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Biochemical and Behavioral Characterization of a Novel Cholinergic Agonist, SR 95639

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M1 selective Partial agonist AF102B Receptor binding Phosphoinositide turnover Cognition Radial maze Dementia Alzheimer's disease

LEARNING and memory processes are, at least in part, regulated by the central cholinergic system (2, 3, 12). Administration of anticholinergic agents such as atropine or scopolamine is known to cause memory impairments in normal humans (13,17) and monkeys (4). In both human (11) and animal studies (1), drugs which increase acetylcholine levels antagonize the memory deficits produced by scopolamine. In addition, one of the primary manifestations of Alzheimer type dementia (DAT) is central cholinergic hypofunction (3, 5, 6, 10, 19, 22, 28, 29). Therefore, one pharmacologic approach to the treatment of SDAT may be the selective enhancement of central cholinergic function. A specific means of increasing central cholinergic function is the direct stimulation of postsynaptic muscarinic receptors.

Current evidence indicates the presence of at least two types of muscarinic receptors in the central nervous system, referred to as M1 and M2 receptors (16, 18, 24–27). Studies have shown that in the brain M2 receptors function on cholinergic terminals as "autoreceptors" to modulate the release of acetylcholine (21). Activation of these receptors inhibits acetylcholine release, not a therapeutically desirable result for the treatment of a disease state characterized by cholinergic hypofunction. In contrast, the M1 receptor in the brain may be localized on postsynaptic nerve cells (19). Also, while the central nervous system contains a high proportion of M1 receptors, muscarinic receptors in the periphery are mainly of the M2 type (25,27). For these general reasons, pharmacotherapy designed to directly enhance central cholinergic function should be targeted toward the M1, rather than the M2 receptor.

Novel tricyclic pyridazine derivatives have recently been described as cholinergic receptor agonists (14). We selected one example (SR 95639) of this series of compounds which appeared to have potential as a selective M1 agonist (23) and characterized it in several biochemical and behavioral tests. SR 95639 was synthesized at Wyeth-Ayerst Research following the patent experimental procedure (14).

METHOD

Male Sprague-Dawley CD rats, approximately 300 g body weight (Charles River, Wilmington, MA), were used in all experiments.

Muscarinic Binding

Hippocampi and cerebella (for M1 and M2 receptor binding, respectively) were dissected and homogenized, using a hand-held teflon-coated pestle, in 20 volume of 0.32 M sucrose (20 strokes at 4°C). After centrifugation (747 × g for 10 min at 4°C), the supernatant was decanted and recentrifuged (18677 × g for 20 min at 4°C). The resultant pellet was resuspended in the original volume of 0.32 M sucrose and frozen. After thawing, the suspension was diluted (1:1 v/v for M1 binding, 1:2 v/v for M2 binding) with 10 mM Na_2HPO_4/KH_2PO_4 buffer (pH = 7.4). [^{3}H]Pirenzepine (PZ) (0.5 nM, 0.04 μCi) (for M1 binding) or [^{3}H]quinuclidinyl benzilate (QNB) (0.23 nM, 0.01 μCi) (for M2 binding) was then incubated with a 100 μl sample of the tissue suspension in the buffer (1 ml final volume). One-half of the tubes contained atropine sulfate [2 μM (for M1 binding) or 100 μM (for M2 binding)]. After 60 minutes of incubation [25°C in the dark (for M1 binding) or 37°C (for M2 binding)] the binding was terminated by vacuum filtration onto Whatman GF/B filters [presoaked for 60 minutes in 0.1% (w/v) polyethylenimine to reduce nonspecific binding (for M1 binding)]. After three washes

TABLE 1

EFFECT OF SR 95639 ON M1 AND M2 RECEPTOR BINDING

Compound	K_i (M2)	K_i (M1)	K_i (M2)/K_i (M1)
Carbachol	2.94 ± 0.88 μM	37.8 ± 19.0 μM	0.08
Oxotremorine	0.14 ± 0.02 μM	0.21 ± 0.03 μM	0.67
McN-A-343	2.43 ± 0.7 μM	1.04 ± 0.05 μM	2.34
SR 95639	5.09 ± 0.37 μM	2.14 ± 0.40 μM	2.38
Atropine	0.86 ± 0.21 nM	0.21 ± 0.04 nM	4.10
Pirenzepine	0.64 ± 0.08 μM	4.45 ± 0.75 nM	143.8

Values shown represent the mean values ± S.E. of at least 3 independent experiments.

with buffer (4°C, 3 ml/wash), the vacuum was allowed to run for two minutes before the filter-trapped radioactivity was assayed by liquid scintillation spectroscopy. Specific binding was defined as total binding, minus binding in the presence of atropine sulfate. K_i values were calculated according to the method of Cheng and Prusoff (9).

Phosphoinositide (PI) Turnover

Hippocampi were dissected and sliced to a thickness of 0.5 mm with a McIlwain tissue chopper. A physiological buffer (KRB; containing 117 mM NaCl, 4.7 mM KCl, 11.5 mM dextrose, 1.25 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4 and 25 mM $NaHCO_3$) was bubbled for 1 h with a mixture of 95% O_2/5% CO_2, warmed to room temperature, and adjusted to pH 7.2–7.25. Next the slices were incubated in oxygenated KRB (1 hippocampus/ml, 37°C). After 1 h, the slices were washed with 0.9% NaCl, and were placed in oxygenated KRB containing 0.35 μM [^{3}H]myoinositol (approximately 5 μCi/ml/hippocampus). After 30-min incubation (37°C) the slices were washed with 0.9% NaCl, and 3 slices were placed in each of 25 tubes containing oxygenated KRB, 10 mM LiCl, and either vehicle, test drug or 1 mM carbachol. Separation of phosphoinositides was performed, with slight modifications, according to the method of Brown et al. (7). Briefly, after 1 h of incubation (37°C), chloroform (333 μl) and methanol (366 μl) were added to each tube. After vortexing, chloroform (300 μl), methanol (300 μl) and water (300 μl) were added to each tube. After additional vortexing, the tubes were centrifuged (138 × g, 5 min), and 0.5 ml of the aqueous layer was placed on Dowex 1 × 8–200 anion exchange resin. To elute phosphoinositides, the columns were washed with: 1) water (6 ml), 2) 5 mM Na tetraborate/60 mM Na formate (9 ml) and 3) 0.1 M formic acid/0.2 M ammonium formate (8 ml). This final 8 ml fraction, which contains [^{3}H]inositol monophosphates, was analyzed by liquid scintillation spectroscopy. After correcting for crossover radioactivity, the percent of total tritium present as [^{3}H]inositol monophosphate was used as an index of phosphoinositide turnover.

Observational Study

Rats were given either vehicle, arecoline (54 mg/kg, IP), pilocarpine (100 mg/kg, IP), oxotremorine (5.4 mg/kg, IP) or SR 95639 (54 mg/kg, IP). Animals were observed immediately preinjection for baseline measures, and at 10-minute intervals for 40 minutes postinjection. The incidence of M2-mediated effects (chromodacryorrhea, lacrimation, salivation, piloerection, tremors, moist rales, convulsions and death) (30) was recorded for each animal.

Body Temperature

Rats received either vehicle or SR 95639 (10 and 30 mg/kg, IP). Rectal body temperatures were monitored (2100 telether-

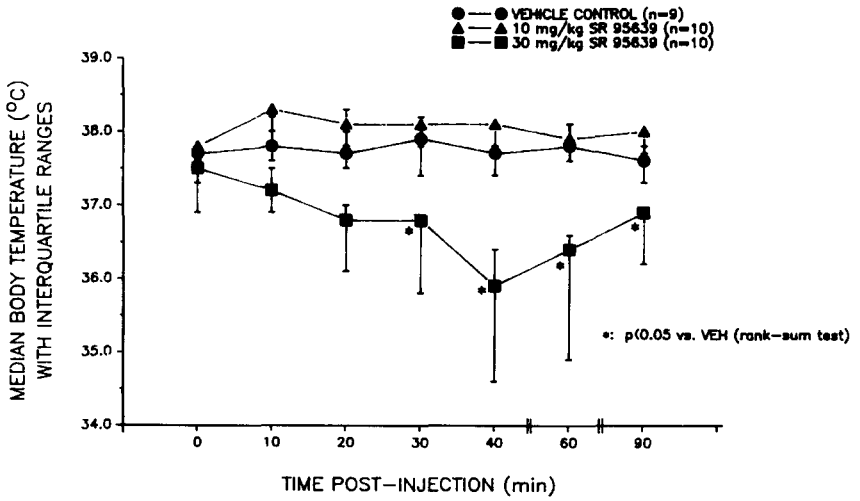

FIG. 1. Significant reductions in rat body temperature are seen after treatment with 30 mg/kg, IP of SR 95639.

TABLE 2

EFFECT OF SR 95639 ON PHOSPHOINOSITIDE (PI) TURNOVER

Compound	Vehicle	Drug	Drug + PZ	Carb.	Drug + Carb.
Oxotremorine	0.8 ± 0.2	1.2 ± 0.4	0.9 ± 0.2	3.6 ± 0.4	1.4 ± 0.3†
(0.5 mM)	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	2.6 ± 0.2	0.8 ± 0.2†
McN-A-343	0.8 ± 0.1	2.4 ± 0.3*	1.6 ± 0.3	3.5 ± 0.3	2.5 ± 0.4
(1 mM)	1.0 ± 0.1	1.8 ± 0.2*	1.4 ± 0.3	4.3 ± 0.8	1.9 ± 0.3†
SR 95639	0.1 ± 0.1	0.3 ± 0.1	—	1.4 ± 0.04	0.4 ± 0.1†
(1 mM)	0.3 ± 0.1	0.7 ± 0.1*	—	2.7 ± 0.4	1.0 ± 0.2†
	0.4 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	2.2 ± 0.2	—
	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	3.0 ± 0.04	—

Values shown represent the mean of at least 2 values ± SE.
Pirenzepine (PZ): 10 μM; carbachol (Carb): 1 mM. *$p<0.05$ vs. vehicle; †$p<0.05$ vs. carbachol.

mometer, Yellow Springs Instr. Co.) at 10-minute intervals for 40 minutes postinjection and again at 60 and 90 minutes.

Scopolamine-Induced Hyperactivity

Rats were placed into an Omnitech Digiscan locomotor activity apparatus and allowed to habituate for 30 min. The animals were then injected with vehicle, scopolamine (1.7 mg/kg, SC) or combinations of scopolamine and SR 95639 (1, 3, 10, 30 or 54 mg/kg, IP), and returned to the apparatus for 1 h. Activity was recorded in four 15-min intervals. Statistical comparisons (ANOVA followed by Dunnett's *t*-tests) were made on total activity (=horizontal + vertical activity) summed over the 1-h period.

Scopolamine-Impaired Inhibitory Avoidance

Rats were given either vehicle alone, scopolamine alone (1.7 mg/kg, SC) or scopolamine plus one dose of SR 95639 (1.0, 1.7, 3.0, 5.4, 10 or 30 mg/kg, IP). SR 95639 was administered 45 minutes pretraining and 15 minutes prior to scopolamine. The apparatus and procedure were modified from DeNoble (10). Each animal was placed in a lit chamber and allowed to enter a dark chamber, in which a continuous 1 mA footshock was administered. After 4 s, the animal was allowed to escape into the lit chamber. Twenty-four hours later each animal was placed in the lit chamber and latency to reenter the dark chamber was recorded (retention latency). A maximum retention latency of 300 s was imposed and therefore nonparametric statistics (Kruskal-Wallis followed by Mann-Whitney U-tests) were utilized.

Radial Maze

The apparatus and procedure were modified from Peele and Baron (20). Rats on a 23-hour food deprivation schedule were trained in an 8-arm radial maze, each session consisting of two maze exposures. After four correct choices with all arms baited, each animal was removed from the maze for one hour, then returned with only the remaining four arms baited. Delay errors (reentry into any of the four arms chosen during the first exposure), current errors (reentry into any of the four arms chosen during the second exposure), and total errors (delay + current) were recorded during the second exposure. Scopolamine HBr (0.3 mg/kg, SC) and SR 95639 (1, 3, 10 mg/kg, IP) were given simultaneously 30 minutes prior to the test.

RESULTS

Muscarinic Binding

Compared to several standard ACh agonists and antagonists (Table 1), SR 95639 was relatively weak at displacing [^{3}H]QNB from cerebellar M2 sites. SR 95639 displaced [^{3}H]pirenzepine from hippocampal M1 sites with affinity close to that of oxotremorine and McN-A-343. This M1 affinity was much less

TABLE 3

OBSERVATIONAL STUDY

	Number of Animals Displaying Symptoms							
	Chromodacryorrhea	Lacrimation	Salivation	Piloerection	Tremors	Moist Rales	Convulsions	Death
Vehicle	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Arecoline (54 mg/kg)	4/8*	0/8	7/8*	3/8	1/8	5/8*	1/8	1/8
Oxotremorine (5.4 mg/kg)	1/3	3/3*	3/3*	0/3	1/3	2/3	0/8	5/8*
Pilocarpine (100 mg/kg)	3/8	8/8*	8/8	2/8	1/8	0/8	0/8	0/8
SR 95639 (54 mg/kg)	0/6	2/6	3/6	0/6	0/6	0/6	4/8*	3/8

*$p<0.05$ versus vehicle, Fisher Exact Test.

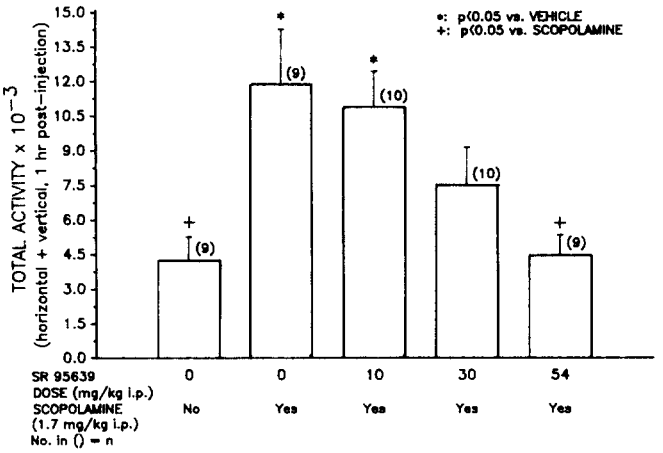

FIG. 2. Hyperactivity induced by scopolamine is significantly and dose-dependently reduced by SR 95639.

than that for pirenzepine, but almost 20-fold greater than that for carbachol. The relative affinities of SR 95639 for M1 and M2 receptor [$K_i(M2)/K_i(M1) = 3$] suggest that the compound is somewhat selective for M1 receptors, but not nearly as selective as the prototypical M1 selective antagonist pirenzepine [$K_i(M2)/K_i(M1) = 182$].

Phosphoinositide Turnover

SR 95639 inconsistently increased PI turnover (not as consistently or with the magnitude of McN-A-343 or carbachol, Table 2). SR 95639 did significantly and consistently reduce carbachol-stimulated PI turnover. This pattern of partial stimulation with reduction of the effects of a known agonist is consistent with partial agonist activity at M1 receptors.

Observational Study

SR 95639 produced a nonsignificant incidence of lacrimation and salivation, two measures which were affected significantly by other known cholinergic agonists (Table 3). SR 95639 did produce a significant incidence of convulsions, and several of the animals which convulsed also died. Others have shown that while lacrimation and salivation can be blocked by cholinergic antagonists the convulsions are not (30). Thus it is concluded that SR 95639 lacks significant M2 effects when administered in vivo.

Body Temperature

SR 95639 reduced body temperature at 30 mg/kg (Fig. 1). This reduction was statistically significant from 30 to 90 min postinjection. The 10 mg/kg dose had no effect on body temperature. These data support a central muscarinic agonist mechanism for SR 95639 (8).

Scopolamine-Induced Hyperactivity

Scopolamine significantly increased total activity compared to vehicle-treated animals (Fig. 2). This hyperactivity was attenuated by the 30 mg/kg dose of SR 95639 (i.e., no increase relative to vehicle) and was significantly reduced by the 54 mg/kg dose. The 10 mg/kg dose (and lower doses, e.g., 3 and 1 mg/kg not shown) had no effect on the scopolamine-induced hyperactivity. These data indicate that the M1 agonist activity of SR 95639 is manifested in vivo.

Scopolamine-Impaired Inhibitory Avoidance

Scopolamine significantly reduced inhibitory avoidance retention latencies (Fig. 3). In one experiment, the 3 mg/kg dose of SR 95639 attenuated the scopolamine impairment (i.e., this

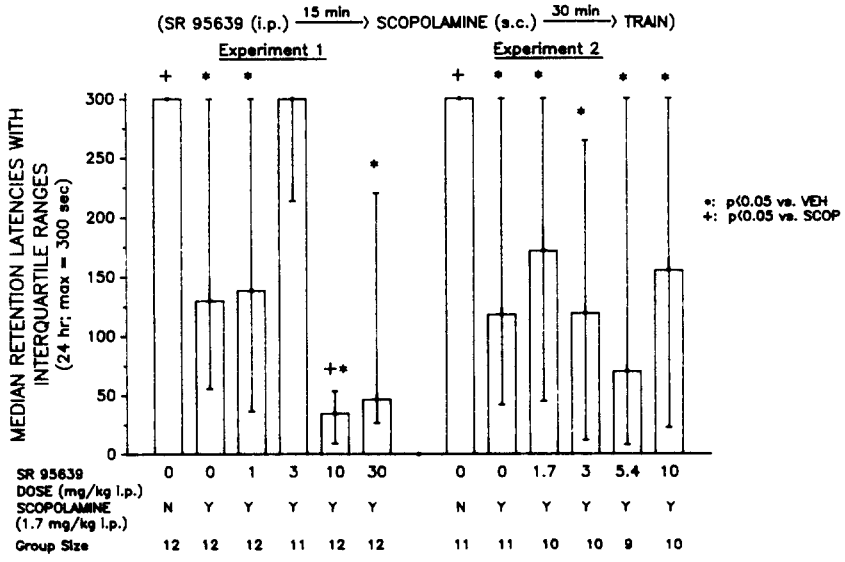

FIG. 3. Inconsistent effects of SR 95639 on scopolamine-impaired inhibitory avoidance suggest a partial agonist effect.

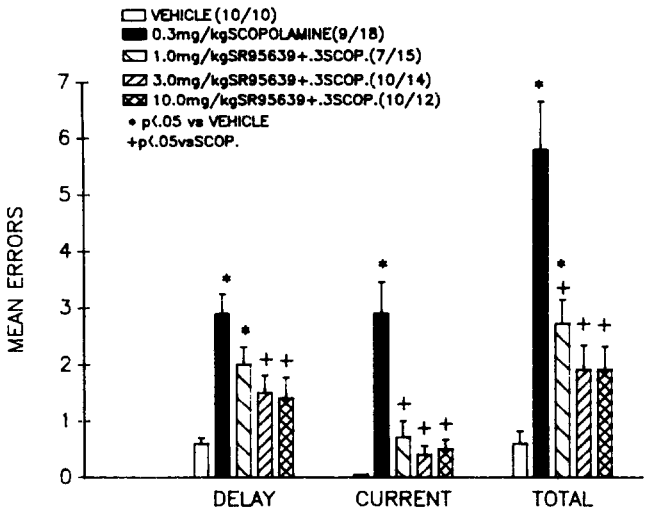

FIG. 4. Radial maze errors induced by scopolamine are significantly reduced by SR 95639.

group was not significantly impaired compared to a vehicle group, but neither was it significantly improved compared to the scopolamine group), while the 10 mg/kg dose significantly reduced latencies below those seen with scopolamine alone. In order to replicate and further characterize these effects, additional doses of SR 95639 were studied in a second experiment. No effects of SR 95639 were seen in this second experiment. Thus, although the results of the first experiment support a muscarinic partial agonist mechanism for SR 95639, these effects are inconsistent over the dose range studied. Additional assessment of higher doses of the compound is warranted.

Radial Maze

Scopolamine significantly increased both delay and current errors and this was reflected in an increase in total errors (Fig. 4). SR 95639 significantly reduced the scopolamine impairment in all three measures at 3 and 10 mg/kg. The 1 mg/kg dose significantly reduced current errors compared to scopolamine, but did not affect delay errors. Thus total errors at this dose were significantly reduced compared to scopolamine, but remained significantly elevated compared to vehicle. These data support the muscarinic agonist profile for SR 95639, and suggest further that cognitive impairments due to cholinergic hypofunction may be reduced by this compound.

DISCUSSION

The results of this study are basically consistent with earlier reports (14,23) that SR 95639 is a muscarinic M1 selective agonist. It is worth pointing out, however, that under our experimental conditions, SR 95639 exhibited lower affinity (2.1 μM) and less selectivity (2-fold) for the M1 (versus the M2) receptor than reported by Sanofi [0.2 μM and ~63 fold, respectively (23)]. It is an overextension to utilize M1 selectivity alone (measured in terms of differential affinity) to predict M1 selective efficacy. Compounds which are equally selective in terms of binding affinity may exhibit varying degrees of efficacy in any number of "functional tests." Also, clearly affinities and efficacies may vary according to the system(s) studied. Definitions of selectivity are best made on a case-by-case (or test-by-test) basis. In this study, we have shown that SR 95639 (a) binds selectively to the M1 receptor, (b) exhibits varying degrees of muscarinic agonism in efficacy tests and, (c) does not elicit significant M2 effects in vivo. Taken together, these data are consistent with the M1 muscarinic agonist profile previously reported for SR 95639.

We have extended the biochemical profile of this compound by examining its effects on PI turnover. Although SR 95639 increased PI turnover inconsistently, it did reduce carbachol stimulated PI turnover. These data suggest that the compound may be a partial agonist at M1 sites. This partial agonist profile was supported by inhibitory avoidance data, which showed an attenuation of a scopolamine impairment at 3 mg/kg but a potentiation of the scopolamine effect at 10 mg/kg. These inhibitory avoidance effects were inconsistent.

We have also extended the behavioral profile of SR 95639 by examining its effects in models of cognitive impairment due to cholinergic hypofunction. Specifically, scopolamine-induced impairments were attenuated in both inhibitory avoidance and radial maze procedures. These data suggest that the compound may have utility in the treatment of dementia, especially when cholinergic hypofunction is implicated (3, 5, 6, 10, 19, 22, 28, 29). The positive effects produced by SR 95639 in the radial maze test were at lower doses than those seen in the hyperactivity test. This may reflect differences in the dose of scopolamine needed to produce the respective behavioral effects (e.g., 0.3 mg/kg in radial maze and 1.7 mg/kg in hyperactivity). The inconsistent effects in inhibitory avoidance may also be due to the scopolamine dose difference. However, it is also possible that hyperactivity and cognition impairment are mediated by different brain regions which are differentially affected by SR 95639. Further characterization of other selective M1 agonists would help to clarify this issue.

At present there are no highly M1-selective muscarinic agonists available, and SR 95639 falls in the same category. Another partial agonist with some M1 selectivity, AF102B, has been reported (15), and shows a similar profile to that seen with SR 95639. For example, AF102B also binds selectively to M1 sites, reduces carbachol-stimulated PI turnover, and attenuates cognitive impairments due to cholinergic hypofunction (15). While the results of the present study are positive, a continued search for muscarinic agonists with higher affinity, greater selectivity and more robust behavioral effects is warranted as an approach to treating cognitive impairments due to cholinergic hypofunction.

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