Characterization of G-Protein-Gated K^+ Channels Composed of Kir3.2 Subunits in Dopaminergic Neurons of the Substantia Nigra

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G-protein-gated K^+ (K_G) channels generate slow inhibitory postsynaptic potentials in the brain. Current opinion suggests that neuronal K_G channels are heterotetramers of Kir3.1 and Kir3.2. In substantia nigra (SN), however, mRNA of Kir3.1 does not express, whereas that of Kir3.2 clearly does. Therefore, we have characterized the K_G channels containing Kir3.2 subunits in SN using biochemical and immunological techniques. We found that they were composed of only Kir3.2 subunits and did not contain significant amounts of either Kir3.1 or Kir3.3. Furthermore, at least some of the K_G channels in SN were assemblies of the splicing variants Kir3.2a and Kir3.2c. The channels were localized specifically at the postsynaptic membrane on

the dendrites of dopaminergic neurons. Kir3.2c, but not Kir3.2a, could bind a PDZ domain-containing protein, PSD-95. The heterologously expressed K_G channels composed of Kir3.2a plus Kir3.2c or Kir3.2a alone were activated by G-protein stimulation, but expression of Kir3.2c alone was not. This study reveals that the Kir3.2 splicing variants play distinct roles in the control of function and localization of some of the K_G channels in dopaminergic neurons of SN.

Key words: substantia nigra; G-protein; inwardly rectifying potassium channel; dopamine; immunohistochemistry; dendrite; postsynaptic density

Dopaminergic neurons of the substantia nigra (SN) are involved in the control of movement and affective behavior by controlling the activity of medium-sized spiny neurons in the striatum (Le Moal and Simon, 1991; Wilson, 1998). Excitability of the dopaminergic neurons is reduced by dopamine and GABA acting at their somatodendritic regions, which may result in decrease of dopamine release from their axonal termini in the striatum. Because deficiency of dopamine release may be responsible for the hypokinetic–hypertonic property of Parkinsonism, regulation of the nigral dopaminergic neurons by these neurotransmitters may play pivotal roles in control of movement (Le Moal and Simon, 1991; Wilson, 1998). In these neurons, it has been shown that stimulation of D_2 or $GABA_B$ receptors generate slow IPSPs by activating an inwardly rectifying K^+ (Kir) channel via pertussis toxin-sensitive G-proteins (Innis and Aghajanian, 1987; Lacey et al., 1988). It is therefore important to identify the molecular

properties of the G-protein-gated Kir (K_G) channel in dopaminergic neurons in SN.

Recently, four cDNAs of Kir subunits encoding mammalian K_G channels have been isolated. They are designated GIRK1/Kir3.1 (Dascal et al., 1993; Kubo et al., 1993), GIRK2/Kir3.2 (Lesage et al., 1994), GIRK3/Kir3.3 (Lesage et al., 1994), and GIRK4/CIR/Kir3.4 (Krapivinsky et al., 1995). Kir3.1 and Kir3.2 have several splicing variants (Lesage et al., 1995; Isomoto et al., 1996; Nelson et al., 1997). The neuronal K_G channel is currently believed to consist of Kir3.1 and Kir3.2 based on *in situ* hybridization analyses (Kobayashi et al., 1995; Karschin et al., 1996) and also on electrophysiological studies of the heterologously expressed channels (Kofuji et al., 1995; Duprat et al., 1995; Slesinger et al., 1996; Velimirovic et al., 1996). In SN pars compacta (SNC) and ventral tegmental area, however, it was found that whereas Kir3.2 mRNA was clearly expressed, Kir3.1 mRNA could hardly be detected (Kobayashi et al., 1995; Karschin et al., 1996), suggesting that the K_G channels in SN may have a different subunit composition.

To date, at least three isoforms of Kir3.2 have been identified. They are GIRK2A/Kir3.2a (Lesage et al., 1994), GIRK2B/Kir3.2b (Isomoto et al., 1996), and GIRK2C/Kir3.2c (Lesage et al., 1995). The primary amino acid sequences show that these isoforms diverge only at their carboxyl terminal tails. In this study, we characterized the K_G channels containing Kir3.2 subunits in SN using immunological and biochemical techniques. Our study indicated that the SN K_G channels were composed of only Kir3.2 subunits and did not contain significant amounts of either Kir3.1 or Kir3.3. At least some of the SN K_G channels were assemblies of the two splicing variants Kir3.2a and Kir3.2c.

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Furthermore, the K_G channel was localized at the postsynaptic membranes on the dendrites of dopaminergic neurons. Functional and biochemical analyses strongly suggested that each splicing variant of Kir3.2 played a distinct role in the control of function and localization of the SN K_G channel.

MATERIALS AND METHODS

Antibodies. Polyclonal antibodies (aG2N-2 and aG2A-5) were raised in rabbit with synthetic peptides, AKLTESMTNVLEGD and WS-VSSKLNQHAEELE, that correspond to amino acids 4–17 and 385–398, respectively, of Kir3.2, whereas the antibody named aG2C-3 was generated in guinea pig against a synthetic peptide, DVANLENESKV, corresponding to amino acids 415–425 of Kir3.2c (Lesage et al., 1995). The antibodies were purified through protein A and their antigenic peptide-coupled resins as described previously for the aG1C-1 antibody against Kir3.1 (Inanobe et al., 1995). A commercially available rabbit polyclonal antibody against Kir3.2 (aGIRK2; Alomone Labs, Jerusalem, Israel) was also used. Guinea pig antisera (aG2B-2 and aG3NC) against Kir3.2b and Kir3.3 were raised with synthetic peptides, GKMGGFALGFL (318–327; Isomoto et al., 1996) and AQENEESPGEPPRRRRGR (2–21; Lesage et al., 1994), respectively. We have also developed an antibody to Kir3.4 (aG4N-10) in rabbit using the antigenic peptide DSRNAMNQDMEIGV (amino acids 4–17 of Kir3.4; Krapivinsky et al., 1995). Mouse monoclonal anti-tyrosine hydroxylase antibody (anti-TH; Boehringer Mannheim, Mannheim, Germany) was used for the identification of dopaminergic neurons. PSD-95 and SAP97 proteins were detected with rabbit antisera (Takeuchi et al., 1997; Y. Horio, in preparation).

Immunoblot and immunoprecipitation analyses of K_G channels. Membrane preparations and biotinylation of Kir3.2 isoform-transfected HEK293T cells or SN and cerebral cortex (Cx) of rat brain were performed as described previously (Inanobe et al., 1995). The membrane proteins were separated with SDS-PAGE (10%) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with 5% (w/v) skim milk and 0.2% (w/v) Lubrol PX in 50 mM Tris-HCl, pH 8.0, and 80 mM NaCl (buffer A) for 30 min at room temperature. Then 1:100 (v/v) of normal goat serum was added to the solution, and the membranes were further incubated for 1 hr at room temperature. The PVDF membranes were overlaid with the antibodies at a concentration of 0.5 μ g/ml in buffer A for 0.5–1 hr at room temperature. After washing three times with buffer A for 10 min each, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (EnVision Plus; Dako, Carpinteria, CA) diluted to 1:300 (v/v) in buffer A, followed by washing three times with 2% Lubrol PX and 0.2% (w/v) SDS in 50 mM Tris-HCl, pH 8.0, and 80 mM NaCl. Immunoreactive bands were developed with SuperSignal chemiluminescence immunostaining kit (Pierce, Rockford, IL) according to the manufacturer's protocol. Immunoprecipitation experiments were performed as described previously (Inanobe et al., 1995).

RT-PCR. To identify the Kir3.0 mRNAs, total RNAs were isolated from rat SN and Cx by the guanidine thiocyanate method and reverse transcribed with SuperScript II RT (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. The cDNAs were amplified by PCR with LA-Taq polymerase (Takara, Tokyo, Japan) using a Kir3.2 sense primer 5'-agattgtgtcctcctgag-3' and a Kir3.2a-specific antisense primer 5'-tccagcgcgactttaagta-3', a Kir3.2b-specific antisense primer 5'-ggcttgatacaaatagc-3', or a Kir3.2c-specific antisense primer 5'-aggctctacactttggactca-3', or using Kir3.1-specific primers (sense, 5'-ctatgctaccgctacatcccg-3'; antisense, 5'-atgagaagcattttctctgctc-3') and Kir3.3-specific primers (sense, 5'-ccatccgagccaagctcatc-3'; antisense, 5'-ctgctggggatgtaccagta-3'). PCR conditions were 30 cycles of 94°C for 1 min, 56°C for 1 min, and then 72°C for 1 min. Subsequently, fragments were sequenced with an automatic sequencer.

Immunocytochemical analysis of Kir3.2 isoform-transfected cells. Each cDNA was subcloned into the expression vector pcDNA3 (Invitrogen, San Diego, CA) and transfected with Lipofectamine (Life Technologies) into HEK293T cells as described previously (Horio et al., 1997). Two days after transfection, the cells were fixed with 4% (w/v) paraformaldehyde (PFA) and incubated with anti-Kir3.2 antibodies at 4°C for 14 hr. Cells were incubated with fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG and anti-guinea pig IgG, and their immunoreactivities were examined with a confocal microscope (model MRC-1024; Bio-Rad, Hercules, CA). Specificity of the antibodies used in this study other than aG2A-5, aG2C-3, and aGIRK2, i.e., aG2N-2, aG1C-1, aG2B-2, aG3NC, and aG4N-10 were also confirmed in this way (data not shown).

Immunohistochemical analysis of rat brain. Wistar rats weighing 100–300 gm were deeply anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and perfused transcardially with 4% PFA in 0.1 M sodium phosphate buffer, pH 7.4. The brains were dissected, post-fixed in the same solution at 4°C for 48 hr, and stored in 30% (w/w) sucrose and 0.05% (w/v) sodium azide in PBS at 4°C. Sagittal and coronal sections (20 μ m) were cut from the frozen brains. Immunostaining of cryosections was performed with free-floating method. Immunoreactivity was developed with the incubation of the individual antibodies indicated in the figure legends, biotinylated goat anti-rabbit or guinea pig IgG of the Vectastain ABC kit (Vector Laboratories, Burlingame, CA), and then with diaminobenzidine (DAB) and nickel ammonium.

Electron microscopic analysis of rat SN. Electron microscopic examination of Kir3.2 immunoreactivity in rat SN was performed as described previously (Morishige et al., 1996). Briefly, rat brain was fixed with periodate-lysine-PFA and cut with a vibratome. Sections were incubated with aGIRK2 antibody and then with biotinylated goat anti-rabbit IgG of the Vectastain ABC kit (Vector Laboratories). After color development with DAB, nickel ammonium, and platinum potassium chloride, the sections were post-fixed in reduced osmium and infiltrated with epoxy resin. The preparations were sectioned with an ultramicrotome, counterstained with uranyl acetate and Reynold's lead citrate, and then examined with an electron microscope (H7100TE; Hitachi, Hitachinaka, Japan).

Heterologous expression of Kir3.0 in *Xenopus* oocytes. The D₂ dopamine receptor was obtained by RT-PCR cloning technique from a mouse brain cDNA library (Todd et al., 1989). For the tandem construction of Kir3.2a-Kir3.2c, a linker of 20 glutamine residues was inserted between the 3' end of the Kir3.2a coding region and the 5' end of Kir3.2c. For the fused clones of Kir3.1, Kir3.2a, and Kir3.2c with mutated GFP (S65A), a *Clal* site was added to the 5' and 3' ends of all Kir3.0 constructs using PCR. The GFP was subcloned into pGEMHE vector, and each PCR product was inserted at the *Clal* site of the 3' portion of GFP. All constructs were inserted into pGEMHE vector with 5' and 3' untranslated regions of *Xenopus* β -globin gene for high expression of their products. Each transcript of different cDNAs was obtained with mRNA capping kit (Stratagene, La Jolla, CA). Fifty nanograms of each cRNA dissolved in 50 nl of sterile water were manually injected into defolliculated *Xenopus* oocytes. After injection, oocytes were incubated in a modified Barth's solution at 18°C, and electrophysiological studies were undertaken 72–96 hr later.

Two-electrode voltage-clamp experiments were performed with a commercially available amplifier (model TEC 01C; Turbo Clamp, Tamm, Germany) with microelectrodes that had resistances of 0.5–1.5 M Ω when filled with 3 M KCl. Oocytes were bathed in a solution that contained (in mM) 90 KCl, 3 MgCl₂, 5 HEPES-KOH, pH 7.4, and 150 μ M niflumic acid to block endogenous chloride current. Voltage steps (1.2 sec in duration) from the holding potential of 0 mV to potentials between +60 and –120 mV with –20 mV increment were delivered every 5 sec. Experiments were performed at room temperature (20–22°C).

Analysis of electrophysiological data. Electrophysiological data were stored on video tapes using a PCM data recording system (NF Electronic Design, Tokyo, Japan) and subsequently replayed for computer analysis (Patch Analyst Pro; MT Corporation, Hyogo, Japan). The data were expressed in means $\pm$ SEM.

RESULTS

Evaluation of Kir3.2 isoform-specific antibodies

To analyze the expression of isoforms of Kir3.2 subunits in SN, we have developed polyclonal antibodies against synthetic peptides as indicated in Figure 1A. Kir3.2a and Kir3.2c are splicing variants from a single gene; Kir3.2c is 11 amino acid residues longer at its C-terminal end than Kir3.2a, which is the only divergent region between these isoforms (Lesage et al., 1995). Therefore, we could only design antigenic peptides that would be either common to both isoforms or only specific for Kir3.2c. Specificity of the developed antibodies (aG2A-5 and aG2C-3) was first examined using HEK293T cells transfected with either Kir3.2a or Kir3.2c (Fig. 1B). As expected, the rabbit polyclonal antibody (aG2A-5) reacted with both Kir3.2a and Kir3.2c (Fig. 1Ba,b), whereas the guinea pig antibody (aG2C-3) recognized only Kir3.2c (Fig. 1Bd,e). The immunoreactivities to these anti-

Figure 1. Characterization of polyclonal antibodies against Kir3.2 isoforms (*A, B*) and immunological analyses of Kir3.2 isoforms expressed in HEK293T cells (*C, D*). *A*, Alignments of amino acid sequence of Kir3.2 isoforms. The antigens for Kir3.2 isoform-specific antibodies are indicated by boxes at the C termini of the proteins with their amino acid sequences represented in single letter code. The shaded regions M1, M2, and H5 indicate the putative transmembrane domains and the pore region of the Kir channel. A putative binding motif for PDZ domain-containing proteins is underlined at the C terminus of Kir3.2c. *B*, Immunofluorescent images of Kir3.2 isoforms obtained with Kir3.2-specific antibodies. HEK293T cells were transfected with Kir3.2a (*a, d, g*) or Kir3.2c (*b, c, e, f, h, i*). After 2 d, the products in cells were immunostained with aG2A-5 (*a–c*), aG2C-3 (*d–f*), and aGIRK2 (*g–i*) antibodies and analyzed with confocal microscopy. Immunoreactivity to each antibody was indicated with green images superimposed with nuclei staining with propidium iodide (red). Immunolabeling was blocked by preincubation with their antigenic peptides (*c, f*) or C terminal of mouse GIRK2 (amino acids 374–414) fused with glutathione *S*-transferase (*i*). Scale bar, 30 μ m. *C*, Immunoblot analysis of Kir3.2 isoforms. Membrane fractions obtained from Kir3.2a- (lane 1), Kir3.2c- (lane 2), or both Kir3.2a- and Kir3.2c- (lane 3) transfected HEK293T cells and the mixture of Kir3.2a and Kir3.2c membranes (lane 4) were separated with SDS-PAGE, transferred to PVDF membranes, and then immunoblotted with aG2A-5 (*top panel*) or aG2C-3 (*bottom panel*) antibodies. *D*, Immunoprecipitation analysis of Kir3.2 isoforms. Kir3.2 isoforms in the membrane fraction of the cells were solubilized and isolated with aG2A-5 and aG2C-3 antibodies (*top* and

bodies in these cells were not detected in the presence of their individual antigenic peptides (Fig. 1*Bc,f*). Similarly, a commercially available antibody (aGIRK2) could detect both isoforms (Fig. 1*Bg,h*), and the signal was diminished with the preincubation of aGIRK2 with the antigen (Fig. 1*Bi*).

The specificity of these antibodies was further evaluated in immunoblot experiments using HEK293T cells transfected with either Kir3.2a or Kir3.2c (Fig. 1*C, lanes 1, 2*). The aG2A-5 antibody detected both Kir3.2a and Kir3.2c, whereas aG2C-3 recognized only Kir3.2c. The molecular weight of Kir3.2c was slightly greater than that of Kir3.2a on the gels. These two antibodies could also be used for immunoprecipitation experiments. Both Kir3.2a and Kir3.2c expressed in HEK293T cells were immunoprecipitated by aG2A-5, whereas only Kir3.2c was by aG2C-3 (Fig. 1*D, lanes 1, 2*). These results were consistent with the immunocytochemical analysis in Figure 1*B*.

We next examined whether Kir3.2a and Kir3.2c could form a protein complex when coexpressed in HEK293T cells (Fig. 1*C, D, lanes 3, 4*). Membranes obtained from the cells cotransfected with both isoforms were used in lane 3, whereas those from cells transfected with either of the isoforms were separately extracted, mixed, and examined in lane 4. In the immunoblot analysis using aG2A-5 (Fig. 1*C*), both bands were detected in both lanes, whereas only the upper band was detected with aG2C-3 antibody. The immunoprecipitants obtained with aG2A-5 either from the membrane fraction of cotransfected cells (Fig. 1*D, lane 3*) or from the mixed membrane preparation (Fig. 1*D, lane 4*) contained two bands. On the other hand, the aG2C-3 antibody immunoprecipitated a single band that corresponded to Kir3.2c from the mixture (Fig. 1*D, lane 4*) but two bands corresponding to Kir3.2a and Kir3.2c from the cotransfected cells (Fig. 1*D, lane 3*). Thus, the immunoprecipitant with aG2C-3 from the cotransfected cells may contain not only homomeric Kir3.2c protein but a protein complex composed of Kir3.2a and Kir3.2c. These results indicate that the Kir3.2 isoforms can be assembled to form a protein complex when coexpressed in the same cells.

RT-PCR and immunoprecipitation analyses of Kir3.2 isoforms in SN and cerebral cortex

We examined the expression of Kir3.1, Kir3.2a, and Kir3.2c mRNAs in rat SN and Cx by using the RT-PCR technique (Fig. 2*A, lanes 1–6*). The mRNAs of both isoforms of Kir3.2 were amplified with their respective specific primers in both SN and Cx, whereas that of Kir3.1 was detected in Cx but not in SN. The mRNA expression of the Kir3.2b and Kir3.3 subunits could also be detected by RT-PCR in both regions (Fig. 2*A, lanes 7–10*). These observations are consistent with the *in situ* hybridization study of various Kir3.0 subunits in the rat brain (Kobayashi et al., 1995; Karschin et al., 1996).

In Figure 2*B*, we analyzed biochemical properties of the immunoprecipitants with aG2A-5 or aG2C-3 obtained from solubilized membrane preparations of rat SN (Fig. 2*Ba*) or Cx (Fig. 2*Bb*). aG2C-3 immunoprecipitated two bands at ~48 kDa from the membrane fraction of SN (Fig. 2*Ba, lane 1*). Both bands were detected by aG2A-5 (lane 2). Similarly, two bands were obtained

bottom panels, respectively). Each Kir3.2 isoform was biotinylated before extraction from the membranes. Biotinylated Kir3.2 isoforms were detected with the incubation of streptavidin–HRP conjugate. Migration positions of Kir3.2a and Kir3.2c on the gels are indicated on the right side of each panel.

Figure 2. *A*, RT-PCR analysis of Kir3.0 mRNAs in rat SN and Cx. Using the Kir3.0-specific primers indicated above each panel, their expression was examined in cDNAs obtained from rat SN (odd numbers) and Cx (even numbers). PCR products (519, 369, 456, and 574 bp) of Kir3.2a, Kir3.2c, Kir3.3, and Kir3.2b were detected in both SN and Cx, whereas that of Kir3.1 (666 bp) was found in Cx but not in SN. *Numbers* indicate the standard markers in base pairs. *B*, Immunoprecipitation analysis of Kir3.2 isoforms in rat SN and Cx. Biotinylated membrane proteins of rat SN (*a*) or Cx (*b*) were incubated with aG2C-3 (lanes 1–3) or aG2A-5 antibodies (lanes 4, 5) as indicated. The immunoprecipitants were detected with streptavidin–HRP (SA–HRP; lanes 1, 4), aG2A-5 (lane 2), aG1C-1 (lane 3), or aG2C-3 antibodies (lane 5). The positions of Kir3.2a (small open arrowheads) and Kir3.2c (large arrowheads) isoforms and Kir3.1 subunits (arrows) are indicated. IgG heavy chains or unknown bands are indicated with asterisks and number signs, respectively. *Numbers* on the left of the panels indicate the molecular weights of the standard markers in kilodaltons. *C*, Detection of Kir3.3 in the aG2A-5 immunoprecipitant from rat Cx. The lysates of HEK293T cells transfected with the plasmids indicated in each lane were examined for specificity of aG3NC and aG2B-2 with immunoblot analysis (*a*). Both antibodies could specifically detect proteins at 41 kDa of Kir3.3 and 38 kDa of Kir3.2b, respectively. When the PVDF membranes blotted with SA–HRP were overexposed to the films until the bands of Kir3.2a and Kir3.2c were indistinguishable (*b*), a faint signal at 40 kDa (small arrows) was detected in the immunocomplex of aG2A-5 obtained from Cx (lane 2), not from SN (lane 1). The 40 kDa protein in the immunoprecipitant has an immunoreactivity to aG3NC. However, signal with aG2B-2 could not be found either in SN or in Cx.

from the same fraction in the aG2A-5 immunoprecipitant (lane 4). Immunoblot analysis showed that the upper band was Kir3.2c (lane 5). These results strongly suggest that Kir3.2a and Kir3.2c are assembled and form a protein complex in SN. A significant protein band was not detected with an anti-Kir3.1 antibody (aG1C-1) (Inanobe et al., 1995) in this SN membrane preparation (data not shown) or in the aG2C-3 immunoprecipitant (lane 3).

In contrast to SN, the immunoprecipitant with aG2C-3 obtained from the membrane fraction of Cx was mainly composed of two bands at ~48 and 65 kDa (Fig. 2*Bb*, lane 1). In immunoblot analysis, the 48 kDa protein was recognized by aG2A-5 (lane 2), whereas the 65 kDa protein was detected by aG1C-1 (lane 3). Therefore, the immunoprecipitant with aG2C-3 from Cx membrane mainly consisted of Kir3.1 and Kir3.2c. On the other hand, the immunoprecipitant obtained with aG2A-5 from Cx was composed of three major bands (lane 4); one at ~65 kDa and the

other two at ~48 kDa. The former was recognized by aG1C-1 (data not shown). Of the latter, the upper band was detected by aG2C-3 (lane 5). Therefore, the aG2A-5 immunoprecipitant from Cx contained Kir3.1, Kir3.2a, and Kir3.2c.

To examine the possibility that other Kir3.0 subunits may exist in the aG2C-3 or aG2A-5 immunoprecipitants, we next exposed the HRP signals of those immunoprecipitants to the films for extremely long period (14 hr). After this long exposure, the two bands of Kir3.2a and Kir3.2c became so strong that we could not separate them. Under this condition, a weak signal with a molecular weight of ~40 kDa could be detected in the aG2A-5 immunoprecipitant obtained from Cx membrane but not in that from SN membrane (Fig. 2*Cb*, top panel). This 40 kDa protein was also coimmunoprecipitated with aG2C-3 antibody from Cx membranes but again not from SN membrane (data not shown). Judging from its molecular weight, either Kir3.3 or Kir3.2b sub-

Figure 3. Immunohistochemistry of Kir3.2 and Kir3.1 subunits in rat brain. Sagittal sections of rat whole brain were immunostained with aG1C-1, aG2A-5, aG2C-3, and aGIRK2 antibodies as indicated in each panel. The photographs illustrate the overview (*A–C*), and sections through the SN (*D–F*) and thalamus (*G–I*). OB, Olfactory bulb; Cx, cerebral cortex; Th, thalamus; Hip, hippocampus; ad, anterior dorsal thalamic nucleus; ld, lateral dorsal thalamic nucleus; SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata; CB, cerebellum. Scale bars: *A–C*, 2 mm; *D–I*, 20 μ m.

unit members of the Kir3.0 subfamily can be the candidate for the signal. Therefore, we produced antibodies against Kir3.3 and Kir3.2b (aG3NC and aG2B-2, respectively). The specificity of these antibodies was examined in the HEK293T cells expressing either Kir3.3 or Kir3.2b by using the immunostaining technique that was shown in Figure 1*B* for other antibodies (data not shown) and also the immunoblotting technique (Fig. 2*A*). aG3NC and aG2B-2 antibodies developed intense signals at 41 kDa for Kir3.3 and at 38 kDa for Kir3.2b on the gels, respectively. We then examined whether the 40 kDa band in the aG2A-5 or aG2C-3 immunoprecipitant could be detected by these antibodies. Figure 2*Cb* shows that the 40 kDa protein was detected by aG3NC but not by aG2B-2. These observations indicate that the K_G channels immunoprecipitated from Cx with aG2A-5 or aG2C-3 contain, in addition to Kir3.1, a small amount of Kir3.3 but not Kir3.2b. Kir3.3 and Kir3.2b could not be detected in immunoprecipitants from SN membrane, although mRNAs of both subunits were detected by RT-PCR (Fig. 2*A*, lanes 7–10).

Therefore, K_G channels in SN seem to be composed of Kir3.2a and/or Kir3.2c isoforms and do not contain significant amounts of either Kir3.3, Kir3.2b, or Kir3.1 subunits. On the other hand, the subunit composition of K_G channels in Cx seems to be more complicated than that in SN. The major components of the immunoprecipitant from Cx with aG2A-5 were Kir3.1, Kir3.2a, and Kir3.2c, and those of aG2C-3 immunoprecipitant were Kir3.1 and Kir3.2c, therefore Cx K_G channels may be mainly composed of either Kir3.1 plus Kir3.2a or Kir3.1 plus Kir3.2c.

Immunohistochemistry of Kir3.2 isoforms in dopaminergic neurons in SN

We compared the distribution of immunoreactivities to aG1C-1, aG2A-5, and aG2C-3 in sagittal sections of rat whole brain (Fig. 3*A–C*, respectively). Intense immunoreactivities from these probes overlapped in many parts in the brain including Cx, hippocampal formation (Hip), and granule cell layer of cerebellum (CB), whereas other regions exhibited more specific antibody immunoreactivity as reported previously (Liao et al., 1996; Ponce et al., 1996; Drake et al., 1997; Murer et al., 1997). In Figure 3, *A* and *B*, strong immunoreactivity to aG1C-1 but not to aG2A-5 was detected in thalamus (Th), and *vice versa* in SN and anterodorsal thalamic nucleus (ad). When the distribution of aG2A-5 immunoreactivity was compared with that of aG2C-3 (Fig. 3*B,C*), their localizations were nearly identical, except in the area of ad, where aG2A-5, but not aG2C-3, immunoreactivity could be detected. The distribution of aGIRK2 immunoreactivity was identical to that of aG2A-5 (data not shown). Figure 3*D–F*, with higher magnification of the SN, shows that the immunoreactivities to the three anti-Kir3.2 antibodies were clearly detected in a somato-dendritic pattern in both SNC and SN pars reticulata (SNR), whereas that to aG1C-1 was not significant in both areas (data not shown). On the other hand, in ad, both aGIRK2 and aG2A-5 immunoreactivities were distributed in a neuropile pattern (Fig. 3*G,H*), whereas no positive immunostaining of aG2C-3 could be detected (Fig. 3*I*). Because *in situ* hybridization indicates that Kir3.2 mRNA is not expressed in ad, it might be the case that the

Figure 4. Colocalization of Kir3.2 isoforms in dopaminergic neurons in rat SN. *A–F*, Immunolocalization of Kir3.2 isoforms was examined with double staining with aG2A-5 (Texas Red; *A, D*) and aG2C-3 (FITC; *B, E*) antibodies in SNC (*A, B*) and SNR (*D, E*). Each pair of micrographs was then merged to show a yellow image for overlapping immunostaining by aG2A-5 and aG2C-3 antibodies (*C, F*). The insets in SNR (*D–F*) present magnified sections of the individual panels. *G–I*, Double immunostaining with anti-TH antibody for the identification of dopaminergic neurons (red; *G*) and with aG2C-3 antibody for Kir3.2c isoform (green; *H*) was performed in SN. *I* is the superimposed image of the immunolocalization of TH and Kir3.2c. Similar expression of Kir3.2a, Kir3.2c, and TH could be also found in dopaminergic neurons of ventral tegmental area (data not shown). Scale bars: *A–F*, 20 μ m; *D–F*, insets, 10 μ m; *G–I*, 50 μ m.

Kir3.2a isoform detected by the antibodies is located at the nerve termini in ad projected from Hip.

In Figure 4, we examined the distribution in SN of the two splicing variants Kir3.2a and Kir3.2c. In SNC, both of the immunoreactivities to aG2A-5 (Fig. 4*A*) and aG2C-3 (Fig. 4*B*) appeared diffusely within the somata and in a granular pattern surrounding them. In SNR, both immunoreactivities were detected as varicosities on dendrites (Fig. 4*D,E*). Superimposition of the images from SNC (Fig. 4*C*) and SNR (Fig. 4*F*) showed that the immunoreactivities of Kir3.2a and Kir3.2c overlapped almost completely in both regions, indicating that these isoforms may exist in close proximity. Immunoreactivities to the antibodies specific for Kir3.1, Kir3.2b (aG2B-2), Kir3.3 (aG3NC), and Kir3.4 (aG4N-10) were not obvious in these regions (Fig. 3*A*; data not shown).

We next examined the immunoreactivity of TH in SN (Fig. 4*G–I*; Liao et al., 1996; Murer et al., 1997). Many somata and dendrites were stained with the anti-TH antibody in SNC and

SNR (Fig. 4*G*). The aG2C-3 immunoreactivity showed a similar distribution (Fig. 4*H*). The merged image for TH and Kir3.2c immunoreactivities (Fig. 4*I*) indicated that Kir3.2c exists in those neurons expressing TH, i.e., dopaminergic neurons. Although the dopaminergic neurons in SN send axonal projections to the striatum, we could not detect obvious immunoreactivity to either aG2A-5 or aG2C-3 at their axonal termini in the striatum (data not shown). These observations suggest that the K_G channels composed of Kir3.2a and Kir3.2c isoforms are localized at the somatodendritic regions of dopaminergic neurons in SN.

Subcellular localization of Kir3.2 subunits in nigral dopaminergic neurons

The subcellular localization of Kir3.2 subunits in SN was examined with immunoelectron microscopy using aGIRK2 antibody (Fig. 5). Dopaminergic neurons have a relatively round nucleus and could be easily identified in SNC (Fig. 5*A*). Only a small amount of DAB–nickel reactant was detected at cytoplasmic

Figure 5. Subcellular localization of Kir3.2 immunoreactivity within SN. The Kir3.2 immunoreactivity developed with DAB–nickel staining was investigated in rat SN. *A*, An electron micrograph of a part of the soma of a dopaminergic neuron that contained only sparse Kir3.2 immunoreactivity (asterisks) in the vicinity of some lamellar structures. There was little DAB reactant at somatic membrane (open arrows), even at a synapse on the soma (filled arrow), whereas Kir3.2-positive unmyelinated processes (arrowheads) were distributed in SNC. Nucleus (*N*) and Golgi apparatus (*G*) are indicated. *B*, A high-magnification photograph of a synapse (filled arrow) on the soma of a dopaminergic neuron in another section. No Kir3.2 immunoreactivity was detected. *C*, Dense reactants were observed on postsynaptic membranes in the dendrites contacted by several axonal termini in SNR. Scale bars: *A*, *C*, 1 μ m; *B*, 0.4 μ m.

lamellar structures (asterisks), indicating that Kir3.2 is probably synthesized in dopaminergic neurons. Immunoreactivity to this antibody could not be detected on the somatic plasma membrane, even at the postsynaptic regions (Fig. 5*A,B*, filled arrows). Immunoreactivity for Kir3.2 was abundant in unmyelinated processes with few vesicles surrounding the soma (Fig. 5*A*, arrowheads). No immunoreactivity was detected either in myelinated axons or in axonal termini containing synaptic vesicles. In SNR, Kir3.2 immunoreactivity was identified in many dendrites (Fig. 5*C*). The immunoreactivity was specifically localized at the postsynaptic membrane of dendrites that faced axonal termini containing many synaptic vesicles. Essentially the same results were obtained using aG2A-5 and aG2C-3 antibodies (data not shown). Therefore, the K_G channels consisting of Kir3.2 isoforms seem to be localized specifically at the postsynaptic membrane on dendrites of dopaminergic neurons in SN.

Kir3.2c does, but Kir3.2a does not, bind PDZ domain-containing anchoring proteins

Various ion channels, including NMDA receptors and voltage-dependent K^+ channels, are localized at postsynaptic regions by interacting with members of the PDZ domain-containing (originally identified in PSD-95, discs large, and ZO-1) anchoring proteins through their C-terminal motif of (E)-S-X-V/I (Sheng, 1996). Some Kir channels (Kir2.1, Kir2.2, Kir2.3, and Kir4.1)

possess the same motif and can interact with the anchoring proteins in neuronal or glial cells (Cohen et al., 1996; Horio et al., 1997).

Because the C-terminal tail of Kir3.2c is E-S-K-V (Fig. 1*A*), it is possible that this Kir3.2c isoform associates with PDZ domain-containing proteins. At present there are no reports concerning PDZ domain-containing proteins localized at the dendrites of dopaminergic neurons in SN. Thus, we examined whether Kir3.2c can interact with PSD-95/SAP90, the prototype of the PDZ domain-containing anchoring proteins existing at the postsynaptic density (Fig. 6; Sheng, 1996). HEK293T cells were cotransfected with various combinations of Kir3.2a, Kir3.2c, and PSD-95. In this series of experiments, immunoprecipitants were obtained with aG2N-2, a polyclonal antibody for the N terminus of Kir3.2 and then immunoblotted with anti-PSD-95 antibody (Takeuchi et al., 1997). The immunoprecipitant obtained from the cells cotransfected with Kir3.2c plus PSD-95 contained PSD-95 immunoreactivity (lane 2), whereas that from the cells with Kir3.2a plus PSD-95 or PSD-95 alone did not (lanes 1 and 3). This result indicates that Kir3.2c has the capability to bind a PDZ domain-containing anchoring protein, PSD-95, but Kir3.2a does not.

Next we examined the distribution of PSD-95 in rat brain. In the CA1 region of Hip, PSD-95 immunoreactivity was detected

Figure 6. Association of the splicing variants of Kir3.2 and PDZ domain-containing protein. *A*, Interaction of Kir3.2c isoform and PSD-95. HEK293T cells were transfected with PSD-95 and either Kir3.2a or Kir3.2c. The immunoprecipitants obtained with aG2N-2 from the transfected cells were immunoblotted with anti-PSD-95 antibody. The immunoprecipitant from the cells with Kir3.2c plus PSD-95 contained PSD-95, whereas those from the cells with Kir3.2a plus PSD-95 or PSD-95 alone did not. *B*, Immunostaining of PSD-95 and SAP97 in rat brain. Localization of PSD-95 (*a*, *b*) and SAP97 (*c*, *d*) were examined with immunofluorescence in the CA1 region of Hip (*a*, *c*) and in the SNC (*b*, *d*). Their immunolocalizations were visualized with green images, and dopaminergic neurons were stained with anti-TH antibody in SNC (red; Fig. 4). *sp*, Substantia pyramidalis; *sr*, stratum radiatum. Scale bars: *Ba*, *c*, *Bb*, *d*, 30 μ m.

prominently at dendrites of pyramidal cells (Fig. 6*Ba*; Cho et al., 1992). However, PSD-95 immunoreactivity was barely detectable in SN (Fig. 6*Bb*). At most, very faint staining in a neuropile fraction could be seen. On the other hand, SAP97, one of the PDZ domain-containing anchoring proteins associated with presynaptic membranes, was reported to be accumulated in SN (Müller et al., 1995). Prominent SAP97 immunoreactivity was

detected in both regions of CA1 of Hip and SNC (Fig. 6*Bc,d*), but they were in neuropile and not in somata. In SNC (Fig. 6*Bd*), many dots of SAP97 immunoreactivity surrounded the somata of dopaminergic neurons, suggesting that they are at the axonal termini. This is consistent with the localization of SAP97 at the presynapse of excitatory synapses in Hip (Müller et al., 1995). These results indicate that neither PSD-95 nor SAP97 are localized at the dendrites of dopaminergic neurons in SN. Therefore, anchoring protein(s) other than PSD-95 and SAP97 may be responsible for the specific localization of SN K_G channels at the postsynaptic membrane of dopaminergic neurons.

Dopamine activates the K_G channels composed of Kir3.2a and Kir3.2c heterologously expressed in *Xenopus* oocytes

Dopamine released from the somatodendritic regions of dopaminergic neurons in SN is known to act on D_2 receptors localized at the same or neighboring neurons and hyperpolarize the membrane (Innis and Aghajanian, 1987; Lacey et al., 1988). We examined whether the K_G channel composed of Kir3.2a and Kir3.2c could be the target of D_2 receptor (Fig. 7).

Different combinations of Kir3.2a and Kir3.2c cRNAs were injected with that of D_2 receptor into *Xenopus* oocytes (Fig. 7*Aa–d*). When Kir3.2a cRNA alone was injected (Fig. 7*Aa*), a significant background activity of Kir current was expressed. Dopamine (10 μ M) further increased the Kir current, whose amplitude was $1.2 \pm 0.2 \mu$ A ($n = 6$) at the end of the voltage step to -120 mV. Both currents ($2.8 \pm 0.4 \mu$ A; $n = 6$) were completely inhibited by Ba^{2+} (1 mM). In the oocytes injected with Kir3.2c alone (Fig. 7*Ab*), there was no significant background Kir activity. Dopamine had little effect. In the oocytes injected with the cRNAs of both Kir3.2a and Kir3.2c (Fig. 7*Ac*), background and dopamine-induced Kir currents equivalent to those of Kir3.2a alone were measured. Because, when cotransfected in HEK293T cells, Kir3.2a and Kir3.2c were found to be assembled to form a protein complex (Fig. 1*D*), it seemed likely that at least some of the background and dopamine-induced Kir currents in these oocytes flowed through heteromeric Kir3.2a-Kir3.2c channels. We therefore constructed a tandem clone of Kir3.2a and Kir3.2c (Kir3.2a-Kir3.2c) and injected its cRNA to oocytes (Fig. 7*Ad*). Both background and dopamine-induced Kir currents expressed in these oocytes were equivalent to those of Kir3.2a alone or of Kir3.2a plus Kir3.2c.

In Figure 7*Ae–h*, we examined the subcellular localization of Kir3.2a and Kir3.2c in *Xenopus* oocytes using the GFP-fluorescence technique. The cDNAs of Kir3.2a and Kir3.2c were fused with GFP at their N termini. The oocytes injected with cRNAs of GFP-Kir3.2a and/or GFP-Kir3.2c expressed Kir currents exhibiting the same electrophysiological properties as those of control Kir3.2a and/or Kir3.2c ($n = 5$ for each of GFP-Kir3.2a alone, GFP-Kir3.2c alone and both). When injected alone (Fig. 7*Ae,f*) or in combination (Fig. 7*Ag*), the intense fluorescence of GFP-Kir3.2a or GFP-Kir3.2c could be detected at the plasma membrane in addition to cytoplasmic regions. There was no significant difference in the distribution of fluorescence between the oocytes injected with GFP-Kir3.2c alone and those with GFP-Kir3.2c plus Kir3.2a (Fig. 7*Af,g*). The localization of Kir3.2c on the plasma membrane was further confirmed by immunogold electron microscopy (data not shown). In noninjected oocytes, no significant fluorescence was detected (Fig. 7*Ah*). These results indicate that, even when Kir3.2c cRNA was injected

Figure 7. Functional expression of Kir3.2 isoforms in *Xenopus* oocytes. *Aa–d*, *Ba–c*, Whole-cell currents recorded from *Xenopus* oocytes expressing various combinations of Kir3.2a, Kir3.2c, and Kir3.1 clones with the D₂ dopamine receptor. The combination is indicated with each family of current traces. The bath solution contained 90 mM K⁺. Voltage steps (1.2 sec in duration) from the holding potential of 0 mV to potentials between +60 and –120 mV with –20 mV increment were delivered at a rate of every 5 sec. Traces show current records obtained in the absence (*Basal*) and in the presence of 10 μ M dopamine (*DA*) and after addition of Ba²⁺ (1 mM), as indicated at the top of each column of current traces. *Ae–h*, *Bd,e*, The distribution of GFP fluorescence in oocytes injected with the cRNAs of GFP-tagged Kir3.0 clones. The GFP-tagged clone was indicated by the asterisk in each figure. GFP-Kir3.2a, GFP-Kir3.2c, and GFP-Kir3.1 were injected either alone or with other clones as indicated. After the fixation with 4% PFA, 10 μ m sections of oocytes were examined by confocal microscopy. Arrows indicate the plasma membrane. Scale bars, 50 μ m. *C*, The amplitudes of Kir currents recorded in the oocytes injected with different combinations of Kir3.2 isoforms and Kir3.1 cRNAs. Each column represents the average current amplitude ($n = 5 \sim 6$) at the end of the voltage step to –120 mV. The amplitude of each Kir current was obtained as the Ba²⁺-sensitive component in the presence or absence of 10 μ M dopamine in those oocytes that expressed the D₂ receptor (closed or open columns) or in the absence of dopamine in those oocytes that coexpressed Kir subunit(s) and G $\beta\gamma$ subunit ($\beta 1$ and $\gamma 2$) (hatched columns). The vertical bars indicate the SE.

alone, the cRNA was translated to Kir3.2c protein, which then was transported to the plasma membrane.

We next compared the effects of Kir3.2 isoforms on the formation of K_G current with Kir3.1 in *Xenopus* oocytes (Fig. 7*Ba–c*). The functional K_G channel current in the oocytes injected with Kir3.1 alone was marginal as reported previously (Fig. 7*Ba*; Kofuji et al., 1995; Duprat et al., 1995; Isomoto et al., 1996; Slesinger et al., 1996; Velimirovic et al., 1996). It was markedly enhanced by coexpression of not only Kir3.2a but also Kir3.2c (Fig. 7*Bb,c*). Consistent with the expression experiments, the fluorescence of GFP-Kir3.1 was only faintly detected at the plasma membrane in the oocytes injected with Kir3.1 alone (Fig. 7*Bd*), it markedly increased when coinjected with Kir3.2a or Kir3.2c (Fig. 7*Be*).

In Figure 7*C*, we summarize the data concerning Ba²⁺-sensitive Kir currents in the oocytes injected with different combinations of Kir3.2a, Kir3.2c, the tandem clone, and Kir3.1. In these experiments, Kir currents were activated either by dopamine applied to oocytes in which D₂ receptor had been coexpressed or by coexpression of $\beta 1\gamma 2$ subunits of G-protein (G $\beta\gamma$). In the latter case, the functional K_G channels would be fully activated by the overexpressed G $\beta\gamma$ even in the absence of an external ligand. The Ba²⁺-sensitive Kir currents recorded from these two groups of oocytes were equivalent. When Kir3.2c alone was expressed in oocytes with either D₂ receptor or G $\beta\gamma$, no significant Kir current was detected even in the presence of dopamine. Kir3.2c could, however, enhance the Kir current when coexpressed with Kir3.1. In contrast, Kir3.2a could

form functional K_G channels either alone, with Kir3.1 or with Kir3.2c.

DISCUSSION

Subunit composition of K_G channels in SN

In this study, we have characterized the K_G channels containing Kir3.2 in rat SN. These K_G channels are composed of the splicing variants of Kir3.2, Kir3.2a, and Kir3.2c (see also Kobayashi et al., 1995; Karschin et al., 1996; Liao et al., 1996). Neither Kir3.1, Kir3.2b, Kir3.3, nor Kir3.4 seem to contribute to the SN K_G channels. Homomeric K_G channels composed of any of these subunits seem unlikely based on the evidence that we have available. A homomeric Kir3.2a channel may not be significant because immunoreactivity to aG2A-5 and aG2C-3 strongly overlapped in the SNC and the SNR (Fig. 4A–F). Immunologically it is difficult to rule out homomeric assembly of the Kir3.2c subunit, although this was not functional when heterologously expressed in *Xenopus* oocytes (Fig. 7). Although it is always possible that novel Kir3.0 subunits will be discovered, it is difficult not to conclude from our data that some, if not all, SN K_G channels are heteromeric assemblies of splicing variants from a single gene, i.e., Kir3.2a and Kir3.2c.

Specific roles of Kir3.2a and Kir3.2c in the assembled K_G channel

Kir3.2a and Kir3.2c isoforms differ only in that Kir3.2c contains an additional 11 amino acids on its C-terminal tail (Fig. 1A). Individual heterologous expression in *Xenopus* oocytes showed that Kir3.2a could form functional K_G channels and Kir3.2c could not. Although the expression of either subunit with Kir3.1 resulted in the known facilitation of Kir3.1 heteromeric K_G channels (Duprat et al., 1995; Kofuji et al., 1995; Slesinger et al., 1996; Velimirovic et al., 1996), either the coexpression or even the expression of tandems of Kir3.2a and Kir3.2c did not facilitate these K_G currents above those shown by the expression of Kir3.2a alone. When tagged with GFP, the Kir3.2c protein was localized at the plasma membrane (Fig. 7A_f). Thus, the Kir3.2c subunit seems to be equipotent to Kir3.2a in generation of current once incorporated into the functional K_G channel, and the specific functional role of the Kir3.2c subunit in any heteromeric structure with Kir3.2a is not clear.

However, Kir3.2c may be responsible for the subcellular localization of Kir3.2 K_G channels in dopaminergic neurons. The C terminus of Kir3.2c contains the sequence E-S-K-V, which corresponds to the (E)-S-X-V/I motif known to interact with PDZ domains of anchoring proteins (Sheng, 1996), which are responsible for the postsynaptic localization of both voltage-dependent K^+ channels and NMDA receptors (Kim et al., 1995; Kornau et al., 1995). Inward rectifier K^+ channels Kir2.1, Kir2.3, and Kir4.1 possess similar domains and interact with PDZ domain-containing anchoring proteins (Cohen et al., 1996; Horio et al., 1997). We show that Kir3.2c can bind the anchoring protein PSD-95 (Fig. 6) but that neither PSD-95 nor SAP97 were colocalized with Kir3.2 in the SN (Fig. 6B). A number of other anchoring proteins that contain the PDZ domain have been recently identified in the brain (Kim et al., 1996; Müller et al., 1996; Chevesich et al., 1997; Dong et al., 1997; Brakeman et al., 1997; Kurschner et al., 1998; Short et al., 1998). Further studies are required to identify which might be localized with the K_G channels in the SN.

Nevertheless, from the present results, it is strongly suggested that, in the heteromeric assembly of Kir3.2a and Kir3.2c, the

former subunit is primarily responsible for formation of a functional K_G channel, and the latter controls subcellular localization of the K_G channel. In other words, each splicing isoform of Kir3.2 may play a respective unique role in the control of the heteromeric Kir3.2a-Kir3.2c K_G channel in the dendrites of dopaminergic neurons of SN.

Physiological implications of the Kir3.2a-Kir3.2c complex G-protein-gated K^+ channel in SN

Stimulation releases dopamine from the somatodendritic regions of dopaminergic neurons in SN, whereas GABA is released in the SN from the axonal termini of GABAergic projections from various parts of brain, including striatum and globus pallidus (Bolam and Smith, 1990). These neurotransmitters can generate slow IPSPs in dopaminergic neurons of SN by acting on D_2 and GABA_B receptors (Innis and Aghajanian, 1987; Lacey et al., 1988), and the K_G channels at the postsynaptic membranes of dendrites of dopaminergic neurons may be the target of these receptors. The K_G channel-mediated slow IPSP decreases the firing rates of the dopaminergic neurons and attenuates dopamine release from their axonal termini in the striatum. Therefore, the Kir3.2a-Kir3.2c heteromeric K_G channel may be an essential element in the interaction between SN and striatum and critically involved in the control of movement and affective behavior. This notion is supported by the discovery that the *weaver* mutant mouse, a mouse model of Parkinsonism, is caused by a point mutation in the pore region of Kir3.2 (Patil et al., 1995). In the *weaver* mouse, the dopaminergic neurons in SN degenerate, and disorders in movement develop. Kir3.2-null mice, however, did not show either any developmental defects in SN or apparent movement disorder (Signorini et al., 1997). Therefore, the physiological role of K_G channels in SN should be further evaluated.

Dopamine D_2 receptors are distributed on the dendrites and the somata of SN dopaminergic neurons (Sesack et al., 1994) and probably also on their axon terminals in the striatum (Cragg and Greenfield, 1997). The K_G channels made up of Kir3.2a and/or Kir3.2c were found only on the dendrites of dopaminergic neurons in this study. Therefore, D_2 receptors in other regions of the cells may be coupled to other signaling systems.

Molecular diversity of the G-protein-gated K^+ channels in the brain

Although neuronal K_G channels have been supposed to be heteromeric complexes of Kir3.1 and Kir3.2 (Duprat et al., 1995; Kofuji et al., 1995; Liao et al., 1996; Velimirovic et al., 1996), by showing the assembly of Kir3.2 in SN this study has raised the possibility that K_G channels in the brain may be more diverse than previously thought. For example, although the expression of Kir3.4 mRNA in the brain was minimal (Karschin et al., 1996), immunoreactivity of Kir3.4 could be detected at axonal termini in rat cerebellar cortex (Iizuka et al., 1997), and Kir3.1 immunoreactivity has been localized at the axonal termini in specific regions of brain (Morishige et al., 1996; Ponce et al., 1996). The subunit composition, subcellular localization, and functional role of neuronal K_G channels at axonal termini have not yet been well characterized. Also, mRNA of Kir3.3 has been shown to be expressed widely in the brain, even in the SN (Karschin et al., 1996). In this study, a small amount of Kir3.3 protein could be detected in the immunocomplex of Kir3.2 subunits obtained from Cx but not from SN (Fig. 2C_b). The distribution and properties of native K_G channels containing Kir3.3 have not yet been well clarified. Because a variety of G-protein-coupled inhibitory re-

ceptors, including M_2 -muscarinic, 5-HT_{1A}-serotonergic, A_1 -purinergic, α_2 -adrenergic, μ - and δ -opioid, and somatostatin receptors activate K_G channels in many regions of brain (for review, see North, 1989; Hille, 1992), further studies on molecular diversity, functional properties, and localization of neuronal K_G channels are required to elucidate the physiological roles of this system in the control of different brain functions.

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