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Dopamine receptor subtypes colocalize in rat striatonigral neurons

(sodium current/neostriatum/substantia nigra/voltage clamp/neuromodulation)

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ABSTRACT **Dopaminergic neurons of the substantia nigra provide one of the major neuromodulatory inputs to the neostriatum. Recent *in situ* hybridization experiments have suggested that postsynaptic dopamine receptors are segregated in striatonigral and striatopallidal neurons. We have tested this hypothesis in acutely isolated, retrogradely labeled striatonigral neurons by examining the neuromodulatory effects of selective dopaminergic agonists on Na currents and by probing single-cell antisense RNA populations with dopamine receptor cDNAs. In most of the neurons examined (20/31), the application of the D_1 dopamine receptor agonist SKF 38393 reduced evoked whole-cell Na^+ current. The D_2 agonists quinpirole and bromocriptine had mixed effects; in most neurons (23/42), whole-cell Na^+ currents were reduced, but in others (8/42), currents were increased. In cell-attached patch recordings, bath application of SKF 38393 decreased currents as in whole-cell recordings, whereas quinpirole consistently (6/10) enhanced currents—suggesting that D_2-like receptors could act through membrane delimited and non-delimited pathways. Changes in evoked current were produced by modulation of peak conductance and modest shifts in the voltage dependence of steady-state inactivation. Antisense RNA probes of dopamine receptor cDNA Southern blots consistently (5/5) revealed the presence of D_1, D_2, and D_3 receptor mRNA in single striatonigral neurons. These findings argue that, contrary to a strict receptor segregation hypothesis, many striatonigral neurons colocalize functional D_1, D_2, and D_3 receptors.**

The role of the dopaminergic nigrostriatal system in controlling the excitability of neostriatal neurons has been intensely studied since it became clear that the loss of this innervation was responsible for the psychomotor symptoms of Parkinson disease (1). Molecular cloning and hybridization studies (2, 3) have revealed that the postsynaptic actions of dopamine are mediated by a family of five G-protein-coupled receptors (D_1–D_5). *In situ* hybridization experiments of Gerfen *et al.* (4, 5) have suggested that two members of this family—the D_1 and D_2 receptors—are segregated in the two major efferent populations of the neostriatum that project to the substantia nigra and pallidum. They have inferred from their findings that these pathways can be selectively modulated by D_1 and D_2 agonists. In its strictest form, this hypothesis requires that striatonigral neurons express only those dopamine receptors with a D_1 pharmacological profile (D_1 and D_5) and that striatopallidal neurons express only those receptors with a D_2 profile (D_2, D_3, and D_4).

This model of receptor segregation is difficult to reconcile with much of the biochemical and physiological literature addressing the effects of dopamine in the neostriatum (6–12). Gerfen *et al.*'s hypothesis implicitly argues that the appearance of convergence is a consequence of the failure to separate direct postsynaptic effects from indirect effects mediated by adjacent neurons. Such a separation is virtually impossible in any preparation that preserves local tissue architecture or fails to isolate individual neurons. Therefore, to provide a clear test of this hypothesis, we have studied acutely isolated striatonigral neurons identified by retrograde labeling. Two approaches were used to determine the extent of receptor colocalization. First, by using whole-cell and cell-attached voltage clamp, the modulatory effects of selective D_1 and D_2 dopaminergic agonists on Na^+ currents were studied. Second, to provide a molecular characterization of the receptors mediating the physiological response, the RNA from individual striatonigral neurons was amplified and used to detect dopamine receptor cDNAs.

MATERIALS AND METHODS

Retrograde Labeling and Cell Isolation. Injections of fluorescent rhodamine-impregnated microbeads (13) into the substantia nigra were made 2–12 days prior to the experiment. Animals were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (100 mg/kg) i.p. A suspension of microbeads in saline (Lumafluor, New York) was injected via a stereotaxically placed microsyringe needle. Three injections (3 μl each) were made on each side, separated by 0.5 mm in the rostrocaudal direction. Bead injections into the substantia nigra typically labeled about half the cells in frontal sections of the striatum (Fig. 1*A*), as well as about half of the cells acutely dissociated from this region (Fig. 1 *B* and *C*). Neurons were acutely isolated as described (14).

Patch-Clamp Recordings. Voltage-clamp recordings were made at room temperature (20–22°C) with conventional techniques (15, 16) from acutely dissociated striatonigral neurons that were retrogradely labeled with rhodamine-impregnated microbeads. Recordings were made in cells that lacked prominent processes. Whole-cell recordings used a dialyzing solution that consisted of (in mM) 72 $TrisPO_4$, 41 Tris base, 10 EGTA, 4 $MgCl_2$, 25 phosphocreatine, 2 Na_2ATP, 0.2 Na_3GTP, and 0.1 leupeptin, adjusted to pH 7.3 with HCl, 265–270 mosmol/liter. Cells were bathed and drugs applied in a solution that consisted of (in mM) 5–50 NaCl, 100–145 Et_4NCl, 2 $CaCl_2$, 1 $MgCl_2$, 0.4 $CdCl_2$, 5 CsCl, and 10 Hepes, adjusted to pH 7.4 with Et_4NOH and to 300–305 mosmol/liter with glucose. Cell-attached patch recordings were obtained with an electrode solution that consisted of (in mM) 125 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 0.4 $CdCl_2$, 10 Hepes, 20 Et_4NCl, 10 4-aminopyridine, adjusted to pH 7.4 with HCl, and to 300 mosmol/liter with glucose. Cells were bathed and drugs were applied in a solution that consisted of (in mM) 140 $KMeSO_4$, 1 EGTA, 10 $MgCl_2$, 10 Hepes, adjusted to pH 7.2 with KOH, 300–305 mosmol/liter. Drugs were delivered with an electronically controlled superfusion system. The junctional potential between the electrode and the cell interior

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Abbreviation: aRNA, antisense RNA.

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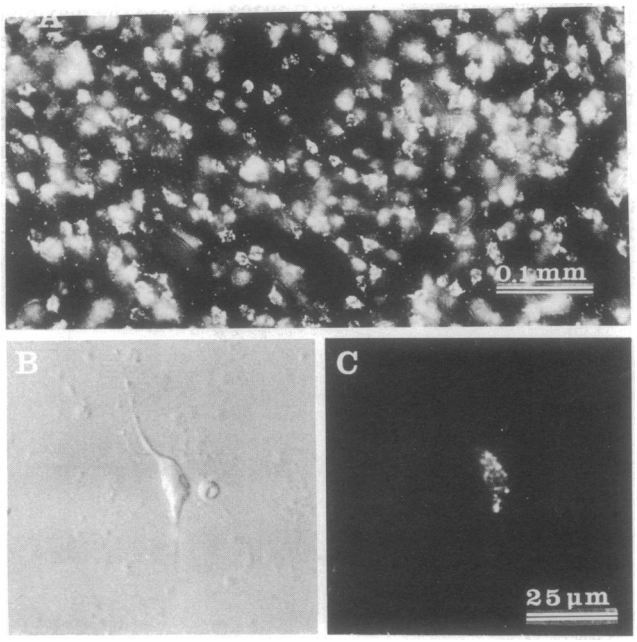

FIG. 1. (*A*) Epifluorescence photomicrograph of a frontal section through the striatum of a retrogradely labeled rat. Note the numerous rhodamine-labeled cell bodies. (*B* and *C*) Acutely isolated neurons from the striatum viewed under normal illumination (*B*) and under epifluorescence (*C*), showing a retrogradely labeled neuron.

was not compensated; capacitative and leak currents were subtracted by using a P/N technique (37). Series resistance compensation of 75–90% was typically employed. Data were low-pass filtered (corner frequency, 5 kHz) and digitized at 50–125 kHz; data were displayed, stored, and analyzed with PCLAMP (Axon Instruments, Burlingame, CA) software. Statistical analyses and nonlinear curve fitting used a commercially available software package (SYSTAT, Evanston, IL). Steady-state inactivation data were fitted with a least-squares criterion to a Boltzmann function of the form $I_{peak} = I_{max}/\{1 + \exp[(V_m - V_h)/V_c]\}$, where I_{max} is maximal current, V_m is prepulse potential, V_h is half-inactivation voltage, and V_c is the slope factor.

RNA Amplification. Striatal neurons were dissociated, identified, and recorded from as above except that the patch-clamp electrode was backfilled with a solution containing dNTPs, oligo(dT) primer, and reverse transcriptase (17, 18); positive pressure was maintained on the electrode during the approach to avoid entry of cellular debris. After seal rupture, the electrode and attached cell were lifted into a stream of control salt solution and the mRNA was sucked into the pipette. First-strand cDNA synthesis and amplification proceeded as described (17, 18). The antisense RNA (aRNA) probe was made by incorporating radiolabeled nucleotides into the second round of aRNA amplification, as described (18). Each of the cDNAs (2 μg) was digested with *Bam*HI (except for the glycine receptor cDNA, which was digested with *Hin*dIII), electrophoresed through a 1% agarose gel, and transferred to nitrocellulose. cDNAs were visualized prior to transfer by staining with ethidium bromide. After prehybridization in 6× standard saline citrate (SSC)/5× Denhardt's solution/60% (vol/vol) formamide with salmon sperm DNA (25 μg/ml) at 37°C overnight, probe hybridization was carried out at 37°C in the same solution. The blots were washed successively in 5× SSC/1% SDS at 37°C for 1 hr, 2× SSC/1% SDS at 50°C for 1 hr, and 0.1× SSC/1% SDS at 50°C for 1 hr. Blots were air-dried and apposed to x-ray film for 72 hr at −70°C with an intensifying screen. For PCR amplification, templates were obtained from reverse transcription of aRNAs that were amplified from single striatonigral neurons (18). Primers used for D_1 receptor RNA detection were 5′-CGCGGATCCACAAGCTTCTA-CAGGATTGCCCAGAAGCAA-3′ (nucleotides 1051–1089) and 5′-CGCGGATCCGAAGCTTTCACTTTAGAACTT-TCGTCTCCCT-3′ (nucleotides 1213–1252). The size of the amplified D_1 cDNA is 203 base pairs (bp). Primers used for D_2 receptor RNA detection were 5′-GAGGCAACCTGAAT-TCACCACTCAAGGATGCTGCC-3′ (nucleotides 823–859) and 5′-TGGTCTGCAGCTAAAAGAACTTGGCAAT CCTGGG-3′ (nucleotides 960–993). The size of the amplified D_2 cDNA is 170 bp; the primers used for D_3 receptor RNA detection were 5′-TGTAATACGACTCACTATAG-3′ and 5′-ATGCATCCAACGCGTTGGGA-3′. The size of the amplified D_3 cDNA is 420 bp. The PCR mixture contained 1/30th of the cDNA template made from a single cell, 50 ng of each primer, 1 unit of *Taq* DNA polymerase, 0.25 mM dNTPs, 1× PCR buffer (Cetus/Perkin–Elmer), and 2 mM $MgCl_2$. Temperature cycling was performed (95°C for 1 min, 53°C for 1 min, 72°C for 1 min) for a total of 35 cycles. After phenol/chloroform extraction and ethanol precipitation, one-third of the total PCR products was analyzed by electrophoresis in a 1.5% agarose gel. For cDNA visualization the gel was stained with ethidium bromide. Photomicrographs of the gels and blots were scanned and digitally processed with software supplied by the National Institutes of Health (IMAGE version 1.41).

RESULTS

The ionic currents seen under our recording conditions exhibited biophysical and pharmacological properties similar to those of Na^+ currents described in other brain neurons (19–22). These currents play an important role in subthreshold integration and spike generation in the neostriatum (23, 24). As described for monoamines in other tissues (25, 26), the application of dopamine (10–50 μM) produced a reversible decrease in the amplitude of Na^+ currents evoked by depolarizing voltage steps. A large percentage (81%, 25/31) of the striatonigral neurons tested responded to the specific D_1 agonist SKF 38393. In most of these cells (80%, 20/25), SKF 38393 (1–5 μM) reduced the amplitude of the evoked Na^+ current (Fig. 2*A*). The modulation by SKF 38393 appeared to be mediated by D_1 receptors, as it was blocked by the specific D_1 agonist SCH 23390 (1 μM) (Fig. 2*A*; $n = 4$) and unaffected by the D_2 receptor antagonist (−)-sulpiride (5 μM) ($n = 6$). Bath application of 8-bromo-cAMP (1 mM) partially mimicked this effect ($n = 2$), suggesting that the modulation was mediated by D_1 receptors that were positively coupled to adenylate cyclase.

In 55% (23/42) of the neurons tested in whole-cell recordings, the application of D_2 agonist, quinpirole ($n = 31$) or bromocriptine ($n = 11$), produced a time-locked decrease in evoked current amplitude (Fig. 2*B*). The decrease was not a consequence of the enhancement of an outward current, as the application of the specific Na^+ channel blocker tetrodotoxin eliminated the evoked current and the change produced by quinpirole ($n = 5$). The response to quinpirole (100 nM) was blocked by the D_2 receptor antagonist (−)-sulpiride (1 μM) (Fig. 2*B*, $n = 5$), suggesting that the modulation was mediated by D_2-like receptors.

In about 20% (8/42) of our whole-cell recordings, the application of D_2 agonists increased the amplitude of the current evoked from −70 mV. (−)-Sulpiride antagonized this response, suggesting that it also was mediated by a D_2-like receptor ($n = 4$). Similar responses to D_2 agonists were observed in more than half (6/10) of cell-attached patch recordings with bath-applied agonists (Fig. 2*C*). D_2 agonists never decreased the evoked current in cell-attached patches. The D_1 agonist SKF 38393, on the other hand, decreased the evoked current in most patches (4/5), as in whole-cell re-

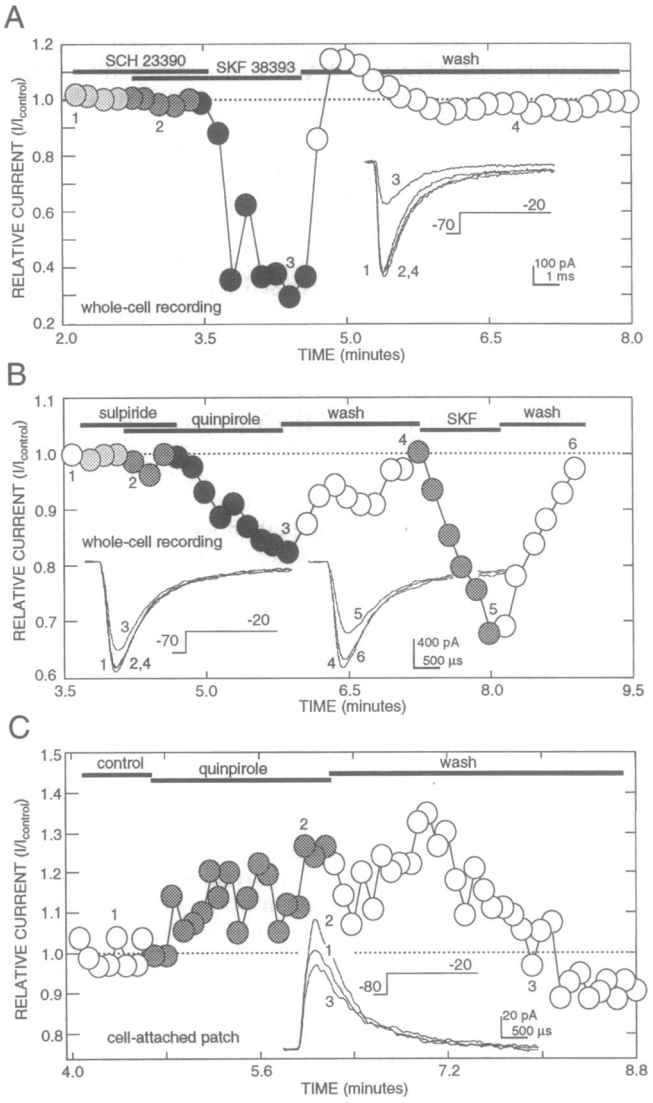

FIG. 2. Both the D_2 agonist quinpirole and the D_1 agonist SKF 38393 modulated the amplitude of evoked Na^+ currents. (*A*) The relative current amplitude evoked by a step to −20 mV from a holding potential of −70 mV is plotted as a function of time after initiating whole-cell recording. Inset are the current traces from relevant points in the record, labeled 1–4. The records show that the reduction in current produced by the specific D_1 agonist SKF 38393 (1 μM) is blocked by the D_1 receptor antagonist SCH 23390 (1 μM). (*B*) Quinpirole (100 nM) produced a similar reduction that was antagonized by the D_2 receptor antagonist (−)-sulpiride (1 μM). After recovery, the specific D_1 agonist SKF 38393 (1 μM) was applied. (*C*) In cell-attached patch recordings, the D_2 agonist quinpirole (1 μM) enhanced Na^+ currents evoked by a step to −20 mV from a holding potential of −80 mV.

cordings. In the cell-attached recording configuration, bath-applied agonists do not interact with the membrane containing the channels being modulated. As a consequence, the modulatory responses seen in this configuration must be dependent upon soluble second messengers produced by activation of receptors *outside* of the recorded patch. These experiments suggest that D_1- and D_2-like receptors can reciprocally modulate Na^+ currents through a soluble second messenger (presumably cAMP) in many striatonigral neurons. These results also suggest that the reduction in Na^+ current produced by D_2 agonists is mediated by a membrane-delimited pathway, as it was not seen in cell-attached patches.

An examination of the biophysical mechanisms underlying the changes in evoked current revealed three patterns of modulation. The D_1 agonist SKF 38393 reduced the maximal Na^+ current and shifted its steady-state inactivation voltage dependence toward more negative potentials, without changing the voltage-dependence of activation. An example of these changes is shown in Fig. 3*A*; in 10 cells, peak current was reduced an average of 22% and the half-inactivation voltage shifted an average of −5.6 mV. This modulation is similar to that produced by cAMP-dependent kinase phosphorylation of Na^+ channels (27). When D_2 agonists in-

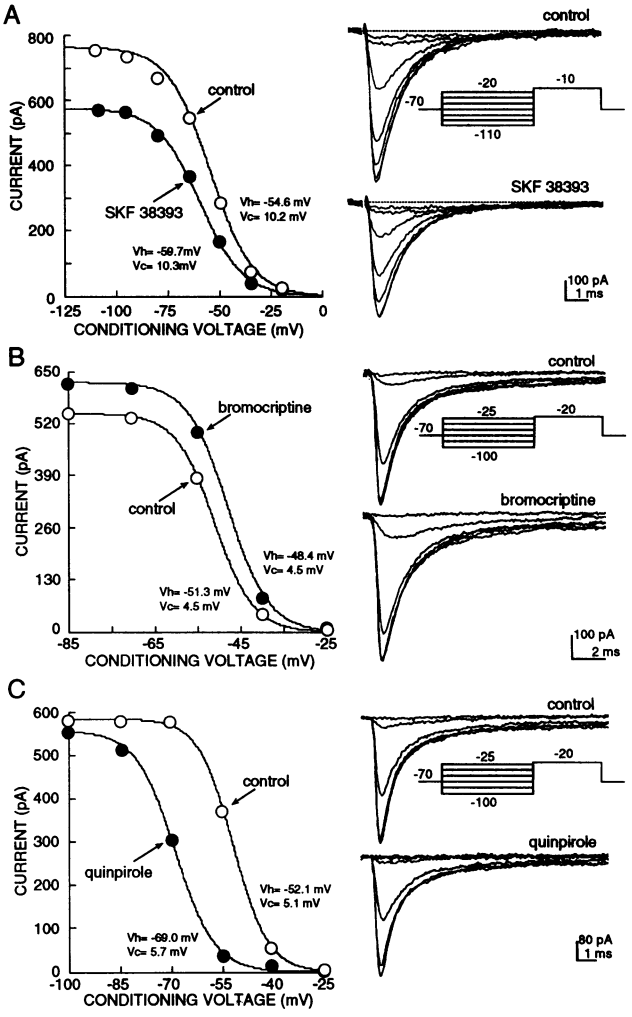

FIG. 3. The biophysical mechanisms by which D_1 and D_2 agonists modulated Na^+ currents differed, suggesting at least three signaling pathways. (*A*) SKF 38393 produced a reduction in peak current and a negative shift in the voltage dependence of steady-state inactivation. On the right, the currents evoked by a step to −10 mV in a standard inactivation protocol (*Inset*) are shown; at the top, the currents evoked under control conditions and, at the bottom, currents evoked in the presence of 1 μM SKF 38393. At the left, the peak currents are plotted as a function of prepulse potential; under both conditions, the data were well fit with a Boltzmann function. Under control conditions (○), I_{max} = 764 pA, V_h = −54.6 mV, and V_c = 10.2 mV; in the presence of SKF 38393 (●), I_{max} = 576 pA, V_h = −59.7 mV, and V_c = 10.3 mV. The dotted line is the zero current level. (*B*) Bromocriptine (500 nM) produced a positive shift in the voltage dependence of steady-state inactivation and increased the maximal current. Plots are as above. In control records, I_{max} = 547.2 pA, V_h = −51.3 mV, and V_c = 4.5 mV; in bromocriptine records, I_{max} = 612.4 pA, V_h = −48.4 mV, and V_c = 4.5 mV. (*C*). Quinpirole (100 nM) produced a negative shift in steady-state inactivation voltage dependence without reducing peak current. Plots are as in *A*. Under control conditions (○), I_{max} = 578 pA, V_h = −52.1 mV, and V_c = 5.1 mV; in the presence of quinpirole (●), I_{max} = 558 pA, V_h = −69.0 mV, and V_c = 5.7 mV. The voltage dependence of activation did not appear to be altered by SKF 38393, quinpirole, or bromocriptine.

creased Na^+ current, the biophysical changes were complementary to those of D_1 agonists. In the example of Fig. 3*B*, bromocriptine (500 nM) shifted inactivation-voltage dependence toward more depolarized values and increased the maximal current. In 4 cells, the half-inactivation voltage shifted an average of 3.2 mV and the peak current increased an average of 19%. In contrast, the membrane delimited modulation produced by D_2 agonists did not change peak current but did shift steady-state inactivation toward more negative potentials (Fig. 3*C*). In 8 neurons where quinpirole (100–500 nM) reduced current amplitudes, the mean half-inactivation voltage shifted an average of −5.1 mV. The maximal current in these cells was not altered significantly nor was the voltage dependence of activation. The modulation produced by quinpirole was not seen in neurons cultured from the embryonic striatum (n = 8; data not shown), suggesting that its mechanism of action differed from that reported for neuroleptics (28).

These findings are not only contrary to a strict segregation of dopamine receptors but suggest that many striatonigral neurons coexpress *two* D_2-like receptors, in addition to a D_1-like receptor. However, at present, the pharmacological tools necessary to differentiate D_2, D_3, and D_4 receptors are not available. As a consequence, we turned to a recently developed technique for amplifying the mRNA of single cells to characterize the receptors (17, 18). Fig. 4*A Upper* shows an ethidium bromide-stained gel containing cDNAs that were transferred to nitrocellulose for probing with radiolabeled aRNA from a single striatonigral neuron. The dopamine receptor cDNAs were constructed from the mRNA sequences coding for nonhomologous regions of the third cytoplasmic domain of the receptors. Fig. 4*A Lower* shows the autoradiogram resulting from aRNA hybridization to the Southern blot. Positive hybridization to cDNAs for D_1, D_2, and D_3 dopamine receptors, the β_1 subunit of the type A γ-aminobutyrate receptor, and the Na^+ channel is apparent; longer development times also revealed hybridization to the neurofilament cDNA. All five of the striatonigral neurons subjected to aRNA amplification had detectable levels of D_1, D_2, and D_3 receptor mRNAs. These receptor signals did not result from amplification of genomic DNA, since aRNA populations did not hybridize to known middle-repetitive sequences, and hybridization intensity was not uniform. Two other experiments have shown that retrogradely labeled cells possess mRNA for substance P but not enkephalin, in agreement with their identification as striatonigral neurons (29–31).

To further test the specificity of the dopamine receptor hybridization, regions of aRNA molecules coding for the third cytoplasmic domain of each receptor were amplified with the PCR and subjected to size analysis. This was accomplished by converting the aRNA into cDNA [by using random primers to prime reverse transcription (18)] and then performing PCR using oligonucleotide primers that were specific for regions of the third cytoplasmic loop of the distinct receptor subtypes. For D_1 receptor aRNA, this should yield a cDNA of 203 bp; D_2 and D_3 receptor aRNAs should yield cDNAs of 170 and 420 bp, respectively. Fig. 4*B* shows the ethidium bromide-stained gel of PCR-amplified cDNA made from the aRNA of two striatonigral neurons. In cell 1, cDNA of the predicted sizes for D_1, D_2, and D_3 receptor mRNA was evident. In cell 2, cDNA of the predicted sizes was seen plainly for D_1 and D_3 receptors whereas that for the D_2 receptor was faint. As the D_2 PCR cDNA was from a region of the D_2 mRNA transcript that is normally spliced, the absence of an unspliced product in either cell provides further evidence that genomic DNA was not amplified. These experiments establish the specificity of the aRNA hybridization and the supposition that mRNA was amplified, not genomic DNA fragments.

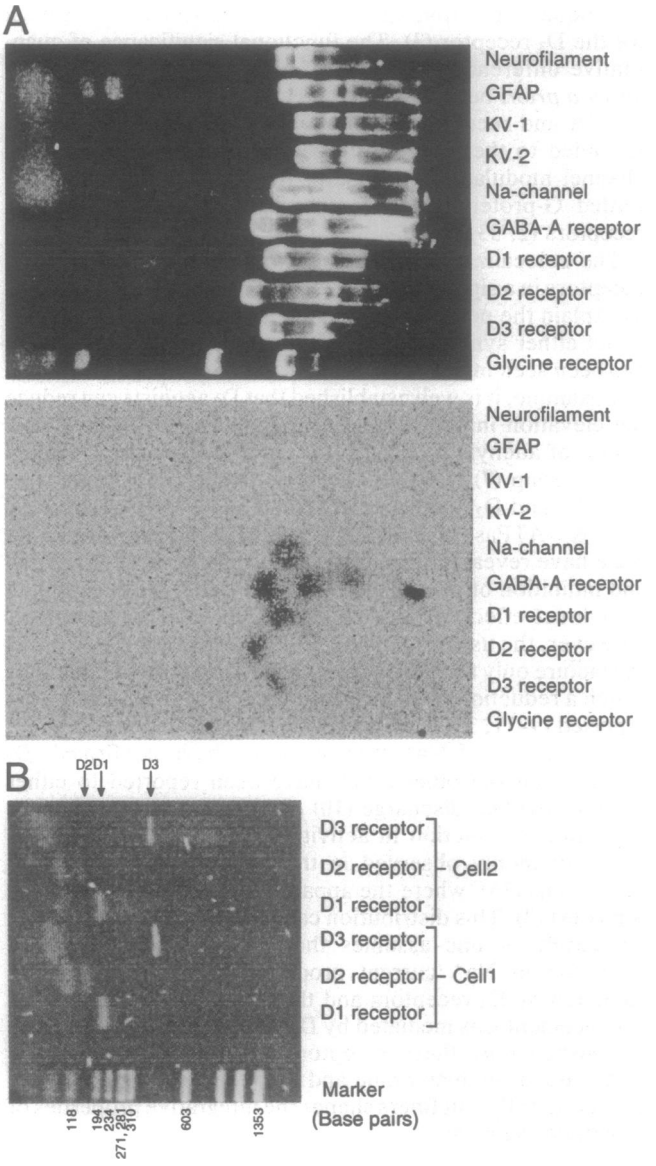

FIG. 4. Expression profiles and specific PCR of D_1, D_2, and D_3 dopamine receptors from single striatonigral neurons. (*A*) (*Upper*) Ethidium bromide-stained agarose gel containing cDNAs for neurofilament, glial fibrillary acidic protein (GFAP), K^+ channels (KV-1, KV-2), Na^+ channel, type A γ-aminobutyrate (GABA-A) receptor, D_1, D_2, and D_3 dopamine receptors, and the glycine receptor. (*Lower*) Autoradiogram resulting from probing of the Southern blot of the gel shown at the top with radiolabeled aRNA from a single neuron. (*B*) An agarose gel containing the PCR-amplified cDNAs for the D_1, D_2, and D_3 dopamine receptors from two neurons (Cell 1 and Cell 2). The marker lane contained ϕX174 DNA digested with *Hae* III; the size in base pairs of each band is as indicated. Arrows at the top of the gel show the approximate positions expected for the D_1, D_2, and D_3 receptor DNAs.

DISCUSSION

We conclude that many striatonigral neurons express D_1, D_2, and D_3 dopamine receptors and that activation of these receptors produces functionally significant alterations in Na^+ currents and excitability. An accurate estimate of the extent of receptor colocalization must await further work, but both physiological and molecular approaches indicate that well over half of the striatonigral population express all three receptors. It is plausible that *in situ* receptor hybridization experiments have failed to detect D_2 mRNA in striatonigral neurons (4, 32) because it is present at levels below the

technique's detection threshold; a similar situation may exist for the D_3 receptor (3). The functional significance of quantitative differences in receptor mRNA levels is difficult to know *a priori* because of the uncertain relationship between mRNA and receptor protein. This uncertainty can also be extended to the relationship between receptor protein and channel modulation because of the signal amplification afforded G-protein-linked receptors, such as the dopamine receptors (2, 33).

The colocalization of both D_2 and D_3 receptors with D_1 receptors in a significant fraction of neostriatal neurons helps to explain the perplexing ability of D_1- and D_2-class agonists to act either synergistically or antagonistically. This duality has been seen in both biochemical and physiological studies. For example, it is well established that D_2 agonists can reduce the elevation in cAMP levels produced by D_1 agonist activation of adenylate cyclase (6). On the other hand, Greengard's group (7) has shown that in isolated neostriatal neurons, D_1 and D_2 agonists can act synergistically to suppress Na^+/K^+ ATPase activity. Physiological studies using dopamine have revealed complex response patterns of excitation and inhibition of evoked activity (8, 34) that were attributed to indirect effects or coactivation of D_1 and D_2 receptors. However, the use of receptor-selective agonists has clarified the picture only for the D_1 receptor-mediated modulation, for which a reduction in evoked discharge has consistently been reported (9–11, 35). Calabresi *et al.* (35) attributed this effect to a reduction of Na^+ current, as we have confirmed. D_2 agonists, on the other hand, have been reported to either enhance evoked discharge (10) or reduce activity (12). The cooperative reduction in activity by D_1 and D_2 agonists is most commonly observed in the ventral striatum/nucleus accumbens (36), where the apparent density of D_3 receptors is highest (3). This distribution can readily be reconciled with our results if one assumes that the membrane-delimited reduction in Na^+ current produced by D_2 agonists was mediated by D_3 receptors and the non-membrane-delimited enhancement was mediated by D_2 receptors. It remains to be established how these receptors are distributed over the somatodendritic membrane and how their activation by dopaminergic afferent fibers shapes the integrative properties of neostriatal neurons.

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