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Differential Modification of Striatal D_1 Dopamine Receptors and Effector Moieties by *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline *In Vivo* and *In Vitro*

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SUMMARY

Both *in vivo* and *in vitro* treatments with the irreversible protein-modifying reagent, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), were used to investigate rat striatal D_1 dopamine receptor/effector interactions. Peripherally administered EEDQ markedly reduced D_1 dopamine receptor binding and D_1 dopamine receptor-stimulated adenylate cyclase in a dose-dependent manner. However, EEDQ administered *in vivo* did not result in functional modification of either the guanine nucleotide-regulatory protein (N_s) or the catalytic subunit of striatal adenylate cyclase as assessed via guanine nucleotide- or forskolin-stimulated cAMP production. Interestingly, the loss in D_1 dopamine receptor binding did not correlate directly with observed reductions in dopamine-stimulated adenylate cyclase activity; 40% of D_1 dopamine receptor binding was lost with no significant reduction in the V_{max} of dopamine-stimulated adenylate cyclase activity. Conversely, the reduction by EEDQ of the adenylate cyclase activity stimulated by the partial agonist SKF38393 was reduced in parallel with EEDQ-induced reductions in the D_1 dopamine receptor B_{max}. However, when SKF38393-stimulated adenylate cyclase activity was potentiated by forskolin, ~30% of receptors could be lost with no significant reduction in cAMP production, resembling the pattern observed utilizing the full agonist dopamine. *In vivo* pretreatment with the specific D_1 antagonist, SCH23390, prevented reductions in dopamine-stimulated adenylate cyclase activity and D_1 dopamine receptor binding, suggesting that EEDQ acts at the ligand recognition site of the receptor. Unlike *in vivo* treatment, *in vitro* EEDQ treatment resulted in dose-dependent decreases in catalytic subunit activity as assessed by forskolin-stimulated cAMP production, indicating that, *in vitro*, the adenylate cyclase catalytic subunit is vulnerable to EEDQ-induced modification. These data indicate that EEDQ is an effective tool for elucidating the mechanisms and biochemistry of D_1 dopamine receptor/effector coupling.

Previous research has demonstrated the existence of two distinct dopamine receptor subtypes (1, 2), possessing unique pharmacologic and biochemical properties. D_1 dopamine receptors mediate stimulation of adenylate cyclase activity (3), whereas D_2 dopamine receptors mediate the inhibition of this enzyme (4–6). The detailed characterization of D_1 dopamine receptors and dopamine-stimulated adenylate cyclase has only recently become possible due to the introduction of selective D_1 agonists and antagonists. The introduction of the high affinity D_1 dopamine receptor-selective antagonist SCH23390 (7) and the D_1 dopamine receptor radioligand [^{3}H]SCH23390 (8, 9) has been particularly useful in D_1 dopamine receptor characterization.

EEDQ has been shown to be an irreversible pharmacologic antagonist at a variety of neurotransmitter receptors including α-adrenergic, serotonergic, and muscarinic receptors (10–12). We recently demonstrated that peripheral administration of EEDQ results in a dose-dependent reduction in both D_1 and D_2 dopamine receptor binding (13). That EEDQ acts as an irreversible antagonist at dopamine receptors is suggested by the maintenance of the significant reduction in receptor density even after extensive *in vitro* wash procedures. The mechanism of action of EEDQ is thought to be due to creation of highly reactive mixed carbonic anhydrides from carboxyl groups which, in turn, interact with nucleophilic groups such as free α-amino groups (14).

Striatal D_1 dopamine receptors have been shown to mediate stimulation of adenylate cyclase activity via a guanine nucleotide-regulatory subunit (1, 2). This adenylate cyclase stimulation may be elicited by a number of reagents such as dopamine, GTP, and forskolin, which act specifically via receptor, N_s, or catalytic subunit, respectively. These reagents may be used as

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ABBREVIATIONS: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; N_s, stimulatory guanine nucleotide-regulatory protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

"biochemical markers" in adenylate cyclase assays to determine the functional integrity of each subunit. In the present study, by using these "markers," the specificity of *in vivo* and *in vitro* EEDQ modification of receptors, N_s, or catalytic subunits was examined in order to investigate D_1 dopamine receptor/effector mechanisms.

Materials and Methods

***In vivo* EEDQ treatment.** Male Sprague-Dawley rats (160–180 g Simonsen) were injected intraperitoneally with EEDQ freshly dissolved in ethanol/water (1:1, v/v) or vehicle. For "protection" experiments, rats were injected subcutaneously 30 min before EEDQ (4 mg/kg) or vehicle administration with 0.5 mg/kg SCH23390 dissolved in saline and a minimal quantity of 1 N HCl (10 μl of HCl/ml of saline). Four hr after treatment, rats were sacrificed by decapitation; the striata were removed, rapidly frozen in liquid nitrogen, and stored (−70°) for no longer than 2 weeks. Striata from EEDQ-treated and control rats were homogenized in 50 volumes (w/v) of ice-cold 10 mM Tris maleate, 2 mM EGTA (pH 7.4 at 37°) using a Tekmar Tissumizer (setting of 7, 10 sec). The tissue was centrifuged (35,000 × *g*, 10 min) and washed once more in this buffer followed by resuspension to a final concentration of 16 mg wet weight/ml in the same buffer.

***In vitro* EEDQ treatment.** Rats were sacrificed by decapitation; the striata were removed, frozen in liquid nitrogen, and stored (−70°) no longer than 2 weeks prior to assay. No change in either binding parameters or adenylate cyclase activity was observed during this time. Tissue was then thawed and homogenized in 50 volumes (w/v) of ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25°), using a Tekmar Tissumizer (setting 7, 10 sec), centrifuged (35,000 × g, 10 min), and resuspended in Tris buffer to a concentration of 20 mg wet weight/ml. Following the addition of 1 mM ATP, which precluded the loss of adenylate cyclase activity which can occur during the extensive tissue preparation, aliquots of tissue were incubated at 37° for 10 min with various concentrations of EEDQ. All aliquots were then diluted 4-fold with ice-cold 50 mM Tris buffer to stop the reaction and centrifuged (35,000 × *g*, 10 min). The pellets were then resuspended in 50 volumes (w/v) of ice-cold 10 mM Tris maleate, 2 mM EGTA (pH 7.4 at 37°), centrifuged, and resuspended in that same buffer to a final concentration of 16 mg wet weight/ml.

Adenylate cyclase assay. For experiments which did not utilize EEDQ treatment, male Sprague-Dawley rats (160–180 g, Simonsen) were sacrificed by decapitation, and the striata were removed, frozen in liquid nitrogen, and stored (−70°) 1 to 2 weeks before assay. Tissue was then thawed and homogenized individually in 50 volumes of ice-cold 10 mM Tris maleate, 2 mM EGTA (pH 7.4 at 37°) using a Tekmar Tissumizer (setting 7, 10 sec). The tissue was centrifuged (35,000 × *g*, 10 min) and washed once more in this buffer, followed by resuspension to a final concentration of 16 mg wet weight/ml in the same buffer. The adenylate cyclase assay medium consisted of 80 mM Tris maleate (pH 7.4 at 37°) 0.4 mM EGTA, 4 mM $MgSO_4$, 1 mM 3-isobutylmethylxanthine, 5 mM phosphocreatine, 50 units/ml creatine phosphokinase, 0.02% ascorbic acid, 1 mM ATP (2–3 × 10^6 cpm of [^{32}P]ATP), and approximately 40 μg of membrane protein in a final assay volume of 200 μl. Agonist-stimulated adenylate cyclase experiments included 0.1 mM GTP. Dopamine-stimulated adenylate cyclase assays were performed in the presence and absence of 50 nM spiperone, a D_2 dopamine receptor antagonist, with no difference in stimulation of adenylate cyclase activity observed. Drugs and reagents were added in 20-μl aliquots of buffer. A 5-min (37°) preincubation of striatal homogenates with assay constituents preceded the reaction which was initiated by the addition of ATP/[^{32}P]ATP. The reaction was allowed to continue for 5 min and was terminated by the addition of 100 μl of a 50 mM Tris-HCl solution containing 45 mM ATP and 2% sodium dodecyl sulfate. No significant loss in enzyme activity was observed by preincubation with agonists for this period of time and enzyme activity was found to be linear up to 20 min. [^{3}H]cAMP (~20,000 cpm/sample) was added to each tube to monitor recovery of cAMP, and the separation of [^{32}P]cAMP from [^{32}P]ATP was accomplished by sequential elution over Dowex and alumina columns. Recovery was consistently greater than 80%. Proteins were determined according to the method of Lowry *et al.* (15).

D_1 dopamine receptor radioligand binding assay. Aliquots of striatal homogenates prepared for adenylate cyclase experiments were used immediately for [^{3}H]SCH23390 saturation analyses. SCH23390 has been shown to be a selective D_1 dopamine receptor antagonist in both adenylate cyclase and binding studies (5, 7) and is a selective D_1 dopamine receptor radioligand in the striatum (8, 9). For saturation analyses, incubations were initiated by adding tissue (2.5 mg of wet weight tissue/tube) to duplicate tubes containing [^{3}H]SCH23390 (80 Ci/mmol; 0.125–2 nM) to yield a 2.5-ml final assay volume in an assay buffer of 50 mM Tris (pH 7.4 at 37°), 5 mM $MgSO_4$, and 0.5 mM EDTA; nonspecific binding was defined by 100 nM *cis*-flupentixol. Tubes were incubated for 30 min at 37° and filtered over Whatman GF/C glass fiber filters which were then washed rapidly with 15 ml (3 × 5 ml) of ice-cold Tris buffer and counted by scintillation spectroscopy at an efficiency of 50%. Saturation data were analyzed by the method of Scatchard (16) in order to determine the affinity (K_D) and maximal number of binding sites (B_{max}).

Where appropriate, dopamine competition for [^{3}H]SCH23390 binding was performed utilizing conditions comparable to those employed in the adenylate cyclase assay. For these assays, tissue was prepared as described previously in the adenylate cyclase methods, and the radioligand binding assay buffer was identical to that utilized in the adenylate cyclase assay described above using unlabeled ATP instead of [^{32}P]ATP. In other experiments, assay buffer was the same as that employed in the saturation analyses. For all competitions, incubations were initiated by adding tissue (2.5 mg of wet weight tissue/tube) to duplicate tubes containing [^{3}H]SCH23390 (80 Ci/mmol, 0.25 nM) and unlabeled dopamine where appropriate to yield a 2.5-ml final assay volume; nonspecific binding was defined by 100 nM *cis*-flupentixol. The assay was completed as described for [^{3}H]SCH23390 saturation analyses. Competition data were analyzed using the iterative, nonlinear, curve-fitting computer program, LIGAND (17).

Compounds were obtained from the following sources: isobutylmethylxanthine, phosphocreatine, creatine phosphokinase, and ATP, Sigma Chemical Co. (St. Louis, MO); dopamine, Research Biochemicals (Wayland, MA); forskolin, Calbiochem (San Diego, CA); [^{3}H]cAMP and [^{32}P]ATP, ICN Radiochemicals (Irvine, CA); and EEDQ, Aldrich Chemical Co. (Milwaukee, WI). SCH23390 and [^{3}H]SCH23390 were generously donated by Drs. L. C. Iorio and A. Barnett of the Schering Plough Corp. (Bloomfield, NJ). *cis*-Flupentixol was generously donated by Dr. J. Hyttel of H. Lundbeck and Co. (Denmark). SKF38393 was donated by Smith, Kline and French (Philadelphia, PA).

Results

Effects of *in vivo* EEDQ administration on the guanine nucleotide-regulatory subunits and catalytic subunits of adenylate cyclase. To determine whether EEDQ, administered *in vivo*, elicited a functional modification of either N_s or the catalytic subunit of adenylate cyclase, guanine nucleotide, NaF-, or forskolin-stimulated cAMP production was assessed in EEDQ-treated and control animals. No significant difference in basal adenylate cyclase activity was observed between EEDQ-treated (111 ± 18 pmol/min/mg of protein) and control rats (119 ± 7 pmol/min/mg of protein). As shown in Fig. 1, a comparable stimulation above basal activity in EEDQ-treated and control animals was observed in the presence of the putative N_s effectors: GTP (0.1 mM), guanosine 5′-(β,γ-imido)triphosphate (0.1 mM), and NaF (10 mM). Likewise, the stimulation by forskolin (1 μM), thought to act directly at the catalytic subunit (18), was comparable in homogenates of con-

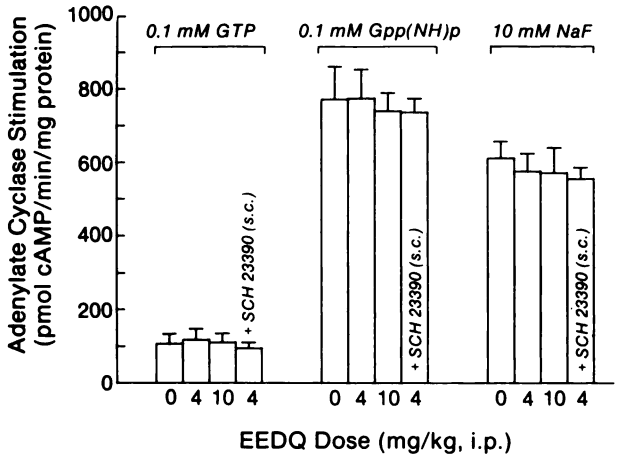

Fig. 1. Effect of *in vivo* EEDQ administration on adenylate cyclase activity stimulated via the guanine nucleotide-regulatory protein (N_s). *Bars* represent the mean + standard error of stimulation by 0.1 mM GTP, 0.1 mM guanosine 5′-(β,γ-imido)triphosphate, and 10 mM NaF above basal activity (n = 4–6). The *4 mg/kg + SCH 23390 (s.c.) bars* denote treatment with 0.5 mg/kg SCH23390 (subcutaneously) prior to 4 mg/kg EEDQ treatment. No significant differences were observed between control and EEDQ treated tissue.

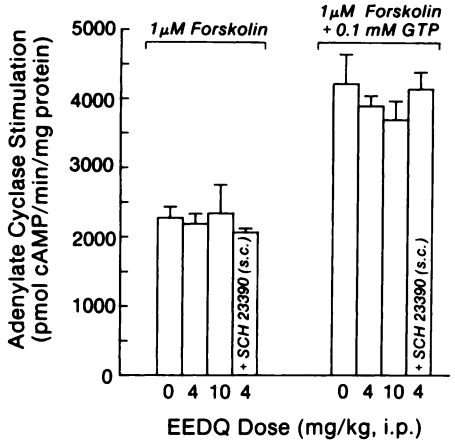

Fig. 2. Effect of *in vivo* EEDQ administration on forskolin-stimulated adenylate cyclase activity. *Bars* represent the mean + standard error of differences between basal activity and 1 μM forskolin (±0.1 mM GTP)-stimulated cAMP production (n = 4–6). The *4 mg/kg + SCH 23390 (s.c.) bars* denote subcutaneous treatment with 0.5 mg/kg SCH23390 prior to 4 mg/kg EEDQ treatment. No significant differences in stimulation were observed between control and EEDQ-treated tissue.

trol and EEDQ-treated animals (Fig. 2). Additionally, the synergistic stimulation of adenylate cyclase by forskolin (1 μM) + GTP (0.1 mM) was unaltered by EEDQ administration (Fig. 2). Control data reported include determinations from both vehicle-injected animals and those animals pretreated subcutaneously with 0.5 mg/kg SCH23390 prior to vehicle injection since there was no significant difference between these two groups.

Reductions in D_1 agonist-stimulated adenylate cyclase activity and D_1 dopamine receptor binding after peripheral administration of EEDQ. [^{3}H]SCH23390 binding was markedly reduced by *in vivo* EEDQ treatment in a dose-dependent manner wherein, even at the lowest dose of EEDQ investigated (0.5 mg/kg), a significant reduction in the [^{3}H]SCH23390 B_{max} was observed (Figs. 3 and 4). However, the K_D of [^{3}H]SCH23390 binding did not change from control values

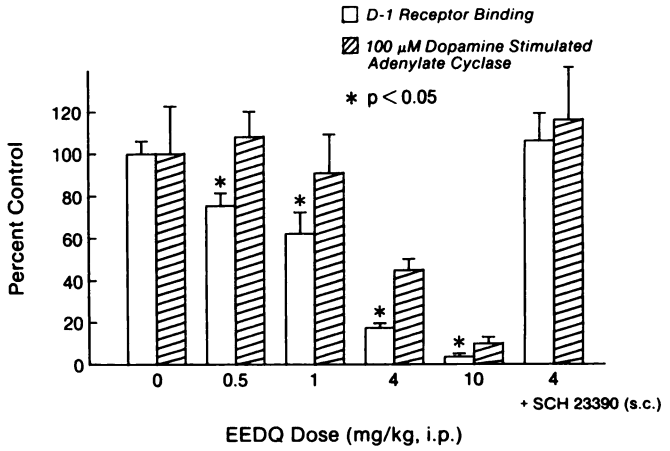

Fig. 3. Dose-dependent reduction in D_1 dopamine receptor binding and receptor-mediated stimulation of adenylate cyclase activity after peripheral administration of EEDQ. □, mean + standard error of [^{3}H]SCH23390 B_{max} values as a percentage of the control B_{max} (47 ± 7 pmol/g of tissue); ▨, mean + standard error of the differences between stimulation by 0.1 mM GTP alone and stimulation by 100 μM dopamine + 0.1 mM GTP as a percentage of stimulation in controls (186 ± 45 pmol of cAMP/min/mg of protein). EEDQ-induced reductions were prevented by subcutaneous pretreatment with SCH23390 (0.5 mg/kg), denoted by the *bar* labeled + *SCH 23390 (s.c.)*. *, percentage of control B_{max} levels which are significantly less than the percentage of control dopamine-stimulated adenylate cyclase activity, $p < 0.05$, *a posteriori* analysis of variance test.

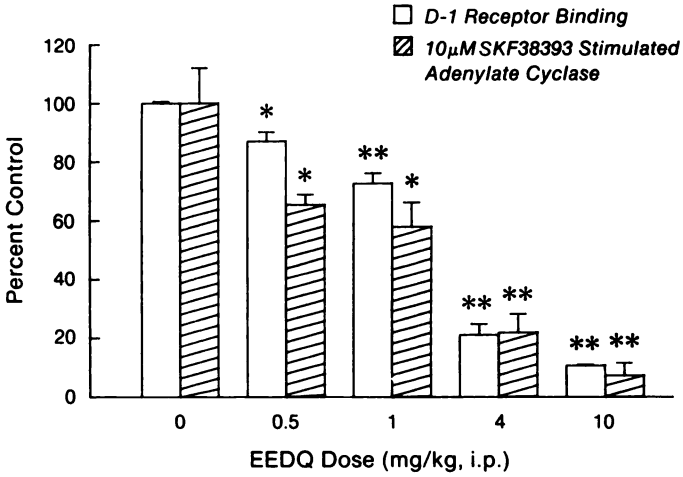

Fig. 4. Dose-dependent reduction in D_1 dopamine receptor binding and partial agonist (SKF38393)-stimulated adenylate cyclase activity following peripheral administration of EEDQ. □, mean + standard error (n = 4–5) of [^{3}H]SCH23390 B_{max} values as a percentage of the control B_{max} (46 ± 3.0 pmol/g of tissue); ▨, mean + standard error (n = 4–5) of SKF38393 stimulation of adenylate cyclase activity (i.e., the difference between stimulation by 0.1 mM GTP alone and stimulation by 10 μM SKF38393 + 0.1 mM GTP) at each EEDQ dose as a percentage of control stimulation by SKF38393 in non-EEDQ-treated rats (166 ± 19 pmol of cAMP/min/mg of protein). Values significantly less than control values are designated by: *, $p < 0.05$; **, $p < 0.005$, Student's t test.

(0.43 ± 0.18 nM) at any dose of EEDQ (Fig. 5). As we and others have previously demonstrated (19, 20) EEDQ-induced reductions in [^{3}H]SCH23390 B_{max} could be prevented by pretreating rats with a catalepsy-inducing dose of SCH23390 (0.5 mg/kg) prior to the 4 mg/kg EEDQ injection (Fig. 3).

The V_{max} of receptor-mediated adenylate cyclase stimulation was assessed by conducting dose-response curves in parallel for dopamine (1 μM–1 mM) or the partial agonist SKF38393 (10 nM–10 μM) (Fig. 6). It is apparent from Fig. 6 that, when

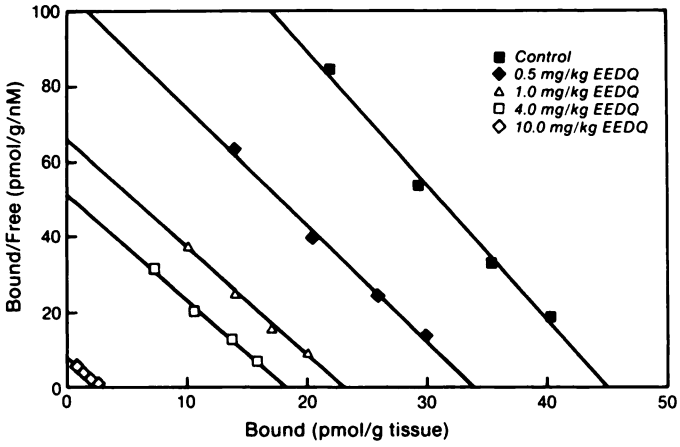

Fig. 5. Scatchard analyses of [^{3}H]SCH23390 binding following peripheral administration of various doses of EEDQ. Data represent single experiments in which the concentration of [^{3}H]SCH23390 ranged from 0.125 to 2 nM and the percentage of specific binding was 90–95%.

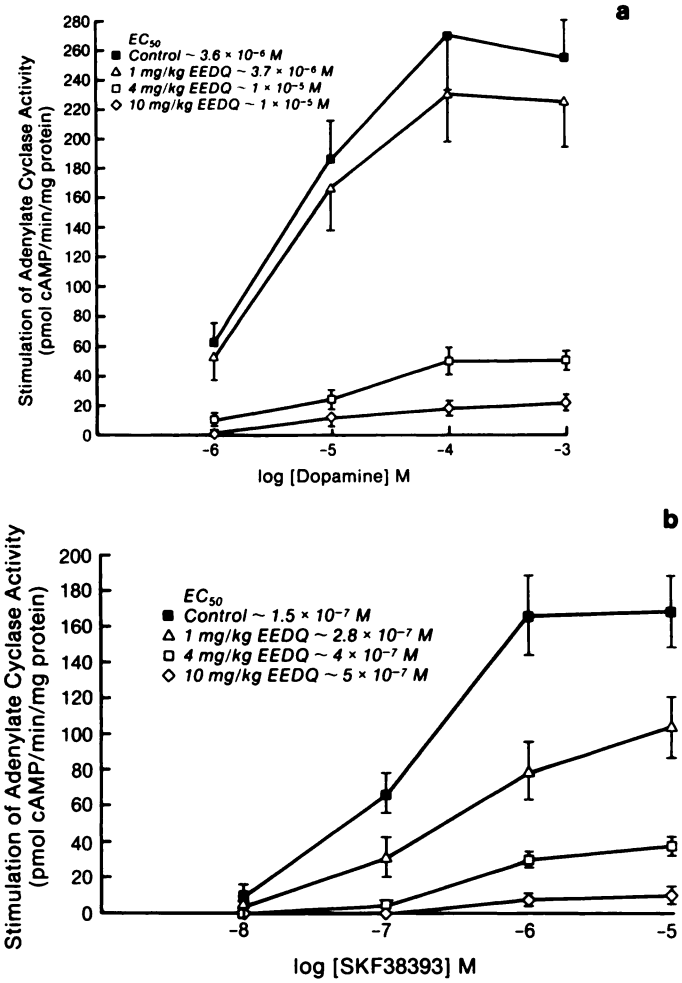

Fig. 6. Concentration-dependent stimulation of D_1 receptor-mediated adenylate cyclase activity by dopamine and SKF38393 performed in parallel following *in vivo* EEDQ administration. Each *point* represents the mean ± standard error (n = 4–5) of the stimulation by dopamine (a) or the partial agonist SKF38393 (b) above the stimulation elicited by 100 μM GTP.

dopamine-stimulated and SKF38393-stimulated adenylate cyclase activity was performed in parallel, SKF38393 was a partial agonist with a V_{max} approximately 60% of the V_{max} of dopamine (Fig. 6), consistent with our previous report (21). Maximal stimulation of adenylate cyclase activity elicited by agonists was also markedly reduced by EEDQ in a dose-dependent manner, whereas the EC_{50} values for these agonists shifted rightward approximately 3-fold at the highest dose of EEDQ. However, unlike the observed reductions in receptor binding, the V_{max} of dopamine-stimulated adenylate cyclase activity was not significantly affected at lower doses of EEDQ (Fig. 3). In fact, at doses of 0.5 and 1 mg/kg EEDQ, dopamine-stimulated adenylate cyclase did not differ significantly from control levels. By contrast, the B_{max} of [^{3}H]SCH23390 binding at these doses was significantly reduced from control levels by 25 ± 6% and 38 ± 10%, respectively. Additionally, the percentage reduction in dopamine-stimulated adenylate cyclase was significantly less than the percentage reduction in D_1 receptor binding at all doses of EEDQ. In concert with the protection of D_1 dopamine receptor binding, pretreatment with SCH23390 protected against the effects of EEDQ-induced reductions in dopamine-stimulated adenylate cyclase activity (Fig. 3). The tissues utilized for the experiment illustrated in Fig. 3 were also utilized for the experiments illustrated in Figs. 1 and 2. Due to tissue availability, dopamine dose response curves for adenylate cyclase activity were undertaken in a different group of animals, accounting for the variability in the V_{max} of dopamine-stimulated adenylate cyclase activity (Figs. 3 and 6a).

In contrast to the effects of EEDQ on dopamine-stimulated adenylate cyclase activity, reductions in adenylate cyclase activity stimulated by 10 μM SKF38393, a partial agonist (21), paralleled the reductions in [^{3}H]SCH23390 binding, i.e., a small reduction in receptor density was reflected by a reduction in SKF38393-stimulated adenylate cyclase activity (Fig. 4). At all doses of EEDQ, D_1 receptor binding and SKF38393-stimulated adenylate cyclase were significantly lower than control values. At a dose of 0.5 mg/kg EEDQ, SKF38393-stimulated adenylate cyclase activity was reduced by 35 ± 7% and [^{3}H]SCH23390 binding was reduced by 23 ± 7%. The reductions in SKF38393-stimulated adenylate cyclase were comparable to the reductions in D_1 receptor binding at all other doses of EEDQ (Fig. 4).

Effect of forskolin on full agonist- and partial agonist-stimulated adenylate cyclase activity after *in vivo* EEDQ administration. Ten μM forskolin was used to potentiate cAMP production in EEDQ-treated tissue. Tissue utilized in these experiments was the *same* tissue assayed in Fig. 4 with the experiments performed simultaneously. In the presence of 10 μM forskolin the stimulation by the partial agonist SKF38393 (11,180 ± 1,295 pmol of cAMP/min/mg of protein) was comparable to that of the full agonist dopamine (11,352 ± 1,293 pmol of cAMP/min/mg of protein). Interestingly, no difference was observed between maximal adenylate cyclase stimulation by the full agonist dopamine and the partial agonist SKF38393 in the presence of forskolin at *any* dose of EEDQ (Fig. 7). Thus, the EEDQ-induced reductions in the stimulation produced by SKF38393 in the presence of forskolin paralleled the pattern of EEDQ-induced reductions of adenylate cyclase stimulated by 100 μM dopamine in the absence of forskolin (Fig. 3): no reduction in receptor-stimulated cAMP production was observed at 0.5 mg/kg or 1 mg/kg EEDQ for either agonist (Fig. 7), although [^{3}H]SCH23390 binding was significantly

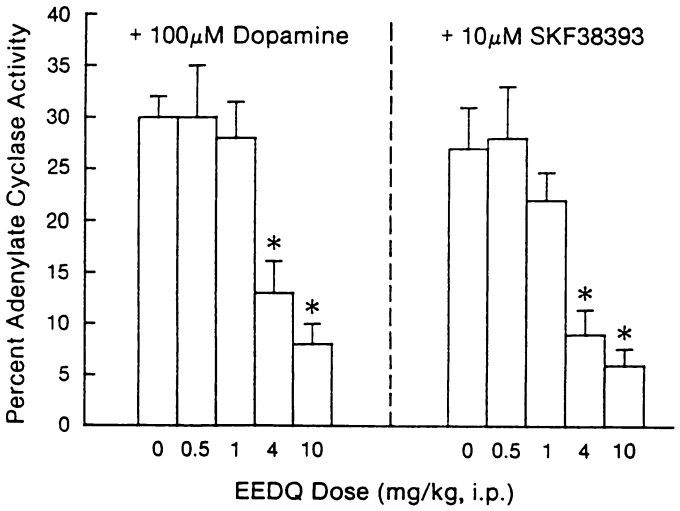

Fig. 7. Reductions in D_1 dopamine receptor-mediated stimulation of adenylate cyclase activity by maximal concentrations of dopamine and SKF38393 potentiated by 10 μM forskolin following *in vivo* EEDQ administration. □, mean + standard error from four to five experiments of the percentage of increase in stimulation by dopamine or SKF38393 in the presence of 0.1 mM GTP + 10 μM forskolin over stimulation by 0.1 mM GTP + 10 μM forskolin (8753 ± 989 pmol of cAMP/min/mg of protein). Values significantly less than control values are designated by *, $p < 0.05$, Student's *t* test.

reduced at these doses as seen in Fig. 4. Significant reductions from control levels in agonist-stimulated, forskolin-potentiated cAMP production were observed at a dose of 4 mg/kg and 10 mg/kg EEDQ for both agonists (Fig. 7).

Dopamine-stimulated adenylate cyclase activity and dopamine competition for [^{3}H]SCH23390 binding performed in parallel. Detailed dose response curves for dopamine-stimulated adenylate cyclase activity and detailed dopamine competition curves for [^{3}H]SCH23390-labeled D_1 dopamine receptors were performed utilizing four concentrations of dopamine per log unit under comparable conditions in untreated control tissue. Maximal receptor-mediated stimulation of adenylate cyclase was consistently elicted by 60 μM dopamine with an EC_{50} = 2.67 ± 0.27 μM (n = 3). We have previously reported that when dopamine competition for [^{3}H]SCH23390 binding is performed in the presence of GTP in 50 mM Tris-HCl, 5 mM $MgSO_4$, 0.5 mM EDTA buffer, the competition curve is biphasic, consisting of a high and a low affinity agonist binding site (9). Interestingly, when dopamine competition for [^{3}H]SCH23390 binding was performed under conditions comparable to those of the adenylate cyclase assay, the competition curve was monophasic and exhibited pseudo-Hill coefficients of approximately 1. Analysis of such curves with LIGAND demonstrated that they were composed entirely of low affinity agonist-binding sites with a K_i for dopamine of 15.2 ± 0.1 μM. Thus, to determine the percentage of fractional occupancy of D_1 receptors labeled by [^{3}H]SCH23390 at that concentration of dopamine which elicits maximal stimulation of adenylate cyclase activity, occupancy was assessed by the equation: $1/1 + (K_D/[D])$, where $[D] = 60$ μM dopamine and K_D of dopamine = 15.2 μM. Although maximal stimulation of receptor-mediated adenylate cyclase activity occurs at 60 μM dopamine, this same concentration of dopamine occupies only 80% of [^{3}H]SCH23390-binding sites.

Effect of *in vivo* EEDQ administration on dopamine competition for [^{3}H]SCH23390 binding. Although EEDQ treatment induced a dose-dependent reduction in [^{3}H]SCH23390 binding, this loss in D_1 dopamine receptors had no significant effect on the characteristics of dopamine's inhibition of [^{3}H]SCH23390 binding in non-cyclase buffer (Table 1). EEDQ administration did not change the dissociation constants of dopamine for either the high (K_H) or low (K_L) affinity sites. Likewise, the proportion of high affinity (R_H) and low affinity (R_L) agonist-binding sites was not altered following EEDQ treatment. Tissue from rats treated with 10 mg/kg EEDQ was not utilized for competition experiments due to the extremely low levels of specific [^{3}H]SCH23390 binding in this tissue.

Effects of *in vitro* EEDQ treatment on adenylate cyclase activity. Initially, we found that the extensive *in vitro* tissue preparation required to wash out the EEDQ after *in vitro* exposure reduced basal adenylate cyclase activity in control tissue to 20% of the basal activity observed in tissue prepared as described for *in vivo* EEDQ treatment experiments. To preclude this loss in enzyme activity, we found that if ATP was added to the *in vitro* tissue preparation, prior to the 10-min incubation at 37°, control tissue adenylate cyclase activity was preserved in a dose-dependent manner with 1 mM ATP preserving up to 80% of the enzyme activity (data not shown). Results similar to those reported below were obtained in preparations where the addition of 1 mM ATP was omitted. However, the marked reduction in basal adenylate cyclase activity after this extensive preparation without ATP did not allow for a clear determination of the effects of *in vitro* EEDQ treatment on basal activity.

Guanine nucleotide-, NaF-, and forskolin-stimulated cAMP production were again used as "markers" to assess functional modification of N_s or the catalytic subunit by EEDQ. As shown in Table 2, *in vitro* treatment with EEDQ induced a dose-dependent decrease in basal adenylate cyclase activity and forskolin-stimulated adenylate cyclase activity. Additionally, *in vitro* EEDQ treatment also resulted in a dose-dependent reduction in [^{3}H]SCH23390 binding. Because the catalytic subunit was directly modified by EEDQ, it is difficult to assess the functional integrity of N_s (i.e., GTP and NaF stimulation of cAMP production) since N_s function in these studies is assessed by catalytic subunit activation (i.e., via cAMP production). However, it is of interest to note that the percentage of adenylate cyclase stimulation by GTP or NaF over basal adenylate cyclase activity and the percentage of adenylate cyclase stim-

TABLE 1

Effect of *in vivo* EEDQ administration on dopamine competition for [^{3}H]SCH23390 binding

Computer-modeled parameters are given for dopamine inhibition of 0.25 nM [^{3}H]SCH23390 binding in rat striatum after intraperitoneal EEDQ treatment. Competition curves were best fit by a two-site computer-derived model where the affinity of [^{3}H]SCH23390 was constrained to be equal (K_D = 0.5 nM) at both high (R_H) and low (R_L) agonist affinity sites. The dissociation constants for high (K_H) and low (K_L) affinity agonist-binding sites were determined while constraining the nonspecific binding parameter defined by 100 nM *cis*-flupentixol. Competition data represent means ± standard errors from four independent experiments.

EEDQ dose	K_H	K_L	R_H	R_L	Control-specific [^{3}H]SCH23390 binding
mg/kg	nM	nM	%	%	%
0	165 ± 22	4062 ± 543	46 ± 3	54 ± 3	100 ± 7.2
0.5	143 ± 12	4270 ± 201	48 ± 1	52 ± 1	93.2 ± 1.5
2.5	146 ± 55	3499 ± 962	37 ± 5	63 ± 5	40.6 ± 5.2
4.0	131 ± 9	3569 ± 827	44 ± 2	56 ± 2	14.2 ± 1.0

TABLE 2

***In vitro* EEDQ-induced reductions in rat striatal adenylate cyclase activity and [^{3}H]SCH23390 binding**

Each value for adenylate cyclase assays represents the mean ± standard error of a single representative experiment with triplicate determinations. [^{3}H]SCH23390 binding values represent data from saturation studies utilizing five concentrations of [^{3}H]SCH23390. Scatchard analyses were performed on the same tissue as that employed in the adenylate cyclase assay. Experiments were performed three times yielding variable levels of control basal enzyme activity due to the extensive tissue preparation. For each experiment, comparable percentage reductions were observed in basal and 10 μM forskolin-stimulated adenylate cyclase activity, and in [^{3}H]SCH23390 binding after *in vitro* EEDQ treatment.

EEDQ	Adenylate cyclase Activity		[^{3}H]SCH23390 binding	
	Basal	+10 μM Forskolin	K_D	B_{max}
M	pmol cAMP/min/mg protein		nM	pmol/g tissue
0	80 ± 10	2263 ± 35	0.33	41.6
10^{-6}	69 ± 1 (86)[a]	2150 ± 38 (95)	0.39	36.1 (86)
10^{-5}	60 ± 5 (75)	1448 ± 27 (64)	0.37	28.7 (69)
10^{-4}	29 ± 1 (36)	402 ± 7 (18)	0.47	10.9 (26)
10^{-3}	0 (0)	0 (0)	ND[b]	ND

[a] Values in parentheses represent the percentage of control levels of adenylate cyclase activity or [^{3}H]SCH23390 binding after *in vitro* EEDQ treatment.
[b] ND, no detectable binding.

ulation by GTP and forskolin over forskolin-stimulated adenylate cyclase activity were comparable in control and EEDQ-treated tissues (data not shown).

Discussion

These results suggest that peripherally administered EEDQ acts in a receptor-specific manner with respect to striatal D_1 dopamine receptors, and leaves the guanine nucleotide-binding protein and catalytic subunits functionally intact. This is reflected in the dramatic decreases observed in adenylate cyclase stimulation by 100 μM dopamine and D_1 dopamine receptor binding of [^{3}H]SCH23390 after *in vivo* EEDQ treatment without reduction in GTP, NaF, or forskolin stimulation of adenylate cyclase activity. That receptor binding modification and reduction in dopamine stimulation of adenylate cyclase by EEDQ can be prevented by pretreatment with the D_1 receptor antagonist SCH23390 indicates that these modifications occur at the receptor recognition site.

The lack of N_s or catalytic subunit modification by EEDQ may be explained in several ways. The mechanism of action of EEDQ is thought to occur via peptide carboxyl group activation. It is possible that neither subunit has carboxyl groups at the active site, making N_s and the catalytic subunit "resistant" to EEDQ-induced modification. However, this is unlikely, not only because EEDQ is a highly reactive molecule, with the capacity for modifying enzymes such as serine proteases (14) as well as receptors, but also because many enzymes analyzed to date have been shown to contain carboxyl groups at the active sites [e.g., lysozyme and carboxypeptidase (22, 23)]. Furthermore, in phosphoric ester hydrolysis, where proton exchanges occur, carboxyl groups act as base catalysts. Alternatively, a more feasible explanation is that peripherally administered EEDQ does not modify N_s or the catalytic subunit because it does not have access to these moieties. That is, neither N_s nor the catalytic subunit of adenylate cyclase contains extracellularly exposed carboxyl groups, making EEDQ-induced modifications sterically impossible. Implicit in this contention is that EEDQ cannot penetrate the cell membrane; hence, *in vivo* administration of EEDQ may not directly modify intracellular function.

This contention is further supported by the *in vitro* EEDQ data presented here. Because *in vitro* EEDQ treatment resulted in D_1 dopamine receptor modification similar to that observed *in vivo*, the mode of action of EEDQ *in vitro* is probably similar, if not identical, to that which occurs *in vivo*. However, EEDQ did modify the catalytic subunit of adenylate cyclase *in vitro*, suggesting that the catalytic subunit contains moieties with which EEDQ may interact. In fact, these carboxyl groups appear to be as sensitive to EEDQ-induced alterations as those carboxyl groups modified at the receptor, since comparable percentage reductions in binding and adenylate cyclase activity occurred for each concentration of EEDQ utilized *in vitro* (Table 2). Additionally, the pseudo-IC_{50} values of EEDQ for receptor and adenylate cyclase modification were comparable, with 50% D_1 receptor binding inhibited and 50% adenylate cyclase activity inhibited at approximately 50 μM EEDQ. That the catalytic subunit of adenylate cyclase is modified by EEDQ *in vitro* but not *in vivo* suggests that EEDQ does not have access to these carboxyl groups unless the tissue is homogenized exposing both inner and outer cellular membranes to EEDQ.

In vivo EEDQ treatment did not alter the stimulation of striatal adenylate cyclase activity by guanine nucleotide, regulatory protein, or catalytic subunit reagents. Because the N_s and catalytic moieties are not functionally modified, peripheral EEDQ administration may be used as a tool to assess the stoichiometry of receptor/effector interactions. That is, by progressively blocking increasing numbers of receptors and monitoring receptor-linked adenylate cyclase activity, the loss of D_1 dopamine receptors may be correlated with decreases in D_1 dopamine receptor-mediated stimulation of adenylate cyclase activity. Interestingly, in the data presented here, the loss in D_1 dopamine receptor binding did not correlate directly with observed reductions in dopamine-stimulated adenylate cyclase activity; approximately 40% of D_1 dopamine receptor binding could be lost with no significant reduction in the V_{max} of dopamine-stimulated adenylate cyclase activity. That is, only approximately 60% of the original D_1 dopamine receptor population was necessary for full *in vitro* agonist stimulation of adenylate cyclase activity.

Paralleling these results, when [^{3}H]SCH23390 radioligand binding and adenylate cyclase assays were performed under comparable conditions, the concentration of dopamine which elicited maximal adenylate cyclase activity (60 μM) inhibited only 80% of [^{3}H]SCH23390 binding. These results again suggest that all D_1 dopamine receptors need not be occupied by dopamine for maximal receptor-stimulated cAMP production. Thus, these data suggest that the D_1 dopamine receptor population is not a stoichiometrically limiting factor in agonist stimulation of adenylate cyclase.

Interestingly, the EEDQ-induced reduction in D_1 dopamine receptor density had no effect on the distribution of high and low affinity agonist binding sites. Although there exist "spare" D_1 dopamine receptors, the percentage of receptors coupled with N_s (hypothesized as represented by R_H, high affinity agonist binding sites in competition experiments) remains constant even after the "spare" receptor pool is reduced. Apparently, then, the proportion of N_s-coupled receptors is independent of the actual receptor density. This indicates that either the population of N_s or the equilibrium established between the receptor and N_s (i.e., the "affinity" of N_s for the receptor) or

both of these may be the stoichiometrically limiting factors in dopamine stimulation of adenylate cyclase activity.

It is important to note that the K_L of dopamine competition for [^{3}H]SCH23390 binding performed under adenylate cyclase conditions is approximately 3–4 times higher than the K_L obtained when the competition is performed in 50 mM Tris-HCl, 5 mM $MgSO_4$, 0.5 mM EDTA (henceforth referred to as radioligand binding buffer). However, the adenylate cyclase medium had no effect on the affinity (K_D) of [^{3}H]SCH23390 for D_1 dopamine receptors as assessed by saturation analysis (data not shown). Additionally, competition curves performed under adenylate cyclase conditions which include GTP are monophasic, composed entirely of low affinity agonist-binding sites. We have previously reported (9) that the addition of GTP to dopamine competition for [^{3}H]SCH23390 binding performed in radioligand binding buffer results in only a partial conversion to low affinity agonist-binding sites with 10–20% of the curve still consisting of high affinity agonist-binding sites. No significant effect of GTP was observed on the affinity of dopamine for the high (K_H) or the low (K_L) affinity agonist-binding states. Thus, the adenylate cyclase assay medium apparently contains a reagent or a combination of reagents which promotes complete conversion to the low affinity agonist-binding state of the receptor and which also decreases the affinity of dopamine for the receptor.

We have previously provided evidence supporting the "spare" receptor hypothesis by assessing the time-dependent recovery of [^{3}H]SCH23390 binding and dopamine-stimulated adenylate cyclase activity following peripheral administration of EEDQ (24). Although dopamine-stimulated enzyme activity returned to control values by recovery day 4, the D_1 dopamine receptor density remained significantly below control levels at this time. Additionally, at all recovery times, the percentage reduction in maximal dopamine-stimulated cAMP production was significantly less than the percentage reduction in D_1 dopamine receptor density. Thus, the stoichiometric ratio between recovering D_1 dopamine receptors and dopamine-stimulated adenylate cyclase activity was not 1:1, and there exists a receptor reserve of D_1 dopamine receptors for the natural neurotransmitter.

In contrast to the results observed with dopamine-stimulated adenylate cyclase activity following EEDQ treatment, a small reduction in the D_1 dopamine receptor density resulted in concomitant reductions in SKF38393-stimulated enzyme activity. Since we have previously demonstrated that SKF38393 is a partial agonist at D_1 dopamine receptors (21), these results are not surprising. As a partial agonist, SKF38393 would require a high fractional occupancy to stimulate cAMP production. However, even at high concentrations, SKF38393 did not produce a maximal response equivalent to that produced by dopamine; consequently no spare receptors are present for SKF38393. Thus, any reduction in D_1 receptor density will be reflected in a reduction in partial agonist-mediated enzyme activity, as was demonstrated herein. These results also demonstrate that the "spare" receptor data obtained utilizing the full agonist, dopamine, was not a product of our methods but rather represents the true enzyme-activating properties of the agonist.

It has been suggested that [^{3}H]SCH23390 identifies binding sites with a pharmacological profile that is similar to that of D_1 dopamine receptors linked to the stimulation of adenylate cyclase but which are not linked to this second messenger system (25, 26), i.e., not "spare" receptors as we propose. However, EEDQ must modify both putative receptor populations as >90% of [^{3}H]SCH23390-binding sites are lost after 10 mg/kg EEDQ administration. If indeed there were no "spare" receptors but a distinct population of non-cyclase-linked receptors, then "spare" receptors would still have been observed using the partial agonist SKF38393 after low dose EEDQ administration as both cyclase-linked and unlinked receptors would both still be present. This is clearly not observed.

Paralleling the present results, Meller *et al.* (27) have recently demonstrated that a large D_2 dopamine autoreceptor reserve exists in rat striata. This study also utilized EEDQ to irreversibly block D_2 dopamine receptors and assessed apomorphine reversal of γ-butyrolactone-induced L-DOPA accumulation. Like the results presented herein, these investigators found that maximal reversal by a full agonist could be maintained after irreversible blockade of a significant portion of the total receptor population. However, maximal reversal by partial agonists was reduced concomitantly with EEDQ-induced blockade of the receptors.

Interestingly, when adenylate cyclase activity stimulated by SKF38393 or dopamine is assessed in the presence of forskolin, the partial agonist appears to simulate the properties of the full agonist. That is, in the presence of forskolin, SKF38393 and dopamine stimulate cAMP production to the same levels, regardless of the degree of receptor reduction induced by EEDQ. Additionally, 40% of D_1 receptors may be lost with no significant reduction in *either* dopamine or SKF38393-stimulated adenylate cyclase activity. Forskolin appears to synergistically influence the interaction of the partial agonist-bound receptor with the adenylate cyclase moiety, potentiating the relative efficacy of SKF38393 and altering the functional constraints imposed on partial agonists. Thus, in the presence of forskolin there exist "spare" receptors for both dopamine and SKF38393. It is unlikely that forskolin interacts with the receptor recognition site directly to alter these constraints as 10 μM forskolin has no effect on [^{3}H]SKF38393 binding in the presence or absence of GTP (data not shown). That forskolin alters these constraints may be attributed solely to potentiation of the catalytic subunit. Alternatively, forskolin may interact directly with N_s to effect these alterations by potentiating the interactions between N_s and the catalytic subunit. In fact, we have previously demonstrated that forskolin acts synergistically with N_s modulators (i.e., GTP, NaF) to greatly stimulate adenylate cyclase activity (21). However, the mechanism responsible for this phenomenon is unclear as N_s is not retained on forskolin affinity columns (28).

The findings presented herein have important neurophysiologic implications. Receptor occupancy is determined by transmitter (or drug) concentration. Since only ~60% occupancy of the normal D_1 dopamine receptor population is required for maximal effector stimulation, the dopamine concentration necessary to elicit full stimulation (~4 × K_D) is considerably less than that concentration necessary for 100% receptor occupancy (100 × K_D). The existence of these "spare" receptors then potentially speeds neurotransmission by reducing the effective concentration of dopamine required to diffuse across the synaptic cleft to achieve a full response.

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