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U-66444B and U-68553B, Potent Autoreceptor Agonists at Dopaminergic Cell Bodies and Terminals

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ABSTRACT

U-66444B was evaluated for pre- and postsynaptic effects in dopaminergic (DA) cell body and nerve terminal regions of chloral hydrate anesthetized rats. U-66444B depressed DA neurons in substantia nigra pars compacta and ventral tegmental area with a potency three times that for apomorphine. With a sufficient dose, cells were completely silenced. Activity was found to reside principally in the (+)-stereoisomer, U-68553B. The effects of U-66444B and U-68553B were reversed by 0.1 mg/kg haloperidol. Apomorphine, but not U-66444B nor U-68553B, depressions were accompanied by rapid tachyphylaxis. After 2 wk of U-66444B 0.6 mg/kg/day, potency was not significantly affected. By using *in vivo* voltammetry, 100 μg/kg U-68553B produced a depression in DA release that was more dramatic and more prolonged than that for 500 μg/kg apomorphine. On DA postsynaptic receptors, iontophoretic U-66444B and apomorphine were approximately equipotent in depressing caudate neuron firing. It is concluded that U-66444B and its active enantiomer, U-68553B, are more potent, longer acting and possibly more selective as DA autoreceptor agonists than apomorphine. The propensity to produce tolerance appears weak.

Currently marketed antipsychotic agents are thought to exert their therapeutic effects *via* a depression of DA transmission (Carlsson and Lindquist, 1963; Matthyse, 1974). This action is exerted by an antagonism of the postsynaptic effects of dopamine (DA; Bunney and Aghajanian, 1978). Acutely, this effect is compromised by an increase in dopamine cell firing and dopamine release. These increases in dopaminergic cellular activity are caused by 1) blockade of a negative feedback system from dopamine-inhibited cells onto the dopamine-containing cells (Bunney and Aghajanian, 1978) and 2) blockade of dopamine autoreceptors, which are inhibitory receptors found on DA cell bodies and terminals (Bunney *et al.*, 1973; Aghajanian and Bunney, 1977). It has been suggested that these increases in DA neuronal activity attenuate the effectiveness of the acute blockade by DA receptors by antipsychotic agents. Chronically, antipsychotics may paradoxically inhibit DA cell activity *via* a depolarization-induced blockade (Chiodo and Bunney, 1983) and/or supersensitization of the autoreceptors (Vogelsang and Piercey, 1985). Under these conditions, the DA systems would be fully turned off, both presynaptically and postsynaptically. If true, the above sequences could explain the delay in therapeutic effects of antipsychotic agents (Johnstone *et al.*, 1978; Meltzer *et al.*, 1978; Crow *et al.*, 1980).

An alternate way to depress the DA system would be to block DA neuronal firing and transmitter release by stimulation of DA autoreceptors (Aghajanian and Bunney, 1977; Clark *et al.*, 1985). Such stimulation would immediately cause DA neurons to cease firing and inhibit both the release and synthesis of the neurotransmitter. These effects might effectively remove the DA neuron from the feedback loop, making such an agent very effective for treating schizophrenia acutely.

Successful efforts to induce antipsychotic activity with dopamine autoreceptor agonists (Corsini *et al.*, 1977; Tamminga *et al.*, 1978, 1986) have supported this hypothesis. However, DA agonists thus far tested have been effective at postsynaptic as well as presynaptic DA receptors. Moreover, rapidly induced tolerance has led to requirements for increasing therapeutic doses resulting in production of side effects. Thus, to fully evaluate the practical utility of the DA autoreceptor agonist approach for antipsychotic activity, it is desirable to obtain a more selective DA agonist with minimal propensity to produce tolerance. We now describe the effects of the dihydrophenalene U-66444B (fig. 1) on firing rates and neurotransmitter release from DA neurons whose cell bodies are located in the substantia nigra pars compacta (SNPC) and ventral tegmental area (VTA) and whose nerve terminals are located in the striatum and mesolimbic area, respectively. From these experiments, it is concluded that U-66444B and its active enantiomer, U-68553B, are very potent DA autoreceptor agonists. Additional experiments demonstrate that these compounds may have somewhat

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ABBREVIATIONS: VTA, ventral tegmental area; SNPC, substantia nigra pars compacta; DA, dopamine; HAL, haloperidol; APO, apomorphine; GLUT, glutamate.

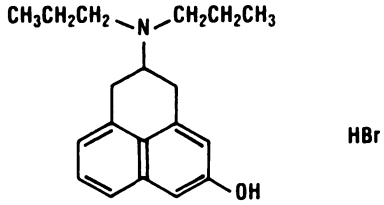

Fig. 1. U-66444B

greater selectivity for presynaptic receptors than apomorphine, the prototypical DA agonist, and have but minimal propensity to produce tolerance.

Some of this work has been previously described in preliminary communications (Piercey *et al.*, 1987a).

Methods

Microelectrode recordings. The electrophysiological techiques are similar to those we have previously described (Vogelsang and Piercey, 1985; Piercey *et al.* 1987b; Lum and Piercey, 1988). Male rats (300–400 g) were anesthetized with chloral hydrate (400–500 mg/kg i.p., supplemented by 150 mg/kg as necessary). The femoral vein and artery were cannulated to administer drug and monitor blood pressure, respectively. Body temperature was maintained at 37°C.

Standard microelectrode recording techiques were used. Drug effects were measured as changes in firing rates as indicated by an integrated rate meter output monitored throughout each experiment. Drug solutions were made in distilled water. The atlas of Paxinos and Watson (1986) was used for electrode placement in the SNPC or VTA. Dopamine neurons were identified by their long duration, positive-negative spikes in slow-firing (4–12 spikes/sec) neurons with a characteristic slow "bursty" firing pattern according to Bunney *et al.* (1973). Histological localization of iontophoresed pontamine sky blue dye spots verified electrode location. In all experiments involving i.v. drugs, only one DA cell was evaluated in each animal, and unless otherwise stated only one drug was evaluated in each.

In experiments evaluating effects of chronic U-66444B, rats were prepared with venous cannulas at least 4 days before use. Rats then received infusion pump-controlled injections of 0.1 mg/kg U-66444B (or an equal volume of the 5% ethanol vehicle) every 4 h for 14 days, resulting in a total daily dose of 0.6 mg/kg i.v., delivered in a volume of 0.2 ml vehicle. After the 2-wk injection, cannulas were checked for patency (Collins *et al.*, 1984), and the animal was returned to its home cage for electrophysiological evaluation 24 h later.

For microiontophoresis experiments, seven-barreled microelectrodes were advanced stereotaxically into the caudate of chloral hydrate- or urethane-anesthetized Sprague-Dawley rats. Dopamine HCl (200 mM, pH 4.0), apomorphine HCl (50 mM, pH 4.4) and U-66444B HBr (10 mM, pH 4.0) were tested for their abilities to inhibit spontaneous or, more usually, excitatory amino acid (200 mM sodium glutamate, pH 8.0)- or 50 mM DL homocysteic acid (pH 8.0)-induced excitations of caudate neurons. Potencies for inhibiting caudate cells were estimated by 1) percent inhibition per nanoampere, 2) relative potencies between DA agonists and 3) ordinate ranking of potencies on each cell.

Dopamine release from nerve terminals. Dopamine release was measured by *in vivo* electrochemistry (semiderivative voltammetry). The electrochemical techniques are similar to those already described (Broderick, 1985a, 1987–1989). Male Wistar rats (250–350 g) were anesthetized for surgery with chloral hydrate (450 mg/kg i.p.); booster shots were administered hourly to maintain adequate anesthesia. The indicator electrode coordinates for anterior striatum were 2.6 mm anterior to Bregma, 2.5 lateral to midline and 4 mm below skull surface. Those for accumbens were the same anteroposteriorally and mediolaterally, and the dorsoventral coordinate was 7.3 mm below skull surface (Pelligrino and Cushman, 1967).

The placement of the indicator electrode was confirmed by cresyl violet stain after the brain was removed from the skull and subsequently fixed in 10% Formalin.

Indicator electrodes are now selective for the detection of dopamine, without the concurrent detection within the same electrochemical signal, of the dopamine metabolites 3,4-dihydroxyphenylacetic acid and ascorbic acid (Blaha and Lane, 1983; Broderick *et al.*, 1983; Gerhardt *et al.*, 1984; Broderick, 1985a, 1987, 1988, 1989; Kelly and Wightman, 1986).

In the present experiments, the graphite paste indicator electrode was chemically altered with stearic acid (1.5 g graphite, 1.24 g Nujol, 100 mg stearic acid). When stearic acid is admixed into the graphite and oil paste, dopamine is detected at an oxidation potential of 0.140 v ± 0.015 v. The stearate electrode does not respond at the same oxidation potential needed to detect dopamine to either the addition of ascorbic acid, 3,4-dihydroxyphenylacetic acid, serotonin, 5-hydroxyindoleacetic acid or uric acid (Broderick, 1987–1989). Electrocatalysis of dopamine by ascorbic acid does not occur with the stearate acids (Broderick, 1989). Details of the fabrication and conditioning of the stearate microelectrode are recently reported by Broderick (1989).

Potentials were applied with respect to a Ag/AgCl reference electrode with a DCV-5 detector (Bioanalytical Systems, West Lafayette, IN) within a range of 0.2–0.5 v. The indicator microelectrodes were calibrated *in vitro* in phosphate buffer solution, pH 7.4 (*cf.* Broderick 1989 for details). Voltammograms were recorded at a scan rate of 10 mv sec^{-1}, every 10 min. Drugs were dissolved in distilled water and injected i.p. after a stable and reproducible basal dopamine release was evident for at least 30 min to 1 h.

Results

Effects of acute U-66444B on DA neuron firing rates. In the first set of experiments, 48 rats were tested with i.v. U-66444B.

Thirty-two of the 33 SNPC neurons identified as DA cells were inhibited after administration of U-66444B (fig. 2). For all cells, firing ceased with doses of >30 μg/kg i.v. For most cells, however, 10 μg/kg was sufficient to silence the cell. Full dose-response curves were obtained with cumulative dosing in nine animals. The ED_{50} for inhibiting SNPC neurons (dose to depress firing rate by 50%) was 3.0 ± 0.94 μg/kg i.v. (±S.E.M., $n = 9$). U-66444B was significantly more potent in inhibiting SNPC neurons than was apomorphine (APO, $ED_{50} = 9.6 \pm 3.2$ μg/kg i.v., $n = 6$, $P < .025$, t test). Haloperidol (HAL), 0.1 mg/kg i.v., promptly and completely reversed the effects of both U-66444B ($n = 8$) and APO ($n = 7$) whenever tested (fig. 2).

In six SNPC cells tested with repeated APO injections, a marked tachyphylaxis was observed at doses that were at first very effective in decreasing cell firing. For example, repeated injections of 100 μg/kg APO, which typically silenced DA cells, were accompanied by a return of firing. Raising the dose to 300 or 1000 μg/kg did not reverse this effect. This tachyphylaxis could be so profound that even very high doses (up to 15 mg/kg i.v.) became ineffective in altering firing rates of cells previously inhibited at very low doses. Tachylphylaxis seemed nonexistent in three cells tested with repeated, pharmacologically active, low doses of U-66444B (30–300 μg/kg). However, four cells given very high doses (1–2 mg/kg i.v.) showed an apparent reversal of inhibitory activity. However, it was often hard to record with high U-66444B doses since the drug appeared to have a simultaneous effect of decreasing the effectiveness of the chloral hydrate anesthesia.

The DA cells in the VTA responded similarly to U-66444B (fig. 3). The ED_{50} for U-66444B in this region (4.9 ± 0.1 μg/kg i.v., $n = 8$) was not significantly different from that for U-66444B in the SNPC. Similarly, the potency for APO to inhibit

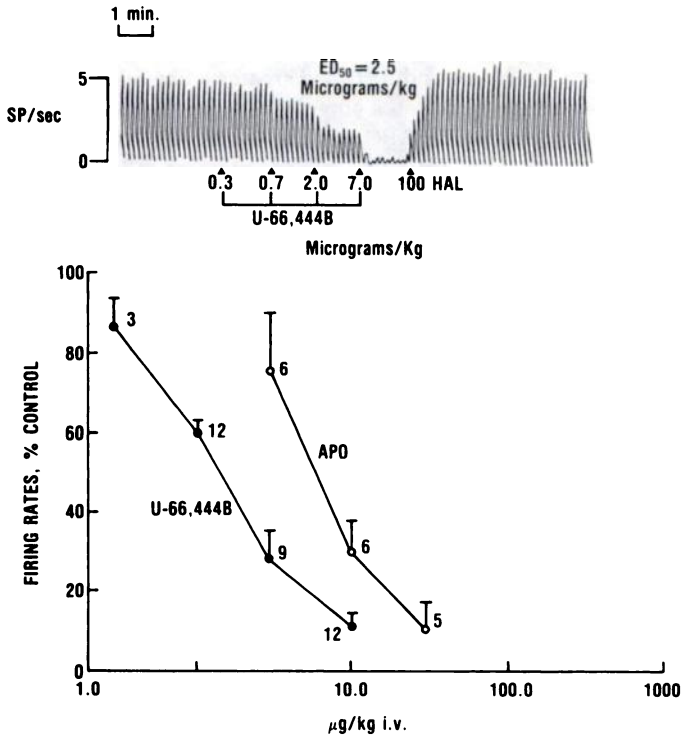

Fig. 2. Inhibition of DA cells in SNPC by U-66444B. Upper panel, effects of U-66444B on firing rate of one SNPC cell and its reversal by HAL. Doses are noncumulative. Note that cell completely ceases firing after a cumulative dose of 10 μg/kg i.v. U-66444B. Bottom panel, population dose-response curves for both U-66444B and APO. Ordinate, the means ± S.E.; abscissa, various cumulative i.v. doses. Numbers, cells evaluated at each adjacent point. SP, spikes.

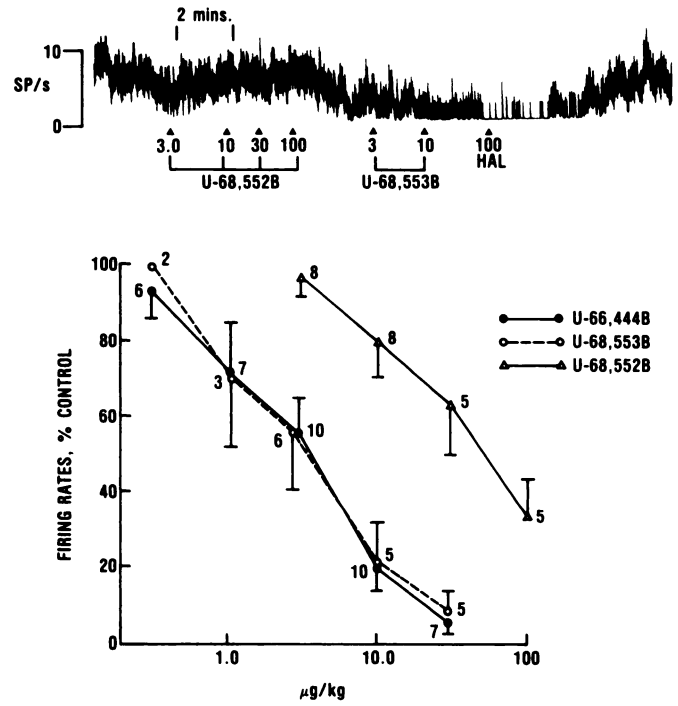

Fig. 3. Effects of U-66444B and its enantiomers on VTA neurons. Upper panel, doses up to 0.1 mg/kg U-68552 [(−)-U-66444B] fail to alter firing rate of this VTA cell, but lower doses of U-68553B [(+)-U-66444B] completely silence the cell by a HAL-sensitive mechanism. Lower panel, graphed dose-response curves for U-66444B, U-68553B and U-68552B on a population of VTA neurons. Layout similar to graph in figure 2.

neurons in the VTA (ED_{50} = 9.4 ± 3.0 μg/kg i.v., n = 4) was statistically indistinguishable from that for APO in the SNPC but was significantly less than that for U-66444B in the VTA ($P < .05$). As in the SNPC, 0.1 mg/kg i.v. HAL reversed the inhibition of VTA cells by U-66444B (n = 5) and APO (n = 4) whenever tested.

U-66444B was also tested for effects on seven non-DA cells outside the SNPC and VTA. These cells lacked the electrophysiological characteristics of DA cells. Most had short-, negative-positive action potentials and firing patterns different from those for DA cells. Doses of U-66444B totally silencing DA neurons failed to show similar effects in non-DA cells.

Effects of chronic U-66444B on DA neurons. Animals chronically treated with 0.2 ml/day vehicle or with 6 mg/kg U-66444B in 0.2 ml/day vehicle for 14 days still responded to the potent DA autoreceptor agonist effects of U-66444B. In both cases, sufficiently high doses of drug completely stopped DA neuron impulse activity. For vehicle-treated animals, the ED_{50} of 2.7 ± 0.6 μg/kg i.v. (n = 5) was not statistically different from the value of 3.2 ± 0.9 μg/kg reported above for naive animals. Nor was the ED_{50} in chronically treated U-66444B animals (6.6 ± 1.7 μg/kg, n = 8) significantly higher than that for acutely treated animals or for animals chronically treated with vehilce (t test for groups with unequal variances, Snedecor and Cochran, 1967). Analysis of the distribution of ED_{50} values among these groups of animals (fig. 4) illustrates that half the cells in the chronically treated animals had U-66444B potencies less than those for acute or vehicle-treated animals. For the remaining U-66444B-treated animals, the ED_{50} values were the same as for animals not receiving drug treatment. Thus, there was some suggestion of tolerance, but it was statistically insignificant.

Effects of U-66444B on caudate neurons. A total of 81

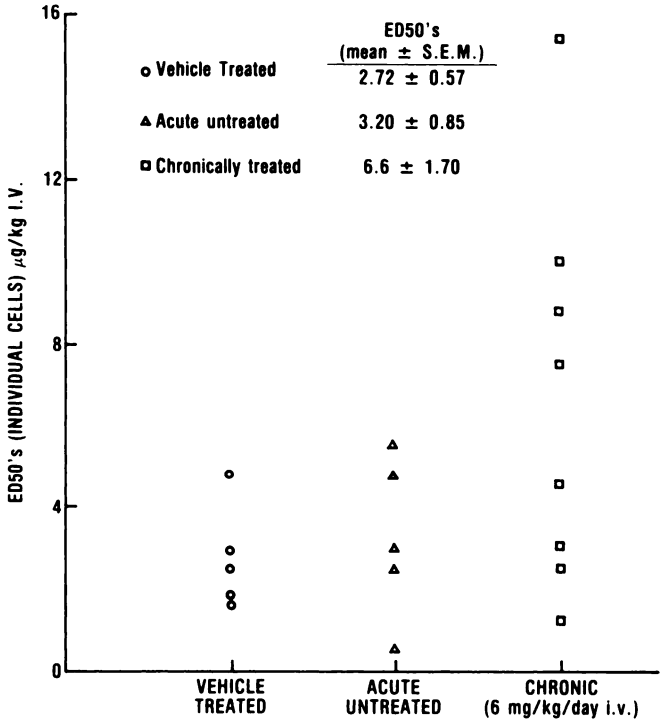

Fig. 4. Distributions of ED_{50} values for U-66444B in depressing VTA DA neurons in animals exposed to chronic vehicle (circles) or chronic U-66444B (squares) compared with those in animals receiving no chronic injections (triangles).

caudate neurons in 27 rats were evaluate (table 1). All were inhibited by U-66444B, APO, or DA (fig. 5). On some cells, U-66444B was the most potent, whereas on others APO or DA might be the most potent. In all 41 cells tested with 5–60 nA iontophoretic U-66444B, firing rates were inhibited 36–100%, the mean percent decrease per nanoampere being 2.58 ± 0.15%. The percent decreases per nanoampere for APO (3.55 ± 0.36, n = 25) or DA (2.68 ± 0.18, n = 66) were not significantly different from that for U-66444B or from each other (table 1).

Because each microelectrode recording has unique geometrical relations to affected cells, the most accurate way to compare potencies of two drugs is to compare their effects on the identical population of cells. When relative efficacies were measured by the percent inhibition produced by identical currents, both U-66444B and APO had greater efficacies than DA, which was arbitrarily given a potency value of 1.0 (table 1). On the 11 cells where both APO and U-66444B were tested with equal currents, U-66444B had a mean relative efficacy compared with APO of 1.03 ± 0.16, which was not statistically distinguishable from unity.

TABLE 1

Effects of dopamine agonists on postsynaptic receptors in caudate

Values are means ± S.E.

	Dopamine	Apomorphine	U-66444B
Potencies relative to dopamine	1.0	1.2 ± 0.1	1.3 ± 0.1
95% Confidence interval		(0.9–1.5)	(1.0–1.6)
n		16	30
Absolute potencies (%depression/nA)	2.7 ± 0.2	3.6 ± 0.4	2.6 ± 0.2
95% Confidence interval	(2.3–3.1)	(2.8–4.3)	(2.3–2.9)
n	66	25	41
Number cells more potent than APO	4		2
Number cells less potent than APO	8		6
Number cells equipotent to APO	4		3
P (binomial distribution)	0.23		0.11

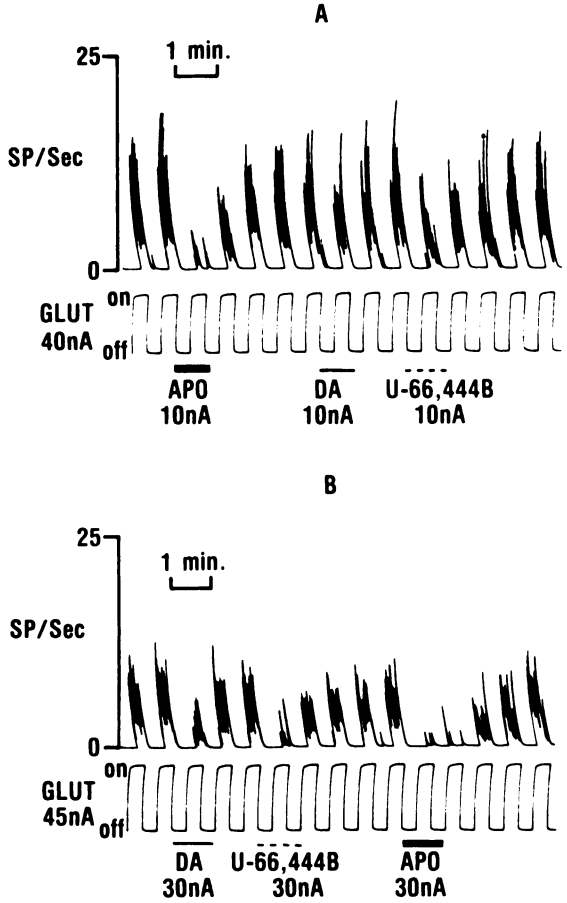

Fig. 5. Effects of microiontophoretic APO, DA and U-66444B on glutamate (GLUT)-evoked firing of caudate neurons. Chart recordings show effects on two different caudate cells; ordinates, firing rates. Times of microiontophoretic application of GLUT, APO, DA and U-66444B can be identified on abscissas. On both these cells, APO was most potent inhibitor. SP, spikes.

Effects of U-66444B enantiomers on VTA neuron firing rates. U-68553B, which is (+)-U-66444B, and U-68552B, which is (−)-U-66444B, were both tested for their effects on DA neuron firing rates in VTA. U-68552B depressed only three of nine VTA cells in a dose-sensitive fashion. In each of these cells, complete inhibition was never attained. In two cells devoid of a dose-response inhibition with U-68552B, similar or lower doses of U-68553B could completely stop the cell from firing. In five other rats, U-68553B inhibited VTA cells with dose-related inhibitions similar to those observed with U-66444B (fig. 3). A covariance analysis of the three sets of data in figure 5 shows that U-68552B was significantly weaker in inhibiting DA cells than either U-66444B or U-68553B, which were equipotent. Thus, U-68553B, the (+)-isomer, is the active stereoisomer of U-66444B. The depression of VTA neurons by U-68553B was reversed by 0.1 mg/kg i.v. HAL (n = 5, fig. 3).

Effects of U-68553B on DA release. Both (−)-apomorphine (500 μg/kg i.p.) and U-68553B (100 μg/kg i.p.) depressed striatal dopamine release as measured with *in vivo* voltammetry (fig. 6). U-68553B, despite being tested with a lower dose, exerted a much more profound and longer lasting effect on striatal DA release than APO. U-68553B suppressed DA release by more than 25% for longer than 50 min after injection. Indeed, the DA signal did not return to base line within 1 h after injection. By contrast, APO effects persisted for only 10 min. The maximal change in the DA signal from U-68553B was 44% compared with only 20% for APO.

U-68553B (100 μg/kg i.p.) also depressed DA release in the nucleus accumbens. After a transient (10-min) but statistically significant ($P < .05$) increase in the accumbens DA signal, there was a dramatic 75% decrease in DA release that was still decreasing even 1 h after injection.

Discussion

The experiments with the enantiomers of U-66444B clearly demonstrate that the (+)-isomer, U-68553B, is the active form

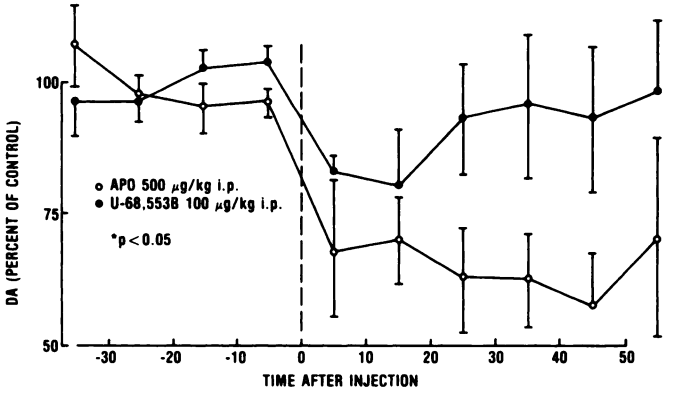

Fig. 6. Effect of U-68553B (100 μg/kg i.p., open circles) and APO (500 μg/kg i.p., solid circles) on DA release from rat caudate. Data represent means ± S.E. (n = 4). Asterisks, points lie outside 95% confidence limits of control mean.

of U-66444B. U-68553B did not demonstrate the theoretical doubling of potency compared with its racemic mixture. One possibility is that the (−)-isomer potentiates the effects of the (+)-isomer, but this is far from certain. Assuming that most of the effects of U-66444B are due to its active enantiomer, this discussion will mention only U-68553B even when drawing conclusions from those experiments using the racemic mixture.

U-68553B is a very potent DA autoreceptor agonist. At the somatodendritic autoreceptor, it is three times more potent in depressing firing rates of DA neurons in the SNPC and VTA than APO, a potent DA autoreceptor agonist standard. Both are much more potent than the selective autoreceptor agonist (−)-PPP (Clark *et al.*, 1985; Hoffmann and Piercey, 1987). At the nerve terminal autorecedptor, U-68553B is even more impressive when compared with APO. Even though the dose of U-68553B was only one-fifth of that for APO, the depression in DA release exceeded that for APO by more than double. Although incomplete, the available evidence suggests that, in contrast to APO, U-68553B may be somewhat selective for the nerve terminal compared with the somatodendritic autoreceptor. Such a possibility is consistent with recent evidence that the aminotetralin, (+)-AJ 76, is a preferential antagonist for the DA nerve terminal autoreceptor (Svensson *et al.*, 1986; Piercey *et al.*, 1988). In addition to being more potent than APO, the release experiments demonstrate that U-68553B is much longer lasting than APO, a factor that could be of some utility in potential clinical applications.

The effects of U-68553B were clearly mediated at the level of the DA autoreceptor. First, the depression of neuronal firing rates was specific for DA neurons. Second, the effects were blocked by HAL, a specific DA antagonist. Finally, activation of negative feedback pathways secondary to an amphetamine-like increase in DA release (Bunney *et al.*, 1973) can be eliminated because the *in vitro* voltammetry experiments demonstrate a decrease, rather than an increase, in DA release. Other behavioral and neurochemical experiments substantiate these conclusions (Schreur and Nichols, 1987; VonVoigtlander *et al.*, 1989; Lahti *et al.*, 1989).

Microiontophoretic applications of U-68553B, APO, and DA depress spontaneous and glutamate-evoked excitations of caudate nucleus neurons. This effect is thought to be mediated by DA postsynaptic receptors (Aghajanian and Bunney, 1977). In contrast to its more potent effects at autoreceptors, U-68553B was no more potent than APO as a postsynaptic agonist. This suggests that U-68553B could be a more selective autoreceptor agonist than APO. However, rigorous quantitative comparisons require determinations of transport numbers (Curtis, 1964). Nonetheless, some behavioral and biochemical data also suggest that U-68553B might be more selective for DA autoreceptors than other DA agonists (VonVoigtlander *et al.*, 1989; Lahti *et al.*, 1989).

Clinical studies with DA agonists have demonstrated promising, but fleeting, antipsychotic activity (Corsini *et al.*, 1977; Tamminga *et al.*, 1978, 1986). The efficacy of these agents may have been limited not only by short durations of action and poor selectivity for autoreceptors compared with postsynaptic receptors but by a pronounced and rapid tolerance. In acute experiments, APO demonstrated a very rapid tachyphylaxis. This effect began to appear with the lowest doses silencing DA neuron firing rates. Similar losses in ability to depress DA release with high APO doses have also been demonstrated (Broderick, 1985b). In contrast, U-68553B did not elicit tachyphylactic effects except at very high doses. Daily injections of 0.6 mg/kg U-68553B for 2 wk did not result in a statistically significant decrease in potency for depression of DA neuron firing rates.

U-68553B is a structurally novel, very potent DA autoreceptor agonist that, because it is more potent, longer-acting, possibly more specific, and less likely to induce tolerance, could have enhanced probability for success when compared with previous autoreceptor agonists in treating schizophrenia. In addition, because it appears to have some postsynaptic agonist effects, U-68553B could have some potential in treating hypodopaminergic states such as parkinsonism.

Acknowledgments

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