.6	47.6 ± 1.8
0.2	60	6	30.0 ± 2.0*	28.9 ± 0.9*
0.4	15	5	51.1 ± 1.9†	50.7 ± 2.0

Significantly different from physostigmine; 0.2 mg/kg 15 min. *$P < 0.01$, †$P < 0.05$.

Results

When injected intravenously in rabbits, flunitrazepam, 1 mg/kg, caused a loss of righting reflex and lack of response to stimuli, within 5–30 min, in all 36 rabbits. In 30 out of 36 rabbits, respiration rate was reduced significantly within 10 min ($P < 0.001$ all groups) and remained depressed for 1.5 to 2 h. All of the animals given flunitrazepam alone recovered consciousness only after 2.5–3 h. Physostigmine, with or without scopolamine, was given only to those animals in which respiration rate had been reduced by flunitrazepam (30 animals).

Physostigmine caused a dose-related increase in consciousness within 5–15 min after its injection. This awakening effect lasted only about 10–15 min after doses of 0.1 and 0.2 mg/kg, and 20–25 min after 0.4 mg/kg of physostigmine (table 1). Pretreatment with scopolamine, 1 mg/kg, completely prevented the restoration of consciousness produced by physostigmine, 0.2 mg/kg, in flunitrazepam-treated rabbits.

Respiration rate, which had been reduced by flunitrazepam, was increased in a dose-related manner by physostigmine. Only the highest dose, 0.4 mg/kg, restored the rate to the control level. This effect of physostigmine was antagonized by scopolamine (table 2). Signs of peripheral cholinergic hyperactivity, such as salivation, defecation, miosis, and muscle fasciculations, were absent at the smallest dose of physostigmine, of mild intensity at

0.2 mg/kg, but very marked at the largest dose, 0.4 mg/kg.

Anticholinesterase Activity of Physostigmine in Rabbit Brain

When incubated at 37° C with a solubilized preparation of AChE derived from the cerebral cortex of untreated rabbits, physostigmine ($1.2–9.5 \times 10^{-8}$ M) caused a dose-related inhibition. The IC_{50} (concentration needed to inhibit enzyme activity by 50%) was found to be 2.4×10^{-8} M.

AChE activity of solubilized preparations of frontal cortex and medulla of rabbits given saline, measured at room temperature (20–22° C), within 15 min of removal from the animal and after incubation of 37° C for 30 min, followed by cooling to room temperature, is shown in table 3. Activity was significant greater in both brain areas when the enzyme was maintained at 37° C than at 21° C, and subsequent cooling reduced this activity to its original value at room temperature. Enzyme prepared from the cortex and medulla of rabbits that had been killed 45 min after injection of flunitrazepam, 1 mg/kg, showed the same activity as those taken from animals given saline. Physostigmine reduced enzyme activity in a dose-related manner. When enzyme preparations taken from physostigmine-treated rabbits were incubated for 30 min at 37° C and then cooled to room temperature, AChE activity was restored to the level found in control rabbits (table 3). The per cent reduction in AChE activity induced by three different doses of physostigmine was calculated from the values obtained before and after removal of the physostigmine inhibition by incubation of the enzyme preparation at 37° C (table 4). Physostigmine caused a dose-related inhibition of AChE in the cortex and medulla, 15 min after its injection.

After 60 min, when the analeptic effect of this agent clearly had worn off, the degree of enzyme inhibition induced by 0.2 mg/kg also was considerably reduced (table 4).

The concentration of cold flunitrazepam required to displace 50% of bound ^{3}H flunitrazepam in cortex homogenates was approximately 4×10^{-9} M. However, physostigmine failed to displace any ^{3}H flunitrazepam in concentrations ranging from 10^{-9}–10^{-4} M (fig. 1).

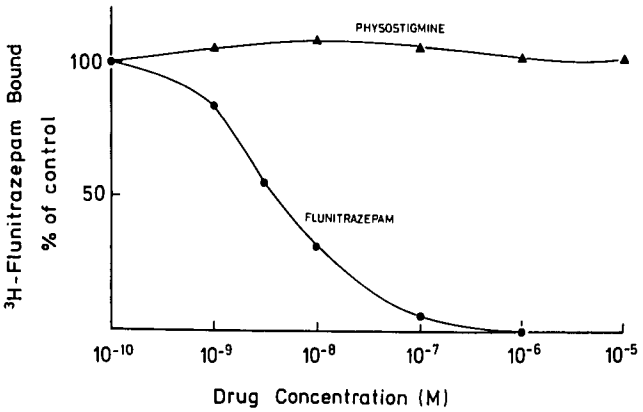

FIG. 1. Failure of physostigmine to displace ^{3}H-flunitrazepam from membrane binding sites in rabbit cortex. Each point represents the mean of three determinations on the membrane preparation from the same rabbit cortex. Note that cold flunitrazepam caused a dose-related displacement of ^{3}H-flunitrazepam bound to receptors in the membrane, while physostigmine did not.

Discussion

Benzodiazepines have been shown to bring about their sedative, anxiolytic, and anticonvulsant effects through interaction with specific receptors.[21] Some of the effects of these agents are believed to be mediated by an increase in the activity of gamma-amino-butyric acid (GABA),[22] while others may be due to a potentiation of the effects of adenosine.[23] Both of these inhibitory neurotransmitters have been shown to reduce cholinergic transmission in several brain areas.[12,13] Experimental sleep induced by electrical stimulation of the ventromedial intrathalamic nuclei (VMIN) in the rabbit also has been associated with a reduction in cerebral acetylcholine release. Conversely, the release of this neurotransmitter is increased during arousal following stimulation of the midbrain reticular formation.[24] Thus, benzodiazepines may induce sleep either by mimicking the effects of stimulation of the VMIN, or by inhibiting activation of the midbrain reticular formation.

Acetylcholine has been shown to play an important role in the control of respiration by specific centers in the medulla.[25] Thus, one may expect that both the hypnotic and respiratory depressant effects of benzodiazepines would be antagonized by drugs that can increase central cholinergic transmission, such as physostigmine.

In this study, flunitrazepam (a more potent benzodiazepine derivative than diazepam)[26] caused a loss of consciousness in all of the rabbits, accompanied by a decrease in respiratory rate in 83% of them. The former effect lasted 2.5–3 h, while the latter usually returned to predrug levels within 2–2.5 h.

Both the loss of consciousness and decrease in respiratory rate were reversed in a dose-dependent manner by physostigmine. This effect of physostigmine was well-correlated with the degree of inhibition of AChE in both the cerebral cortex and medulla, 15 min after its injection. At 60 min, when consciousness again was lost, the degree of enzyme inhibition after 0.2 mg/kg of physostigmine also decreased below the value required to cause a significant wakening effect. (Compare values for enzyme in-