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Tight binding dopamine reuptake inhibitors as cocaine antagonists

A strategy for drug development

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The experiments reported in this study tested the hypothesis that tight binding dopamine (DA) reuptake inhibitors might act as cocaine antagonists. Binding studies demonstrated that the high affinity dopamine reuptake inhibitor, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine (GBR12909) produced a wash-resistant inhibition of the DA transporter in rat striatal membranes as labeled by [^{3}H]cocaine or [^{3}H]1-[2-(diphenyl-methoxy)ethyl]-4-(3-phenylpropyl)piperazine ([^{3}H]GBR12935), indicative of tight binding. In vivo microdialysis experiments showed that administration of 25 mg/kg GBR12909 to rats produced a modest, but not statistically significant, increase in the extracellular levels of striatal DA, while this same dose of GBR12909 inhibited the ability of cocaine to elevate extracellular DA levels by 64%. These data suggest that tight binding DA reuptake blockers may provide a fruitful approach for developing a cocaine antagonist.

Cocaine; Dopamine reuptake inhibitor; Microdialysis, in vivo; Dopamine

1. INTRODUCTION

Cocaine, a major drug of abuse, is thought to produce its effects primarily by inhibiting the reuptake of biogenic amines [1]. Although cocaine inhibits the reuptake of norepinephrine and serotonin, its ability to inhibit the reuptake of dopamine (DA) is thought to be the main neurochemical mechanism responsible for its addictive and euphorogenic properties [2]. Several lines of evidence support the DA hypothesis, including a major role of dopaminergic systems in reward mechanisms [3] and a correlation between the potency of cocaine-like drugs to inhibit DA reuptake and their potency as self-administered agents in animals [2].

However, not all DA reuptake inhibitors are abused by humans. For example, mazindol, nomifensine and bupropion are clinically effective antidepressants which have not been reported to have euphorogenic effects in humans [4-8]. Moreover, benztropine, a drug widely prescribed for treatment of Parkinson's disease and for treatment of extrapyramidal side-effects resulting from administration of antipsychotic drugs, is also a potent inhibitor of DA reuptake [9] which is not reported to have cocaine-like effects in humans [10]. Interestingly, whereas mazindol and nomifensine are self-administered by animals [11-13], bupropion is not [14]. To reflect these differences, we term DA-reuptake inhibitors abused by humans 'type 1', and those not abused 'type 2'.

The ability of agents to inhibit DA reuptake in vitro or be self-administered by animals does not always predict their abuse liability in humans [4-8]. Published data suggest that their ability to elevate extracellular levels of DA (ECDA) in vivo does [15]. For example, cocaine and amphetamine, as well as other drugs of abuse, produce large elevations in ECDA as measured by the technique of in vivo microdialysis [15]. In contrast, type 2 agents such as nomifensine and benztropine produce much lower and inconsistent elevations of ECDA levels [16].

Based on these data, we hypothesized that, defining elevation of ECDA as the dependent variable (which does not specify a mechanism of action), type 1 and type 2 agents are full and partial agonists, respectively. A direct prediction of this hypothesis is that occupation of the DA reuptake complex by a type 2 reuptake inhibitor should attenuate the effects of cocaine, and potentially be useful in humans as antagonists of type 1 agents. A competitive antagonist would be of limited value, since a person would simply self-administer more of the type 1 agent, overcoming the inhibition, and in-

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Abbreviations: GBR12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine; GBR12935, 1-[2-(diphenyl-methoxy)-ethyl]-4-(3-phenylpropyl)piperazine; DA, dopamine; ECDA, extracellular dopamine; aCSF, artificial CSF

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creasing the risk of peripheral side-effects such as cardiac arrhythmias. Alternatively, administration of a tight binding (slowly dissociating) type 2 agent should produce an insurmountable (noncompetitive) inhibition over the time period which it remains bound to the reuptake complex.

To test this hypothesis, we chose GBR12909, a potent DA reuptake inhibitor [9] which does not by itself produce large elevations in ECDA [17]. Its high affinity for the DA reuptake complex [9] and our experiments suggesting that it binds tightly to the DA reuptake complex [18] led us to select it as a prototypic type 2 agent. The results reported here demonstrate that administration of GBR12909 to rats: (i) produces a pseudoirreversible inhibition of [^{3}H]cocaine and [^{3}H]GBR12935 binding to the DA transporter assayed using striatal membranes in vitro; and (ii) antagonizes the ability of cocaine to elevate ECDA in vivo.

2. MATERIALS AND METHODS

2.1. *Preparation of striatal membranes*

GBR12909 (25 mg/kg) dissolved in dimethylsulfoxide (DMSO)/saline was administered i.p. to 3 male Sprague-Dawley rats while 3 other control rats received vehicle. One hour later the rats were sacrificed and the caudate nuclei dissected and stored at −70°C. On the day of assay, caudate nuclei were homogenized with a polytron in ice-cold 55.2 mM sodium phosphate buffer, pH 7.4 (buffer 1). The homogenate was centrifuged for 10 min at 39000 × *g*, at 4°C. The pellets were resuspended in ice-cold buffer and recentrifuged. The pellets were then resuspended in ice-cold buffer 1 for the [^{3}H]GBR12935 binding assay (approximately 100 μg protein/ml), or with ice-cold 25 mM sodium phosphate buffer, pH 7.4, for the [^{3}H]-cocaine binding assay (approximately 1.2 mg protein/ml).

2.2. *Ligand binding assays*

To label [^{3}H]cocaine binding sites [19], striatal membranes were incubated in 12 × 75 mm polystyrene test tubes for 20–60 min at 0°C (equilibrium) in 25 mM sodium phosphate buffer, pH 7.4, with 10 nM [^{3}H]cocaine (spec. act. = 28.5 Ci/mmol) in a final vol. of 1 ml. The nonspecific binding was determined using 10 μM cocaine. Incubations were terminated by rapid filtration, using a single manifold, over Whatman GF/B filters presoaked in 1% polyethylenimine, followed by a wash with 7 ml ice-cold 25 mM phosphate buffer, pH 7.4, containing 400 mM NaCl.

Binding sites associated with the DA transporter were also labeled with [^{3}H]GBR12935 [20]. The membrane suspensions were incubated in a final vol. of 1 ml for 2 h at 0°C (equilibrium) in 55 mM sodium phosphate buffer containing EDTA (10 μM), EGTA (10 μM), chymostatin (2.5 μg/ml) and leupeptin (2.5 μg/ml). Nonspecific binding was determined by incubations in the presence of 1 μM GBR12909. Incubations were terminated by rapid filtration through Whatman GF/B filters presoaked in 2% polyethylenimine.

2.3. *In vivo microdialysis*

Male Sprague-Dawley rats (300–350 g, Taconic Farms, MD) were anesthetized with chloral hydrate 400 mg/kg and placed in a stereotaxic frame. Anesthesia was maintained at a level throughout each experiment whereby corneal reflexes were abolished. A 3 mm microdialysis probe (Carnagie Medicine) was lowered into the striatum at stereotaxic coordinates of AP +9.9, ML +3.2, DV +3.0 [21]. Artificial CSF (aCSF) containing NaCl (8.6 g), KCl (0.3 g), $CaCl_2 \cdot 2H_2O$ (0.335 g) and ascorbic acid (0.004 g) in distilled water (1 liter) was pumped through the probe at a flow rate of 1.0 μl/min using a microinfusion pump (CMA/100). After the probe was inserted, 20 μl samples were collected every 20 min into polyethylene tubes containing 10 μl perchloric acid (1 M). Immediately after collection samples were injected into a high pressure liquid chromatography system and analyzed using electrochemical detection. After 2 h, 3 baseline samples were collected and then the pharmacological treatment was started.

Treatment consisted of either GBR12909 (25 mg/kg) or saline injected i.p. 1 h afterwards, the same rats were infused through the probe with cocaine (1 mM) dissolved in aCSF. After 20 min, perfusion of the probe with normal aCSF was resumed and samples were collected for an additional hour.

The HPLC system used in these studies included a BAS LC-4B electrochemical detector with a glassy carbon working electrode set at 0.730 V. A Brownlee 10 cm reversed-phase C_{18} column, and a mobile phase comprised of sodium acetate (2.0 g), citric acid (3.0 g), sodium octylsulphate (350 mg), EDTA (88 mg) and methanol (70 ml) in distilled water (1 liter) were used to isolate the DA in the samples.

2.4. *Statistics*

Statistical differences among groups in the ligand binding experiments were determined using the Student's *t*-test. In vivo microdialysis data were analyzed by one-way analysis of variance (ANOVA).

2.5. *Chemicals*

[^{3}H]GBR12935 (spec. act. = 30.5 Ci/mmol) and [^{3}H](−)-cocaine (spec. act. = 28.5 Ci/mmol) were purchased from New England Nuclear. Unlabeled (−)-cocaine was supplied by Dr Rapaka of NIDA. GBR12909-$(HCl)_2$ was synthesized in Laboratory of Medical Chemistry, NIDDK, as described [22]. Other reagents were purchased from Sigma Chemical Co.

3. RESULTS

In the first series of experiments, GBR12909 (25 mg/kg) was administered to rats. Caudates were dissected 60 min after the injection, and striatal membranes were prepared as described in section 2. As shown in fig.1, this dose of GBR12909 produced a

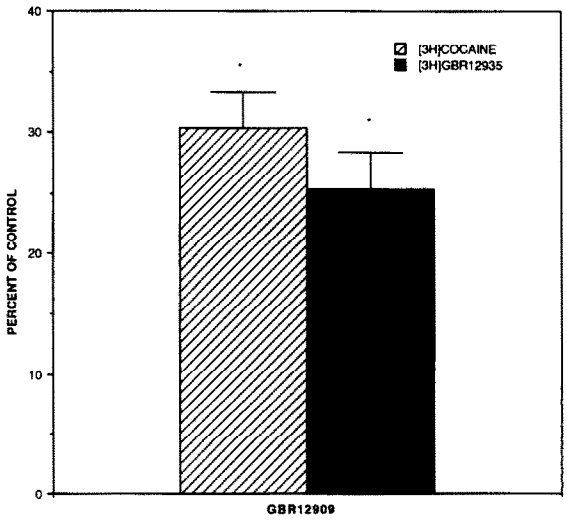

Fig.1. GBR12909 (25 mg/kg) or vehicle was administered to rats and, as described in section 2, striatal membranes were prepared 60 min later. The specific binding of 9.6 nM [^{3}H]cocaine and 2.5 nM [^{3}H]-GBR12935 was determined as described in section 2. The results are reported as the percent of control ± SD ($n = 3$). *$P < 0.001$ when compared to control.

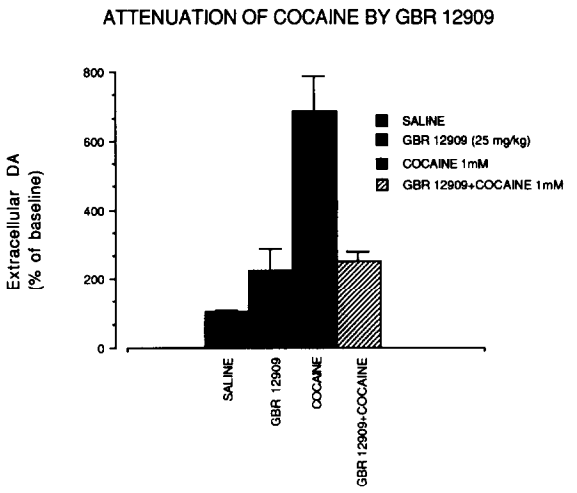

Fig.2. The effects of saline or GBR12909 (25 mg/kg) administered i.p. to rats on the levels of striatal extracellular DA produced by cocaine (1 mM) delivered through the microdialysis probe are reported above. The effect of a drug was calculated as a percent of the preexisting baseline value. One-way analysis of variance revealed a significant difference among treatment groups ($F = 15.6$, df 3/18, $P < 0.001$). Duncan's multiple range tests indicated that the increase in extracellular DA induced by cocaine was significantly different from that induced by saline ($P < 0.01$) as well as the GBR12909 + cocaine group ($P < 0.01$). Each value is the mean ± SE ($n = 5$).

74.7% and 69.6% inhibition of [^{3}H]GBR12935 and [^{3}H]cocaine binding, respectively. Subsequent experiments demonstrated that administration of GBR12909 (25 mg/kg) inhibited [^{3}H]GBR12935 binding by decreasing the B_{max} from 10900 to 5200 fmol/mg protein, and increasing the K_d from 2.0 to 6.6 nM. Moreover, washing membranes an additional two times by centrifugation and resuspension did not reverse these changes in the B_{max} or K_d (data not shown).

Administration of 25 mg/kg GBR12909 to rats produced a stable, modest, and long-lasting (at least 6 h) elevation of ECDA levels (221% of control) which did not achieve statistical significance. Administration of 100 mg/kg GBR12909 increased ECDA levels to 357 ± 179 (mean ± SD, $n = 3$) percent of control (data not shown). As reported in fig.2, administration of cocaine (1 mM) through the microdialysis probe increased ECDA levels 686% over baseline levels. However, in rats pretreated with GBR12909 (25 mg/kg), cocaine increased ECDA levels only 248% over the preexisting GBR12909-induced baseline, leading to the observed 63.8% reduction in the ability of cocaine to elevate ECDA levels.

4. DISCUSSION

Data presented in this study demonstrate that administration of GBR12909 to rats produces a wash-resistant inhibition of both [^{3}H]GBR12935 and [^{3}H]cocaine binding. Saturation binding studies with [^{3}H]-GBR12935 demonstrated that the inhibition was due to a decrease in the B_{max}, as well as an increase in the K_d. Further investigation revealed that the increase in the K_d was due to GBR12909 carried over into the assay, and that washing the membranes by repeated centrifugation and resuspension did not remove the GBR12909 (data not shown). Similar experiments were not done with [^{3}H]cocaine because of the large amounts of membrane protein required by that assay. Our interpretation of these results is that the decrease in the B_{max} results from GBR12909 tightly binding to the reuptake complex, and that the increase in the K_d results from free GBR12909. It is also possible that a higher fraction of binding sites were occupied in vivo, but not detected in vitro due to dissociation of GBR12909.

We found that administration of GBR12909 at a dose sufficient to tightly bind to about 50% of the DA transporters, produced modest increases in ECDA levels, which did not achieve statistical significance, and which were much lower than the increases in ECDA levels typically produced by cocaine. Consistent with persistent occupation of the DA transporter by GBR12909, the ECDA levels remained elevated for at least 6 h. Moreover, GBR12909 attenuated the ability of cocaine to increase ECDA levels. The occurrence of local anesthetic effects at higher cocaine concentrations complicates the generation of complete dose-response curves, thus it may not be possible to prove the non-competitive nature of the inhibition.

The ability of GBR12909 to antagonize the ability of cocaine to increase ECDA levels by binding tightly to the DA transporter suggests that it might also antagonize the actions of other drugs of abuse. For example, amphetamine must be transported by the DA reuptake complex into the DA nerve terminal to release DA. Similarly, to the extent that the psychotomimetic and addictive properties of phencyclidine (PCP) relate to inhibition of DA reuptake [23,24], GBR12909 may act as a PCP antagonist. Future experiments will test this hypothesis.

Psychopharmacological treatment of the cocaine abuser is a complex matter under investigation by many research groups. Although administration of antidepressants such as desipramine and mazindol [25–27] to humans helps attenuate the craving for cocaine, this effect takes several weeks to develop, and does not appear related to a direct antagonism of the cocaine. The demonstration that GBR12909 acts as a cocaine antagonist, in a system measuring the neurotransmitter considered to be the brain's addictive signal, suggests that it might act as a cocaine antagonist in humans, which would be of considerable value in the treatment of cocaine abuse and addiction. Even if GBR12909 were found to be unsuitable for use in humans, we believe that the strategy of using tight binding type 2 DA reuptake inhibitors represents a potentially fruitful approach for developing a cocaine antagonist.

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