Blockade of Cannabinoid Type 1 Receptors Augments the Antiparkinsonian Action of Levodopa without Affecting Dyskinesias in 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Treated Rhesus Monkeys

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ABSTRACT

Drugs acting at cannabinoid type 1 receptors (CB1) have modulatory effects on glutamate and GABA neurotransmission in basal ganglia; thus, they potentially affect motor behavior in the parkinsonian setting. Preclinical trials with diverse cannabinoid agents have shown varied results, and the precise effects of blocking cannabinoid CB1 receptors remain uncertain. We tested behavioral effects of the selective antagonist 1-[7-(2-chlorophenyl)-8-(4-chlorophenyl)-2-methylpyrazolo[1,5-a][1,3,5]triazin-4-yl]-3-ethylaminoazetidine-3-carboxylic acid amide benzenesulfonate (CE) as monotherapy and in combination with L-DOPA in treatment-naive and L-DOPA-primed 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated rhesus monkeys with moderate and severe parkinsonism. Motor disability and L-DOPA-induced dyskinesias were scored with a standardized scale after subcutaneous drug administration, and plasma levels of L-DOPA were determined by high-performance liquid chromatography/electrochemical detection.

CE doses ranged from 0.03 to 1 mg/kg, and L-DOPA methyl ester doses were selected as optimal and suboptimal doses (maximal and 50% of maximal responses, respectively). CE had no intrinsic effects on motor behavior regardless of the degree of parkinsonism (moderate or severe groups) or previous drug exposure (“de novo” or after L-DOPA priming). Initial CE administration did not affect development of L-DOPA antiparkinsonian responses. In coadministration trials, CE, in a dose-dependent manner, increased responses to L-DOPA (suboptimal doses). These effects were seen in both moderate and severely parkinsonian monkeys as a 30% increase of, predominantly, response duration with no effects on L-DOPA pharmacokinetics. CE did not modify levodopa-induced dyskinesias. These results suggest that selective cannabinoid CB1 antagonists may enhance the antiparkinsonian action of dopaminomimetics and possibly facilitate the use of lower doses, thereby reducing side effects.

Parkinson’s disease (PD) is characterized principally by progressive neurodegeneration of the nigrostriatal dopamine system and its accompanying motor dysfunction: tremor, rigidity, and bradykinesia. Dopamine replacement with the dopamine precursor L-DOPA improves motor symptoms, although long-term therapy causes disabling side effects, such as varied motor complications (response fluctuations and dyskinesias) (Nutt, 2000; Obeso et al., 2000). Because of these shortcomings, therapies that act as either adjuncts or alternatives to L-DOPA by modulating its effects and reducing adverse reactions may help in restoring normal function in the late-stage disease. Putative bases for developing new therapies lie in the interaction of dopamine with other neurotransmitter systems in basal ganglia. In fact, pathogenic mechanisms of L-DOPA-induced motor complications involve the glutamate system as the major transmitter driving the activity of striatal neurons (Chase and Oh, 2000). Other

ABBREVIATIONS: PD, Parkinson’s disease; GPe, globus pallidus external segment; CB1, cannabinoid type 1 receptor; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; CE, 1-[7-(2-chlorophenyl)-8-(4-chlorophenyl)-2-methylpyrazolo[1,5-a][1,3,5]triazin-4-yl]-3-ethylaminoazetidine-3-carboxylic acid amide benzenesulfonate; BSA, bovine serum albumin; SR 141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboximide hydrochloride; CP 55,940, (1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; GTPγS, guanosine 5′-O-(3-thio)triphosphate; LD, L-DOPA; ANOVA, analysis of variance; r/l, right/left; V, vehicle.
neurotransmitters may also play a role, and in recent years, nondopaminergic symptomatic therapies have been extensively sought for ameliorating L-DOPA motor complications (Papa and Chase, 1996; Grondin et al., 1999; Papa et al., 2004).

The cannabinoid system seems to have important influences in dopamine-mediated mechanisms within basal ganglia (Di Marzo et al., 1998). CB1 receptors and the endocannabinoids anandamide and 2-arachidonoyl glycerol (Herkenham et al., 1990; Di Marzo et al., 2000) are particularly abundant in striatum and the striatal terminals in the globus pallidus (globus pallidus in rodents; globus pallidus external segment (GPe) in primates, and substantia nigra pars reticulata/internal globus pallidus, the “indirect” and “direct” striatal output pathways, respectively). In striatum, presynaptic CB1 receptors regulate glutamate release and reuptake, and the combination of these effects results in reduction of glutamate-mediated postsynaptic excitability (Gerdenman and Lovinger, 2001; Brown et al., 2003). In addition, striatal CB1 receptors seem to regulate dopaminergic and serotonergic (serotonin receptor 1B) signaling (Hermann et al., 2002), and increased binding of CB1 receptors and activity of G protein coupling have been demonstrated in the striatum of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treated marmosets and PD patients (Lastres-Becker et al., 2001). In the internal globus pallidus segment/substantia nigra pars reticulata, the net effects of CB1 stimulation are uncertain because of combined inhibitory interaction with GABA release and uptake from striatal terminals as well as glutamate release from the subthalamic nucleus (Szabo et al., 2000; Wallmichrath and Szabo, 2002). However, in GPe, the main effects are thought to be mediated by inhibition of GABA reuptake that results in increased GABA transmission and inhibition of the subthalamic nucleus (Maneuf et al., 1996). Through this mechanism in the indirect striatal output pathway, CB1 antagonists may synergize the L-DOPA antiparkinsonian effects. Nevertheless, the effects of cannabinoid agonists and antagonists after systemic administration remain unclear due to their complex actions in multiple basal ganglia sites.

Earlier studies of cannabinoid drugs in rodents have shown marked motor effects, although data derived from agonists and antagonists have not correlated with findings of equivalent tests in primates (Meschler et al., 2000a,b). Studies of CB1 receptor antagonists in nonhuman primates have reported contradictory results (Di Marzo et al., 2000; Meschler et al., 2001; van der Stelt et al., 2005), whereas in clinical trials they did not show positive effects (Mesnage et al., 2004). Recently, the selective CB1 antagonist rimonabant (SR 141716A) was shown to have antiparkinsonian actions per se and to reduce L-DOPA-induced dyskinesias in MPTP-treated primates (van der Stelt et al., 2005). These data suggest that CB1 receptor-mediated transmission plays a functional role in different motor behaviors developed in the chronic course of the disease. However, the effects of selective CB1 receptor antagonists have not been studied thoroughly in primates.

In this study, a selective cannabinoid CB1 antagonist, 1-[7-(2-chlorophenyl)-8-(4-chlorophenyl)-2-methylpyrazolo[1,5-a]-[1,3,5]triazin-4-yl]-3-ethylaminoazetidin-3-carboxylic acid benzenesulfonate (CE) (Fig. 1), was used for a series of trials in parkinsonian monkeys. CE is a highly selective full antagonist for the CB1 receptor that is well absorbed and readily gains access to the brain. We used MPTP-treated rhesus monkeys in the following CE trials: 1) monotherapy for dose-response curves; 2) monotherapy for comparison with L-DOPA as “de novo” treatments in a crossover design trial (also CE effects were tested before and after L-DOPA priming); 3) coadministration with L-DOPA in moderately parkinsonian monkeys; 4) coadministration with L-DOPA in severely parkinsonian monkeys with L-DOPA-induced dyskinesias; and 5) determination of CE interaction with L-DOPA pharmacokinetics in tests of coadministration. These trials produced the data for a complete behavioral profile of CB1 antagonists in parkinsonian primates.

**Materials and Methods**

**Subject Preparation**

Seven adult monkeys (*Macaca mulatta*), two males and five females, weighing between 5 and 8 kg, were kept in controlled housing conditions with constant temperature, relative humidity, and a 12-h light/dark cycle. Animals had free access to food, fresh fruit supplements, and water. All studies were conducted in accordance with the Institute of Laboratory Animal Resources (1996), and they were approved by the Institutional Animal Care and Use Committee. Monkeys received 0.5 to 0.8 mg/kg i.v. MPTP once weekly for 6 months or longer until they had a stable parkinsonism of moderate-to-severe degree. Four new animals were rendered moderately parkinsonian for this study to compose the de novo group. The other three monkeys were severely parkinsonian, received L-DOPA as regular treatment before entering this study, and formed the group of dyskinetic monkeys. L-DOPA was given orally [25–50 mg; carbidopa/levodopa (Sinemet 25/100)] twice daily until development of consistent and reproducible dyskinesias. Motor disability was scored using a standardized scale for parkinsonian primates (see below), and scores are shown in Table 1.

**CE Pharmacological Characterization**

**CB1 and CB2 Receptor Binding.** Human embryonic kidney 293 (CB1) or Chinese hamster ovary (CB1 and CB2) cells (American Type Culture Collection, Manassas, VA) were stably transfected with the human CB1 or CB2 receptors, and membranes were prepared as described previously (Bass et al., 1996). A BCA protein assay kit (Pierce Chemical, Rockford, IL) was used to determine protein concentrations. CE was diluted in buffer (25 mM Tris, 5 mM MgCl2, and 1 mM EDTA, pH 7.4) containing 0.5% BSA and 10% dimethyl sulfoxide, and then 25 μl of these solutions was added to 96-well polystyrene plate. [3H]SR 141716A (1.2 nM final concentration) was diluted in a buffer containing 0.5% BSA, and 25 μl was added to the plate. The plates were covered, and then they were placed in an incubator at 30°C for 60 min. The reaction was stopped by addition of 125 μl of buffer containing 10% BSA, and then membranes were collected onto GF/C filter plates (PerkinElmer Life and Analytical Sciences, Boston, MA) presoaked in buffer containing 0.5% BSA. Filters were washed twice and then dried overnight. Filters were counted on a PerkinElmer Wallac Trilux counter. CB2 receptor bind-
ing was assayed under the same conditions but using [3H]CP 55,940 (10 nM final concentration) as radioligand.

[35S]GTP[S] Binding Assays at CB1 Receptors. [35S]GTP[S] binding assays were performed in a 96-well FlashPlate format (PerkinElmer Life and Analytical Sciences) in duplicate using 100 pM [35S]GTP[S] and 10 µg of membrane per well in assay buffer composed of 50 mM Tris-HCl, pH 7.4, 3 mM MgCl2, pH 7.4, 10 mM MgCl2, 20 mM EGTA, 100 mM NaCl, 30 µM GDP, 0.1% bovine serum albumin, and protease inhibitors (100 µg/ml bacitracin, 100 µg/ml benzamidine, 5 µg/ml aprotinin, and 5 µg/ml leupeptin). The assay mix was incubated with increasing concentrations of CE (10−15–10−8 M) for 10 min, and then they were challenged with the cannabinoid agonist CP 55,940. Assays were performed at 30°C for 1 h. The FlashPlates were then centrifuged at 2000 g for 10 min. Stimulation of [35S]GTP[S] binding was then quantified using a PerkinElmer Wallac Microbeta plate counter.

Cannabinoid Tetrad. Cannabinoids have long been associated with inducing a set of four well characterized, centrally mediated behaviors in rodents: hypothermia, antinociception, hypolocomotion, and ring immobility (catalepsy), known as the in vivo tetrad (Little et al., 1988). The efficacy and potency of CE to reverse the effects mediated by the agonist CP 55,940 were examined in groups of male mice (17–19 g). Animals were given vehicle or 0.3, 1.0, or 3.0 mg/kg s.c. CE followed 15 min later by vehicle or 0.78 mg/kg s.c. CP 55,940. Twenty-five minutes after CP 55,940 administration, mice were placed in a hot-plate apparatus (Columbus Instruments, Columbus, OH), and we recorded the latency to flick or lick a hind paw, or to jump from the hot-plate, after which animals were removed (40-s cut-off). Twenty minutes after the hot-plate test, temperatures (to the nearest 1/10 of a degree) were recorded using a small thermostat probe inserted 2 to 2.5 cm into the rectum. Fifteen minutes later, catalepsy was assessed by placing animals on a horizontal 6.5-cm steel ring attached to a ring stand at a height of 30.5 cm. The animal was suspended in the gap of the ring with fore and hind paws gripping the perimeter. The time of remaining completely motionless (except for respiratory movements) was recorded over a 3-min period. An immobility rating was calculated as the percentage of the motionless period from the total time of observation.

In vitro pharmacological profiling showed that CE exhibits both high-affinity binding to and functional antagonism of the human CB1 receptor expressed in Chinese hamster ovary cells. CE displaces binding of [3H]SR 141716A to the human CB1 receptor, with a Kᵢ of 0.33 nM. In contrast, the compound has a lower affinity for the human CB2 receptor, with a Kᵢ = 10,000 nM that indicates a 30,000-fold selectivity. CE is also 2000-fold selective for CB1 receptor binding over a panel of 53 receptors, ion channels, and uptake sites expressed in the central nervous system. Following agonist binding to the CB1 receptor, G protein activation occurs, and GTP or analogs bind the receptor complex with high affinity. Thus, CB1 agonist-induced increase in [35S]GTP[S] binding was used as a measure of CB1 receptor activation, and inhibition of [35S]GTP[S] binding was used to measure the antagonist potency and efficacy of CE. CE blocked CB1 agonist CP 55,940-stimulated [35S]GTP[S] binding, with a Kᵢ = 0.07 nM. Inhibition was surmountable and of a mixed competitive/noncompetitive type (Kᵢ = 0.63 nM; slope = 5.8). In vivo CE testing in the cannabinoid tetrad confirmed central CB1-mediated effects. CE dosed at 0.3 mg/kg s.c. significantly reversed the effects of a centrally acting cannabinoid agonist (0.78 mg/kg i.p. CP 55,940) in three of the four components of the tetrad, and in all four components at 1 mg/kg (Table 2).

Drugs
CE is described in Griffith (2007) (Fig. 1). Human CB1 and CB2 receptor cDNAs and/or cell lines were the gift of Dr. Debra Kendall (University of Connecticut, Storrs, CT). CP 55,940 was synthesized at Pfizer Global Research & Development (Groton, CT). [3H]CP 55,940 (158 Ci/mmol) and [35S]GTP[S] were purchased from PerkinElmer Life and Analytical Sciences. [3H]SR 141716A (44.0 Ci/mmol) was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). L-DOPA methyl ester, benserazide, and MPTP were purchased from Sigma-Aldrich (St. Louis, MO). L-DOR benserazide was dissolved in Cavasol solution. L-DOR methyl ester and benserazide were dissolved in saline.

Experimental Drug Tests
CE Dose-Response Curve. To determine the intrinsic effects of CE on parkinsonian motor symptoms, we tested CE as monotherapy at different doses (dose-response curve) in a group of three severely parkinsonian monkeys. At the time of testing, animals had a stable parkinsonian motor syndrome, which was characterized by a stable, moderately parkinsonian motor score. At the time of testing, animals had a stable, moderately parkinsonian motor score.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Activity</th>
<th>Analgesia</th>
<th>Hypothermia</th>
<th>Catalepsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 mg/kg s.c.</td>
<td>65</td>
<td>49*</td>
<td>41*</td>
<td>60*</td>
</tr>
<tr>
<td>1.0 mg/kg s.c.</td>
<td>136*</td>
<td>116*</td>
<td>80*</td>
<td>85*</td>
</tr>
<tr>
<td>3 mg/kg s.c.</td>
<td>70*</td>
<td>80*</td>
<td>108*</td>
<td>87*</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. 0.78 mg/kg of CP 55,940 alone (n = 5–7 animals/group).
larly with oral L-DOPA, which was withdrawn for 48 h before each CE test.

All tests were performed mid-morning. In every test, monkeys received two immediately successive injections; the first injection was a certain dose of CE, and the second injection was always L-DOPA vehicle (for subsequent comparisons with L-DOPA effects if CE had intrinsic effects). We tested four doses of CE: 0.03, 0.1, 0.3, and 1 mg/kg, and all injections were given subcutaneously. Thus, CE tests were 1) CE vehicle + L-DOPA vehicle; 2) 0.03 mg/kg CE + L-DOPA vehicle; 3) 0.1 mg/kg CE + L-DOPA vehicle; 4) 0.3 mg/kg CE + L-DOPA vehicle; and 5) 1 mg/kg CE + L-DOPA vehicle. Each test was repeated at least once, and results from two or three tests were averaged to generate each animal data points for statistical analysis.

Comparison of CE and L-DOPA as de Novo Treatments. In this test, we used the group of four monkeys that had a milder parkinsonism, and they were naive of antiparkinsonian treatment. One aim of this experiment was to detect any discrete intrinsic effects of CE that could not be identified in tests of severely impaired monkeys. Another aim was to determine whether previous development of antiparkinsonian responses with L-DOPA induced CE effects as a priming mechanism that has been described with other agents. To accomplish these aims, the study had a crossover design that compared the effects of CE and L-DOPA as de novo treatments, i.e., two treatment series that were switched across two groups of monkeys (group 1, monkeys A and B; and group 2, monkeys C and D). In treatment series 1, the first injection was CE vehicle, and the immediate second injection was one of the two doses of L-DOPA (LD-low or LD-high). In treatment series 2, the first injection was either of the two doses of CE (0.3 or 1 mg/kg), and the immediate second injection was L-DOPA vehicle. Table 3 shows the crossover sequences; monkeys A and B were tested first with L-DOPA (treatment series 1); subsequently, they were tested with CE (treatment series 2). Monkeys C and D started with CE tests, and then they were switched to L-DOPA tests. Each test was repeated at least once, and results of two or three tests were averaged. Several weeks elapsed in between the two treatment series, so there were no residual effects of CE or L-DOPA. CE doses were selected from the highest doses of previous tests where CE had no intrinsic effects (dose-response curve), i.e., 0.3 and 1 mg/kg. L-DOPA (LD) doses were selected as an “optimal” dose, the minimal dose that produced maximal effects (LD-high, usually 100–200 mg), and a “suboptimal” dose, the dose that produced considerably lower but unequivocally measurable effects (LD-low, usually 50–100 mg). These two doses of subcutaneous L-DOPA methyl ester plus benserazide were determined at the time of beginning L-DOPA testing in each group.

Coadministration of CE and L-DOPA. In this test, we examined the effects of CE on the antiparkinsonian responses to L-DOPA in both groups: moderately (one monkey was not included because of unstable responses to L-DOPA) and severely parkinsonian monkeys. Optimal and suboptimal L-DOPA doses were selected as described above. In the moderately parkinsonian group, the two highest doses of CE (0.3 and 1 mg/kg) were coadministered with L-DOPA. In the severely parkinsonian group, three doses of CE were used to include a lower dose for full assessment of effects on dyskinesias (0.1, 0.3, and 1 mg/kg). Vehicle injections were also tested here to update the control results. In these tests, animals received first, CE vehicle at 0.1, 0.3, or 1 mg/kg, and second, LD-low or LD-high. All tests had immediately successive injections. Tests were repeated at least once, and they were separated by intervals of 3 days at minimum as drug washout periods. Results are the average of two or three experiments.

Behavioral Assessment

Motor behavior was assessed with a standardized motor disability scale developed for MPTP-treated primates that has two parts: part I, motor disability (see Table 1 for components) and part II, drug-induced adverse reactions (Papa and Chase, 1996). Motor behavior is itemized in the scale according to expression of parkinsonian symptoms; thus, the sensitivity of the scale is higher than most motor tasks in parkinsonian macaques. In addition, this type of scale was developed for macaques as equivalent to the rating scales used in clinical trials for patients. Scores were taken just before drug injections (time 0) and afterward starting at 30 min and continuing every 20 min until there was a return to baseline. Dyskinesias were assessed at the same time points using part II of the scale that rates dyskinesias with a wide range within the following major categories: 0, absent; 1, mild (fleeting, rare, present less than 30% of the observation period); 2, moderate (interfering with normal activity, present less than 60% of observation period); and 3, severe (disabling, replacing normal activity, present more than 90% of observation period). All tests were performed after an overnight fast. Animals were videotaped for subsequent rating by a blinded investigator. Each test in all sets of experiments was repeated. Data were averaged to yield a mean from two to three data points for each treatment in each monkey.

Plasma Levels of L-DOPA

To determine CE interactions with L-DOPA pharmacokinetics, the plasma levels of L-DOPA were assessed when coadministered with CE at the highest dose of CE tested in these studies (1 mg/kg) in two chair-trained monkeys. L-DOPA, at a dose at which effects were modified by the addition of CE (LD-low; 75 mg), was administered immediately following the CE injection. Both drugs were administered subcutaneously as in previous tests. Blood samples were collected beginning with time 0 for baseline (before drug administration) and thereafter at 45, 90, 105, 120, 135, and 150 min. In each monkey, blood samples were taken in three repeated experiments for control treatment (CE vehicle + L-DOPA) and in three repeated experiments for CE treatment (1 mg/kg CE + LD-low). Whole blood was centrifuged, and separated plasma samples were stored at −80°C until analysis. L-DOPA levels were determined based on modification of previously published methods (Blandini et al., 1997). In brief, plasma was deproteinized by addition of equal volume of 1.2 M HClO4, and then it was diluted 1:5 in H2O. After centrifugation for 20 min until there was a return to baseline. Dyskinesias were assessed at the same time points using part II of the scale that rates dyskinesias with a wide range within the following major categories: 0, absent; 1, mild (fleeting, rare, present less than 30% of the observation period); 2, moderate (interfering with normal activity, present less than 60% of observation period); and 3, severe (disabling, replacing normal activity, present more than 90% of observation period). All tests were performed after an overnight fast. Animals were videotaped for subsequent rating by a blinded investigator. Each test in all sets of experiments was repeated. Data were averaged to yield a mean from two to three data points for each treatment in each monkey.

**TABLE 3**

Comparison of CE and L-DOPA treatments (crossover)

<table>
<thead>
<tr>
<th>Initial Treatment</th>
<th>Subsequent Treatment</th>
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<tbody>
<tr>
<td>CE V + LD-low*</td>
<td>A: 4.3 ± 0.5</td>
</tr>
<tr>
<td>CE V + LD-high*</td>
<td>A: 2.0 ± 0.05</td>
</tr>
<tr>
<td>CE 0.3 + LD V</td>
<td>C: 10.8 ± 0.5</td>
</tr>
<tr>
<td>CE 1 + LD V</td>
<td>C: 11 ± 0.5</td>
</tr>
</tbody>
</table>

* p < 0.003 vs. 0.3 CE mg/kg + LD V or 1 mg/kg CE + LD V.

To determine CE interactions with L-DOPA pharmacokinetics, the plasma levels of L-DOPA were assessed when coadministered with CE at the highest dose of CE tested in these studies (1 mg/kg) in two chair-trained monkeys. L-DOPA, at a dose at which effects were modified by the addition of CE (LD-low; 75 mg), was administered immediately following the CE injection. Both drugs were administered subcutaneously as in previous tests. Blood samples were collected beginning with time 0 for baseline (before drug administration) and thereafter at 45, 90, 105, 120, 135, and 150 min. In each monkey, blood samples were taken in three repeated experiments for control treatment (CE vehicle + L-DOPA) and in three repeated experiments for CE treatment (1 mg/kg CE + LD-low). Whole blood was centrifuged, and separated plasma samples were stored at −80°C until analysis. L-DOPA levels were determined based on modification of previously published methods (Blandini et al., 1997). In brief, plasma was deproteinized by addition of equal volume of 1.2 M HClO4, and then it was diluted 1:5 in H2O. After centrifugation for 30 min (15,000 rpm at room temperature), the supernatant was collected for analysis by high-performance liquid chromatography with electrochemical detection. Ten microliters of supernatant was applied to an in-line preoxidation electrode (ESA 5020 guard cell electrode set at 0.20V; ESA Inc., Chelmsford, MA) followed by a reverse-phase C18 Thermo (Thermo Fisher Scientific Inc., Waltham, MA) column (150 × 3 mm; 3-μm particle size; pore size, 120 Å). The column was eluted with an aqueous mobile phase consisting of 50 mM KH2PO4, 0.7 mM SDS, and 0.3 mM EDTA, containing 12%
acetonitrile (pH 2.9 with glacial acetic acid). Mobile phase flow rate was 0.200 ml/min, controlled by a Shimadzu LC-10AD pump (Shimadzu USA Manufacturing Inc., Camby, OR). L-DOPA was detected in the column effluent using an ANTEC electrochemical detector (ANTEC Leyden, Zoeterwoude, The Netherlands) with a working electrode potential of 0.30 V. Data were processed by Empower Pro 2 software from Waters (Milford, MA). L-DOPA level in each sample was determined from the peak height in comparison with a curve generated using three concentrations of authentic L-DOPA as standard. Plasma samples are expressed as nanograms per milliliter.

**Statistical Analysis**

Scores of motor disability and dyskinesias were graded within wide ranges; thus, data composed continuous variables. All results are expressed as mean values ± S.E.M. Two-factor analysis of variance (ANOVA) for repeated measures, followed by the post hoc Fisher’s protected least significant difference test when the F value indicated significance, was used to compare every treatment over the time course after drug administration. Significance was taken at p < 0.05.

**Results**

**Intrinsic Effects of CE.** CE as monotherapy had no measurable effects on parkinsonian monkeys with severe motor impairment. Motor disability scores remained unchanged during the 2 h after administration of every CE dose. Scores averaged 15.5 ± 2.5, 15.2 ± 2, 15.1 ± 2, and 15.6 ± 2.2 after CE vehicle, 0.03, 0.1, 0.3, and 1 mg/kg, respectively (p > 0.05). Although monkeys had a tendency to relax, motor disability was not worsened (unchanged scores in the scale). Monkeys did not exhibit retching, vomiting, or changes in social interaction. The highest doses of CE produced slight somnolence in two animals. This effect was observed irregularly, and it did not affect the interaction of the animal with the examiner.

CE was also tested as monotherapy in moderately parkinsonian monkeys during the study of comparison with L-DOPA (crossover). CE per se did not have motor effects in this group of monkeys either. Since in this study CE monotherapy was also evaluated after monkeys received L-DOPA tests, results show that monotherapy with CE had no effects in treatment-naive or L-DOPA-primed monkeys, i.e., responses to CE did not change between the groups (Table 3). Monkeys that received CE treatment after developing antiparkinsonian responses to L-DOPA had no antiparkinsonian response to CE, just as monkeys that received CE treatment initially. L-DOPA and CE responses were clearly different (p < 0.001; Table 3). Alternatively, the antiparkinsonian effects of L-DOPA before and after CE administration were similar in all animals. Therefore, CE did not potentiate initial responses to L-DOPA. Nor did it modify the course of development and stabilization of L-DOPA responses. Together, these results indicated that CE had no intrinsic effects on motor behavior of parkinsonian monkeys.

**Effects of CE on Antiparkinsonian Action of L-DOPA.** Coadministration of CE with L-DOPA resulted in enhanced antiparkinsonian effects. Responses to the suboptimal dose of levodopa were prolonged by the addition of CE in parkinsonian monkeys with different degrees of disability. In the severely parkinsonian group, a significant effect (CE + LD-low) was found on motor disability scores accumulated over the whole antiparkinsonian response, total scores (Fig. 2A). These effects were dose-dependent, and they were clearly manifested at the two higher doses, 0.3 and 1 mg/kg (p < 0.05 compared with baseline response of L-DOPA low dose, CE vehicle + LD-low). The reduction of total disability scores by adding CE derived from extended duration of low scores as shown in Fig. 2B (p < 0.01 for differences of scores at late individual intervals). Prolongation of L-DOPA responses varied among animals from 20 to 40 min after coadministration of CE, and this time represents 30% increase of response duration for L-DOPA low doses, which average 100 min. Coadministration of CE with L-DOPA optimal doses resulted in slighter effects in severely parkinsonian monkeys, suggesting that a maximal effect was reached (difference of scores at individual time points did not attain significance). The minimal scores obtained at the peak of L-DOPA effects (LD-low and LD-high) did not significantly change with the addition of any dose of CE.

In the moderately parkinsonian group, a significant effect of CE in combination with LD-low was also found on total motor disability scores accumulated during the antiparkinsonian response (p < 0.01; Fig. 3A). The addition of CE at the

![Fig. 2. Effects of coadministration of CE with L-DOPA on motor disability scores in severely parkinsonian monkeys. A, total motor disability scores. Each bar represents the effect of each treatment, CE vehicle (V) + LD-low, 0.1 mg/kg CE + LD-low, 0.3 mg/kg CE + LD-low, and 1 mg/kg CE + LD-low; and the same CE combinations with LD-high. Values are the average of the sum of each interval total scores during the whole antiparkinsonian response (from 30 min after treatment to the last interval of scores 50% or lower than baseline) from all animals. *, p < 0.05 versus CE vehicle + same dose of L-DOPA. B, time course of responses. Each curve represents the effects of each treatment combination of CE and LD-low, as described in A. Values are the average of total motor disability scores for individual intervals from all monkeys. “OFF” represents the baseline score taken just before drug injections (time 0), and after injection, scoring starts at 30 min and follows thereafter every 20 min. **, p < 0.01 for differences in same time point between 0.3 mg/kg CE + LD-low or 1 mg/kg CE + LD-low and CE vehicle and LD-low (ANOVA for repeated measures; n = 3). Data points are mean ± S.E.M.
highest dose (1 mg/kg) had a tendency to reduce motor disability to lower scores at the peak effect, and it extended the reversal of parkinsonism from 80 to 110 min (Fig. 3B). L-DOPA high doses produced a marked response in this group that remained unchanged by the addition of CE.

On average from all parkinsonian monkeys, a substantial difference between treatments is demonstrated at 110 min from the injections, and it is maintained at 130 min. Figure 4 shows changes of response duration with coadministration of CE and LD-low in every monkey. Scores are also lower during the peak effect with the combination of 1 mg/kg CE and LD-low, although these differences were inconsistent.

**Effects of CE on L-DOPA-Induced Dyskinesias.** CE had no effects on L-DOPA-induced dyskinesias. Severely parkinsonian monkeys had stable dyskinetic responses to L-DOPA. Dyskinesias were typically peak-dose and of choreic type, although dystonic dyskinesias were also seen with the tested doses of L-DOPA. Mostly neck and trunk dystonia together with choreic movements of the limbs was present with the higher doses of L-DOPA. Dystonia was seen in the legs less frequently. Figure 5 shows scores of dyskinesias in each interval for all treatment combinations of CE and L-DOPA. A slight difference (nonsignificant) in values is present in the late intervals; thus, this effect is probably related to the prolongation of levodopa action by the addition of CE. The peak values of dyskinesias were not changed with the addition of CE. CE had no significant effect on individual scores of dyskinesias at any given time point (Fig. 5, A and B).

**Plasma Levels of L-DOPA.** CE had no effects on L-DOPA pharmacokinetics. Following systemic administration of L-DOPA plus benserazide at the suboptimal doses, L-DOPA concentration in plasma increased steadily until reaching its peak at 45 min, and then it gradually decreased during the remaining 100 min of behavioral effects (Fig. 6). Curves of plasma levels of L-DOPA were similar following coadministration of CE vehicle and the highest CE dose (1 mg/kg) that prolonged L-DOPA responses. No significant differences in plasma concentrations of L-DOPA were found in samples taken at any time during repeated determinations in two monkeys (Fig. 6). In fact, plasma levels of L-DOPA were slightly lower after administration of CE than after vehicle. Thus, the effects of CE on prolonging the response to L-DOPA were not due to prolongation of L-DOPA plasma concentration.

**Discussion**

Results of this study demonstrate that CE potentiates the antiparkinsonian action of levodopa in MPTP-treated rhesus monkeys. CE, in a dose-dependent manner, augmented L-DOPA responses by lowering scores of motor disability at the peak effect, and more markedly, by prolonging effects by 30% of the usual duration after a suboptimal dose of L-DOPA. This prolongation of L-DOPA response was not related to pharma-
monkeys (RGi6 and RLt6). Each data point is the average of L-DOPA and CE vehicle, 12,901 \pm 2010 in RGl6 and 10,539 \pm 999 in RLt6; 2) L-DOPA + 1 mg/kg CE, 10,916 \pm 1179 in RGl6 and 8106 \pm 745 in RLt6 (nonsignificant differences).

These data originally show that blockade of CB1 receptors augments the antiparkinsonian effects of L-DOPA in the rhesus monkey. The study also shows that CB1 receptor antagonism has 1) no intrinsic antiparkinsonian effects and 2) no antidyskinetic effects. Previous studies of cannabinoid agonists and antagonists have shown conflicting results where, for example, antagonists were found to be effective as monotherapy and to have antidyskinetic effects (Fernandez-Espejo et al., 2005; van der Stelt et al., 2005). Because we used MPTP-treated macaques that closely mimic motor behavior and basal ganglia anatomy of humans, and we used the inclusive experimental design from monotherapy to multiple aspects of levodopa coadministration, this study provides critical data to clarify the potential role of CB1 antagonists in the therapy of PD. Most previous work in primates derives from trials with the CB1 antagonist rimonabant (SR 141716A) in marmosets. Despite a homologous neuroanatomy (Hardman et al., 2002), definite differences between these nonhuman primate species are evidenced by the sensitivity to MPTP and the responsiveness to L-DOPA. In marmosets, a stable parkinsonism is rapidly produced (5 days), and dyskinesias are induced after short drug exposure (10 days) (Pearce et al., 1995), as opposed to several months of both MPTP and L-DOPA treatments required in macaques. In addition, different motor behaviors of marmosets (high baseline activity) is commonly assessed with locomotor activity and range of movement. Results from these measurements are not equivalent to our scoring system using standardized scales that were designed to quantify specific parameters of parkinsonian disability, such as tremor, posture, and stability. Therefore, this study presents unequivocal data that demonstrate potentiation of L-DOPA antiparkinsonian effects by CB1 antagonists. These drugs have barely been tested in parkinsonian patients by limited trials of a single challenge with a single dose (Mesnage et al., 2004). Our results support a thorough clinical evaluation of CB1 antagonists.

CE had antiparkinsonian effects in our two groups of monkeys that differed not only in their motor impairment but also in their responsiveness to L-DOPA. High sensitivity in severe conditions leads to a similar high effect close to a “ceiling” effect with different doses of L-DOPA (“all or none” response) (Nutt and Holford, 1996). Severely parkinsonian monkeys also have motor fluctuations typically characterized by shortened responses, and they may have a reduced capability to adjust the response duration to different doses (Nutt, 2001). Thus, motor responses to dopamine in the advanced stages of the disease involve altered dopamine-mediated mechanisms, and results of CE in both conditions of parkinsonism suggest a cannabinoid mechanism that is independent of direct interaction with a certain dopamine signaling. Lack of intrinsic effects of CB1 antagonists was also found in cynomolgus monkeys with rimonabant (Meschler et al., 2001). Here, CE was tested in monkeys naive of antiparkinsonian treatment to reveal slighter motor effects that are overshadowed after development of full responses to dopamine stimulation in conditions of marked motor impairment.
onists have no antiparkinsonian action per se. Moreover, effects of l-DOPA high doses that approximated maximal responses in both groups of monkeys remained unchanged by the addition of CE, which further supports the notion that CE antiparkinsonian effects depend on modulation of dopamine responses.

Lack of antidysonetic effects of CE may be in line with the opposite effects of the cannabinoid agonist nabilone (Sierradzan et al., 2001; Fox et al., 2002). It remains unclear where cannabinoid antagonists and agonists mediate these opposing behavioral effects among the various locations of CB1 receptors in basal ganglia. The status of endocannabinoid transmission after dopamine denervation remains controversial. Changes of striatal endocannabinoids have been reported in opposite directions (Gubellini et al., 2002; Ferrer et al., 2003). In addition, CB1 receptor binding and mRNA were found to increase in striatum and other basal ganglia regions (Mairell and Vanderhaeghen, 1993; Lastres-Becker et al., 2001). However, the precise function of this system through presynaptic and postsynaptic receptors remains obscure. CB1 receptors have modulatory effects on GABAergic, glutamatergic, and other activities of transmitters that may scurse. CB1 receptors have modulatory effects on GABAergic, glutamatergic, and other activities of transmitters that may scurse. CB1 receptors have modulatory effects on GABAergic, glutamatergic, and other activities of transmitters that may scurse.

In summary, results of this study indicate that the specific effects of CB1 receptor blockade by systemic administration of selective antagonists consist of enhancement of antiparkinsonian responses to dopaminergic stimulation. These effects are expressed in moderately as well as severely parkinsonian rhesus monkeys that have altered responses to dopaminergic drugs. Therefore, selective CB1 receptor antagonists may prove useful as an adjuvant to dopamine replacement therapy in patients with various disease stages. Furthermore, combined treatment with cannabinoid antagonists may help adjust l-DOPA doses to delay or reduce disabling motor complications.

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