0026-895X/91/050712-05$03.00/0

MOLECULAR PHARMACOLOGY, 40:712-716

Antipeptide Antibodies Localize *N*-(4-Azido-3-[^{125}I] iodophenethyl)spiperone Binding to the Carboxyl-Terminal Portion of the D_2 Dopamine Receptor

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Received November 13, 1990; Accepted July 16, 1991

SUMMARY

Antibodies against synthetic peptides of the D_2 dopamine receptor were used, in combination with photoaffinity labeling, to localize the region of ligand binding in the receptor. Specific antibodies to peptide sequences 221–234 and 259–272 and to the carboxyl-terminal peptide 402–415, all corresponding to cytoplasmic regions in the D_2 dopamine receptor, were elicited. After photoaffinity labeling with *N*-(4-azido-3-[^{125}I]iodophenethyl)spiperone ([^{125}I]NAPS), all three antibodies specifically immunoprecipitated the 90-kDa D_2 dopamine receptor. Differential reactivity of the antipeptide antibodies with various proteolytic fragments indicates that [^{125}I]NAPS binds covalently to a 13-kDa fragment of the D_2 dopamine receptor. This fragment is immunoprecipitated with anti-peptide 402–415 and not with the other two antipeptide antibodies, indicating that the photoaffinity ligand binds to a fragment that begins beyond amino acid 272 and extends through the carboxyl-terminal end of the receptor.

Dopamine has been recognized to play a central role in central nervous system neurotransmission. Its action is transmitted through at least two types of receptors, known as D_1 and D_2, which have been classified by their ability to inhibit or stimulate adenylate cyclase, respectively, and by pharmacological differences (reviewed in Refs. 1 and 2). Until the recent cloning of the D_2 dopamine receptor, little was known about the molecular nature of this receptor, which has been implicated in key functions such as control of movement, behavior, and endocrine and cardiovascular function (3). Various drugs that are pharmacologically related to D_2 ligands are being used in the treatment of disorders such as Parkinson's disease and schizophrenia, although the exact site of their action within the D_2 receptor has not yet been elucidated.

The rat D_2 dopamine receptor (4) was predicted from its hydropathy plot to have seven transmembrane regions, characteristic of a member of a family of G protein-coupled receptors (5). Its subsequent cloning in human (6) and bovine tissues (7) showed that the receptor has >95% homology between species and also identified that there are two isoforms of the receptor that differ by the presence of an 87-base pair "insert" in the sequence originally published. These two isoforms, termed, among other names, "long" and "short," were shown to be expressed in the same regions of both rat and human brain and pituitary (4, 6, 7). It is still not clear what functional differences, if any, are present between them.

The binding sites for agonists and/or antagonists in the D_2 receptor have yet to be identified. [^{125}I]NAPS, a derivative of the D_2 antagonist spiperone, is a well characterized high affinity photolabel reagent for the D_2 dopamine receptor (8, 9). In the present study, we have developed specific antibodies directed against synthetic peptides corresponding to defined sequences in the D_2 receptor and have used them, in conjunction with photoaffinity labeling, to localize the region of [^{125}I]NAPS binding. We present evidence that this photoaffinity reagent is binding close to the carboxyl-terminal portion of the D_2 dopamine receptor. Preliminary results of this study have been reported recently (10).

Experimental Procedures

Materials

[^{125}I]NAPS (2200 Ci/mmol) was purchased from New England Nuclear (Boston, MA). (+)-Butaclamol and mianserin were obtained from Research Biochemicals, Inc. (Wayland, MA). EDCI, *Staphylococcus aureus* V8 protease, soybean trypsin inhibitor, benzamidine, leupeptin, trypsin, phenylmethylsulfonyl fluoride, HEPES, Triton X-100, and

This research was supported by grants from the Rockfeller-Weizmann Collaboration Trust Fund and from the Yeda Fund.

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; [^{125}I]NAPS, *N*-(4-azido-3-[^{125}I]iodophenethyl)spiperone; EDCI, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline (0.14 M NaCl, 0.01 M phosphate buffer, pH 7.2); RIPA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 10 mM sodium phosphate buffer, pH 7.5; SDS, sodium dodecyl sulfate; CFA, complete Freund's adjuvant.

BSA were all purchased from Sigma Chemical Co. (St. Louis, MO). Protein A-Sepharose and Sephadex G-25 were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). [3H]Spiperone was purchased from Amersham (Buckinghamshire, England). CFA and incomplete Freund's adjuvant were purchased from Difco (Detroit, MI). All other reagents were of the highest analytical grade.

Fresh bovine striata were obtained from a local butcher and used immediately or frozen at −80° until use.

Methods

Membrane preparation. Tissue samples of bovine striatum were homogenized in 10 volumes of ice-cold 50 mM HEPES, pH 7.4, containing 0.25 M sucrose and the following protease inhibitors: 20 mM EDTA, 15 μg/ml benzamidine, 5 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 1000 × *g* for 10 min, and the supernatant was recentrifuged at 40,000 × *g* for 50 min. The resulting pellet was resuspended in 50 mM HEPES buffer, pH 7.4, containing 100 mM NaCl and protease inhibitors (assay buffer), to yield a final concentration of approximately 5 mg/ml, representing 0.5–1.0 nM [3H]spiperone binding.

Photoaffinity labeling. Labeling of membranes was carried out by a modification of the procedure described (11). Membranes (1 ml) were incubated for 15 min at 37° in 9 ml of assay buffer, in the presence or absence of 10^{-6} M (+)-butaclamol or mianserin as indicated. [125I]NAPS (1 ml) was then added to yield a final concentration of 50–100 pM, equivalent to the concentration of spiperone binding sites in the membranes. After a 90-min incubation at 22°, the membranes were centrifuged at 40,000 × *g* at 4° for 15 min, washed once in ice-cold assay buffer containing 0.5% BSA, recentrifuged, and resuspended in 1 ml of assay buffer (4°). Photolysis (45 sec, 4°) was carried out with a mercury lamp at a distance of 10 cm. [125I]NAPS-labeled membranes were sedimented at 15,000 × *g*, washed with assay buffer, and processed for immunoprecipitation as described below.

Antibody preparation. The following three synthetic peptides derived from the predicted amino acid sequence of the rat D_2 dopamine receptor (4) were synthesized in the Laboratory of Peptide Synthesis of the Weizmann Institute of Science, by the solid-phase method of Merrifield (12): KRVNTKRSSRAFRA (residues 221–234), SPPERTRYSPIPPS (residues 259–272), and NIEFRKAFMKILHC (residues 402–415). The sequences of the first two peptides are identical in rat, human, and bovine D_2 receptors. In the third peptide (residues 402–415), methionine-410 of the rat receptor is replaced by a leucine in the human and bovine D_2 receptors (6, 7). Peptides were purified by chromatography on Sephadex G-25 and conjugated to BSA essentially as described (13). Briefly, the peptide was dissolved in 2 ml of PBS and was added to BSA in a 40-fold molar excess. After 10 min of stirring at 22°, the coupling reagent EDCI (dissolved in 0.5 ml of H_2O) was added in a 10-fold molar excess over the peptide. The mixture was stirred at 22°, dialyzed against PBS, aliquoted, and kept frozen at −20°. New Zealand White rabbits were immunized by multisite intradermal injections of 1 mg of peptide conjugate in 0.5 ml of PBS emulsified in 0.75 ml of CFA. A booster injection (0.5 mg in CFA) was similarly administered 2–3 weeks later. Additional booster injections were given at 2-week intervals in incomplete Freund's adjuvant. Rabbits were bled by ear vein puncture 10–14 days after the booster injection. Antisera were assayed for their ability to interact specifically with the free peptide by a solid-phase radioimmunoassay (14). Typically, two booster injections were enough to yield suitable antipeptide titers of over 1/10,000; the animals were subsequently bled every 2 weeks and the sera were frozen at −20°.

Immunoprecipitation. Photolabeled membranes were sedimented by centrifugation at 15,000 × *g*. For solubilization, the membranes were resuspended in an equal volume of RIPA buffer (15) and rotated at 4° for 1 hr. The solution was centrifuged for 15 min at 15,000 × *g*, and the supernatant (RIPA-solubilized membranes) was collected. Unless indicated otherwise, 200 μl of RIPA-solubilized membranes were incubated with 20 μl of antiserum. After incubation overnight at 4°, 40 μl of 50% (v/v) Protein A-Sepharose CL-4B (preswollen in PBS) were added and rotated for 1 hr at 4°. The beads were centrifuged at 15,000 × *g* for 2 min and washed twice in PBS, once in PBS-0.5% Triton X-100, and once in PBS. The beads were vortexed in SDS-polyacrylamide gel electrophoresis sample buffer (25 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromphenol blue) and heated at 37° for 30 min. The beads were recentrifuged, and the supernatants were loaded onto gradient 5–15% polyacrylamide gels or 15% gels (16), as indicated. The gels were dried, autoradiographed with AGFA Curix RP2 film, and exposed with an intensifying screen (Cronex Quanta III; Du Pont) at −80°. For inhibition of immunoprecipitation by synthetic peptides, the antiserum was incubated with 20 μM tested peptide for 1 hr at 22° before the incubation of the antiserum with the RIPA-solubilized membranes.

In some cases, the antibodies were prebound to Protein A-Sepharose for 1 hr at 4° before their addition to the solubilized membranes. After overnight rotation at 4°, the beads were washed and treated as described above. There was no difference in immunoprecipitation efficacy between the two methods.

Proteolytic digestions. For trypsinization, photolabeled membranes were resuspended in assay buffer devoid of protease inhibitors, and 0.3 mg of trypsin was added per 1 ml of membranes. After a 2-hr incubation at 22°, the membranes were spun down and solubilized, and immunoprecipitation was carried out as described above. Where V-8 protease is indicated, sample buffer eluates of immunoprecipitation were loaded onto the gel, and 1.6 μg of enzyme was added per 10-μl sample. The samples were allowed to enter the gel and then stopped for 1 hr, while digestion was carried out in the stacking gel. The gel was then completed as previously described.

Results

Specific immunoprecipitation of [125I]NAPS-labeled D_2 dopamine receptors by antipeptide antibodies. Photoaffinity labeling of striatal membranes from various species using [125I]NAPS has been shown to specifically label D_2 dopamine receptors (8, 9). We have used this reagent to label the dopamine receptor in bovine striatum and have used antipeptide antibodies to immunoprecipitate the receptor. [125I]NAPS labeled primarily a 90-kDa protein. Some labeling was also detected in proteins of 40, 20, and 14 kDa (data not shown). The D_2 specificity of labeling was verified by the observation that all four bands were not labeled if the membranes were preincubated with (+)-butaclamol and were labeled in the presence of the serotonergic antagonist mianserin.

We developed antipeptide antibodies against three synthetic peptides, corresponding to residues 221–234, 259–272, and 402–415 of the rat D_2 dopamine receptor. The first two peptides were chosen to be from unique D_2 sequences in the putative third cytoplasmic loop of the receptor, and they show no homology with other members of the G protein-coupled receptor family. Peptide 221–234 encompasses a consensus site for protein kinase A phosphorylation, and peptide 259–272 is rich in proline residues (five prolines of 14 amino acids). The third peptide (residues 402–415) corresponds to the carboxyl-terminal end of the D_2 receptor, which is proposed to be cytoplasmic as well. All three peptides are in regions that were suggested to be important for coupling to G proteins in other receptors of this family (reviewed in Ref. 17).

Antibodies to the three peptides specifically immunoprecipitated the photolabeled D_2 receptor. Immunoprecipitation of the labeled 90-kDa protein by anti-peptide 221–234 (Fig. 1A), anti-peptide 259–272 (Fig. 1B), and anti-peptide 402–415 (Fig. 1D) was not detected if the membranes were photolabeled in

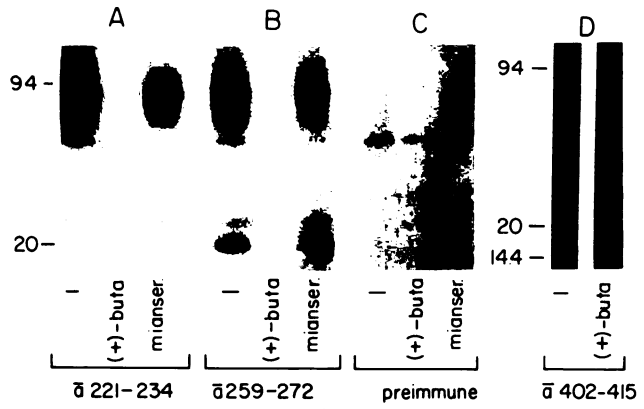

Fig. 1. Immunoprecipitation of photolabeled D_2 dopamine receptors from bovine striatum. Striatal membranes were labeled with [^{125}I]NAPS, in the absence (–) or presence of (+)-butaclamol or mianserin, and then solubilized and immunoprecipitated by the various antisera, as outlined in Experimental Procedures. Immunoprecipitates from 200 μl (A, B, and C) or 100 μl (D) of membranes were electrophoresed on a 5–15% gradient polyacrylamide gel and autoradiographed. The molecular masses of known prestained standards are shown in kDa.

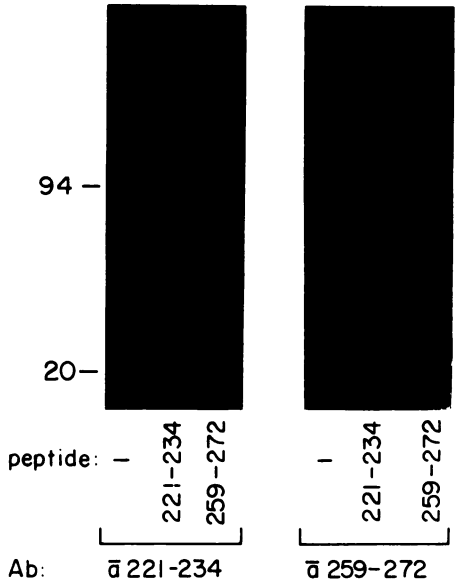

Fig. 2. Inhibition of immunoprecipitation of photolabeled bovine striatal membranes by specific peptides. Bovine striatal membranes were labeled with [^{125}I]NAPS and solubilized as outlined in Experimental Procedures. Immunoprecipitation was performed with the antisera in the absence (–) or presence of 20 μM indicated peptide. Samples were electrophoresed on a 5–15% gradient polyacrylamide gel and autoradiographed.

the presence of (+)-butaclamol. After photoaffinity labeling in the presence of mianserin, the 90-kDa protein was detectable (Fig. 1, A and B), giving additional evidence that this is the D_2 receptor. In addition to the 90-kDa band, anti-peptide 259–272 immunoprecipitated the smaller, specifically photolabeled 20-kDa fragment (Fig. 1B), and anti-peptide 402–415 immunoprecipitated this band in addition to a 14-kDa band (Fig. 1D). A fainter labeled band (~55 kDa), which was occasionally observed, appears to be nonspecific, because it was labeled in the presence of (+)-butaclamol and was immunoprecipitated by control preimmune serum as well (Fig. 1C).

The specificity of the interaction of the antibody with its peptide epitope on the D_2 dopamine receptor was verified by inhibition experiments (Fig. 2). In the presence of the corresponding immunizing peptide the immunoprecipitation of the photolabeled 90-kDa band was completely inhibited, whereas in the presence of a different peptide from the sequence no inhibition of the immunoprecipitation was observed. As seen in Fig. 2, the smaller labeled fragment of 20 kDa, which was immunoprecipitated by anti-peptide 259–272, was also inhibited by its respective peptide. Thus, the antibodies are recognizing well defined regions of the D_2 dopamine receptor, and conclusions can be drawn as to the nature of the smaller labeled fragments that are being immunoprecipitated by certain anti-peptide antibodies.

***Differential reactivity of the antipeptide antibodies with various proteolytic fragments of the D_2* dopamine receptor.** As mentioned above, all three antipeptide antibodies immunoprecipitated the [^{125}I]NAPS-labeled 90-kDa band, probably representing the fully glycosylated D_2 dopamine receptor (9). However, anti-peptide 259–272 and anti-peptide 402–415 precipitated smaller labeled fragments as well (Fig. 3A). Both sera precipitated the 20-kDa band, whereas anti-peptide 402–415 also precipitated a 14-kDa fragment. In order to find out whether these smaller fragments could be derived from the larger 90-kDa band, the immunoprecipitated products were subjected to digestion by V-8 protease (Fig. 3B). According to the published sequence (7), there are many possible cleavage points in the bovine D_2 receptor for V-8, which cleaves peptide bonds after aspartic and glutamic acid residues. Indeed, after partial digestion with V-8 enzyme, the 90-kDa immunoprecipitate of anti-peptide 221–234 (Fig. 3*A*, *first lane*) yielded both the 20-kDa and 14-kDa bands (Fig. 3B, *first lane*). Some digestion products of lower molecular masses were also seen. Similarly, the immunoprecipitation products of the other two antibodies yielded the same profile, with increased amounts of the 20-kDa and 14-kDa fragments (Fig. 3). This supports the notion that the smaller fragments originally immunoprecipitated by anti-peptide 259–272 and anti-peptide 402–415 were derived from the 90-kDa protein and may represent products of partial proteolysis. Because only anti-peptide 402–415 immunoprecipitated the 14-kDa fragment (Fig. 3A, *third lane*), we concluded

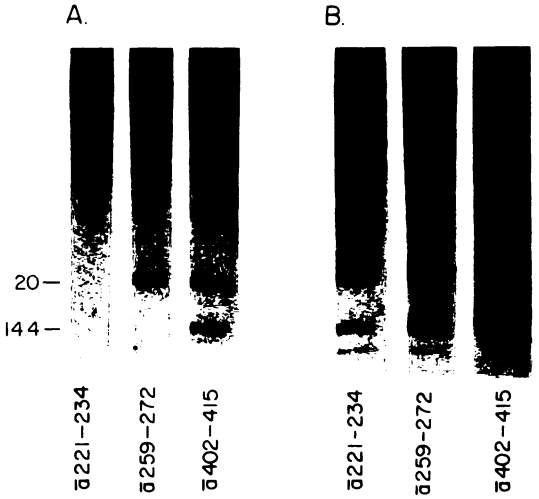

Fig. 3. Immunoprecipitation and V-8 digestion of immunoprecipitates from [^{125}I]NAPS-labeled membranes. A, Immunoprecipitation was carried out with three different antisera as indicated, as outlined in Experimental Procedures. B, Immunoprecipitates, solubilized in sample buffer, were treated with V-8 protease as outlined in Experimental Procedures. The samples were electrophoresed on a 15% polyacrylamide gel and autoradiographed.

at this point that the photolabel is localized somewhere within a carboxyl-terminal fragment of the D_2 receptor, which is not longer than 14 kDa.

In order to attempt the localization of the affinity labeling site in the D_2 receptor to even smaller fragments, we have subjected the membranes to tryptic digestion before immunoprecipitation with the various antipeptide antibodies (Fig. 4). When membranes were digested with trypsin, all the label was localized in small fragments (Fig. 4B, *first lane*). Of the three antipeptide antibodies tested, only the antibody against the carboxyl-terminal peptide (residues 402–415) could immunoprecipitate a labeled fragment. This experiment indicates that the photolabel is bound to a residue(s) that is beyond amino acid 272 of the D_2 receptor. The fragment containing the photolabel is of approximately 13 kDa and should, therefore, represent the product of cleavage toward the end of the third cytoplasmic loop of the protein. This implies that the photoaffinity ligand binds close to a residue(s) located within the sixth or seventh transmembrane segments or in the extracellular stretch between them.

Discussion

In this report we have used antipeptide antibodies in combination with photoaffinity labeling and immunoprecipitation in order to localize the region of [125I]NAPS binding to the D_2 dopamine receptor. We used two different approaches in order to look at immunoprecipitation of labeled fragments of the receptor, immunoprecipitation of fragments already present in the photolabeled membrane preparation and immunoprecipitation of fragments after proteolysis. Using the first strategy, we showed that only antibodies to the carboxyl-terminal peptide (residues 402–415) could immunoprecipitate a 14-kDa labeled fragment (Fig. 3A, *third lane*). Digestion of the immunoprecipitates with V-8 protease supported the notion that this fragment was indeed derived from the major 90-kDa labeled protein, which probably represents the fully glycosylated receptor. Thus, this fragment is most likely the product of partial proteolysis that takes place despite the protease inhibitors used. It should be noted that addition of such inhibitors to the RIPA buffer during the solubilization step had no effect on the appearance of these fragments.

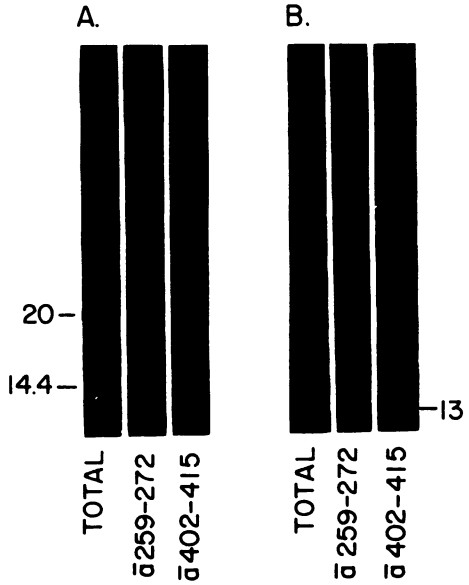

Fig. 4. Immunoprecipitation of [125I]NAPS-labeled membranes after trypsinization. Membranes were photolabeled and either not treated (A) or digested (B) with trypsin. Immunoprecipitation was carried out as described in Experimental Procedures. *TOTAL*, 20 μl of RIPA-solubilized membranes. The samples were electrophoresed on a 15% polyacrylamide gel and autoradiographed.

In the second set of experiments, we digested the membranes with trypsin, in order to identify even smaller labeled fragments of the receptor. Indeed, after trypsinization, all of the label was localized in small fragments (Fig. 4B, *first lane*). Again, of the three antibodies tested, only the carboxyl-terminal antibody was capable of immunoprecipitating a labeled fragment. Thus, the fragment of approximately 13-kDa molecular mass extends through the carboxyl-terminal end of the receptor. Because neither anti-peptide 221–234 nor anti-peptide 259–272 immunoprecipitated this fragment, the tryptic cleavage should be in the third cytoplasmic loop, beyond amino acid 272. The calculated molecular mass of a fragment extending from residue 273 to the carboxyl-terminal end (residue 415) is 16 kDa. This means that the cleavage site should be located approximately 20–30 amino acid residues carboxyl-terminal to position 272. Indeed, according to the available sequence (7), there are several lysines and arginines in this region, which may be candidates for tryptic cleavage.

It should be noted that anti-peptide 402–415 could not precipitate all the labeled fragments after trypsinization. Such nonprecipitable fragments may be the products of cleavage between amino acids 402 and 415 and, therefore, not recognized by the antibody. Alternatively, it is possible that the photoaffinity reagent also labels other portions of the receptor that are spatially at the right distance from the ligand-binding region. We have some preliminary evidence that antibodies to the putative first cytoplasmic loop immunoprecipitate some small labeled fragments as well.[1]

By analogy to other G protein-linked receptors, the putative seven transmembrane segments are likely to be bundled such that the ligand fits into a pocket and contacts more than one residue simultaneously. These contact points may be in regions that appear to be distal, according to the amino acid sequence (3). Indeed, various studies on other G protein-linked receptors suggest that there may be several contact points involved in ligand binding. Involvement of the putative seventh transmembrane region in ligand binding was proposed for rhodopsin (18) and the β_1-adrenergic receptor (19). Other studies indicated that conserved aspartates in the putative second and third transmembrane domains and serines in the fifth transmembrane domain interact with catecholamine ligands (20–22). It is also possible that not all ligands contact the same points in a given receptor (23).

To our knowledge, this is the first report on localization of a ligand binding site in the D_2 dopamine receptor. Our study demonstrates that the sixth and/or seventh transmembrane regions are involved in or close to the site of antagonist binding. Further fragmentation and sequencing of this labeled fragment must be carried out in order to determine the exact point of labeling. In addition to the possibility that one reagent may label more than one residue, various isoforms and subtypes of the same receptor may be labeled differently. In this respect, it would be interesting to find out whether the long and short D_2

[1]C. David and S. Fuchs, unpublished data.

receptors and the newly described D_3 (24) and D_4 (25) receptors bind [^{125}I]NAPS at the same site. The antipeptide antibodies described here, as well as others developed in our laboratory, prove to be most useful tools to clarify these questions.

Acknowledgments

We wish to thank Dr. Z. Elazar and Dr. H. Kanety for fruitful discussions.

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