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The dopamine D_1 receptor: partial purification of a digitonin-solubilized receptor–guanine nucleotide binding complex

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Dopamine D_1 receptors are known to be associated with dopamine-stimulated adenylate cyclase activity [1, 2] and, more recently, with a dopamine-adenosine 3′:5′-monophosphate regulated phosphoprotein [3]. More extensive *in vitro* pharmacological characterization of the dopamine D_1 receptor has been hampered by the poor selectivity of available radiolabeled antagonist ligands [4–6]. The recent development of the selective D_1 receptor antagonist, SCH-23390 [7, 8], and its radiolabeled analog [9, 10] has now made the molecular characterization of the receptor feasible. As a first step towards attaining this aim we have solubilized and characterized the striatal dopamine D_1 receptor [11]. Digitonin-solubilized receptor preparations bound [^{3}H]SCH-23390 with high affinity (5 nM) and retained all the pharmacological characteristics of membrane bound D_1 receptors. In this report, we document that: (a) the digitonin-solubilized D_1 receptor can be partially purified by lectin affinity chromatography, and (b) the partially purified receptor is closely associated with a guanine nucleotide-binding protein, presumably N_s*.

Methods

Materials. [^{3}H]SCH-23390 (7.78 Ci/mmole) was synthesized by Drs. I. Pri-Bar and O. Buchman of the Nuclear Research Center-Negev, Israel. Digitonin was obtained from the Sigma Chemical Co. (St. Louis, MO) and WAKO Chemicals (Dallas, TX). Sephadex G-50 (fine), WGA- and Con-A-Sepharose were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Dopamine, Gpp(NH)p and β-D-GlcNAc were from the Sigma Chemical Co., and (+)-butaclamol (BTC) was from Ayerst Research Laboratories (Montreal, Quebec).

Membrane preparation. Striata were dissected from partially thawed canine brains (PEL-Freez Biologicals, Rogers, AR) and homogenized (Brinkmann Polytron, setting no. 6, 20 sec) in 20 ml of 50 mM Tris–HCl buffer containing 1 mM EDTA (acid), 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$ and 120 mM NaCl (where indicated), pH 7.4, at 4°. All procedures were carried out at 0–4°. Homogenates were centrifuged for 20 min at 48,000 *g*; the resulting pellets were resuspended in an original volume of buffer and recentrifuged. Membrane pellets were resuspended in Tris–ion buffer (as above, buffer A) containing 1% digitonin (w/v), pH 7.4 (4°), with a membrane to detergent ratio of 100 mg tissue/10 mg digitonin per ml of solution. The suspension was stirred slowly for 30–40 min at 4° with the solution subsequently centrifuged for 60 min at 115,000 *g*. The supernatant fraction, containing soluble receptors, was aspirated and placed on ice for immediate use.

Binding of solubilized receptors to immobilized lectins. Soluble preparations (2 ml) were incubated for 90 min, with gentle mixing, at 4° with 0.8 ml of lectin-linked gel previously equilibrated with buffer A and 0.1% digitonin (1 hr). Sepharose 6B gel (containing no immobilized lectin) was used to determine the non-specific retention of receptors to gel alone. Following incubation, the gel was centrifuged and the supernatant fraction was decanted. Gels were washed subsequently with 3 × 35 ml of buffer A containing digitonin (0.1%) and resuspended in 2 ml of 0.1 M β-D-GlcNAc (WGA-Sepharose) or 0.1 M α-D-Man (Con-A-Sepharose) for 60 min at 4°. Following centrifugation, aliquots of eluted material were assayed for dopamine D_1 receptor activity. Briefly, 0.3 ml (~ 70 μg) of eluted receptor was incubated in triplicate with [^{3}H]SCH-23390 (20 nM) for 90 min at 22° in a total assay volume of 0.45 ml, in the presence or absence of 10 μM (+)-butaclamol. Bound receptor was separated from free ligand by Sephadex G-50 chromatography. Aliquots (0.3 ml) of the assay mixture were applied to a 4 ml column (0.5 × 12 cm) of Sephadex G-50, pre-equilibrated at 4° with 50 mM Tris–HCl buffer, pH 7.4, and 0.1% digitonin. Receptors were eluted in the void volume at a flow rate of 0.5 ml/min at 4°. Fractions (0.5 ml) were collected and monitored for tritium on a Packard 460 C liquid scintillation spectrometer at 33% efficiency.

In experiments where agonist/[^{3}H]SCH-23390 competition curves were conducted on WGA-Sepharose exposed receptors, striatal membranes were first solubilized in 50 mM Tris, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM KCl, 2 mM EDTA containing 0.1% ascorbate, 12 μM nialamide (buffer B), and 1% digitonin, pH 7.4. Following solubilization, receptor preparations (20 ml) were applied to WGA-Sepharose (8 ml) as described above. Following incubation (1 hr at 4°), non-adsorbed material was decanted and the resin washed with 8 × 35 ml of buffer B containing 0.1% digitonin and the gel subsequently resuspended in 20 ml of 0.1 M β-D-GlcNAc. Aliquots of eluted receptor preparations (0.3 ml) were incubated with increasing concentrations of dopamine (10^{-12}–10^{-4} M) and 8 nM [^{3}H]SCH-23390 for 90 min at 22°. Non-specific binding was determined in the presence of 10 μM (+)-butaclamol. Bound ligand was separated from free by Sephadex G-50 chromatography as described. Data were analyzed for one- and two-site fits by the computer program LIGAND [12] with control versus experimental parameter estimates compared for statistically significant differences by simultaneous analysis, as previously described [11].

Protein determinations. Protein concentrations were determined by a modified Lowry assay [13] using bovine serum albumin as a standard. Solubilized samples were dialyzed or treated with SM-2 beads (BioRad) to remove excess detergent with protein values corrected for the presence of digitonin.

Results and discussion

The glycoprotein nature of solubilized dopamine D_1 receptors was assessed by WGA- and Con-A-Sepharose lectin affinity chromatography. Solubilized receptor preparations could be adsorbed (60%) and bound receptor specifically eluted from WGA-Sepharose gels by 100 mM (1–4)-D-GlcNAc. Adsorption of soluble receptor to Con-

* Abbreviations: N_s, guanine nucleotide stimulatory protein; Gpp(NH)p, guanylylimidodiphosphate; WGA, wheat-germ agglutinin-Sepharose 6B; Con-A, Concanavalin-A-Sepharose 4B; GlcNAc, *N*-acetylglucosamine; Gal, glactose; and Man, mannose.

A-Sepharose yielded lower retention (~15%) of receptors as indicated by the specific elution with 0.1 M α-D-Man, and was probably due to the interference by digitonin.

As shown in Fig. 1, the specificity of solubilized D_1 receptor interaction with WGA-Sepharose was investigated with a number of different carbohydrates. Sucrose, α-D-Man or Gal failed to significantly elute bound receptors from WGA-Sepharose as compared to buffer alone. β-D-GlcNAc, however, was successful in eluting bound receptors in each case. The carbohydrate specificity of eluted receptor from WGA-Sepharose indicates that the receptor is probably composed of complex-type oligosaccharide substituents with available *N*-acetylglucosamine and/or sialic acid residues [14, 15]. The presence of these carbohydrate side chains appears to be a general property of a number of neurotransmitter receptor systems including the dopamine D_2 [16–18], adrenergic [19], cholinergic [20] and opiate [21] receptor. The binding of D_1 receptor to Con-A most likely demonstrates the existence of high-mannose containing carbohydrates [22]. It should be noted, however, that the assessment of oligosaccharide specificity of D_1 receptors under conditions of digitonin solubilization is a problem. This is especially true of receptor binding to Con-A-Sepharose since digitonin micelles themselves may bind to the lectin via carbohydrate moieties found on digitonin molecules.

As shown in Fig. 1, a 10- to 12-fold increase in the specific binding activity of [^{3}H]SCH-23390 was observed following elution of bound receptor with *N*-GlcNAc and suggests the use of WGA-Sepharose lectin-affinity chromatography as an initial step towards the purification of the D_1 receptor

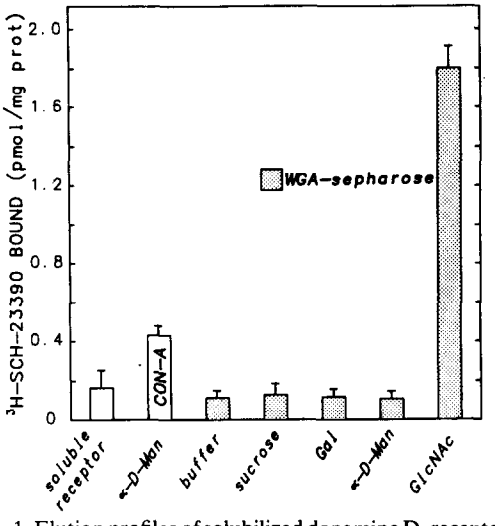

Fig. 1. Elution profiles of solubilized dopamine D_1 receptors from Con-A- and WGA-Sepharose immobilized lectins. Striatal membranes were solubilized and incubated with either Con-A- or WGA-Sepharose as described in Methods. Extensively washed gels were incubated subsequently with either 0.1 M α-D-Man (Con-A-Sepharose) or various carbohydrates (WGA-Sepharose) for 1 h at 4°. Supernatant fractions were collected and assayed for [^{3}H]SCH-23390 receptor binding activity. WGA-Sepharose gels were subsequently washed (20 ml) and incubated with 0.1 M β-D-GlcNAc for 1 additional hour, and supernatant fractions were collected and assayed for dopamine D_1 receptor activity with 20 nM [^{3}H]SCH-23390. The data are means (± S.E.) of triplicate determinations for three independent experiments. Specific binding represented approximately 1000 cpm/column in solubilized receptor preparations and 500–800 cpm/assay following elution from WGA-Sepharose with β-D-GlcNAC. Non-specific binding accounted for ≤20% of the total binding.

protein. A smaller increase in the specific binding activity (2- to 3-fold) of [^{3}H]SCH-23390 was noted following elution of bound receptors from Con-A-Sepharose with 100 mM α-D-Man. Prelabeling of solubilized dopamine D_1 receptors with [^{3}H]SCH-23390 (30-40 nM) or [^{3}H]dopamine (10 nM) did not interfere with either the adsorption or specific elution of bound receptors from WGA-Sepharose, suggesting that the glycoprotein side chain is distinct from the active binding site of the D_1 receptor. Similar results have been obtained with the dopamine D_2 receptor [16–18].

We reported previously that the agonist high-affinity form of the D_1 receptor can be solubilized by digitonin [11]. To assess further the molecular properties of the solubilized agonist high-affinity form of the D_1 receptor, agonist/[^{3}H]SCH-23390 competition experiments were done on WGA-Sepharose exposed receptor. As depicted in Fig. 2, dopamine/[^{3}H]SCH-23390 competition curves were clearly biphasic with estimated dissociation constants of 1 ± 0.2 and 150 ± 50 nM for the agonist high- and low-affinity forms of the receptor respectively. The K_D of dopamine for the D_1 high-affinity state of the solubilized receptor corresponds well to that observed in native membranes (1–5 nM; [5, 6, 10]). Of the total receptor population labeled, dopamine recognized approximately 67–70% as existing in the high-affinity form. To ascertain whether the agonist high affinity state of WGA-Sepharose eluted receptor is conferred by the same guanine-nucleotide-sensitive receptor complex found in brain membranes [5, 10, 11] agonist/[^{3}H]SCH-23390 competition curves were conducted on WGA-Sepharose-eluted receptor preparations that had been preincubated with the guanine nucleotide, Gpp(NH)p, prior to solubilization. As seen in Fig. 2, WGA-Sepharose-eluted receptors, which had been pretreated with 100 μM Gpp(NH)p, existed entirely in a form displaying low affinity for agonists with an observed K_D of 106 ± 10 nM, a result similar to that observed in native membranes [11]. These data (Fig. 2) clearly indicate that: (a) the agonist high-affinity form of the solubilized receptor is due to the coupling of a putative guanine nucleotide-binding protein (N_s) and the receptor and (b) that the same N_s–receptor complex conferring agonist high-affinity binding in brain membranes is solubilized and partially purified following WGA-Sepharose.

These results (Fig. 2) contrast with those reported for the D_2 dopaminergic receptor system, where agonist high-affinity binding is apparently lost following *digitonin* solubilization [16, 23, 24]. The molecular mechanism responsible for this difference is not clear at present but the data suggest that a significant proportion of dopamine D_1 receptors must exist in close association with N_s in order to resist the disruptive effects of digitonin solubilization. Similar results have been observed recently for the digitonin-solubilized adenosine (A_1) receptor system [25].

In vitro binding studies have shown that magnesium ions appear to increase the apparent affinity of the receptor for agonists in both the adrenergic and D_2 dopaminergic receptor systems [26–30]. The exact mechanism whereby magnesium ions modulate agonist affinity for receptors is unknown but appears to require functional components of a guanine nucleotide-binding protein [29, 30].

To further test the hypothesis that the agonist high-affinity form of the digitonin-solubilized D_1 receptor is due to functional receptor–N_s coupling, the effect of Mg^{2+} on agonist/[^{3}H]SCH-23390 competition was examined following receptor elution from WGA-Sepharose. As illustrated in Fig. 2, the agonist high-affinity form of the receptor was absolutely dependent on the addition of Mg^{2+} to the incubation medium (control), since removal of Mg^{2+} resulted in the complete transition of the agonist high-affinity form of the receptor to one displaying only low affinity for agonists with an observed K_D of 90 ± 8 nM. These results contrast with those obtained on the digitonin-solubilized β-adrenoceptors where either the addition or

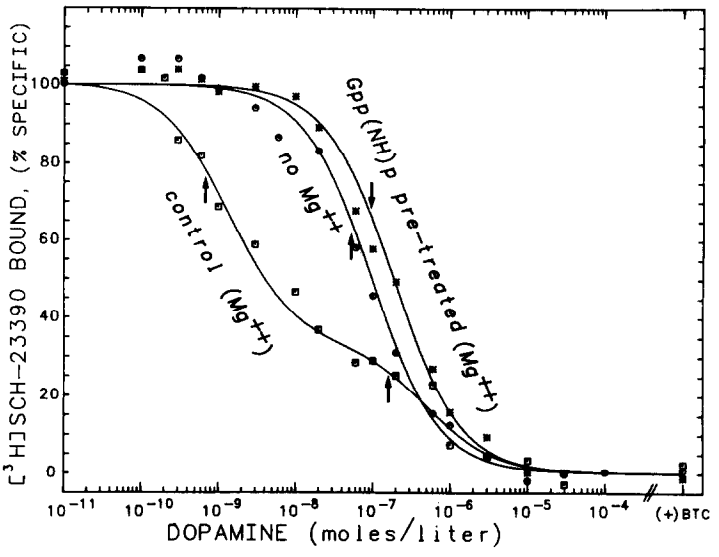

Fig. 2. Dopamine/[^{3}H]SCH-23390 competition to digitonin-solubilized receptors following exposure to WGA-Sepharose. Canine striatal membranes were incubated in the absence or presence of 100 μM Gpp(NH)p for 60 min at 22°. Following centrifugation, membrane pellets were resuspended in solubilization buffer as described in Methods. Solubilized receptor preparations (20 ml) were applied to WGA-Sepharose (8 ml) for 90 min at 4°. Non-absorbed material was decanted and the resin washed with 280 ml of buffer containing 0.1% digitonin in the absence or presence of 100 μM Gpp(NH)p. Specifically bound receptor was eluted in Gpp(NH)p-free buffer containing 0.1 M β-D-GlcNAc in the presence or absence of Mg^{2+} (10 mM). Aliquots of eluted receptor (0.3 ml) were incubated with various concentrations of dopamine and 8 nM [^{3}H]SCH-23390 for 90 min at 22°. Bound ligand was separated from free by Sephadex G-50 chromatography. Data were analyzed for one and two sites by the computer program LIGAND as described in Methods. The data are representative of three independent experiments. K_D values of dopamine (indicated by arrows) are listed in the text.

removal of Mg^{2+} does not change significantly the apparent dissociation constant of agonist binding [26]. Biochemical evidence has documented that the digitonin-solubilized β-adrenoceptor is not associated with a guanine nucleotide-binding protein unless receptors are occupied with agonist prior to solubilization.

The data presented in this report suggest that digitonin-solubilized striatial dopamine D_1 receptors can be partially purified by WGA-Sepharose, and that partially purified receptor preparations retain the ability to bind agonists with high affinity as a result of tight N_s-receptor coupling.

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Antiallergic effect of trimetoquinol analogs on actively sensitized guinea pig lung tissue, *in vitro*

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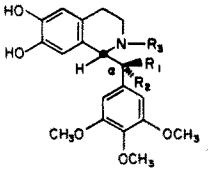
β-Adrenoceptor agonists are drugs widely used for the treatment of allergic bronchial asthma [1, 2]. Trimetoquinol [1-(3,4,5-trimethoxybenzyl)-6-7-dihydroxy-1,2,3,4 tetrahydroisoquinoline (TMQ)] exists as two optical isomers, and *S*(−)-TMQ, is a β_2-selective adrenoceptor stimulant which is currently used in Japan for treatment of moderate bronchial asthma [3, 4]. We found that TMQ is 7 times more selective than isoproterenol as a bronchial relaxant versus a cardiostimulant in guinea pig tracheal and atrial preparations respectively [5]. Our recent studies of chemical modification of TMQ at the α-benzyl carbon atom has led us to compounds with greater β_2-agonist potency and tissue selectivity [5, 6]. Using mono- and dimethyl substituted analogs of TMQ (see Fig. 1), we observed a stereoselectivity and rank order of potency as agonists of tracheal relaxation of *threo*-α-methylTMQ > TMQ > isoproterenol > *erythro*-α-methylTMQ > *N*-methylTMQ > α-dimethylTMQ [5]. Moreover, *N*-methylTMQ, *threo*-α-methylTMQ and *erythro*-α-methylTMQ were 6, 106 and 27 times more selective as β_2-agonists than isoproterenol.

Only a few reports on the effects of TMQ and congeners on antagonism of hypersensitivity reactions are available [7, 8]. Studies of passive cutaneous and systemic anaphylaxis in rodents *in vivo* show that TMQ is more potent than isoproterenol and newer β_2-selective agonists [7]. Blockade of antigen-sensitized histamine release in rat mast cells by TMQ is mediated by β-adrenoceptor activation [7]. Belcheva *et al.* [8] recently found that tetrahydroprotoberberin, a rigid congener of TMQ, is less potent as an antagonist of histamine release *in vivo*. These workers proposed that conformational orientation and flexibility of the 3,4,5-trimethoxybenzyl group of TMQ play an important role in the antiallergic activity of this chemical class of β-adrenoceptor agents.



Compd	R_1	R_2	R_3
Trimetoquinol (TMQ)	H	H	H
Threo-α-methyl TMQ	H	CH_3	H
Erythro-α-methyl TMQ	CH_3	H	H
α-Dimethyl TMQ	CH_3	CH_3	H
N-Methyl TMQ	H	H	CH_3

Fig. 1. Chemical structures of trimetoquinol (TMQ) and methyl-substituted TMQ analogs. The solid circle indicates the presence of the asymmetric carbon atom in the tetrahydroisoquinoline nucleus.

In the present work we have examined the concentration-dependent relationships of these promising TMQ analogs (Fig. 1) on antigen-sensitized histamine release from guinea pig lung tissue. Assessment of structure–activity relationships will allow us to determine if β-adrenoceptor activation is involved for the action of the TMQ analogs in this experimental system.

Male albino Hartley guinea pigs (300–400 g, Glenn Carr, Columbus, OH) were sensitized with chicken egg albumin (ovalbumin) as described by Wong and Buckner [9]. Guinea pigs were killed 3–4 weeks after sensitization. Endogenous catecholamines were depleted by a single intraperitoneal injection of reserpine (5 mg/kg) 12 hr before the experiments. Guinea pigs were killed by exsanguination, and lungs were removed, washed with physiological salt solution, excised free from large blood vessels and bronchi, and chopped into 1-cm fragments by a McIlwaine tissue chopper. Lung slices were filtered and washed thoroughly to remove excess blood. Samples of tissue (200–250 mg) were placed in polypropylene tubes containing 1 ml of Krebs–Henseleit solution of the following millimolar composition; NaCl, 118; KCl, 4.7; $MgCl_2 \cdot 6H_2O$, 6; $CaCl_2 \cdot 2H_2O$, 2.5; $NaHPO_4 \cdot 2H_2O$, 1; $NaHCO_3$, 25; and dextrose, 11. Tissues were incubated with 10^{-5} M phenoxybenzamine for 30 min in a metabolic incubator at 37° aerated with 95% O_2 and 5% CO_2. At this time medium was replaced with a fresh solution containing tropolone (10^{-4} M) and preincubated for 15 min, and then for another 15 min in the presence of various drug concentrations. These pretreatments ensured blockade of extraneuronal uptake and inhibition of catechol-*O*-methyl transferase activity. Histamine release was evoked by the addition of ovalbumin, and the samples were incubated at 37° for 15 min for maximum release of histamine. Preliminary experiments indicated that the addition of 50 μg/ml of ovalbumin was sufficient to induce maximum histamine release. The reaction was terminated by placing samples in an ice-cold bath. Each sample was centrifuged at 100 *g* for 15 min at 4° using a Sorvall refrigerated centrifuge. Histamine content in the supernatant fraction was extracted [10] and assayed according to the fluorometric method of Hakanson and Ronnberg [11]. Total histamine content of the lung slices was determined by homogenization of samples followed by boiling for 10 min and centrifugation. Aliquots of the supernatant fraction were assayed for total histamine content. In each experiment, histamine release from triplicate tissue samples for a given concentration of