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Idaverine, an M_2- vs. M_3-Selective Muscarinic Antagonist, Does Not Prevent Motion Sickness in Cats

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LUCOT, J. B., K. J. VAN CHARLDORP AND M. TH. M. TULP. *Idaverine, an M_2- vs. M_3-selective muscarinic antagonist, does not prevent motion sickness in cats.* PHARMACOL BIOCHEM BEHAV **40**(2) 345–349, 1991.—In this study, the affinity profile of idaverine for the M_1- (neuronal tissue), M_2- (heart) and M_3- (glandular tissue/nonvascular smooth muscle) muscarinic receptors was examined by means of radioligand binding and in vitro organ bath experiments in order to use the compound for the investigation of the muscarinic receptor subtype involved in motion sickness. In the profile study a comparison was made with the muscarinic antagonists atropine, pirenzepine (M_1-selective) AF-DX 116 (M_2-selective) and 4-DAMP (high affinity for M_1- and M_3-binding sites). The affinity of idaverine appeared to be equally high for the M_1- and M_2-binding sites. However, the affinity for the M_1-binding sites should be interpreted cautiously since the Hill slope deviated from unity. Idaverine showed a 20-fold selectivity for the M_2-binding sites over the M_3-binding sites, whereas it showed a small selectivity (<5-fold) for the M_2-receptors compared to the ileal and tracheal smooth muscle receptors. Thus idaverine appears to be M_2 over M_3 selective. However, in contrast to AF-DX 116, it is not clear whether idaverine is also M_2 over M_1 selective. In experiments with cats, idaverine failed to prevent motion sickness at doses from 0.03 to 3 mg/kg. These results are interpreted to implicate M_3-receptors in the motion sickness suppressant effect of antimuscarinic drugs.

Antimuscarinic Atrium Cat Emesis Hippocampus Ileum Motion sickness
Muscarinic receptor subtypes Radioligand binding Submandibular gland Trachea

THE potency of antimuscarinic drugs at preventing motion sickness in squirrel monkeys correlates with the potency of the drugs at displacing [^{3}H]QNB from structures in the brainstem that are associated with motion sickness (19). Autoradiographic studies using a two-site model with N-[^{3}H]methylscopolamine as the ligand describe a preponderance of M_2-receptors in the human brainstem (4,5). The high correspondence between these muscarinic binding sites and brainstem structures associated with motion sickness has led to the suggestion that selective blockade of M_2-receptors should prevent motion sickness while producing fewer side effects than traditional nonselective antimuscarinic drugs (13,17).

However, the displacement of [^{3}H]QNB from brainstem binding sites by carbachol, the measure of M_2 binding used to reach this conclusion, had a Hill slope significantly lower than unity (17). The binding of N-methylscopolamine in the brainstem was subsequently described in terms of two sites, R_1 and R_2, with the R_1 predominating (16). The R_1 and R_2 subtypes correspond to the M_2 and M_3 subtypes, respectively, described by others (8,10) and a preponderance of the M_2 over the non-M_1, non-M_2 subtype in the medulla and pons has been verified (11). Thus the suggested role for M_2-receptors was based on a two receptor model, while a three-receptor model, in which the M_2 is further subdivided into M_2- and M_3-receptors, may be more appropriate.

In the present study, the drug idaverine (Fig. 1) was characterized and found to be selective for M_2- over M_3-receptors. This profile seemed favorable for the prevention of motion sickness, due to the preponderance of M_2- over M_3-receptors in the brainstem.

METHOD

Radioligand Binding Experiments

Male Wistar rats (150–200 g) were killed, the hippocampus, heart and submandibular gland were dissected out and homoge-

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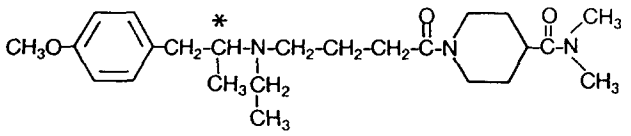

FIG. 1. Chemical structure of idaverine.

nized in 5–10 ml ice-cold HEPES-buffer, composed as follows (mM): HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] 20.0; NaCl, 100.0; and $MgCl_2 \cdot 6H_2O$, 10.0 (pH = 7.5 at 37°C). Homogenisation was performed with an Ultraturax (setting 6, 10 s) followed by a Potter teflon homogenizer (10–20 strokes). Hippocampus tissue was homogenized by the Potter homogenizer only (15 strokes). All homogenates were filtered through two layers of cloth gauze and were centrifuged at 50,000 × g for 10 min. The pellets were either used immediately or stored at −20°C.

The pellets were rehomogenized and diluted to a tissue concentration of 4 mg/ml (hippocampus), 5 mg/ml (atrium) and 12 mg/ml (submandibular gland). Aliquots of 500 μl of membrane suspension were incubated at 25°C for 45 min with [^{3}H]-pirenzepine (specific activity 74.4 Ci/mmol) or at 37°C for 20 min with [^{3}H]-NMS ([^{3}H]-N-methylscopolamine, specific activity 73.8 Ci/mmol) in the presence of various concentrations of nonlabelled muscarinic antagonists in a total volume of 1 ml HEPES-buffer. Incubations were terminated by rapid filtration through Whatman GF-B filters. Filters were washed by three 5-ml portions of ice-cold HEPES-buffer and placed in scintillation vials, containing 10 ml of Hydrocount®, in order to solubilize for 24 h. Radioactivity was determined with a liquid scintillation spectrometer.

The specific binding was assessed as the excess over blanks containing 10^{-7} M atropine or 10^{-6} M dexetimide. Binding curves for the muscarinic antagonists were derived indirectly from the competition experiments against 2.5 nM [^{3}H]-pirenzepine in the hippocampus or against 0.4 nM [^{3}H]-NMS for the other tissues.

K_i values were calculated as follows: $K_i = IC_{50}/(1 + L/K_D)$, where IC_{50} is the concentration of unlabelled drug required to inhibit 50% of the specific radioligand binding, L is the concentration of radioligand used and K_D is the dissociation constant of the radioligand-binding site complex (obtained via Scatchard plot analysis) (2). [^{3}H]-Pirenzepine revealed a K_D of 10.7 nM for the binding sites in the hippocampus. The K_D values of [^{3}H]-NMS for the binding sites present in the atrium and the submandibular gland were 0.66 and 0.36 nM, respectively. B_{max} values in pmol/g were: 21.0 (hippocampus), 16.6 (atrium) and 12.8 (submandibular gland). pK_i values are presented as the means of 3 experiments. Hill coefficients were calculated by linear regression analysis and assessed for deviation from unity using Student's *t*-test.

Functional Experiments

Atrium. Male Wistar rats (200–300 g) were killed, the heart excised and the left atrium suspended isometrically under a tension of 5 mN in 10 ml organ baths filled with oxygenated (95% O_2 + 5% CO_2) Krebs-Henseleit solution, composed as follows (mM): NaCl, 117.5; KCl, 5.6; $MgCl_2 \cdot 6H_2O$, 1.5; $CaCl_2 \cdot 2H_2O$, 1.8; $NaH_2PO_4 \cdot H_2O$, 1.2; $NaHCO_3$, 25.0; and glucose, 11.1 (pH = 7.4, 32°C). The atrium was electrically paced at a rate of 3 Hz (5 ms, 30 mA). During the experiment, the Krebs-Henseleit solution was changed every 20 min. After 30 min of equilibration, a cumulative concentration response curve (CRC) was made with methacholine. A second CRC was made after pretreatment (30 min) with atropine or idaverine. Control experiments showed that two successive CRC's to the agonist could be obtained without a significant change in sensitivity, allowing 45 min between each curve.

The negative inotropic response induced by methacholine was expressed as a percentage of the initial force of contraction. Log-CRC's were constructed by plotting the response versus the logarithm of the concentration of methacholine.

The affinity of each antagonist was assessed by means of the pA_2-value (1). The data are presented as the means of 14 experiments.

Ileum. Male rats (200–300 g) were killed, then the distal part of the ileum was excised. Longitudinal muscle strips were suspended isotonically under 10 mN tension in 10 ml organ baths filled with oxygenated (95% O_2 + CO_2) Krebs-Henseleit solution composed as described above with exception that the concentration of $CaCl_2 \cdot 2H_2O$ was 2.5 mM, the concentration of glucose was 5.6 mM and the temperature was 37°C. During the experiment, the Krebs-Henseleit solution was changed every 20 min. After 60 min of equilibration, a CRC was made with methacholine. A second CRC was made after pretreatment (30 min) with atropine or idaverine. Control experiments showed that two successive CRC's to the agonist could be obtained without a significant change in sensitivity, allowing 45 min between each curve.

The response was expressed as a percentage of the maximal contraction produced by methacholine in the first CRC (E_{max}). The data were calculated as described above and represent the means of 12 experiments.

Trachea. Bovine trachea were obtained from a local abattoir and transported in ice-cold (0–4 °C) oxygenated (95% O_2 + 5% CO_2) Krebs-Henseleit solution. Smooth muscle strips were prepared (2 mm width, 15 mm length) and suspended isotonically under 10 mN tension. The experimental protocol was similar to that described for the ileal tissues.

Motion Sickness

Subjects. A total of 27 cats were housed in the University Laboratory Animal Resources facility. All displayed normal free-fall righting and vestibulo-ocular reflexes. Female cats were used exclusively because they tolerate long-term experiments better than males. All had free access to food and water until the time of testing. The research conforms with the "Guidelines for the Use of Animals in Neuroscience Research" approved by the Society for Neuroscience and the "NIH Guide for the Care and Use of Laboratory Animals," NIH Pub. 85-23 (revised 1985).

Twenty cats were selected for the motion sickness study based on their susceptibility to the stimulus on five screening tests. On the five screening tests, five vomited on all five, two vomited on four, five vomited on three, six vomited on two and two vomited on one.

Seven cats that were not susceptible to motion sickness were used to test the safety of doses selected and to gather evidence for penetration of idaverine into the central nervous system.

Motion testing. The motion stimulus was provided by a motor-driven device described elsewhere (6). Briefly, the cats rode in clear plastic boxes suspended from each end of a 0.89 m beam that rotated about a central axle at 0.28 Hz (17 rpm). Motion tests lasted for 30 min of motion plus one min of observation at rest. Tests were separated by at least two weeks to prevent habituation to the motion stimulus (7).

TABLE 1

INHIBITION CONSTANTS (pK_i-VALUES) OF THE ANTAGONISTS ON THE MUSCARINIC BINDING SITES IN THE HIPPOCAMPUS, ATRIUM AND SUBMANDIBULAR GLAND

	Hippocampus (M_1) pK_i	Atrium (M_2) pK_i	Submand. Gland (M_3) pK_i
Atropine	9.18	8.46	8.80
	(0.13)	(0.02)	(0.05)
Idaverine	8.34	8.43	7.10
	(0.09)	(0.02)	(0.04)
Pirenzepine	7.86	6.28	6.92
	(0.11)	(0.04)	(0.14)
AF-DX 116	6.27	7.07	5.84
	(0.01)	(0.03)	(0.01)
4-DAMP	8.85	7.92	8.74
	(0.10)	(0.01)	(0.06)

Values are the mean of 3 experiments, SE in parentheses. Hill slopes were not significantly different from unity except for 4-DAMP [M_1: $n_H = 0.64$ (0.06)] and idaverine [M_1: $n_H = 0.60$ (0.07)].

TABLE 2

AFFINITY (pA_2-VALUES) OF ATROPINE AND IDAVERINE FOR THE MUSCARINIC RECEPTORS IN THE ATRIUM, ILEAL AND TRACHEAL SMOOTH MUSCLE

	Atrium (M_2) pA_2	Ileum (M_3) pA_2	Trachea (M_3) pA_2
Atropine	8.33	8.77	8.57
	(0.07)	(0.08)	(0.08)
Idaverine	8.24	7.61	7.99
	(0.10)	(0.10)	(0.06)
Pirenzepine	6.20	6.28	6.92
	(0.03)	(0.05)	(0.08)
AF-DX 116	7.03	6.39	6.30
	(0.03)	(0.04)	(0.07)
4-DAMP	7.71	8.61	9.03
	(0.06)	(0.03)	(0.05)

For comparison, the data for pirenzepine, AF-DX 116 and 4-DAMP on the atrium, ileum (9) and trachea (20) are also presented. Values are the mean of at least 12 experiments. In all cases the slope of the Schild plot did not deviate from unity.

Emetic responses during motion testing were analyzed by Cochran's Q-test (3). The latency to the first retch in those that responded were analyzed by ANOVA (21). The latency to the first retch for all tested were analyzed using a procedure for right-censored data based on the two-parameter Weibull distribution (18).

Materials. Methacholine (acetyl-B-methylcholine chloride) and atropine sulphate (Sigma Chemical Co., St. Louis, MO); idaverine (+)-1-[4-ethyl[2,-(methoxyphenyl)-1-methylethyl]amino-1-oxobutyl]-N,N-dimethyl-4-piperidinecarboxamide (Duphar BV, Weesp, The Netherlands); AF-DX 116 (11-[[2-[(dimethylamino)methyl]-1-piperidinyl]-acetyl]5,11-dihydro-6H-pyridol[2,3-b][1,4]-benzodiazepine-6-one) and pirenzepine·2HCl (Dr. Karl Thomas, GmbH, Biberach a.d. Riss, FRG); 4-DAMP (4-diphenylacetoxy-N-methyl-piperidine methiodide) (gift Prof. Dr. R. B. Barlow, Bristol, UK); HEPES (Janssen Pharmaceutica, Beerse, Belgium); [^{3}H]-N-methylscopolamine and ^{3}H-pirenzepine (New England Nuclear, Boston, MA). In the binding and functional studies, drugs were dissolved in distilled water and dilutions were made in the buffer solutions used. For the motion sickness studies, idaverine was prepared before each day of testing by placing it in a small amount of sterile injection water (with 0.9% benzyl alcohol as a bacteriostatic agent; Rugby, Rockville, NY) plus 4 μl of 0.1 N HCl per mg of drug. These were gently sonicated in solution and sterile bacteriostatic water was added to yield an injection volume of 0.1 ml/kg. Injections were SC. The order of doses tested, in mg/kg, was control, 1, 3, 0.3, control, 0.1, 0.03, 0.1 (30-min pretreat) and control.

RESULTS

Radioligand Binding Experiments

The inhibition of the specific binding of [^{3}H]-pirenzepine to the binding sites in the hippocampus resulted in steep curves for 3 of the 5 muscarinic antagonists studied. The displacement curves of 4-DAMP and idaverine were rather flat and their Hill coefficients significantly less than one (pK_i-values and Hill coefficients in Table 1).

The specific binding of [^{3}H]-NMS to the M_2- and M_3-binding sites in homogenates of the atrium and submandibular gland, respectively, was effectively displaced in a concentration-dependent manner by the antagonists studied (Table 1). All compounds produced steep inhibition curves; the Hill plots were linear with Hill coefficients not significantly different from unity ($p<0.05$, data not shown).

Atropine did not discriminate between the binding sites studied (maximal 5-fold difference, between M_1 and M_2). The affinity of pirenzepine for the M_1-binding sites was 38-fold higher than for the M_2-binding sites and 9-fold higher than for the M_3-binding sites. AF-DX 116 discriminated between the M_2-binding sites and both the M_1- and M_3-binding sites (6- and 17-fold, respectively). 4-DAMP revealed a similar affinity for the M_1- and M_3-binding sites, which was about 8-fold higher than for the M_2-binding sites. The affinity of idaverine for the binding sites in the hippocampus and atrium was similar and about 20-fold higher than for the binding sites in the submandibular gland.

Functional Experiments

The agonist-induced cumulative concentration-response curves on the atrial, ileal and tracheal preparations were shifted parallely to the right in a competitive manner by pretreatment with atropine (10^{-8}–10^{-6} M) or idaverine (10^{-8}–10^{-6} M). The pA_2 values of the corresponding Schild plots are shown in Table 2. None of the slopes significantly deviated from unity ($p>0.05$).

Atropine did not discriminate between the muscarinic receptors studied. Idaverine appeared to have a somewhat higher affinity for the atrial receptors compared to the ileal and tracheal smooth muscle receptors (4- and 2-fold, respectively), whereas AF-DX 116 was about 5-fold selective.

Effects of Idaverine on Motion Sickness

The first test with idaverine (1 mg/kg) used a 30-min pretreatment time and yielded negative results. A probe test in two cats at the dose of 10 mg/kg revealed behavioral changes 85 min after the injection and vomiting even later. As a result, subsequent motion tests used a 60-min pretreatment time. Following administration of 3 mg/kg, three cats vomited before motion

TABLE 3

EFFECFTS OF IDAVERINE ON THE INCIDENCE OF MOTION SICKNESS AND THE LATENCY TO THE FIRST RETCH IN CATS

Treatment*	#Vomit/ #Tested	Mean Latency (SE)	Estimated Median Lat.†
Control	17/20	10.17 (1.93)	9.88
0.3 + 60′	14/20	7.64 (2.05)	10.85
1.0 + 30′	17/20	8.26 (2.02)	7.24
3.0 + 60′	17/20	8.14 (2.24)	7.45
Control	15/20	9.59 (2.17)	11.59
Control	8/10	8.39 (2.94)	8.89
0.03 + 60′	9/10	11.06 (3.95)	8.02
0.1 + 60′	7/10	11.23 (4.69)	13.88
Control	8/10	12.46 (3.98)	12.92
Control	15/20	9.59 (2.17)	11.59
0.1 + 30′	13/20	13.03 (2.80)	18.91
Control	16/20	13.57 (2.50)	14.59

*Dose in mg/kg + pretreatment time in min.

†Estimated group median latency to the first retch, 90% confidence interval omitted for clarity.

testing began and six had more than one bout of retch/vomits during motion testing, suggesting that this high dose may have emetic properties. No statistical measure reached significance (Table 3).

After testing the first three doses, evaluation of the data suggested that the higher doses were contributing to motion-induced emesis. Ten of the cats were used to evaluate the effect of lower doses. The failure to decrease motion sickness at either 0.03 or 0.1 mg/kg led to further probe tests with the dose of 3 mg/kg to evaluate whether the drug was penetrating into the central nervous system. The occurrence of motor effects starting from 15 to 33 min after injection led to the decision to retest the dose of 0.1 mg/kg using only a 30 min pretreatment time. This also failed to decrease motion sickness.

Behavioral effects of idaverine on cats at high doses. Two cats received the dose of 10 mg/kg and were observed for 180 min. Both exhibited behavioral alterations and subsequently vomited. These two cats and five others received the dose of 3 mg/kg and were observed for 180 min. Behavioral changes such as head shakes (3 cats), myoclonus (1 cat), head shakes plus myoclonus (1 cat), abnormal bobbing (1 cat) and character change (1 cat) were observed.

DISCUSSION

In the present study, the affinity of idaverine was established for the M_1-, M_2-, and M_3-binding sites as well as for atrial, ileal and tracheal muscarinic receptors. In addition, a comparison was made with the pharmacologically important muscarinic antagonists atropine, pirenzepine, AF-DX 116 and 4-DAMP, for which binding affinities were in agreement with published values (10). The radioligand binding experiments clearly showed that idaverine had a higher affinity for the hippocampal and atrial than for the glandular binding sites (about 20-fold), whereas idaverine did not seem to discriminate between the M_1- and M_2-binding sites. In contrast to idaverine, AF-DX 116 did discriminate between the M_1- and M_2-binding sites, although to a lesser extent (6-fold) than between the M_2- and M_3-binding sites (17-fold). Idaverine, therefore, was less cardioselective than AF-DX 116. In accordance with the literature, pirenzepine appeared to be M_1-selective, whereas 4-DAMP had a high affinity for both the M_1- and M_3-binding sites.

The slope of the Hill plot for idaverine deviated significantly from unity for the M_1-binding sites. This was also observed with 4-DAMP, whereas the pK_i-value of 4-DAMP appeared in good agreement with the literature (10). It is not very likely that the low Hill slopes indicate the presence of more than one known binding site since a mixture of (M_1 + M_2)- or (M_1 + M_3)-binding sites would have been clear from the experiments with pirenzepine and AF-DX 116. This implicates a mixture of (M_1 + M_x)-binding sites. However, 4-DAMP has often been reported to bind to one binding site in rat hippocampus [e.g., (10)]. Another theoretical explanation for the low Hill slopes might be the presence of multiple affinity states of the receptor. In view of the fact that 4-DAMP as well as idaverine are full antagonists, however, this explanation is the least likely. Further investigation has to be done to explain the low Hill slopes of idaverine and 4-DAMP.

The affinities of the antagonists for the M_2-binding sites were in good agreement with those on the atrial M_2-receptors (Tables 1 and 2). The muscarinic receptors involved in the contraction of the ileum have been suggested to adhere to the M_3-subtype, although the M_3-binding sites represent only a minority of the binding sites [the majority being of the M_2-subtype (15)]. 4-DAMP and pirenzepine revealed a high affinity for the M_3-binding sites, which is in accordance with their high affinity for the smooth muscle receptors as reported by others. Idaverine, however, showed a rather small selectivity for the atrial receptors compared to the ileal and tracheal smooth muscle receptors (4- and 2-fold, respectively), in contrast to its marked selectivity for the M_2-binding sites. The selectivity of AF-DX 116 for the atrial receptors over the smooth muscle receptors (4- to 5-fold) was also less pronounced than observed in the binding studies between M_2- and M_3-binding sites. Thus the present study indicates that idaverine is a competitive muscarinic antagonist. Both in radioligand binding experiments and in functional experiments it reveals a selectivity (comparable with AF-DX 116) for the M_2-subtype over the M_3-subtype.

In the second part of the study, idaverine clearly did not prevent motion sickness over the dose range of 0.03 to 3.0 mg/kg. This failure to obtain a response was unexpected but provides useful clues regarding the identity of the muscarinic receptor subtype involved in the anti-motion sickness effects of scopolamine. The ratio of the binding affinity of idaverine (Table 1) to that of scopolamine (9) at M_1, M_2, and M_3 sites is 12, 0.5 and 72, respectively. The ratio of pA_2 values of idaverine (Table 2) to those of scopolamine (12,14) at M_2- (atrium) and M_3- (ileum) receptors is 6.9 and 61.7, respectively. Doses of idaverine (on a molar basis) in this study were as high as 25 times the ED_{50} of scopolamine at preventing motion sickness in cats (unpublished observations). The blockade of M_1- and M_2-receptors by idaverine in the present study was at least equivalent to that produced by doses of scopolamine that are effective in preventing motion sickness. Thus these receptors are unlikely to be involved in the motion sickness process. In contrast, the presence of emetic effects by an unknown mechanism beginning at 3 mg/kg of idaverine prevented the use of doses adequate to produce a blockade of M_3-receptors equivalent to that of the ED_{50} of scopolamine. Thus the failure to prevent motion sickness in the presence of blockade of M_1- and M_2- but not M_3-receptors suggests that the M_3-receptor is the critical site of action. This conclusion will of course have to be evaluated when a selective M_3 antagonist that enters the central nervous system becomes available.

Alternative explanations for the failure of idaverine to pre-

vent motion sickness were evaluated. One is that it does not penetrate into the central nervous system despite its hydrophobicity and low molecular weight. This possibility was evaluated by observing cats in separate cages after administration of idaverine. The presence of myoclonus of the forelimbs and of head shakes strongly suggest actions in the central nervous system. Another possible explanation is that antimuscarinic blockade does not protect the cat from motion sickness. This had previously been evaluated. Scopolamine prevents motion sickness in cats with an ED_{50} of 0.085 mg/kg (unpublished observations). Further, five of the cats in the present experiment were in the previous study and had been protected from motion sickness by scopolamine. Thus the current study is an adequate evaluation of the roles of muscarinic receptor subtypes in the motion sickness process.

In summary, the results from these and other studies make a role for the M_1- and M_2-receptors in the motion sickness process extremely unlikely, leading to the suggestion that the M_3-subtype may be critical. If this suggestion is verified, then selective blockade of M_3 sites would prevent motion sickness while producing far fewer side effects than antimuscarinics in current clinical use.

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