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Glycoprotein Nature of Dopamine D1 Receptors in the Brain and Parathyroid Gland

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SUMMARY

Dopamine D1 receptors can be covalently labeled with the photoaffinity ligand (±)-7-[^{125}I]iodo-8-hydroxy-3-methyl-1-(4-azidophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine ([^{125}I]IMAB) and visualized following sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. In brain membranes, [^{125}I]IMAB labels a polypeptide of apparent $M_r \cong 74{,}000$ as the major ligand binding subunit of D1 receptors and two minor polypeptides of $M_r \cong 64{,}000$ and 52,000. In contrast, [^{125}I]IMAB labels a single polypeptide of apparent $M_r \cong 64{,}000$ in bovine parathyroid glands. In this study, the carbohydrate nature of dopamine D1 receptors from the brain and parathyroid gland were examined using specific exo- and endoglycosidases and lectin affinity chromatography. [^{125}I]IMAB-labeled brain and parathyroid D1 receptors were sensitive to treatment with the exoglycosidases neuraminidase or α-mannosidase, suggestive of the existence of terminal sialic acid and oligomannose residues. Photolabeled D1 receptor polypeptides are not however, associated with distinct populations of complex-type or high mannose-containing carbohydrate chains because 1) wheat germ agglutinin and concanavalin A lectin chromatography of solubilized and photolabeled neuronal D1 receptors followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography revealed no differences in the electrophoretic mobility of column pass-through and specifically eluted [^{125}I]IMAB-labeled polypeptides, and 2) [^{125}I]IMAB-labeled D1 receptors specifically bound to and eluted from concanavalin A-Sepharose were neuraminidase sensitive, indicative of the colocalization of oligomannose- and complex-type glycans. Removal of these terminal glycan residues did not affect the binding of [^{3}H]SCH 23390 to dopamine D1 receptors. Complete *N*-linked deglycosylation of photolabeled D1 receptors from both the brain and parathyroid with peptide *N*-glycosidase F resulted in the migration of a single major labeled polypeptide of apparent $M_r \cong 46{,}000$. These data suggest that, despite differences observed in the electrophoretic mobility and glycosylation patterns of brain and parathyroid D1 receptor polypeptides, the protein backbones of central and peripheral dopamine D1 receptors display similar if not identical molecular weights.

The dopamine D1 receptor, as originally defined by Kebabian and Calne (1), is linked to the stimulation of the enzyme adenylate cyclase. The prototypic dopamine D1 receptor, located in the bovine parathyroid gland, mediates the release of parathyroid hormone (see Refs. 2 and 3). In the central nervous system, dopamine D1 receptors appear to mediate some behavioral responses, modulate the activity of dopamine D2 receptors, and regulate neuron growth and differentiation (4–6). The major ligand binding subunit of the dopamine D1 receptor resides on a $M_r = 74/72{,}000$ polypeptide that has been identified in rat striatum using photoaffinity cross-linking (7) and, more recently, in canine, bovine, and porcine striatum using [^{125}I]IMAB (8), an aryl azide derivative of the selective D1 antagonist SCH 23390 (9). [^{125}I]IMAB specifically labels, in addition, minor neuronal polypeptides of apparent $M_r \cong 62{,}000$ and 51,000 (8), both of which share structural homology with the $M_r \cong 74{,}000$ protein and are probably derived from the $M_r \cong 74{,}000$ polypeptide by endogenous proteolysis (10). The neuronal dopamine D1 receptor has been shown to be a glycoprotein by virtue of its ability to bind to and be specifically eluted from various lectin columns (11). The D1 receptor of the parathyroid gland, however, resides in a polypeptide of apparent $M_r = 64{,}000$–$62{,}000$ (10).

In order to assess the possibility that the differences in subunit composition of neuronal versus peripheral D1 receptors are related to alterations in posttranslational processing, we have probed the carbohydrate nature of the photolabeled subunits of the dopamine D1 receptor. Using specific exo- and endoglycosidase treatments and lectin affinity chromatogra-

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ABBREVIATIONS: [^{125}I]IMAB, (±)-7-[^{125}I]iodo-8-hydroxy-3-methyl-1-(4′-azidophenethyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; NP-40, Nonidet P-40; Con A, concanavalin A; WGA, wheat germ agglutinin; PNGase-F, glycopeptide-*N*-glycosidase F; SCH-23390, (R)-(+)-8-hydroxy-7-chloro-2,3,4,5,-tetrahydro-3-methyl-1-phenyl-1*H*-3-benzazepine; NANA-lactose, *N*-acetyl-neuraminosyl-D-lactose; PNA, *Arachis hypogaea* (peanut) agglutinin; SBA, glycine max (soybean) agglutinin; DMAN, 1-deoxymannojirimycin; GlcNAc; *N*-acetylglucosamine.

phy, we show that the apparent electrophoretic diversity of photolabeled D1 receptor subunits is a result of glycan microheterogeneity and that, upon complete *N*-linked deglycosylation, both the brain and parathyroid D1 dopamine receptor migrate as a single labeled polypeptide of apparent M_r = 46,000.

Experimental Procedures

Materials. (±)-[^{125}I]IMAB (2200 Ci/mmol) was prepared as previously described (8). Protease inhibitors and proteases were from Sigma and prestained molecular weight protein standards were from Amersham. Electrophoresis reagents were obtained from Bio-Rad. Dopaminergic drugs and other reagents were either purchased or were generously donated by various pharmaceutical firms, as previously described (3, 8, 10). Neuraminidase (purified Type X), α-mannosidase (jack bean), GlcNAc, α-D-mannoside, and galactose were obtained from Sigma. *O*-Glycanase was obtained from Genzyme, and PNGase-F (glycopeptidase F), endoglycosidase H, NANA-lactose, and DMAN were purchased from Boehringer Mannheim. WGA-Sepharose and Con-A-Sepharose were purchased from Pharmacia and PNA-Sepharose and SBA-Sepharose were obtained from E-Y Labs (San Mateo, CA).

Membrane preparation. Calf brains were obtained fresh from a local abattoir (Bocknek, Inc.) and were frozen at −70° until use. Canine brains (Pel-Freez, Rogers AR) were also kept at −70°. Striata were dissected from thawed tissue and immediately Teflon-glass homogenized (10 up and down strokes) in 20 volumes of ice-cold 25 mM Tris·HCl buffer containing 250 mM sucrose, in the presence of the following protease inhibitors: 20 mM EDTA, 15 μg/ml benzamidine, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 1 mM PMSF (pH 7.4 at 4°). Homogenates were centrifuged for 10 min at 600 × *g* and the supernatants were collected and recentrifuged at 48,000 × *g* for 20 min. The resulting membrane pellets were resuspended in 25 mM Tris-HCl buffer, containing 100 mM NaCl and the above indicated protease inhibitors, to a protein concentration of 2–4 mg/ml. Calf parathyroid glands were treated equivalently, with the exception that the tissues were trimmed of excess fat and minced before homogenization (30 up and down strokes). Homogenates were subsequently filtered through four layers of cheesecloth before the initial centrifugation.

Photoaffinity labeling. Membranes (1 ml) were incubated in the dark with approximately 250 pM [^{125}I]IMAB, at a D1 receptor concentration of 50–100 pM, in a total volume of 5 ml, for 90 min at 22°, in the absence or presence of the indicated concentration of dopaminergic agents. Following incubation, membranes were sedimented at 48,000 × *g* for 20 min and pellets were resuspended in buffer (as above) containing 0.5% bovine serum albumin and were recentrifuged. The resulting pellets were resuspended in 1 ml of 25 mM Tris·HCl buffer containing 100 mM NaCl and protease inhibitors and were irradiated for 35 sec, as previously described (8).

Exoglycosidase treatments. For neuraminidase treatment, [^{125}I]IMAB-labeled membranes were washed twice in 30 ml of 100 mM sodium acetate buffer, pH 5.1 at 22°. Membranes were then resuspended in the same buffer at 0.5–1.0 mg/ml, to which neuraminidase was added at 2 units/ml, and incubated in the presence or absence of NANA-lactose (at the indicated concentrations), for up to 1 hr at 37°. Incubations were terminated by placing the samples on ice and by diluting the samples in the buffer appropriate for the next treatment. For treatment with α-mannosidase, [^{125}I]IMAB-labeled membranes were washed twice in 30 ml of 50 mM sodium citrate buffer, pH 4.5 at 22°, containing 100 μM PMSF. Incubations were carried out in the presence or absence of the α-mannosidase inhibitor DMAN for 24 hr at 22°, with the indicated concentration of α-mannosidase. For sequential exoglycosidase treatment, membranes were washed twice with 30 ml of the appropriate buffer and then treated with the first enzyme, as detailed above. Incubation was terminated with two 30-ml washes in the buffer appropriate for incubation with the subsequent enzyme (as detailed above). Following incubation, membranes were pelleted (12,000 × *g* for 7 min) and then prepared for SDS-PAGE as described below.

Ligand binding. The saturable binding of [^{3}H]SCH-23390 (40–4000 pM) to exoglycosidase-treated membranes and subsequent nonlinear curve-fitting analysis using LIGAND were carried out as previously described (3, 12).

Lectin affinity chromatography. Briefly, [^{125}I]IMAB-labeled and treated membranes were washed before solubilization (where applicable) with 30 ml of 50 mM Tris·HCl (pH 7.4 at 22°). Washed pelleted (48,000 × *g*, 20 min) membranes were then solubilized (by stirring) in 0.8% NP-40, 10 mM Tris·HCl, 150 mM NaCl (pH 7.4 at 22°), for 1 hr at 22°. The solubilized membrane preparation was then centrifuged (48,000 × *g* for 30 min), and the supernatant containing the solubilized receptors was collected and passed through a 0.22-μm Millex filter and diluted to a final NP-40 concentration of 0.16%, as described by Stiles *et al.* (13). Lectin columns were preequilibrated with 0.1% NP-40, 10 mM Tris·HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.4 at 22°, with the exception of the WGA-Sepharose column, which was preequilibrated with the same buffer in the absence of $CaCl_2$. Chromatography was carried out at 4° as follows: 0.9 ml of the solubilized preparation was added to the column, with the eluate being discarded, and the remainder of the solubilized preparation (approximately 8.5 ml) was slowly run into the column and allowed to equilibrate for 10 min. The eluate was reapplied and allowed to equilibrate for an additional 10 min. The columns were washed with 0.5-ml fractions of the appropriate equilibration buffer until the [^{125}I]IMAB radioactivity declined to baseline. Elution was carried out using 0.5-ml aliquots of the appropriate sugar (WGA, GlcNAc; Con A, α-methyl-D-mannoside; SBA, PNA, galactose), at a concentration of 300 mM in the equilibration buffer, and continued until radioactivity returned to baseline. Aliquots of the pass-through, wash, and elution fractions were desalted on Sephadex G-50 columns that were preequilibrated with 0.2% SDS, 20 mM Tris·HCl, pH 6.8 at 22°, lyophilized, and prepared for SDS-PAGE as described below.

Endoglycosidase treatments. [^{125}I]IMAB-labeled membranes were washed twice in 30 ml of 200 mM sodium phosphate buffer (pH 8.1 at 22°) containing 20 mM EDTA and were preincubated for 1 hr at 22° in the above buffer that was supplemented with 0.5% SDS and 100 mM β-mercaptoethanol. Aliquots (60 μl, approximately 1 mg of protein) were incubated with 1.25% NP-40 and PNGase-F (up to 60 units/ml) for 20 hr at 37°, in a final volume of 90 μl (final concentration of SDS = 0.3%). Treatment with *O*-glycanase was carried out following neuraminidase and PNGase-F treatment and consisted of a 24-hr incubation at 37° with the enzyme at a final concentration of 500 milliunits/ml. Treatment with endoglycosidase H was carried out on neuraminidase-treated membranes essentially as described above for PNGase-F, with the exception that the buffer used was 50 mM sodium acetate, pH 5.6 at 22°, containing 0.5 mM PMSF and that incubations were for 18 hr at 22°. Following incubation, samples were pelleted (13,000 × *g* for 8 min) and then prepared for SDS-PAGE as described below.

SDS-PAGE and autoradiography. Electrophoresis was performed by the method of Laemmli, as previously described (8, 10). Briefly, photolabeled and treated samples were solubilized in 50 mM Tris·HCl (pH 6.8 at 22°), 10% SDS, 10% glycerol, and 5% β-mercaptoethanol for 1 hr at 22°. Aliquots (approximately 100–300 μg of protein) were loaded onto 1.5-mm slab gels containing a 12% separating gel (approximately 16 cm) and a 6% stacking gel and were electrophoresed overnight. Following electrophoresis, gels were dried and exposed to Kodak X-AR film with one intensifying screen at −70°. Molecular weight estimates of the photolabeled receptor subunits were determined graphically by reference to prestained standards. Results shown are typical of experiments carried out at least twice.

Results

Exoglycosidase treatment of brain D1 receptors. The effects of neuraminidase digestion of photolabeled canine striatal D1 receptors are illustrated in Fig. 1A. In control lanes, [^{125}I]IMAB-labeled receptors migrated as diffuse bands of apparent M_r = 74,000 and 52,000. The M_r = 64,000 polypeptide

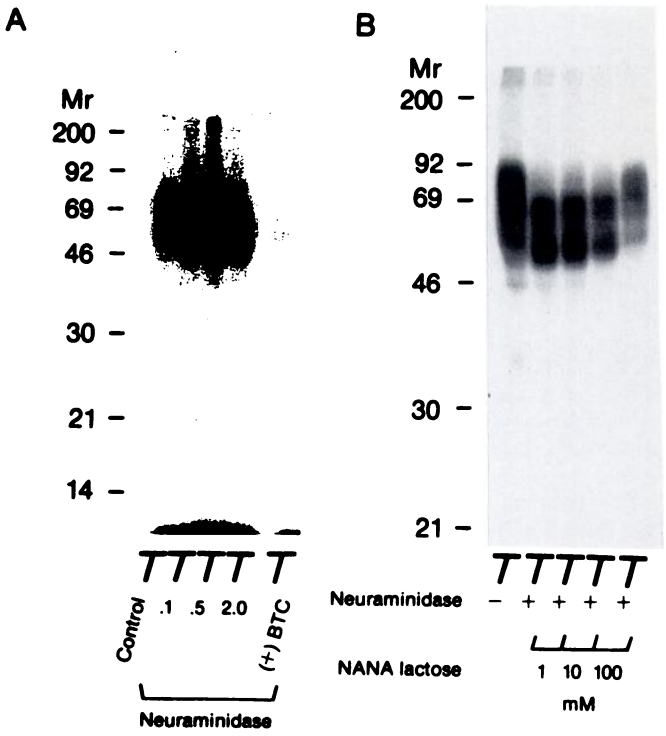

Fig. 1. Effect of neuraminidase on SDS-PAGE migration pattern of [^{125}I]IMAB-labeled brain D1 receptors. A, Canine brain membranes were photolabeled with [^{125}I]IMAB as described in Experimental Procedures. A lane in which nonspecific binding was defined by photolabeling in the presence of 1 μM (+)-butaclamol [(+)*BTC*] is included. The labeled receptors were then exchanged into 100 mM sodium acetate buffer (pH 5.0 at 37°) and incubated in the absence or presence of neuraminidase, at the indicated concentrations, for 1 hr at 37°. After treatment, membranes were sedimented and prepared for SDS-PAGE and autoradiography, as described in Experimental Procedures. This experiment is representative of three such experiments. B, Canine brain membranes, photolabeled with [^{125}I]IMAB as described in Experimental Procedures, were treated with neuraminidase (2 units/ml/for 15 min at 37°), in the absence or presence of the neuraminidase substrate NANA-lactose at the concentrations indicated. Samples were then prepared for SDS-PAGE and autoradiography as detailed in A. Results are representative of an experiment conducted twice. Relative molecular weight is shown × 10^{-3}.

observed previously in control membranes (8) was not consistently resolved from the M_r = 74,000 polypeptide under the treatment conditions utilized (37° and pH 5.0) and may possibly be due to proteolysis. Treatment of photolabeled membranes with neuraminidase increased the electrophoretic mobility of the photolabeled binding subunits to apparent M_r = 60,000 and 48,000 at each of the concentrations tested. These photolabeled bands are specific, because incubation in the presence of 1 μM (+)-butaclamol blocks photoincorporation. This increase in receptor mobility is not the result of nonspecific proteolysis, because enzyme treatment in the presence of NANA-lactose inhibits, in a concentration-dependent manner, the neuraminidase-induced increase of [^{125}I]IMAB-labeled receptor mobility on SDS-PAGE (Fig. 1B). Because neuraminidase specifically cleaves terminal sialic acid residues, the data suggest that all major photolabeled D1 receptor polypeptides contain terminal sialic acid. Moreover, because neuraminidase treatment did not result in the migration of a single [^{125}I]IMAB-labeled polypeptide chain but rather altered the migration patterns of the existing photolabeled bands, the observed heterogeneity of D1 receptor photolabeled polypeptides on SDS-PAGE (Fig. 1A) is not solely attributable to the presence or absence of terminal sialic acid residues.

In order to assess whether other terminal carbohydrate moieties are associated with [^{125}I]IMAB-labeled D1 receptor polypeptides, the exoglycosidase α-mannosidase was used to assess the presence of terminal mannose residues. As depicted in Fig. 2A, α-mannosidase, at a concentration of 12 units/ml, appeared to alter the migration pattern of photolabeled D1 receptor polypeptides. A significant proportion (approximately 80%) of this photolabeled material appeared to migrate with a slightly decreased electrophoretic mobility, compared with control material, with an apparent $M_r \cong 77{,}000$. The remaining component (approximately 20%) now migrated as a distinct and sharp band with increased electrophoretic mobility at M_r = 58,000. These photolabeled bands are specific, because incubation in the presence of 1 μM (+)-butaclamol blocks photoincorporation. It is of interest to note that the intensity of the M_r = 52,000 protein in control preparations is markedly diminished under these conditions (incubation in sodium citrate buffer, pH 4.5) and this diminution may be due to proteolysis (also see Table 1). As illustrated in Fig. 2B, the effect of α-mannosidase can at

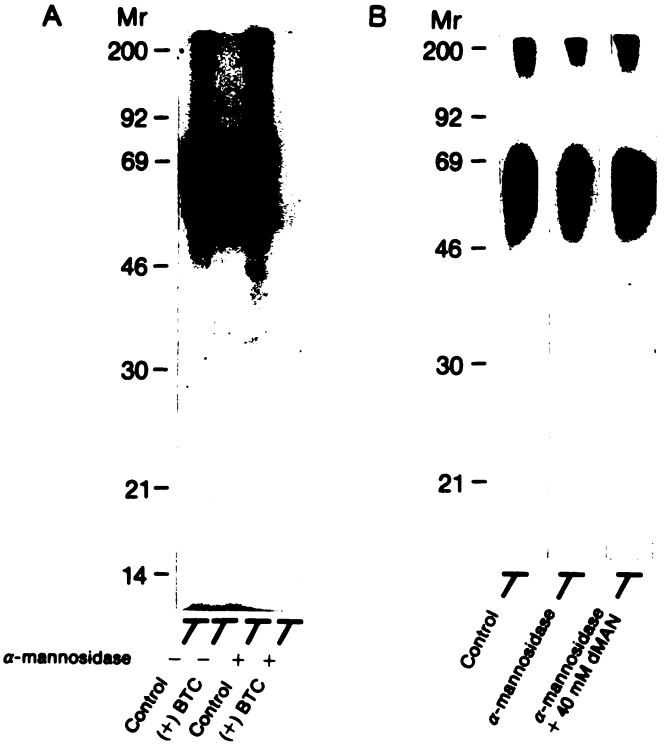

Fig. 2. Effect of α-mannosidase on SDS-PAGE migration pattern of [^{125}I]IMAB-labeled brain D1 receptors. A, Canine brain membranes were photolabeled with [^{125}I]IMAB, as described in Experimental Procedures. Lanes in which nonspecific binding was defined by photolabeling in the presence of 1 μM (+)-butaclamol [(+)*BTC*] are included. The labeled receptors were then exchanged into 50 mM sodium citrate buffer, pH 4.5 at 22°, containing 100 μM PMSF and were incubated in the absence or presence of α-mannosidase at a concentration of 12 units/ml, for 24 hr at 22°. After treatment, membranes were sedimented and prepared for SDS-PAGE and autoradiography, as described in Experimental Procedures. Results are typical of experiments carried out several times. B, Canine brain membranes, photolabeled with [^{125}I]IMAB as described in Experimental Procedures, were treated in the absence or presence of α-mannosidase (as above), in the presence or absence of the α-mannosidase inhibitor deoxymannojirimycin at a concentration of 40 mM. Results are representative of experiments conducted twice. Relative molecular weight is shown × 10^{-3}.

least be partially reversed by the α-mannosidase inhibitor DMAN, suggesting that these effects are due to the trimming of terminal mannose residues. Because we could not completely resolve all [^{125}I]IMAB-labeled polypeptides following exoglycosidase treatment, it remains unclear which of the labeled polypeptides ($M_r \cong 74{,}000$ or 64,000) are α-mannosidase sensitive and so we cannot ascribe the presence or absence of oligomannose-type chains to a labeled protein subunit of a specific molecular mass. In any event, it appears clear that the $M_r = 74{,}000/64{,}000$ labeled polypeptide is composed of differentially glycosylated subunits (i.e., those displaying either increased or decreased electrophoretic mobility following α-mannosidase treatment) and that the glycan microheterogeneity associated with the major ligand binding subunit of D1 receptors may be responsible for the existence of multiple [^{125}I]IMAB-labeled D1 receptor polypeptides.

The glycan microheterogeneity associated with the $M_r = 74{,}000/64{,}000$ labeled polypeptide of the D1 dopamine receptor was further confirmed in experiments where photolabeled receptors were treated sequentially with both neuraminidase and α-mannosidase. As seen in Fig. 3, sequential neuraminidase and α-mannosidase treatment increased the electrophoretic mobility of the $M_r = 74{,}000/64{,}000$ labeled polypeptide to apparent $M_r \cong 58{,}000$, similar to that observed after neuraminidase treatment alone and similar to the apparent M_r of α-mannosidase-sensitive D1 receptor populations of $M_r \cong 58{,}000$ (Figs. 2 and 3). Identical results were obtained when the order of enzyme treatment was reversed (data not shown). The fact that sequential treatment with these enzymes produced nonadditive effects on the electrophoretic mobility of labeled D1 receptor polypeptides, compared with neuraminidase alone, appears to suggest that remnant sialic acid residues are responsible for the observed migration patterns of [^{125}I]IMAB-labeled receptor subunits following α-mannosidase digestion. Once sialic acid residues are removed (i.e., following neuraminidase treatment), α-mannosidase does not differentially affect the migration pattern of the major D1 receptor binding subunit of $M_r = 74{,}000/64{,}000$ (Fig. 3). As already mentioned above, because control membranes were treated and subjected to the same experimental procedures (except for the addition of enzyme) as those treated with both neuraminidase and α-mannosidase, control labeled preparations could not be resolved into three distinct polypeptides of $M_r = 74{,}000/64{,}000/52{,}000$, probably due to the harsh treatment conditions utilized (see Experimental Procedures).

Lectin chromatography of native and exoglycosidase-treated D1 binding subunits. The data presented above support the contention that, although the glycans associated with the major ligand binding subunits of the D1 receptor exhibit microheterogeneity, the differences in glycan structure do not appear to be associated with distinct protein receptor populations. In order to gain further support for this assertion, lectin chromatography was used to demonstrate that the photolabeled D1 receptor polypeptides were not separable into distinct subpopulations on the basis of their differential affinity for the lectins studied. As illustrated in Fig. 4 (*top*), photolabeled and NP-40-solubilized canine striatal membranes can be adsorbed to and specifically eluted from WGA-Sepharose by GlcNAc. Subsequent SDS-PAGE and autoradiography (Fig. 4) failed to demonstrate any differences in the estimated molecular mass of soluble, pass-through, wash, and GlcNAc-eluted

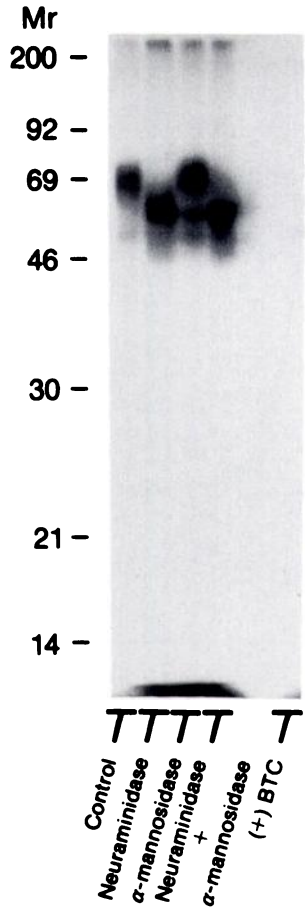

Fig. 3. Effect of sequential neuraminidase and α-mannosidase treatment on SDS-PAGE migration patterns of [^{125}I]IMAB labeled brain D1 receptors. Canine brain membranes were photolabeled with [^{125}I]IMAB, as described in Experimental Procedures. The labeled receptors were then exchanged into 100 mM sodium acetate buffer (pH 5.0 at 37°) and incubated in the absence (control) or presence of neuraminidase at a concentration of 2 units/ml, for 1 hr at 37°. After treatment, membranes were sedimented and then exchanged into 50 mM sodium citrate buffer, pH 4.5 at 22°, containing 100 μM PMSF and were incubated in the absence (control and neuraminidase alone) or presence of α-mannosidase at a concentration of 12 units/ml, for 24 hr at 22°. A nonspecific lane in which photolabeling was done in the presence of 1 μM (+)-butaclamol [(+)*BTC*] before treatment with both exoglycosidases is included. After treatment, membranes were sedimented and prepared for SDS-PAGE and autoradiography as described in Experimental Procedures. Results are typical of experiments carried out three times. Relative molecular weight is shown × 10^{-3}.

fractions. These data indicate that no differences are apparent in the SDS-PAGE mobilities of those polypeptides that do or do not bind tightly to WGA-Sepharose. Similar experiments were carried out examining the adsorption and specific elution of the photolabeled canine striatal D1 polypeptides to a Con A lectin column (Fig. 4, *bottom*). Similar to that observed with the WGA column, differences are not apparent in the electrophoretic mobilities of photolabeled receptors present in the fractions collected. Qualitatively equivalent results were obtained with the adsorption of either neuraminidase- or α-mannosidase-treated receptors to WGA or Con A columns (respectively), with the exception that a greater proportion (from 20–70%, respectively) of the photolabeled material was associated with the wash and pass-through fractions. Subsequent SDS-PAGE and autoradiography of pass-through, wash,

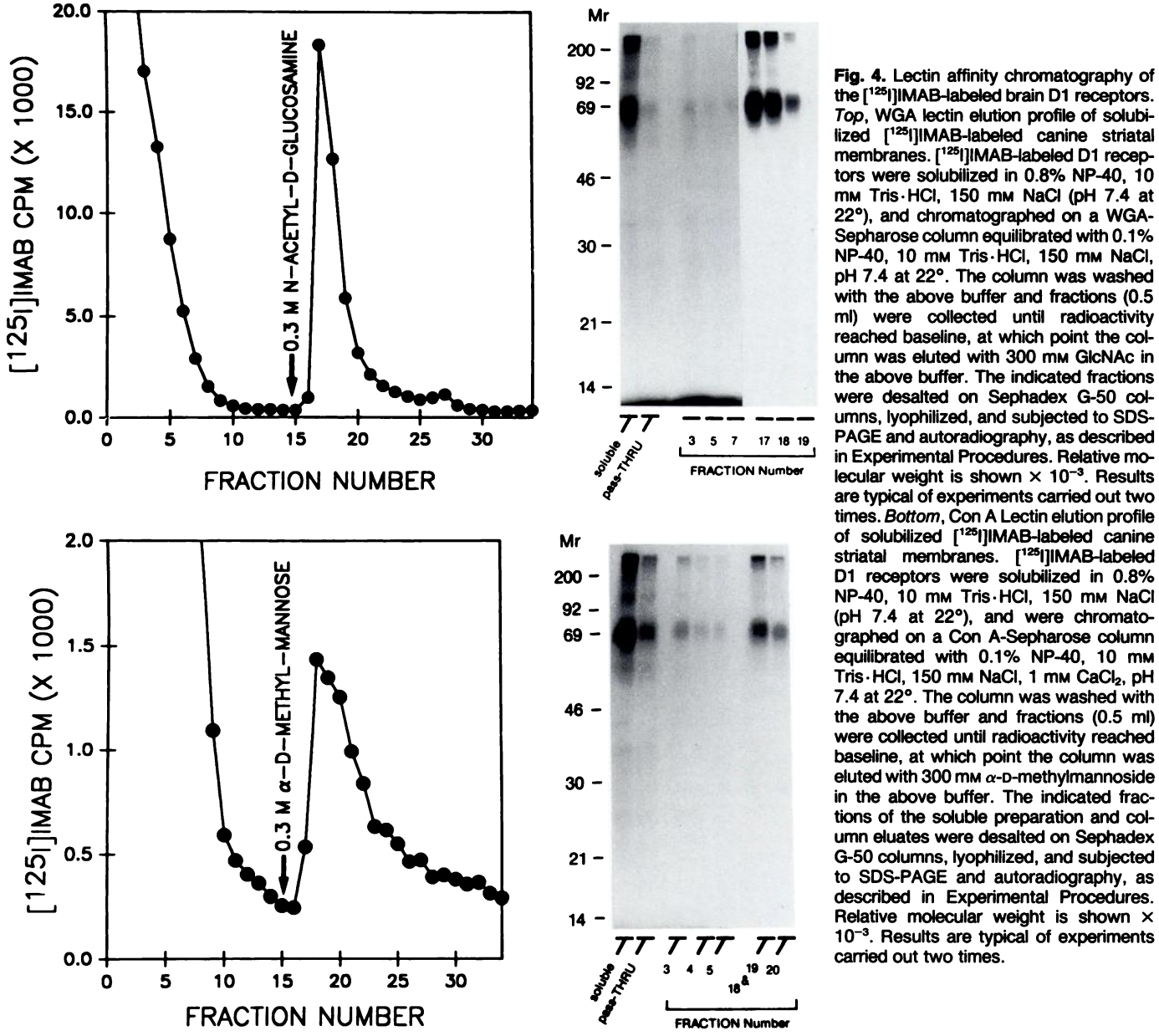

Fig. 4. Lectin affinity chromatography of the [^{125}I]IMAB-labeled brain D1 receptors. *Top*, WGA lectin elution profile of solubilized [^{125}I]IMAB-labeled canine striatal membranes. [^{125}I]IMAB-labeled D1 receptors were solubilized in 0.8% NP-40, 10 mM Tris·HCl, 150 mM NaCl (pH 7.4 at 22°), and chromatographed on a WGA-Sepharose column equilibrated with 0.1% NP-40, 10 mM Tris·HCl, 150 mM NaCl, pH 7.4 at 22°. The column was washed with the above buffer and fractions (0.5 ml) were collected until radioactivity reached baseline, at which point the column was eluted with 300 mM GlcNAc in the above buffer. The indicated fractions were desalted on Sephadex G-50 columns, lyophilized, and subjected to SDS-PAGE and autoradiography, as described in Experimental Procedures. Relative molecular weight is shown × 10^{-3}. Results are typical of experiments carried out two times. *Bottom*, Con A Lectin elution profile of solubilized [^{125}I]IMAB-labeled canine striatal membranes. [^{125}I]IMAB-labeled D1 receptors were solubilized in 0.8% NP-40, 10 mM Tris·HCl, 150 mM NaCl (pH 7.4 at 22°), and were chromatographed on a Con A-Sepharose column equilibrated with 0.1% NP-40, 10 mM Tris·HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.4 at 22°. The column was washed with the above buffer and fractions (0.5 ml) were collected until radioactivity reached baseline, at which point the column was eluted with 300 mM α-D-methylmannoside in the above buffer. The indicated fractions of the soluble preparation and column eluates were desalted on Sephadex G-50 columns, lyophilized, and subjected to SDS-PAGE and autoradiography, as described in Experimental Procedures. Relative molecular weight is shown × 10^{-3}. Results are typical of experiments carried out two times.

and eluate fractions failed to demonstrate any differences in the apparent M_r of the photolabeled D1 binding subunits (data not shown). Further evidence favoring a colocalization of oligomannose- and complex-type chains was obtained by treating the Con A pass-through, wash, and eluate fractions with neuraminidase. As illustrated in Fig. 5, all fractions were sensitive to neuraminidase digestion, demonstrating that terminal sialic acids were present on both the photolabeled binding subunits that passed through and those that were specifically eluted from the Con A column by α-methyl-D-mannoside. The effect of neuraminidase on the Con A eluate was shown to be inhibited by NANA-lactose (data not shown).

Exoglycosidase treatment of bovine parathyroid D1 binding subunits. Because the M_r = 74,000 protein is absent in tissues from the bovine parathyroid gland (10), it was of interest to determine the extent to which glycans contribute to the molecular mass of the M_r = 64,000 D1 receptor binding subunit in this tissue. The effect of treating photolabeled parathyroid tissue with the exoglycosidase neuraminidase is shown in Fig. 6A. Neuraminidase treatment resulted in a slight increase in the mobility of the predominantly labeled M_r = 64,000 polypeptide to apparent M_r = 61,000. The smaller M_r = 46,000 labeled peptide (which may be a proteolytic breakdown product) is not sensitive to neuraminidase and, as such, lacks terminal sialic acid residues. Unlike that seen with brain D1 binding subunits, treatment of parathyroid homogenates with the exoglycosidase α-mannosidase results in an increase in the electrophoretic mobility of the major $M_r \cong$ 64,000 [^{125}I]IMAB-labeled polypeptide to apparent M_r = 58,000. After α-mannosidase treatment, the smaller peptide of M_r = 46,000 now migrates at apparent M_r = 43,000 (Fig. 6B). These data clearly suggest that, unlike the brain D1 receptor, a majority of the peripheral glycans associated with the parathyroid D1 dopamine receptor are of the oligomannose type. As demonstrated in Fig. 6C, the effects of α-mannosidase treatment alone appeared to be very similar to the effects of sequential treatment with neuramini-

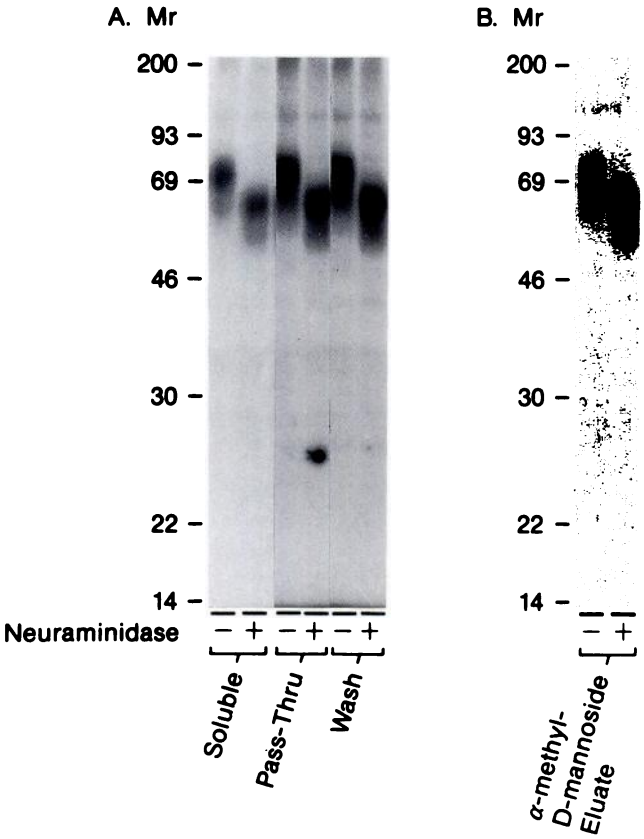

Fig. 5. Effect of neuraminidase on the SDS-PAGE migration pattern of the Con A lectin fractions of [^{125}I]IMAB-labeled brain D1 receptors. [^{125}I]IMAB-labeled D1 receptors were solubilized in 0.8% NP-40, 10 mM Tris·HCl, 150 mM NaCl (pH 7.4 at 22°), and were chromatographed on a Con A-Sepharose column equilibrated with 0.1% NP-40, 10 mM Tris·HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.4 at 22°. The column was washed with the above buffer and fractions (0.5 ml) were collected until radioactivity reached baseline, at which point the column was eluted with 300 mM α-D-methylmannoside in the above buffer. Selected pass-through and wash (A) and eluted (B) fractions were pooled before being treated with neuraminidase at a concentration of 2.8 units/ml for 1 hr at 37°. Incubations were terminated by desalting over Sephadex G-50 columns preequilibrated with 20 mM Tris, pH 6.8 at 22°, 0.2% SDS. Samples were subsequently lyophilized and subjected to SDS-PAGE and autoradiography, as described in Experimental Procedures. Relative molecular weight is shown × 10^{-3}.

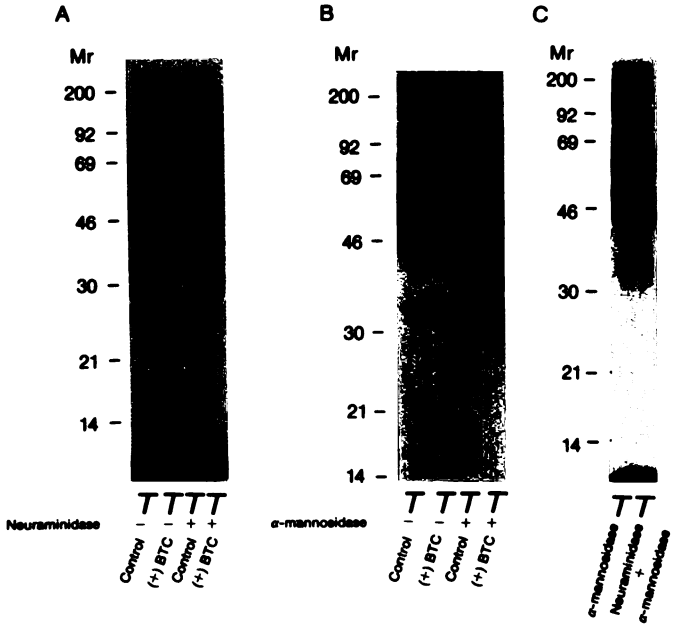

Fig. 6. Exoglycosidase treatment of bovine parathyroid photolabeled D1 receptors. A, Bovine parathyroid membranes were photolabeled with [^{125}I]IMAB, as described in Experimental Procedures. Lanes in which nonspecific binding was defined by photolabeling in the presence of 1 μM (+)-butaclamol [(+)*BTC*] are included. The labeled receptors were then exchanged into 100 mM sodium acetate buffer (pH 5.0 at 37°) and incubated in the absence or presence of neuraminidase at 2 units/ml, for 1 hr at 37°. After treatment, membranes were sedimented and prepared for SDS-PAGE and autoradiography, as described in Experimental Procedures. This experiment is representative of three such experiments. B, Bovine parathyroid membranes were photolabeled with [^{125}I]IMAB, as described in Experimental Procedures. Lanes in which nonspecific binding was defined by photolabeling in the presence of 1 μM (+)-butaclamol are included. The labeled receptors were then exchanged into 50 mM sodium citrate buffer, pH 4.5 at 22°, containing 100 μM PMSF and were incubated in the absence or presence of α-mannosidase at a concentration of 12 units/ml, for 24 hr at 22°. After treatment, membranes were sedimented and prepared for SDS-PAGE and autoradiography, as described in Experimental Procedures. Results are typical of experiments carried out two times. C, Bovine parathyroid membranes were photolabeled with [^{125}I]IMAB, as described in Experimental Procedures. The labeled receptors were then exchanged into 100 mM sodium acetate buffer (pH 5.0 at 37°) and were incubated in the presence of neuraminidase at a concentration of 2 units/ml, for 1 hr at 37°. After treatment, membranes were sedimented and then exchanged into 50 mM sodium citrate buffer, pH 4.5 at 22°, containing 100 μM PMSF and were incubated in the absence or presence of α-mannosidase at a concentration of 12 units/ml, for 24 hr at 22°. After treatment, membranes were sedimented and prepared for SDS-PAGE and autoradiography, as described in Experimental Procedures. Results are typical of experiments carried out two times. Relative molecular weight is shown × 10^{-3}.

dase and α-mannosidase (M_r = 58,000) and, similar to that observed in canine striatal tissues, the effects of neuraminidase and α-mannosidase do not appear to be additive.

Effects of exoglycosidase treatment on the binding of [^{3}H]SCH-23390. Although it is clear that the glycans associated with the dopamine D1 receptor are important determinants of the migration profile of the photolabeled polypeptides on SDS-PAGE, the significance of these glycans for the binding characteristics of the receptor is unknown. The results of the analysis of the equilibrium binding of [^{3}H]SCH-23390 to exoglycosidase-treated canine striatal homogenates were assessed and are presented in Table 1. Removal of sialic acids with neuraminidase produced only modest changes in the binding of [^{3}H]SCH-23390. These changes consisted of slight decreases in binding affinity and capacity. Similarly, although incubation with sodium citrate buffer is seen to produce modest decreases in binding affinity and a large decrease in the binding capacity (consistent with the loss of photolabeled peptides seen in Figs. 2 and 3), α-mannosidase treatment does not appear to affect the binding of [^{3}H]SCH-23390 to D1 receptors, relative to control preparations.

Deglycosylation of the parathyroid and brain D1 binding subunits. Having demonstrated, using exoglycosidases, the extent to which terminal sugar moieties contribute to the observed apparent M_r of dopamine D1 receptors from parathyroid and brain, it was of interest to determine whether differences exist in the electrophoretic mobility of the peptide backbones of these polypeptides. These studies were conducted using [^{125}I]IMAB-photolabeled membranes that were treated with PNGase-F before SDS-PAGE and autoradiography, and the results are depicted in Fig. 7. Complete *N*-linked deglycosylation of canine (Fig. 7, *left*) or bovine brain (data not shown)

TABLE 1

[^{3}H]SCH-23390 binding to exoglycosidase-treated D1 receptors

Striatal membranes were prepared and treated with 2 units/ml neuraminidase or 12 units/ml α-mannosidase in the appropriate buffer, as described in Experimental Procedures. Following treatment, membranes were extensively washed in Tris·HCl buffer, incubated with [^{3}H]SCH-23390 (0.01–4000 pM), and assayed for D1 receptor activity, as previously described (3, 12). B_{max} and K_D values for [^{3}H]SCH-23390 binding were derived from computer-assisted analysis of the data (LIGAND) and are representative of two separate experiments.

Condition	K_D	B_{max}
	pM	
Control	98	44
Neuraminidase-treated	126	36
Control	199	5.8
α-Mannosidase-treated	200	5.3

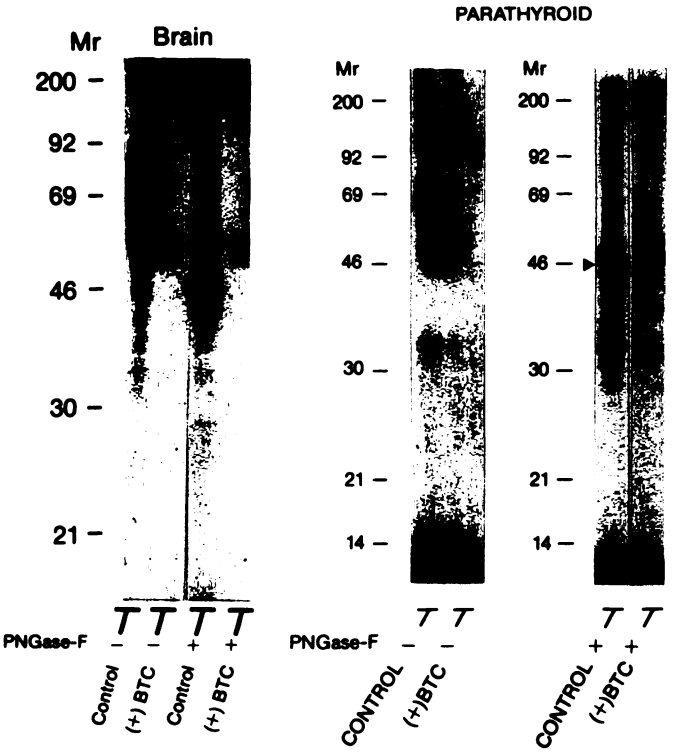

Fig. 7. Endoglycosidase treatment of photolabeled canine brain and bovine parathyroid D1 binding subunits. Canine brain (*left*) and bovine parathyroid (*right*) membranes were photolabeled with [^{125}I]IMAB, as described in Experimental Procedures. Lanes in which nonspecific binding was defined by photolabeling in the presence of 1 μM (+)-butaclamol [(+)*BTC*] are included. The labeled receptors were then exchanged into 200 mM sodium phosphate, 20 mM EDTA buffer (pH 8.0 at 37°), and were incubated in the absence or presence of PNGase-F at a concentration of 60 units/ml, for 24 hr at 37°. After treatment, membranes were sedimented and prepared for SDS-PAGE and autoradiography, as described in Experimental Procedures. This experiment is representative of at least three experiments. Molecular weights are shown × 10^{-3}. *Arrowhead* indicates specifically labeled polypeptide following deglycosylation ($M_r \cong 46{,}000$).

and calf parathyroid gland (Fig. 7, *right*) D1 receptors resulted in the increased mobility of the labeled polypeptides in both tissues to apparent M_r = 46,000. Dose-response experiments (0–80 units/ml) indicate that this change appears to be the maximum induced by PNGase-F (data not shown) and as such represents the completely deglycosylated form of the D1 receptor. Moreover, the binding subunits of the dopamine D1 receptor do not appear to possess additional carbohydrates, such as *O*-linked glycan chains. This conclusion is based upon the absence of changes in the electrophoretic mobility of neuraminidase- and PNGase-F-treated photolabeled D1 receptors following treatment with *O*-glycanase and the absence of specific binding of [^{125}I]IMAB-labeled polypeptides to or elution from SBA and PNA lectin columns (using 0.3 M galactose) (data not shown). Attempts at chemical *O*-linked deglycosylation with sodium hydroxide/sodium borohydride also failed to support the existence of *O*-linked glycans associated with the dopamine D1 receptor binding subunits (data not shown). As such, it appears that treatment of photolabeled D1 receptor binding subunits with PNGase-F renders them completely deglycosylated and that the protein backbone of D1 receptors from both canine and bovine brain and calf parathyroid gland display similar, if not identical, molecular masses.

Discussion

A great deal of information has been accumulated recently regarding the structure and functional significance of the glycans associated with integral membrane proteins (14). Comparable studies examining the dopamine D1 receptor have not been carried out, due to the absence of methods for specifically labeling the binding subunit. Using a D1-selective photoligand, [^{125}I]IMAB (8), we have used lectin chromatography and specific endo- and exoglycosidases to probe the glycans associated with the D1 dopamine receptor.

As a result of these studies we have determined that 1) the predominantly labeled D1 receptor binding subunits of both calf parathyroid and canine brain are sialylated; 2) the glycans associated with these subunits are α-mannosidase sensitive and, as such, have terminal mannose residues; 3) there is some degree of neuronal D1 receptor glycan microheterogeneity and the neuronal and parathyroid D1 receptor appear to be differentially glycosylated; and 4) the sialylated and oligomannose-containing glycans appear to be colocalized on each of the binding subunits. Furthermore, we have demonstrated that the removal of sialic acid or oligomannose residues from the dopamine D1 receptor does not affect the binding characteristics of the D1-selective ligand [^{3}H]SCH-23390. Finally, we have observed that both canine brain and calf parathyroid gland D1 receptor binding subunits, upon complete deglycosylation, migrate on SDS-PAGE with apparently identical estimated molecular masses of $M_r \cong 46{,}000$. These data are consistent with the existence of one or more potential sites of *N*-linked glycosylation associated with either complex and/or hybrid-type glycans. Species- and tissue-specific differences in the estimated molecular weights of photolabeled receptor binding proteins have been previously observed for a number of receptor systems, including benzodiazepine (15), corticotropin releasing factor (16), A_1 adenosine (17), and dopamine D2 receptors (18, 19). Most of the differences observed are due to differential glycosylation patterns.

The existence of neuronal D1 receptor glycan microheterogeneity within the M_r = 74,000/64,000 labeled polypeptide, as delineated by α-mannosidase sensitivity, does not imply, however, the existence of distinct receptor populations differentiable by their glycan constituents. Evidence in support of this contention was obtained from lectin binding of solubilized [^{125}I]IMAB-labeled D1 receptors to WGA- and Con A-Sepharose. The absence of an appreciable difference between the electrophoretic mobilities of the soluble, pass-through, and eluted [^{125}I]IMAB-labeled protein from WGA-Sepharose indicates

that the binding subunits are not differentiable in terms of the presence or absence of either GlcNAc or sialic acid residues. Similar results were observed when receptor preparations were treated with neuraminidase before WGA-Sepharose chromatography, consistent with the role of the chitobiosyl-core of *N*-linked glycans in the interaction with WGA lectins (20–23). Moreover, SDS-PAGE of soluble, pass-through, wash, and eluate fractions of the Con A column demonstrated that the estimated M_r of binding subunits did not differ between fractions. Additionally, it was observed that all of the Con A fractions were equivalently sensitive to the exoglycosidase neuraminidase. This result indicates that complex-type glycans are present on even those photolabeled polypeptides that bind to and are specifically eluted from the Con A lectin. From these results, it is clear that those binding subunits that differ in terms of their affinity for the Con A lectin do not differ in their apparent M_r on SDS-PAGE or with respect to the presence or absence of terminal sialic acids. Moreover, treatment of photolabeled membranes with α-mannosidase before Con-A column chromatography produced a decrease in the retention (~70%) and subsequent elution of [^{125}I]IMAB-labeled polypeptides from the column; however, subsequent SDS-PAGE and autoradiography of the Con A column fractions indicated that no significant differences exist in the apparent M_r of the [^{125}I] IMAB-labeled peptides (data not shown). The residual specific binding of α-mannosidase-treated receptors to the Con A lectin column may indicate that terminal mannose residues are not required for association with the Con A lectin (see Refs. 23 and 24), or, perhaps, that under the conditions utilized α-mannosidase from jack bean did not promote the complete hydrolysis of all mannose residues (24). The results of these studies demonstrate that, although there appears to be microheterogeneity in the complement of glycans associated with D1 receptors, these do not appear to be associated with distinct classes of D1 receptor binding subunits of different estimated molecular masses. These results contrast with those obtained on the β-adrenergic receptor, where it has been shown that β-adrenergic receptors from a variety of tissues contain both high mannose- and complex-type carbohydrate moieties but that only one type of glycan is associated with a given receptor polypeptide (13, 25).

The functional significance of receptor glycosylation for the guanine nucleotide-binding protein-linked family of transmitter receptors is unclear at present. Glycosylation does not appear to be required for either ligand binding or coupling to guanine nucleotide-binding proteins for numerous receptor systems (17, 18, 25–33) including the D1 dopamine receptor, although it appears to be required for proper protein expression on the cell surface and in receptor transport through the cell (31–37).

After total *N*-linked deglycosylation, the predominantly labeled subunit of calf parathyroid and canine and bovine dopamine D1 receptors migrates as a single M_r = 46,000 polypeptide. A minor labeled peptide migrating at M_r = 28,000 probably represents a proteolytic degradation product of the higher molecular weight polypeptide. The binding subunits of the D1 receptor, in these tissues and species, do not appear to have *O*-linked glycans and, as such, the photolabeled protein of M_r = 46,000 observed after treatment with PNGase-F represents a totally deglycosylated D1 receptor. This estimate of the molecular mass of the D1 dopamine receptor falls well within the predicted molecular weight of other guanine nucleotide regulatory protein-linked receptors, based on their predicted amino acid sequence from isolated cDNA. The fact that the D1 receptor binding subunits migrate on SDS-PAGE with an apparently identical molecular mass supports the assertion that the peptide backbone of dopamine D1 receptors from different tissues and species appears to be homologous, consistent with previous peptide-mapping experiments (10). The recognition that D1 binding subunits from different tissues and species appear to be structurally conserved lends support to the contention that the initially observed differences in the SDS-PAGE mobility of photolabeled dopamine D1 receptors are possibly due to posttranslational modifications, primarily in glycosylation.

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