Dopamine D1 Receptors of the Calf Parathyroid Gland: Identification of a Ligand Binding Subunit with Lower Apparent Molecular Weight but Similar Primary Structure to Neuronal D1 Receptors†

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ABSTRACT: The ligand binding subunit of the calf parathyroid D1 dopamine receptor was visualized by autoradiography following photoaffinity labeling with (±)-7-[^{125}I]iodo-8-hydroxy-3-methyl-1-(4′-azidophenyl)-2,3,4,5-tetrahydro-1*H*-benzazepine ([^{125}I]IMAB) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The protein comprising the D1 binding subunit migrated with an apparent $M_r \cong 62\,000$. Photoincorporation of [^{125}I]IMAB into the $M_r \cong 62\,000$ polypeptide required the presence of protease inhibitors and was stereoselectively antagonized by dopaminergic agonists and antagonists with an appropriate pharmacological specificity for D1 receptors. The electrophoretic mobility of the [^{125}I]IMAB-labeled receptor was not altered by the absence or presence of urea or thiol-reducing/oxidizing reagents. The $M_r \cong 62\,000$ protein representing the ligand binding subunit of bovine parathyroid D1 receptors corresponds to one of three D1 receptor binding subunits (M_r = 74 000, 62 000, and 51 000) identified in bovine brain. Peptide map comparisons of radiolabeled D1 receptors from calf parathyroid and brain following limited proteolytic digestion with *Staphylococcus aureus* V8 and papain revealed marked structural similarities. These data suggest that, despite tissue-specific differences in overall molecular weight, both parathyroid and neuronal D1 dopamine binding subunits appear to be pharmacologically and structurally homologous and that the molecular mechanism(s) responsible for the apparent lack of a one to one correspondence in the subunit composition of the D1 receptor in these tissues probably reflect(s) tissue-specific posttranslational modifications.

As originally proposed by Kebabian and Calne (1979), the bovine parathyroid gland contains the prototypical dopamine D1 receptor. The D1 dopamine receptor in both the brain and periphery has been identified and defined on the basis of pharmacological and biochemical criteria [see Seeman (1980), Stoof and Kebabian (1984), Niznik (1987), and Seeman and Niznik (1988) for review] which include the ability to stimulate the enzyme activity of adenylate cyclase and to bind or to respond to dopaminergic agonists and antagonists, specifically of the benzazepine class, with high affinity and specificity. In dispersed cells of the bovine parathyroid gland, dopamine, as well as other selective D1 receptor agonists (SKF-38393[1] and SKF-82526), stimulates the activity of adenylate cyclase and effects the transient release of immunoreactive parathyroid hormone (Brown et al., 1977, 1980, 1988; Attie et al., 1980). These actions are stereoselectively blocked by the selective D1 receptor antagonist SCH-23390 but not (or with much lower potency) by selective D2 receptor antagonists (Brown et al., 1980; Niznik et al., 1988a). Moreover, data from radioligand binding experiments using well-defined dopaminergic agonists and antagonists suggest that bovine parathyroid and neuronal D1 receptors display strong pharmacological homology (Niznik et al., 1988a). In order to assess whether this homology extends to the molecular level as well, we identified the ligand binding subunit of the parathyroid D1 receptor with the recently developed specific D1 photoaffinity probe [^{125}I]IMAB (Niznik et al., 1988b).

Photoaffinity labeling of bovine, canine, or porcine striatal membranes with [^{125}I]IMAB has revealed a polypeptide of apparent $M_r \cong 74\,000$ to represent the major ligand binding subunit of neuronal D1 dopamine receptors (Niznik et al., 1988b) similar to a $M_r \cong 72\,000$ polypeptide identified in rat brain (Amliaky et al., 1987). [^{125}I]IMAB, however, labeled in addition two smaller molecular weight proteins of apparent M_r = 62 000 and 51 000 with a pharmacological profile indicative of the D1 receptor subtype (Niznik et al., 1988b). The molecular relationship between these three binding subunits is currently unknown.

In this paper we document that the parathyroid D1 dopamine binding protein appears to be comprised of only one of the three binding subunits identified in neural tissue with an apparent M_r = 62 000 and which shares structural homology with neural D1 receptors.

Experimental Procedures

Materials. (±)-[^{125}I]IMAB (2200 Ci/mmol) was prepared as previously described (Niznik et al., 1988b; Baindur et al.,

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[1] Abbreviations: [^{125}I]IMAB, (±)-7-[^{125}I]iodo-8-hydroxy-3-methyl-1-(4′-azidophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SCH-23390, (*R*)-(+)-8-hydroxy-7-chloro-3-methyl-7-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF-38393, 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1*H*-3-benzazepine; SKF-82526, fenoldopam; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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1988). Protease inhibitors and proteases were from Sigma, and prestained molecular weight protein standards were from Amersham. Electrophoresis reagents were obtained from Bio-Rad, and X-ray films (XAR-5) and developing solutions were from Kodak. Dopaminergic drugs and other reagents were either purchased from or generously donated by various pharmaceutical firms as previously descirbed (Niznik et al., 1988a,b).

Membrane Preparation. Calf parathyroid glands were frozen at −70 °C until use. Tissues were thawed, trimmed of excess fat, minced, and immediately Teflon–glass homogenized (30 up and down strokes) in 20 volumes of ice-cold 25 mM Tris-HCl buffer containing 250 mM sucrose in the absence or presence of the following protease inhibitors: 20 mM EDTA, 5 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor, 15 μg/mL benzamidine, and 1 mM PMSF (pH 7.4 at 4 °C). Homogenates were filtered through four layers of cheesecloth and were centrifuged for 10 min at 600*g*. The supernatant was collected and recentrifuged at 48000*g* for 20 min. The resulting membrane pellets were resuspended in 25 mM Tris-HCl buffer containing 100 mM NaCl in the absence or presence of protease inhibitors (as indicated), to a protein concentration of 2–4 mg/mL.

Photoaffinity Labeling. Membranes (1 mL) were incubated in the dark with ~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 °C in the absence or presence of the indicated concentration of dopaminergic agents. Following incubation, membranes were sedimented at 48000*g* for 20 min and pellets resuspended in buffer (as above) containing 0.5% bovine serum albumin and recentrifuged. The resulting pellets were resuspended in 1 mL of 25 mM Tris-HCl buffer containing 100 mM NaCl and protease inhibitors (unless stated otherwise) and irradiated for 35 s as previously described (Niznik et al., 1988b). [^{125}I]IMAB-labeled membranes were subsequently sedimented for 10 min at 12000*g*.

SDS–PAGE and Autoradiography. Electrophoresis was performed by the method of Laemmli (1970) as previously described (Niznik et al., 1988b). Briefly, photolabeled membranes were solubilized in 50 mM Tris-HCl, 10% SDS, 10% glycerol, and 5% β-mercaptoethanol (where indicated), pH 6.8, for 1–2 h at 22 °C. Aliquots (~100–300 μg of protein) were loaded onto 1.5 mm thick slab gels containing a 12% separating gel (~16 cm) and a 6% stacking gel and electrophoresed overnight. Following electrophoresis gels were dried and exposed to Kodak XAR film with one intensifying screen at −70 °C for ~72 h. Molecular weights of photolabeled receptor subunits were determined graphically as previously described (Niznik et al., 1988b). The values given are means of several experiments.

Peptide Mapping. Second-dimension gel electrophoresis was performed according to the method of Bordier and Crettol-Jarvinen (1979) as modified by Stiles et al. (1983). Briefly, the gel from the first dimension was incubated for 40 min with 125 mM Tris, pH 6.8, containing 0.1% SDS at 22 °C. The lanes containing the D1 binding subunits that were to be simultaneously compared by peptide mapping were excised and trimmed so that the area of interest $M_r \cong 80000$–48000 remained. Subsequent autoradiography confirmed that the appropriate sections were taken. The two sections were then introduced (horizontally) between the glass plates of the second gel containing a 50-mm stacking gel and a 115-mm separating gel of 17.5% acrylamide. A molten solution of 1% agarose in 125 mM Tris and 0.1% SDS, pH 6.8, was added to the level of the gel sections and allowed to solidify. A 1-mL solution of 50 mM Tris, pH 6.8, 2% SDS, and 10% glycerol was then added with the indicated concentrations of proteinase and carefully overlayed with running buffer. Electrophoresis was carried out at 4 mA/gel for ~16 h. The separating phase of the electrophoresis was carried out at 20 mA/gel. As previously described by Stiles et al. (1983), this technique allows for the simultaneous proteolysis and analysis of at least two receptor peptides of similar molecular weight under identical conditions. In control experiments, where no proteinase was added, only the original photolabeled receptor migrated in the second-dimension gel with no proteolytic products seen. Upon completion of the run, gels were dried and processed for autoradiography as described above. Estimates of the molecular mass of proteolyzed receptor fragments were derived from prestained standards run on identically prepared gels, in the absence of proteinase.

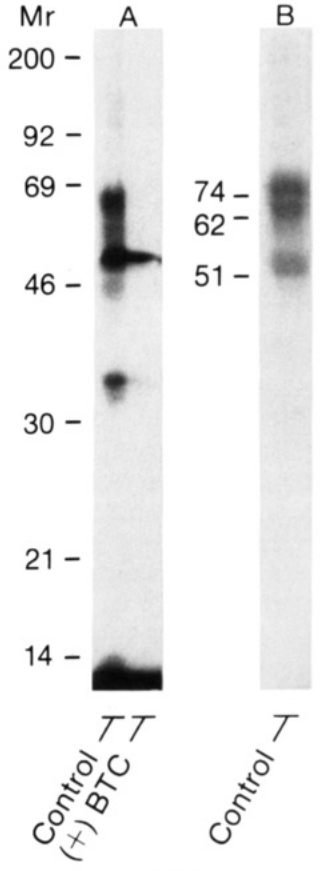

FIGURE 1: Photoincorporation of [^{125}I]IMAB into calf parathyroid and striatal membranes. Homogenates from calf parathyroid (A) and brain (B) were prepared and incubated with ~250 pM [^{125}I]-IMAB alone or in the presence of 100 nM (+)-butaclamol, photolyzed, and subjected to SDS–PAGE and autoradiography as described under Experimental Procedures. The results shown are representative of many experiments. The M_r of known prestained molecular weight standards are shown × 10^{-3}.

Results and Discussion

Figure 1A depicts the results obtained when bovine parathyroid membranes were incubated with [^{125}I]IMAB, photolyzed, and subjected to SDS–PAGE and autoradiography. A band at apparent $M_r \cong 62000$ was labeled. The specificity of labeling was shown by virtue of the fact that covalent photoincorporation of [^{125}I]IMAB into the $M_r \cong 62000$ protein was blocked by 100 nM (+)-butaclamol. In addition, a nonspecific band of apparent $M_r \cong 51000$–50000 was also labeled as well as bands at apparent $M_r \cong 35000$ and 33000. For comparative purposes, Figure 1B also depicts the results

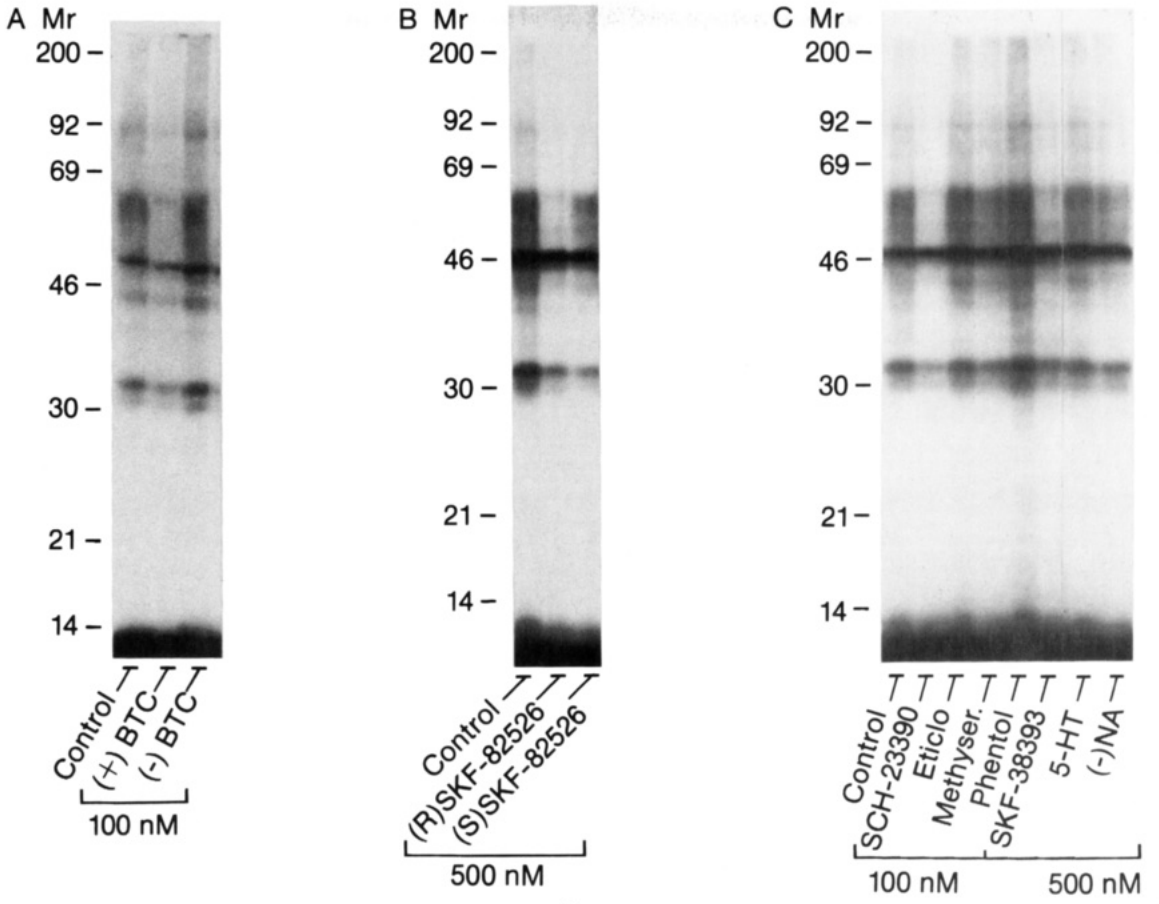

FIGURE 2: Photoaffinity labeling and pharmacological specificity of [^{125}I]IMAB incorporation into membranes of the calf parathyroid gland. Membranes were prepared and photoaffinity labeled with [^{125}I]IMAB (~250 pM) alone or in the presence of the indicated concentrations of competing ligands as described under Experimental Procedures. Samples were solubilized and electrophoresed on a 12% acrylamide gel and subjected to autoradiography. The M_r of prestained molecular weight standards are shown $\times 10^{-3}$. The results shown are representative of three similar experiments. Abbreviations: BTC, butaclamol; ETIC, eticlopride; METHYSER, methysergide; 5-HT, serotonin; NA, noradrenaline.

obtained when bovine striatal membranes were photolabeled with [^{125}I]IMAB and subjected to SDS–PAGE and autoradiography. As previously described (Niznik et al., 1988b) three radiolabeled bands were apparent with electrophoretic mobilities of M_r = 74 000, 62 000, and 51 000. It appears, therefore, that only the M_r = 62 000 labeled protein is shared in these tissues.

The pharmacological specificity of [^{125}I]IMAB photoincorporation was further assessed by examining the ability of various dopaminergic agents to block covalent labeling of the M_r = 62 000 peptide from bovine parathyroid glands. As seen in Figure 2A,B the photolysis-dependent labeling of the M_r = 62 000 protein was stereoselectively blocked by (+)-butaclamol but not (–)-butaclamol and by the selective D1 receptor agonist (*R*)-SKF-82526. Moreover, as seen in Figure 2C, the selective D1 receptor antagonist, SCH-23390, and agonist SKF-38393 blocked covalent labeling of the M_r = 62 000 protein, while the selective D2 receptor antagonist (eticlopride), serotonergic antagonist (methysergide) or agonist (5HT), and adrenergic antagonist (phentolamine) had little effect on [^{125}I]IMAB photoincorporation. Interestingly enough, noradrenaline at a concentration of 500 nM appeared to partially prevent the photoincorporation of [^{125}I]IMAB and appears to reflect this ligand's higher affinity for parathyroid D1 (K_i ~ 20 nM) rather than neuronal (~1000 nM) D1 receptors as assessed by radioligand binding (Niznik et al., 1988a). Taken together, the data suggest that the $M_r \cong$ 62 000 labeled protein represents the ligand binding subunit of the parathyroid D1 dopamine receptor.

In many receptor systems, failure to prevent endogenous protease activity during photoaffinity labeling may result in the generation of lower molecular weight protein fragments displaying the same pharmacological profile as the native receptor (Benovic et al., 1983; Leeb-Lundberg et al., 1984; Niznik et al., 1986; Amliaky & Caron, 1986; Nissenson et al., 1987; Poland & Glover, 1988; Yamada et al., 1987). In order to ascertain whether the [^{125}I]IMAB-labeled polypeptide of $M_r \cong$ 62 000 was a partially proteolyzed receptor fragment of a higher molecular weight protein, membranes of the calf parathyroid gland were prepared and photolyzed in either the complete absence or presence of multiple protease inhibitors previously shown to be useful in preventing receptor degradation. As illustrated in Figure 3 photoaffinity labeling of the apparent $M_r \cong$ 62 000 polypeptide was entirely dependent upon the presence of protease inhibitors since the specific photoincorporation of [^{125}I]IMAB into this polypeptide was completely abolished when membranes were prepared, photolyzed, and processed for SDS–PAGE in Tris buffer alone. Instead, minor and partially specific labeled fragments of apparent $M_r \cong$ 53 000 and 45 000 were seen. These data contrast sharply with those obtained in neural tissue of the same species, where the specific photoincorporation of [^{125}I]IMAB into the polypeptides of apparent $M_r \cong$ 74 000, 62 000, and 51 000 did not appear to be dependent on the presence of protease inhibitors (Niznik et al., 1988b).

The exact class or combination of protease inhibitor(s) responsible for the protection of the $M_r \cong$ 62 000 polypeptide from endogenous degradation was not ascertained, but previous work on other receptor systems has suggested that the metalloprotease inhibitor EDTA alone may be sufficient (Benovic

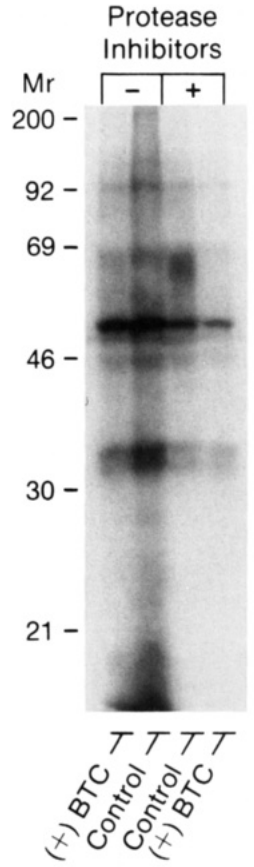

FIGURE 3: Effect of protease inhibitors on the photoaffinity labeling pattern of [^{125}I]IMAB into calf parathyroid D1 dopamine receptors. Membranes were prepared and photolyzed in the complete absence (Tris buffer alone) or presence of multiple protease inhibitors and subjected to SDS–PAGE and autoradiography as described under Experimental Procedures. The results shown are typical of three similar experiments. Included in the gel are lanes demonstrating nonspecific labeling as defined by 1 μM (+)-butaclamol (BTC). M_r of molecular weight standards are shown $\times 10^{-3}$.

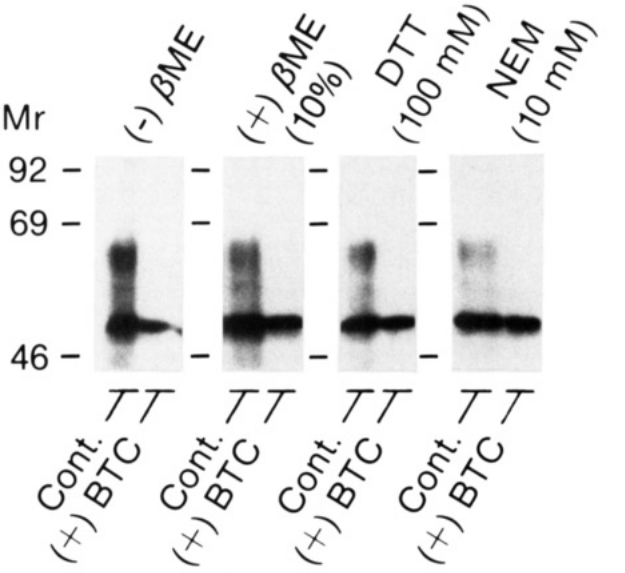

FIGURE 4: SDS–PAGE migration pattern of [^{125}I]IMAB-labeled D1 receptors following thiol-reducing or sulfhydryl-alkylating reagents. Photoaffinity-labeled membranes of the calf parathyroid gland were solubilized for 2 h at 22 °C in buffer containing 10% SDS in the absence (control) or presence of the indicated concentration of thiol-reducing or sulfhydryl-alkylating reagents and processed for SDS–PAGE and autoradiography as described under Experimental Procedures. Included in this gel are lanes demonstrating nonspecific labeling [1 μM (+)-butaclamol] for each condition. M_r of some molecular weight standards are shown $\times 10^{-3}$.

et al., 1983; Leeb-Lundberg et al., 1984). Given the rather extreme sensitivity of the $M_r \cong 62000$ labeled polypeptide to proteolytic degradation, it is difficult to assess whether any additional high molecular weight D1 receptor subunits exist in the parathyroid gland that correspond to the $M_r = 74000$ labeled D1 receptor subunit in neural tissue of the same or different species. In any event, the data presented here appear to suggest that the calf parathyroid $M_r = 62000$ labeled protein is the only major D1 receptor binding subunit observed in this tissue and corresponds to one of three distinguishable binding subunits identified in calf brain under identical experimental conditions.

Previous work on the insulin (Helmerhorst et al., 1986), β-adrenergic (Moxham & Malbon, 1985; Moxham et al., 1988), and neurotensin (Mills et al., 1988) receptor has shown that the apparent molecular mass of these receptors in SDS–PAGE can vary considerably depending on the oxidation–reduction state of the protein. In order to assess the possible subunit composition of the apparent $M_r \cong 62000$ polypeptide, the ability of DTT, β-mercaptoethanol, NEM, or urea to alter the migration pattern of photolabeled parathyroid D1 receptors was investigated. As illustrated in Figure 4 neither β-ME (10%), DTT (100 mM), nor NEM (10 mM) altered the migration pattern of the photolabeled D1 receptor subunit of apparent $M_r = 62000$ as compared to control samples. Similar results were obtained when [^{125}I]IMAB-labeled membranes were treated with these reagents prior to solubilization in SDS buffer or in the presence of 8 M urea during SDS–PAGE (data not shown). These data indicate that the $M_r \cong 62000$ labeled subunit of the parathyroid D1 receptor probably does not contain intramolecular disulfide bridges and is similar to that observed for neuronal D1 receptor binding subunits of the calf striatum (Niznik et al., 1988b).

In order to ascertain whether the [^{125}I]IMAB-labeled D1 receptor binding subunits from bovine parathyroid gland and striatum share structural similarities, peptide maps of photolabeled receptors from both tissues were constructed. Analyzing partial proteolytic peptide maps of heterogeneous receptor populations has been used, for example, to establish structural similarities or differences for numerous proteins (Stiles et al., 1983; Stiles, 1986; Takayanagi et al., 1987; Poland et al., 1986; Regan et al., 1986). Figure 5A depicts the results obtained when photolabeled D1 receptor subunits of $M_r = 62000$ from the bovine parathyroid gland and the $M_r = 74000$, 62000, and 51000 labeled polypeptides from bovine brain striatum were simultaneously digested at the carboxyl side of glutamic and aspartic acid residues (Stiles et al., 1983) with *Staphylococcus aureus* V8 proteinase. Following digestion, multiple [^{125}I]IMAB-labeled fragments were generated, ranging in molecular weight from completely unproteolyzed material ($M_r = 74000/62000$) to apparent $M_r = 14000$. Clearly, the peptide fragments generated from both the brain and parathyroid D1 receptor are similar, suggesting that both proteins share structural homologies despite differences in their starting apparent molecular weight and in their subunit composition. As expected, all photolabeled material entered the second-dimension gel and was therefore susceptible to proteolysis.

It is of interest to note that the $M_r = 62000$ and 51000 labeled neuronal D1 receptor peptides, seen in the first dimension, generate proteolytic receptor fragments similar to the fragments produced from the $M_r = 74000$ peptide. This observation appears consistent with the contention that these lower molecular weight subunits are derivatives from the larger

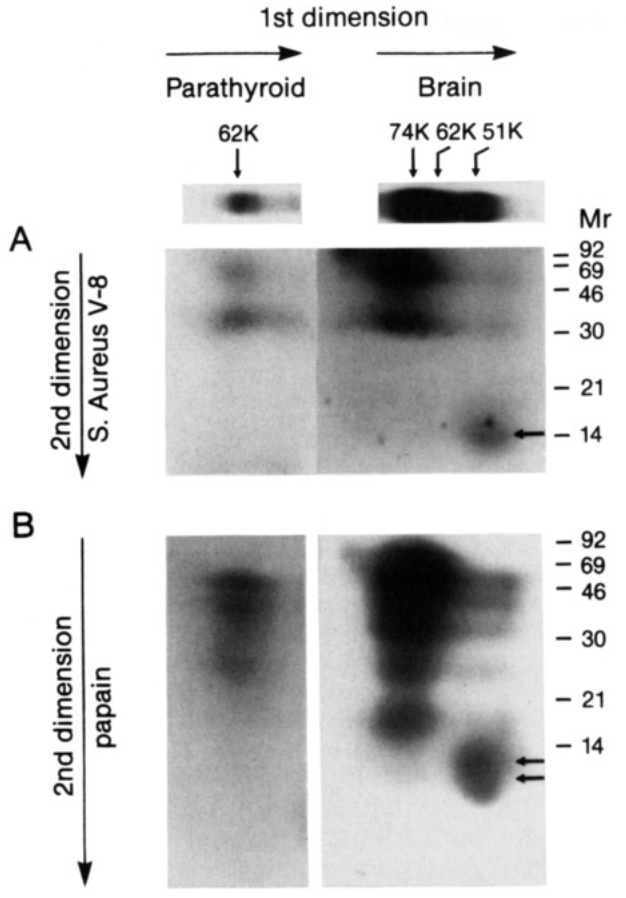

FIGURE 5: Peptide map comparisons of [^{125}I]IMAB-photolabeled D1 dopamine receptor binding subunits from calf parathyroid and brain striatum using *S. aureus* V8 proteinase (A) and papain (B). At the top of the figure are representative slices of the first-dimension gel electrophoresis containing [^{125}I]IMAB-labeled proteins from the indicated tissue and their relative mobilities. The second-dimension electrophoresis depicts the peptide fragments generated by 300 μg of *S. aureus* V8 proteinase (A) and 200 μg of papain (B) present in the overlaying buffer as described under Experimental Procedures. The results are representative of three similar experiments with identical results. Molecular weight protein standards are shown $\times 10^{-3}$ on the right. Arrows indicate the nonhomologous peptide fragments generated from the M_r = 51 000 labeled protein.

M_r = 74 000 polypeptide. In addition, although it is evident that the fragments generated from the M_r = 74 000, 62 000, and 51 000 labeled neuronal D1 receptor have many similarities, the M_r = 51 000 subunit consistently produced fragments (indicated by arrows) that were not seen in either the M_r = 62 000 labeled parathyroid D1 receptor or the M_r = 74 000 and 62 000 labeled subunits of bovine brain. At present, it is difficult to ascertain whether these differences most likely represent the presence of a D1 receptor subtype or simply the presence or absence of various posttranslational modifications (e.g., carbohydrate moieties) of the labeled fragments. In any event, these data point out the difficulty in comparing peptide maps of receptor fragments with different apparent molecular masses.

In order to extend our observations further, we next generated peptide maps of [^{125}I]IMAB-labeled D1 receptors from both brain and parathyroid with the use of papain, an enzyme with markedly different proteolytic specificity. As illustrated in Figure 5B, a papain-specific pattern of multiple [^{125}I]-IMAB-labeled receptor fragments was generated from each binding subunit. Once again, there appeared to be correspondence in the peptide fragments generated from both the parathyroid and brain D1 receptor ranging in apparent molecular weight from M_r = 74 000/62 000 (unproteolyzed material) to $M_r \cong$ 10 000, although some of the lower molecular weight fragments generated from the bovine parathyroid are too faint to be seen. The distribution pattern of radioactive peptide fragments generated is clearly distinct from that seen with *S. aureus* V8 proteinase, indicative of their different proteolytic specificities. As outlined above, many of the fragments generated from the M_r = 62 000 and 51 000 labeled neuronal subunits following proteolysis with papain are common to that of the larger M_r = 74 000 peptide. However, the M_r = 51 000 labeled subunit displayed some clearly distinct peptide fragments (shown by arrows) not seen with either the parathyroid or brain M_r = 74 000 and 62 000 proteins.

The purpose of this study was to compare the subunit composition and structure of dopamine D1 receptors found in calf parathyroid and brain striatal tissues. It is apparent that D1 receptors from different tissues within the same species display many homologous characteristics. This conclusion is supported by the observations that: (a) D1 receptors from both the brain and parathyroid display similar pharmacological specificities as defined by radioligand binding studies (Niznik et al., 1988a), (b) both tissues share a common $M_r \cong$ 62 000 ligand binding subunit which displays typical D1 dopaminergic receptor characteristics, and (c) the peptide fragments generated simultaneously from photoaffinity-labeled parathyroid and neuronal D1 receptor binding subunits with two distinct proteinases (*S. aureus* V8 or papain) are very similar (Figure 5). Thus, the overall primary structure of the D1 receptor appears to be conserved in different tissues. It should be emphasized that data generated from peptide mapping experiments of photolabeled D1 receptors are applicable only to those regions of the receptor that have been labeled by the probe and are visible by virtue of this radioemission.

Two interesting findings from this investigation are worth mention. One is that the parathyroid and neuronal D1 receptors do not appear to be comprised of an identical number of binding subunits. The second is that the lower molecular weight polypeptides of M_r = 62 000 and 51 000 of brain striata seen in the first-dimension electrophoresis display peptide maps which are very similar to the maps derived from the larger M_r = 74 000 peptide. Taken together these data would appear to suggest that the lower molecular weight photolabeled subunits are derived from the M_r = 74 000 protein either by endogenous proteolysis or via tissue-specific posttransitional modifications. As such, it would appear that the apparent lack of a one to one correspondence in the subunit composition of parathyroid and neuronal D1 receptors most likely represents either one of these two processes. However, it is of interest to note that while peptide maps of the M_r = 51 000 binding subunit of neuronal origin reveal marked structural similarities to those of brain and parathyroid D1 binding subunits, there were distinct differences. Whether this reflects the existence of a D1 receptor subtype, displaying similar pharmacological features to peripheral D1 receptors but with altered sensitivity to dopaminergic ligands (Niznik et al., 1988b; Bzowej et al., 1988) or possibly other biochemical indexes of D1 receptor function (Andersen & Braestrup, 1986; Hess et al., 1987), remains to be established. The future isolation of cDNA clones for both neuronal and peripheral D1 receptors should resolve these issues.

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