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Involvement of Receptor Reserve in D_1 Agonistic Action of (−)-Stepholidine in Lesioned Rats

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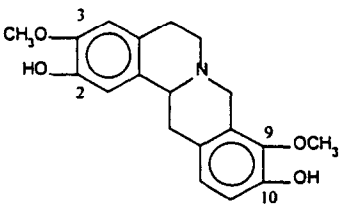
ABSTRACT. (−)-Stepholidine (SPD) is a natural product. Previous studies had demonstrated that SPD displayed D_1 agonism in unilaterally 6-hydroxydopamine (6-OHDA)-lesioned rats and D_1 antagonism in reserpinized rats and normal rats. The aim of the present study was to explain this peculiar pharmacological action based on behavioral and biochemical experiments. In the unilaterally 6-OHDA-lesioned rats, SPD (4 mg/kg, s.c.) induced contralateral rotation as did apomorphine (APO), but the rotation response to SPD was 60% lower than that to APO (0.5 mg/kg, i.p.). Coadministration with APO (0.5 mg/kg, i.p.) and SPD (0.5 to 10 mg/kg, s.c.) produced a biphasic action curve. At low doses (0.5 or 1 mg/kg), SPD potentiated APO action; at high doses (4 or 10 mg/kg), however, SPD suppressed APO. In striatal homogenate of the unilaterally lesioned rats, SPD stimulated cyclic AMP (cAMP) formation and produced a maximal response comparable to that of dopamine (DA) in the denervated striatum, but 70% lower than that of DA in the intact striatum. Coadministration of 10 μM DA with various concentrations of SPD yielded different results, with a biphasic response in the intact side and a synergistic effect in the denervated side. Furthermore, based on the determination of receptor-mediated cAMP formation, the D_1 receptor reserve was analyzed in both denervated and intact striatum by using the DA receptor inactivator *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). The results showed that following EEDQ administration, the receptor density [revealed by [^{3}H]*R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine ([^{3}H]SCH-23390) binding] and the agonist-stimulated adenylate cyclase (AC) activity (revealed by cAMP formation) were reduced concurrently. In the intact striatum, the reduction in SPD-stimulated AC activity paralleled the receptor loss, indicating the absence of receptor reserve, while in the denervated striatum the reduction in AC activity was less than the receptor loss, indicating a significant level of receptor reserve (estimated 16.4%). By comparison, receptor reserve for DA was 45.7 and 25.3% in the denervated and intact striatum, respectively, representing an 80% increase of receptor reserve. In conclusion, SPD is a D_1 partial agonist, and receptor reserve permits SPD to display its D_1 agonistic action in the unilaterally 6-OHDA-lesioned rats. BIOCHEM PHARMACOL **54**;2: 233–240, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. (−)-stepholidine; D_1 dopamine receptor; partial agonist; receptor reserve; adenylate cyclase; rotational behavior

SPD†, isolated from the Chinese herb *Stephania intermedia* Lo, is a leading compound of the tetrahydroprotoberberines [1]. SPD and its analogs share the common structure of an isoquinoline ring and methoxyl groups or hydroxyl groups at position C_{2-3} and C_{9-10}, as shown at right.



Chemical structure of SPD

Previous binding studies have demonstrated that SPD possesses selective affinity for both D_1 and D_2 receptors with a preference for D_1 receptors [2]. In normal rats, SPD antagonizes APO-induced stereotypy [3], reverses APO-induced inhibition of the firing activity of nigral DA neurons [4], increases striatal L-dopa accumulation [5] and DA release in the striatum [6], and stimulates prolactin secretion tonically inhibited by DA *in vivo* [7]. All these data consistently establish SPD as a D_2 antagonist. However, as for the D_1 action of SPD, previous studies have reported controversial observations. In rats treated for 6 days with reserpine, SPD reverses D_1 agonist 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1*H*-benzazepine (SKF

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† *Abbreviations:* AC, adenylate cyclase; APO, apomorphine; cAMP, cyclic adenosine 3′,5′-monophosphate; DA, dopamine; DTT, dithiothreitol; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; L-dopa, levodopa; 6-OHDA, 6-hydroxydopamine; SNr, substantia nigra pars reticulata; and SPD, (−)-stepholidine.

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38393)-induced inhibition of firing activity of nigral DA cells although SPD *per se* has no action on the firing [8], indicating a D_1 antagonistic action. Nonetheless, in rats with unilateral nigral lesions induced by 6-OHDA, SPD produces a significant contralateral rotation in a manner similar to that of the D_1 agonist SKF 38393 [9, 10], and the rotation can be antagonized by the D_1 selective antagonist *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH-23390), all indicating a D_1 agonist action. Recently, an *in vitro* receptor binding assay using calf striatal membrane preparations has confirmed that SPD binds to the D_1 receptor with two sites and can be regulated by GTP, strongly supporting a D_1 agonistic activity [11]. Based on these observations, a partial agonism of SPD at D_1 receptors is proposed.

According to the theory of receptor occupancy, a full agonist merely needs to occupy a part of the receptor pool for its maximal effect, thus leaving a receptor reserve or spare receptors, while a partial agonist acts with no or less receptor reserve [12, 13]. Therefore, examining the receptor reserve would help to characterize a partial agonist. In this study, following the studies of SPD-induced behavioral and biochemical effects, the receptor reserve for SPD in both sides of the striatum of the lesioned rats was analyzed, using a receptor inactivator, EEDQ [14, 15]. For comparison, a classical dopamine receptor agonist, APO or DA, was included in the experiments.

MATERIALS AND METHODS

Drugs and Reagents

(−)-SPD (m.p. 161–162°, $[\alpha]_D$ −440° in pyridine), supplied by the Shanghai Institute of Materia Medica, was dissolved in a small amount of *N*,*N*-dimethylformamide and then diluted with the reaction medium. DA hydrochloride (Fluka Chemie AG, Switzerland), SCH-23390 and spiperone (RBI, U.S.A.), Na_2ATP and Na_2GTP (Sigma Chemical Co., U.S.A.), theophylline (Shanghai No. 2 reagent factory, China), DTT (Serva Feinbiochemica, Heidelberg/New York), APO (Shengyang No. 1 pharmaceutic factory, China), creatine phosphate and creatine phosphokinase (Shanghai biochemical reagent factory, China), forskolin and 6-OHDA–HCl (Sigma) were used. cAMP radioimmunoassay kits were purchased from the Shanghai Second Medical University.

Preparation of 6-OHDA-Lesioned Rats

Male Sprague-Dawley rats (Shanghai Experimental Animal Center, Chinese Academy of Sciences), weighing 200–220 g, were used. After anesthetization with pentobarbital (40 mg/kg, i.p.), rats were fixed on a stereotaxic frame and injected in the unilateral substantia nigra with a saline solution (4 μL) containing 9.7 μg 6-OHDA–HCl (equivalent to 8 μg free base) and 1 μg ascorbic acid at the rate of 1 μL/min.

Rotation Behavior Assay

Three weeks after nigral lesioning, rats were placed in a bowl (diameter 24 cm) for screening. Following 30 min of habituation, the rats were injected with APO (0.2 mg/kg, i.p.) or SPD (4 mg/kg, s.c.) and the turns each rat circled in 15 min were recorded. Only rats showing contralateral rotation at a speed of more than 5 turns/min in response to both APO and SPD were retained. For the rotation study, the rats were pretreated with the D_2 antagonist spiperone (0.5 mg/kg, s.c.) 30 min earlier to block D_2 sites, and then were injected with SPD (s.c.) or APO (0.5 mg/kg, i.p.) alone and together. The rotation response of each rat was recorded as total turns in 15 consecutive min after rats started to circle.

Synaptosomal Preparation

The synaptosomes were prepared according to the method of Waggoner *et al.* [16] with a minor modification. Briefly, the rats, with or without EEDQ pretreatment, were decapitated, and the striata were rapidly dissected out on ice. The pooled striata were homogenized in a blender with 10 vol. of ice-cold 50 mM Tris–HCl (pH 7.45) containing 0.32 M sucrose, 1.2 mM EGTA, 1 mM DTT. The homogenate was centrifuged at 1000 *g* for 15 min to remove nuclei and cell debris. The supernatant was then centrifuged at 20,000 *g* for 20 min at 4°. The resulting pellets were washed once more with the same buffer, followed by final resuspension in an assay buffer for estimating the activity of AC or in a binding buffer for the [^{3}H]SCH-23390 binding assay. Protein concentrations were determined according to the method of Bradford [17], using BSA as a standard.

D_1 Receptor Binding Assay

[^{3}H]SCH-23390 binding was assayed according to a method described elsewhere [18]. Eight concentrations (0.01 to 10 nM) of the radioligand were used. Binding isotherms were analyzed with computer software InPlot (GraphPad Inc., San Diego, CA, U.S.A.), which provides non-linear fitting and an estimate for B_{max}.

AC Assay

AC activity was measured according to the method of Orr *et al.* [19] with minor modification. Fifty micrograms of protein was added into a 300 μL total volume composed of assay buffer containing (final concentrations) 80 mM Tris–HCl (pH 7.45), 5 mM $MgSO_4$, 0.6 mM EGTA, 1 mM DTT, 10 mM theophylline, 50 μM GTP, 5 mM creatine phosphate, 50 U/mL of creatine phosphokinase, and six concentrations of tested drugs. Ten micromolar spiperone was used to preclude D_2 receptor-mediated inhibition of AC activity. In some assay tubes, 10 μM SCH-23390 was used to define the specificity of agonist action. The reaction was initiated by adding 0.5 mM ATP and was carried out at

30° for 10 min, followed by termination by boiling for 3 min. After centrifugation of the reaction mixture at 3000 *g* for 10 min, 100 μL supernatant was used to determine the content of cAMP according to a radioimmunoassay method. Dose–response curves for agonist stimulation of striatal cAMP formation were fitted using InPlot to obtain the estimates for EC_{50} and E_{max} (maximal effect). AC activity was expressed as picomoles of cAMP formed per milligram of protein per minute.

Receptor Reserve Analysis

Receptor reserve for agonist-induced response was estimated by evaluating fractional receptor response against fractional receptor occupancy. The fractional receptor occupancy was calculated from the apparent dissociation constant (K_A), and the K_A was obtained by the partial receptor inactivation method of Furchgott and Bursztyn [20]. According to Furchgott and Bursztyn, the relationship between the effects elicited by an agonist before and after irreversible receptor inactivation may be described by equation 1:

$$1/[A] = 1/q[A] + (1 - q)/qK_A \quad (1)$$

where [A] is the concentration of agonist necessary to produce a specific level of response before inactivation, [A′] is the concentration needed to produce the same response after inactivation, and q is the fraction of receptors left active. [A] and [A′] were obtained from the respective dose–response curves at five levels of response (corresponding to 30, 40, 50, 60, and 70% of the maximal effect after EEDQ treatment). The determination of both K_A and q may be made by plotting the reciprocals of [A] and [A′]. The resulting straight line has a slope of $1/q$ and a K_A value of (slope − 1)/y-intercept.

The fractional receptor occupancy (f) at a particular agonist concentration [A] was then calculated from the law of mass action (equation 2):

$$f = [RA]/[R_T] = [A]/(K_A + [A]) \quad (2)$$

where [RA] is the concentration of the receptor–agonist complex and $[R_T]$ is the total concentration of active receptors. Fractional receptor occupancy at each concentration was plotted against fractional response at several chosen doses. The level of receptor reserve was equal to subtraction of percent of receptor occupancy required for maximal response from 100%.

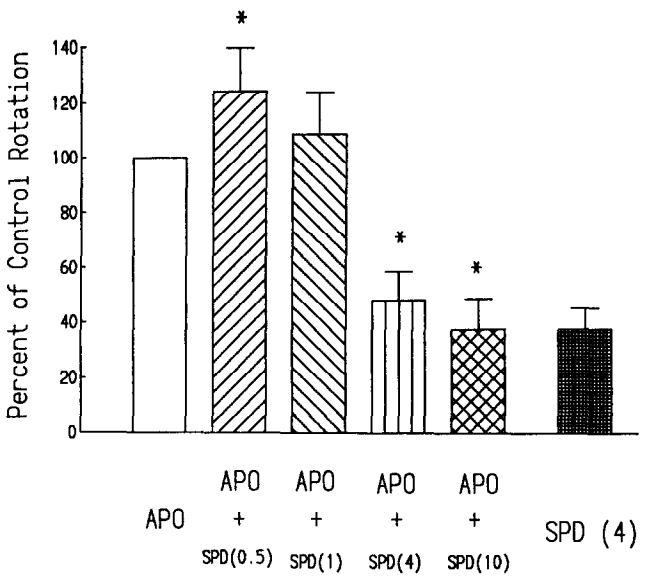

FIG. 1. Effects of APO (0.5 mg/kg, i.p.) or SPD (4 mg/kg, s.c.) alone and coadministration of APO (0.5 mg/kg, i.p.) with SPD (0.5 to 10 mg/kg, s.c.) on rotation in 6-OHDA-lesioned rats (N = 7). The rats were pretreated 30 min before with 0.5 mg/kg (s.c.) spiperone, a D_2 antagonist. Turning activity for each rat was recorded in 15 consecutive min. APO alone induced 66–260 circles of rotation during the assay time, representing a control value of 100%. Numbers in parentheses represented doses of drugs assayed. Values are means ± SD of seven rats. Key: (*) $P < 0.05$ vs control.

RESULTS

D_1 Action of SPD on Rotation in the Lesioned Rats

In unilaterally lesioned rats, SPD (4 mg/kg, s.c.) induced a contralateral rotation as did the classic agonist APO (0.5 mg/kg, i.p.), but the maximal response to SPD was 60% lower than that to APO. Increasing the dose of SPD up to 20 mg/kg was not followed by increased rotational response (data not shown). Coadministration of APO (0.5 mg/kg) with SPD (0.5 to 10 mg/kg) produced a biphasic effect, with

TABLE 1. Effects of DA and SPD on cAMP formation in the denervated and intact striatum

	DA		SPD	
Striatum	E_{max} (pmol cAMP formed/ mg protein/min)	EC_{50} (μM)	E_{max} (pmol cAMP formed/ mg protein/min)	EC_{50} (μM)
Denervated	254.6 ± 20.9	6.91 ± 0.35*†	244.7 ± 31.7‡	5.40 ± 0.38‡
Intact	250.4 ± 24.5†	8.01 ± 0.53	149.2 ± 18.3	8.37 ± 0.65

Data are means ± SEM of three independent experiments.
* $P < 0.05$ vs intact.
† $P < 0.01$ vs SPD.
‡ $P < 0.01$ vs intact.

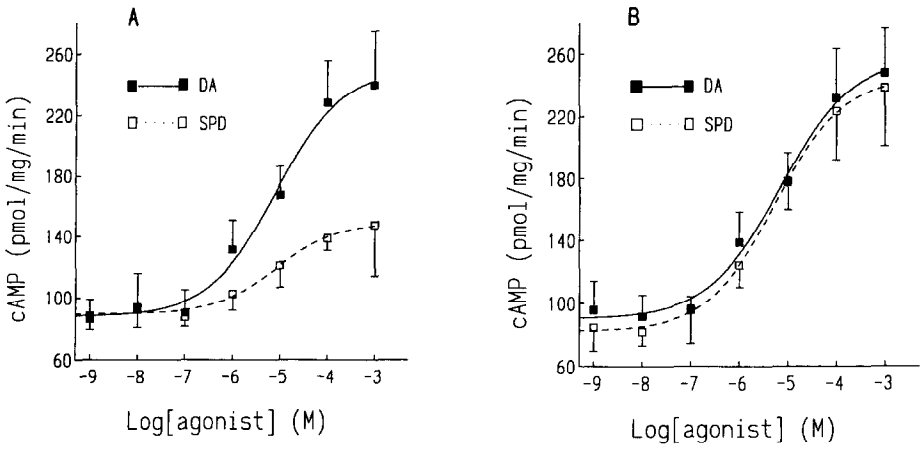

FIG. 2. Comparison between effects of DA and SPD on cAMP formation in the intact (A) and denervated (B) striatum of rats with unilateral SNC lesions. Curves were simultaneously fitted by computer software GPIP (Graph Inplot). Each point is the mean ± SD from three independent determinations.

SPD increasing APO response at 0.5 and 1 mg/kg by 24.4% ($P < 0.05$) and 8.9%, respectively, and suppressing APO action at 4 and 10 mg/kg by 51.8% ($P < 0.05$) and 62.4% ($P < 0.05$), respectively (Fig. 1).

Augmentation of SPD-Stimulated Striatal AC Activity by Nigral Lesion

In the presence of spiperone, the basal AC activity was 82.5 ± 8.8 pmol cAMP/mg protein/min, without significant difference from that obtained in the absence of spiperone (85.3 ± 10.7 pmol cAMP/mg protein/min). In the presence of spiperone, DA concentration-dependently stimulated cAMP formation with a comparable maximal effect in both sides of the striatum despite the fact that the EC_{50} was decreased in the denervated side (Table 1). SPD, given at concentrations from 1 nM to 1 mM, also concentration-dependently stimulated cAMP formation in both sides of the striatum (significant concentrations: 1 μM to 1 mM). However, in the denervated side, SPD caused a more potent and more maximal response; the EC_{50} and E_{max} were 5.40 μM and 244.7 pmol cAMP/mg/min, respectively, whereas those values in the intact side were 8.37 μM and 149.2 pmol cAMP/mg/min, respectively (Table 1). As compared with DA, SPD induced a maximal effect 40% weaker in the intact side (Fig. 2A), but comparable in the denervated side (Fig. 2B, $P > 0.05$, see Table 1). When coadministered with DA, SPD concentration-dependently antagonized the DA-stimulated cAMP formation in the intact side (Fig. 3A). In the denervated side, however, SPD concentration-dependently increased DA action at 1 nM to 10 μM and slightly, but not significantly, antagonized DA effect at concentrations higher than 10 μM (Fig. 3B).

Effects of EEDQ on D_1 DA Receptor Density and Agonist-Stimulated AC Activity

As seen from Table 2, EEDQ induced the reduction of D_1 receptor density but did not change the affinity of radioligand for D_1 receptors when determining the [^{3}H]SCH-23390 binding. Therefore, the reduction of receptor density completely represented the loss in receptor number or receptor pool. A low dose of EEDQ (0.5 mg/kg, i.p.) caused consistent receptor loss (about 16%, see Table 2) in both sides of the striatum, although basal receptor density was 20% higher, due to nigral lesions, in the denervated side than in the intact side. In the intact striatum, the 16% receptor loss was followed by a comparable reduction (15%) in maximal cAMP formation (E_{max}) stimulated by SPD (Fig. 4A). However, in the denervated side, the same extent of receptor loss did not induce any change in SPD-stimulated maximal AC activity except for an initial rightward shift in its dose–response curve (Fig. 4B). This was also the case for DA; following 0.5 mg/kg EEDQ treatment, the DA-stimulated maximal effects were unchanged in both intact and denervated striatum (Fig. 5). Increasing the dose of EEDQ to 6 mg/kg produced more

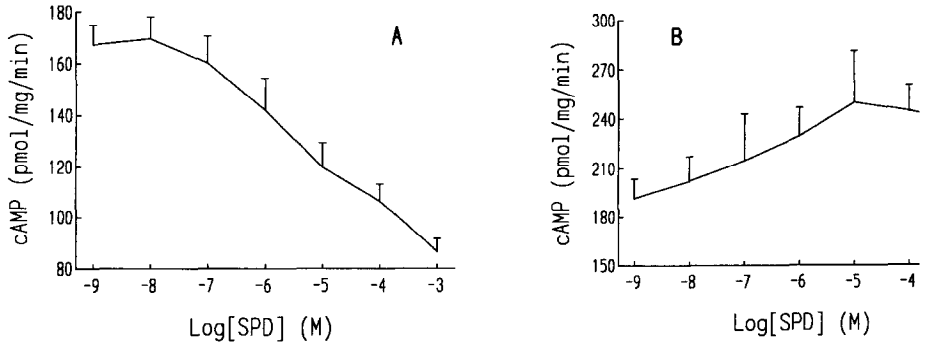

FIG. 3. Effect of coadministration of DA (10 μM) and various concentrations of SPD on cAMP formation in the intact (A) and denervated (B) striatum of unilaterally lesioned rats. Each point is the mean ± SD from three independent determinations.

TABLE 2. Effect of EEDQ treatment on D_1 receptor density (B_{max}) and equilibrium dissociation constant (K_d) in the intact and lesioned sides of unilaterally lesioned rats

Treatment	Intact		Denervated	
	B_{max} (fmol/mg protein)	K_d (μM)	B_{max} (fmol/mg protein)	K_d (μM)
Vehicle	365.8 ± 50.6	1.24 ± 0.19	441.3 ± 45.6*	1.20 ± 0.22
EEDQ (0.5 mg/kg)	310.7 ± 27.3	1.19 ± 0.25	374.5 ± 40.4*†	1.18 ± 0.15
EEDQ (6.0 mg/kg)	78.5 ± 23.2‡	1.33 ± 0.27	91.5 ± 25.8‡	1.31 ± 0.25

Data are means ± SD of five independent determinations.
* $P < 0.05$ vs intact.
† $P < 0.05$ vs vehicle.
‡ $P < 0.01$ vs vehicle.

profound (80%) receptor loss (Table 2), which caused a reduction of E_{max} of SPD by 80% (Fig. 4A) in intact striatum and 55% in denervated striatum (Fig. 4B). In contrast, E_{max} of DA was reduced by only 55% in intact striatum (Fig. 5A) and 40% in denervated striatum (Fig. 5B). These results indicated that different levels of receptor reserve may exist for both SPD- and DA-stimulated AC activity in both sides of the striatum.

Effect of Nigral Lesion on Receptor Reserve for Agonist-Stimulated AC Activity

Following profound receptor loss (at least 40%), which was induced by 6 mg/kg EEDQ in the present study, receptor reserve can be estimated. According to equation 1, the double-reciprocal plot of equieffective doses for DA-stimulated AC activity in the denervated striatum after and before receptor inactivation is given in Fig. 6. The resulting q and K_A values were 0.24 and 34.5 μM, respectively. The q (percent receptor remaining active) was consistent with 80% receptor loss revealed by [^{3}H]SCH-23390 binding. From equation 2, receptor reserve for DA-stimulated cAMP formation was estimated to be 45.7% in the denervated striatum. Based on similar calculations and comparison with DA-induced response in the denervated side, receptor reserve for DA in the intact striatum was 25.3%, and for SPD was 16.4% in the denervated side and none (only 30% maximal response relative to DA) in the intact side (Figs. 7 and 8). Taken together, nigral lesion increased the receptor reserve for both DA and SPD.

DISCUSSION

SPD had been demonstrated previously to possess structural elements of a D_1 agonist [11]. Recently, an electrophysiological study showed that SPD behaved as a D_1 partial agonist, because it induced a firing inhibition of SNr neurons like the D_1 selective agonist SKF 38393, but partially reversed SKF 38393-induced firing inhibition in unilaterally 6-OHDA-lesioned rats [21]. The present data provided behavioral and biochemical support for D_1 partial agonist activity of SPD. According to Hoyer and Boddeke [22], "a partial agonist may simply be: a compound which displays a large range of intrinsic activities at the same receptor depending on the conditions and model used." In the determination of agonist-stimulated cAMP formation, SPD displayed a weaker intrinsic activity than DA in the intact striatum; however, in the denervated striatum, the response induced by SPD was comparable to that by DA, indicating a strong activity. Furthermore, SPD, when co-administered with APO in the behavioral assay, displayed

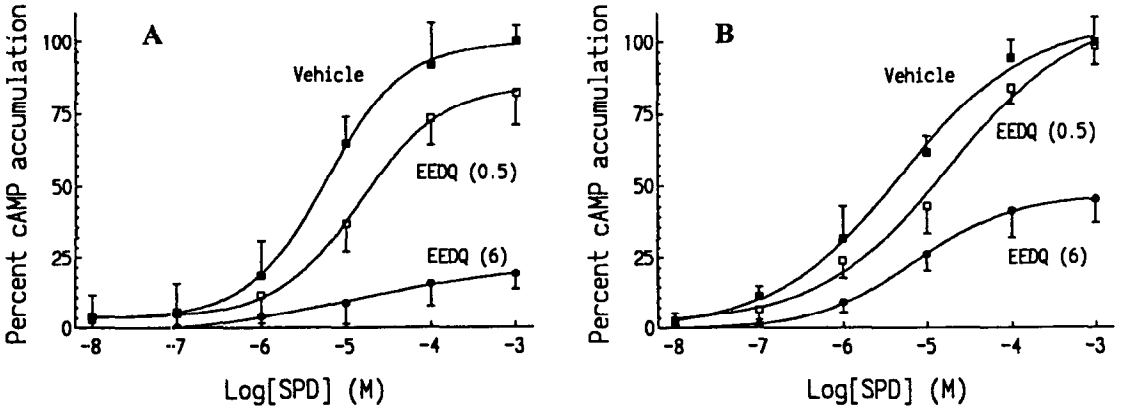

FIG. 4. Representative dose-response curves for SPD-stimulated AC activity in the intact (A) and denervated (B) striatum of vehicle- or EEDQ-treated (0.5 or 6 mg/kg, 6 hr before) rats with unilateral SNC lesions. Curves were fitted simultaneously using GPIP (Graph Inplot). Each point is the mean ± SEM of seven rats.

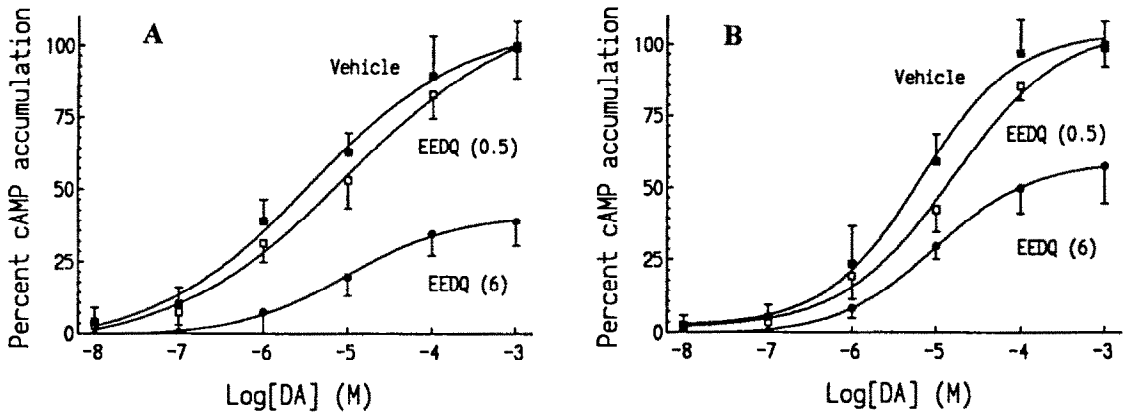

FIG. 5. Representative dose–response curves for DA-stimulated AC activity in the intact (A) and denervated (B) striatum of vehicle- or EEDQ-treated (0.5 or 6 mg/kg, 6 hr before) rats with unilateral SNC lesions. Curves were fitted simultaneously using GPIP (Graph Inplot). Each point is the mean ± SEM of seven rats.

D_1 agonism at low doses but D_1 antagonism at high doses, respectively. Similarly, SPD was demonstrated to potentiate DA action (D_1 agonism) in the denervated striatum but to antagonize DA (D_1 antagonism) in the intact striatum when DA was coadministered with SPD in the stimulation of striatal cAMP accumulation. These results suggested that the intrinsic activity of SPD could be changed qualitatively or quantitatively depending on the sensitivity of the receptor studied and the doses used.

Carlsson [23] proposed that the intrinsic activity of a partial agonist appears to depend upon the degree of previous receptor occupation. Thus, in situations characterized by a low receptor occupancy (e.g. after 6-OHDA lesions), partial agonists will actually act as agonists, while under high endogenous transmitter tone (e.g. in intact striatum) or exogenous agonist treatment, partial agonists only have a lower intrinsic activity and act mainly as antagonists. That may be the reason why SPD displayed variable intrinsic activity when coadministered with the full agonists DA or APO.

Using the irreversible inactivator EEDQ to study the receptor reserve of striatal D_1 receptors, SPD displayed no receptor reserve in the intact striatum, which was another feature of a partial agonist. However, there have been many controversial reports on receptor reserve of striatal D_1 receptor. Meller *et al.* [14] reported that striatal D_1 receptors had no receptor reserve, but Hess *et al.* [24] and Battaglia *et al.* [25] reported 30% D_1 receptor reserve for DA-stimulated cAMP formation in striatum. We found that the level of D_1 receptor reserve for DA was 25.3%, which was in agreement with the results of Hess *et al.* Interestingly, the striatal

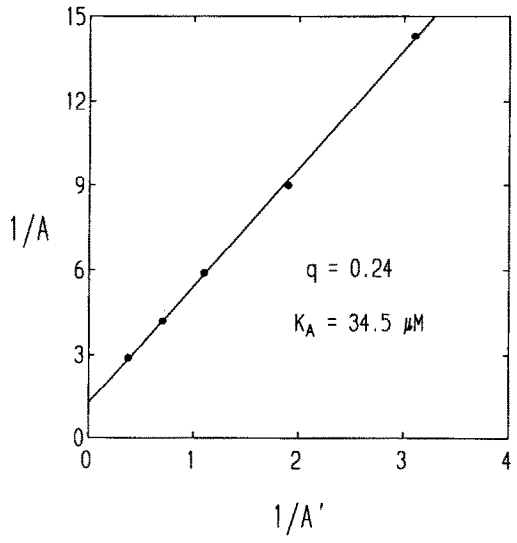

FIG. 6. Double-reciprocal plot of equieffective doses of DA required to stimulate cAMP formation in the denervated striatum in vehicle (A) and EEDQ (A') treated rats. Doses were obtained at five levels of effect [corresponding to 30, 40, 50, 60, and 70% of the maximal response in EEDQ (6 mg/kg) treated rats from the data shown in Fig. 5B].

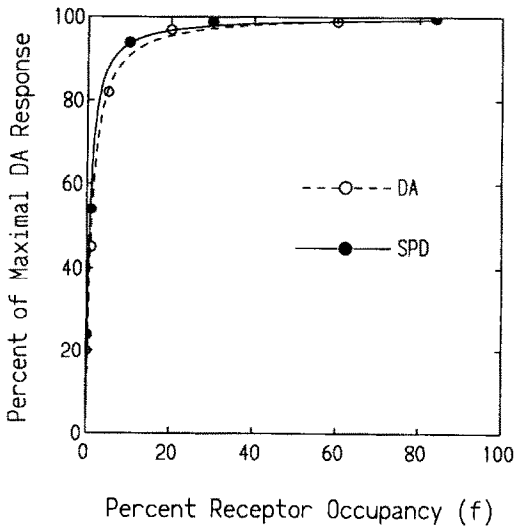

FIG. 7. Comparison between maximal responses for DA- and SPD-stimulated cAMP formation expressed as a function of receptor occupancy in the denervated striatum. Receptor reserve for DA and SPD was estimated to be 45.7 and 16.4%, respectively.

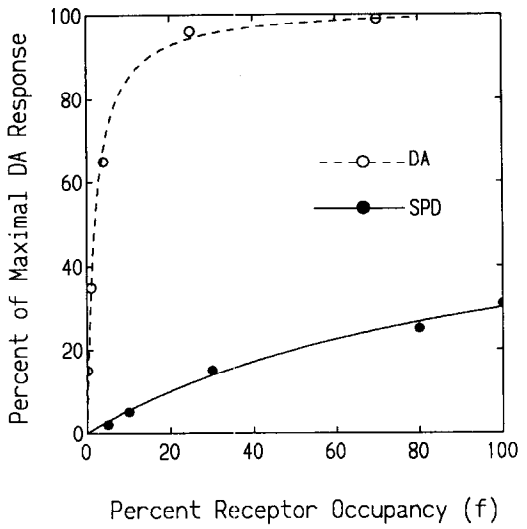

FIG. 8. Comparison between maximal responses for DA- and SPD-stimulated cAMP formation expressed as a function of receptor occupancy in the intact striatum. Receptor reserve of 25.3% for DA was estimated, but SPD displayed a weaker response with the absence of receptor reserve.

receptor reserve for both DA and SPD was increased significantly following nigral lesions. According to the receptor theory, an increase in receptor reserve may result from an increase in receptor number or coupling efficiency between receptor and response. Our study reported a 20% increase in receptor density (Table 2). In addition, other work documented nigral lesion-induced supersensitive responses of striatal D_1 receptors, including an increase in receptor density [26, 27] and an up-regulation of transduction mechanisms of D_1 receptors [28, 29]. Carlsson [23] pointed out that response to an agonist depended not only on the number of receptor molecules, but also on the responsiveness of the receptor. Nigral lesions increased receptor number and coupling efficiency and actually also increased receptor reserve, resulting in alteration of drug activity. A study conducted on cultured cells overexpressing rat 5-HT_{1B} receptor (producing a high receptor reserve) demonstrated that some compounds, previously characterized as antagonists, would display full agonism or partial agonism [30], indicating that receptor reserve affected drug activity.

In conclusion, SPD is a D_1 receptor partial agonist, and the increase in receptor reserve after striatal denervation allows SPD to display an agonistic activity.

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