Kv3.1–Kv3.2 Channels Underlie a High-Voltage–Activating Component of the Delayed Rectifier K⁺ Current in Projecting Neurons From the Globus Pallidus

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INTRODUCTION

A large number of subunits of mammalian K⁺ channels expressed in the CNS have been identified after the cloning of the Shaker gene in Drosophila in 1987 (reviewed in Chandy and Gutman 1995; Coetzee et al. 1999; Jan and Jan 1997; Pongs 1992; Rudy et al. 1991a). This work has revealed the existence of an extraordinary diversity of molecular components of voltage-gated K⁺ channels, predicting a functional diversity well beyond that expected from prior functional studies (Rudy 1988). The cloning studies have allowed enormous progress in the understanding of the molecular mechanisms of channel function, including the recent crystallization and high resolution structural analysis of a K⁺ channel (Doyle et al. 1998). Less progress has been obtained in understanding the physiological significance of the molecular diversity. A major task of future research is to identify physiological roles of the cloned proteins, starting with the identification of native channels containing specific types of cloned subunits.

Among the cloned subunits are the members of the Kv and KCNQ (or KQT) families of K⁺ channel proteins, which are pore-forming components of voltage-gated K⁺ channels (Chandy and Gutman 1995; Coetzee et al. 1999; Jan and Jan 1997; Pongs 1992; Rudy et al. 1991a). The Kv family is divided into several subfamilies based on sequence similarities. Nearly 30 Kv proteins classified in eight subfamilies (Kv1–Kv6 and Kv8–Kv9) are known to date (Coetzee et al. 1999).

The goal of this study was to identify the currents mediated by channels containing proteins of the Kv3 subfamily in neurons. There are four known Kv3 genes (Kv3.1–Kv3.4). In heterologous expression systems, Kv3.1 and Kv3.2 proteins express tetraethylammonium (TEA)-sensitive delayed rectifier type currents, whereas Kv3.3 and Kv3.4 proteins form transient, TEA-sensitive, A-type K⁺ channels (reviewed in Vega-Saenz de Miera et al. 1994). However, native channels may differ from those formed by a given Kv subunit in heterologous expression systems. Kv proteins can form heteromeric channels with novel properties with other members of the same subfamily (Christie et al. 1990; Covarrubias et al. 1991; Isacoff et al. 1990; K. McCormack et al. 1990; Ruppersberg et al. 1990; Weiser et al. 1994). Moreover the functional characteristics of K⁺ channels, including those of the Kv family, also can be modified by accessory subunits and postranslational modifications (Barhanin et al. 1996; Coetzee et al. 1999; Covarrubias et al. 1994; Heinemann et al. 1996; McDonald et al. 1997; Sanguinetti et al. 1996; Serodio et al. 1994, 1996).
expression systems blocked a fraction of the K⁺ current from hippocampal interneurons expressing Kv3.1b proteins that resembles Kv3.1 currents in activation and deactivation properties (Du et al. 1996). However, limitations associated with voltage clamping intact neurons in slices prevented a detailed comparison of the properties of the putative Kv3.1-mediated currents in these cells with the properties of Kv3.1 currents in heterologous expression systems. Both Kv3.1 and Kv3.2 proteins are strongly expressed in neurons of the globus pallidus (GP), suggesting that these cells might be a good system to study the properties of native Kv3.1–Kv3.2 channels using voltage-clamp methods (Moreno et al. 1995; Weiser et al. 1994, 1995). Moreover, methods to dissociate these neurons from rat brain have been developed (Stefani et al. 1992, 1995; Surmeier et al. 1994). Freshly dissociated and short term cultured cells are a good system for this kind of study, allowing improved conditions for space clamp and pharmacological analysis.

We have used whole cell patch-clamp methods to analyze the voltage-dependent K⁺ currents of freshly dissociated rat GP neurons to determine whether they contain currents with properties similar to those carried by Kv3.1–Kv3.2 channels. Because of its central roles in movement control and perhaps also cognitive functions, there is great interest in the anatomic and physiological characterization of the GP (Chang et al. 1987; Chudler and Dong 1995; DeLong 1971, 1972; Difiglia and Rafols 1988; Graybiel and Ragsdale 1979; Hauber et al. 1998; Kita 1992, 1994; Kita and Kitaï 1991, 1994; Morizumi and Hattori 1992; Nambu and Linhas 1994; 1997; Parent and Hazrati 1995a, b; Park et al. 1982; Schneider et al. 1985; Stefani et al. 1992; Surmeier et al. 1994). The present studies contribute novel information on the classification and cellular properties of pallidal neurons.

Previous electrophysiological analysis of the K⁺ currents of pallidal neurons, in the same species, revealed a low voltage-activating fast inactivating current (Iₐ), a component with slower inactivation and slow recovery from inactivation that is blocked by micromolar concentrations of 4-AP (Edwards et al. 1994; Nambu and Llinas 1994, 1997; Parent and Rafols 1988; Graybiel and Ragsdale 1979; Hauber et al. 1998; Kita 1992, 1994; Kita and Kitaï 1991, 1994; Morizumi and Hattori 1992; Nambu and Linhas 1994; 1997; Parent and Hazrati 1995a, b; Park et al. 1982; Schneider et al. 1985; Stefani et al. 1992; Surmeier et al. 1994). None of these components resembles Kv3 currents. These observations do not necessarily indicate that Kv3 currents are either absent in pallidal neurons or have properties different from those of Kv3 currents in heterologous expression systems because the methods that are used in a given study to isolate individual components of the total K⁺ current are tailored to the goals of the particular investigation. Therefore electrophysiological experiments specifically designed to search for native currents with properties similar to those of Kv3.1–Kv3.2 currents in vitro are required before we can conclude whether or not native Kv3.1–Kv3.2 channels in pallidal neurons have properties similar to those in heterologous expression systems.

In this study, we first analyzed the expression of Kv3.1 and Kv3.2 proteins in the rat GP with specific antibodies. We also determined the developmental expression of these proteins, allowing us to select tissue at developmental stages in which the proteins are robustly expressed in pallidal neurons. We used pharmacological and electrophysiological protocols on freshly dissociated neurons appropriate for the isolation of Kv3-like currents and compared these currents to those recorded under identical conditions from mammalian cells transfected with Kv3.1 and Kv3.2 cDNAs. The expression of Kv3 transcripts in the same cells was confirmed by single cell RT-PCR. The studies described here have been previously presented in abstract form (Hernández-Pineda et al. 1996; Pineda et al. 1998).

**METHODS**

**Antibodies to Kv3.1 and Kv3.2 proteins**

Site-specific antibodies against Kv3.1b proteins were prepared by injecting into rabbits the peptide CTPDLIGGDPGDDEDLDGKR coupled via the cysteine to keyhole limpet hemocyanin (KLH). The peptide corresponds to the carboxyl terminal sequence of the Kv3.1b protein (residues 567–585) (Weiser et al. 1995). The characterization of this antibody was described previously (Weiser et al. 1995). To raise antibodies against Kv3.2 proteins, rabbits were injected with the peptides: CTPDLIGGDPGDDEDLGKR and CTPDLIGGDPGDDEDLAAKR coupled via the cysteine to KLH (Chow et al. 1999). The peptides correspond to a sequence present in the constant region of the rat and mouse Kv3.2 proteins, respectively (residues 171–189 plus an N-terminal cysteine added to facilitate coupling), before the first membrane-spanning domain in an area not conserved among different K⁺ channel proteins (Vega-Saenz de Miera et al. 1994; see T. McCormack et al. 1990 for the rat sequence). The mouse sequence has not been published, but it is identical to that in rat except for the substitution of glycines (186–187 by alanines). For affinity purification, the respective peptides were coupled to Sulfolink Sepharose resin (Pierce, Rockford, IL) via the cysteine residue and the sera purified following supplier’s protocols.

**Immunofluorescence labeling**

Male Sprague-Dawley rats (2–3 wk old) or male C57Bl6 mice (6–8 wk old), as well as Kv3.1 and Kv3.2 (Ho et al. 1997) or Kv3.2 (Lau et al. 1999) mice, were anesthetized with an injection of pentobarbital sodium (120 mg/kg ip) and perfused transcardially with 10–20 ml Heparin (1 U/ml) in phosphate-buffered saline (PBS: 0.06 M sodium phosphate buffer, 0.85% sodium chloride, pH 7.35) at room temperature followed by 100–200 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brain was dissected out and blocked coronally into ~5 mm portions, postfixed for 30 min in the same fixative at room temperature and placed in 30% sucrose in PBS for 12–24 h at 4°C. When the tissue had sunk in the sucrose solution, 50-μm sections were produced using a freezing microtome and collected in PBS. The sections were washed twice for 15 min in PBS and incubated in a blocking solution containing 10% normal goat serum (Jackson Immuno Research), 1% bovine serum albumin (Jackson Immuno Research), 0.2% cold water fish gelatin (Sigma Chemicals), and 0.2% Triton X-100 (Sigma Chemicals) in PBS for 1 h to minimize nonspecific binding. The sections then were incubated with primary antibody at the appropriate dilution in a working buffer (0.1× blocking solution in PBS) for 12–24 h at 4°C. For double-labeled sections, a primary rabbit antibody, anti-Kv3.1b or anti-Kv3.2, and a primary mouse antibody, anti-parvalbumin (Sigma Immunochemicals) were added simultaneously. After three 15 min washes in PBS, secondary antibodies diluted in working buffer were applied for 15 min at room temperature. The following secondary antibodies were used, Cy2-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG (Jackson Immuno Research). After two 15 min washes in PBS, the sections were mounted onto glass slides and coversontered with elvanol.

The following primary antibody concentrations were used: antibodies against Kv3.1b (Weiser et al. 1995) at 1:50, Kv3.2 (Chow et al. 1999) at 1:50, parvalbumin (Sigma Immunochemicals) at 1:400. Secondary fluorescent antibodies were used at 1:500. The atlas by Paxinos and Watson (1986) and the book edited by Paxinos (1995) were used to perform the microdissection of the various structures examined.
used as guides to identify CNS neuronal populations and axonal projections.

Images were taken either with a Zeiss Axioskop fluorescent microscope or an Axiovert 35 M confocal microscope, with a ×40 (NA 1.3) or ×63 (NA 1.4) objective lenses. Fluorescent images were recorded using a scanning laser attachment (MRC-600 and MRC-1000, Bio-Rad Laboratories) and a krypton/argon mixed gas laser. Images were collected digitally and transferred to a graphics program (Adobe Photoshop 4.0). After brightness and contrast adjustments, the image files were printed on a Tektronix printer (Phaser 440).

Immunoblots

Rat brain membrane extracts were prepared from a P3 fraction of tissue homogenate (Hartshorne and Catterall 1984) solubilized for 1 h in a 2% Triton X-100 solution containing (in mM) 50 potassium phosphate buffer, pH 7.4; 50 KCl; 2 EDTA; 1 pepstatin A, 1,10-phenanthroline, 0.2 phenylmethylsulfonyl fluoride (PMSF), and 1 iodoacetamide to inhibit proteases. The suspension was spun at 100,000 g to remove nonsolubilized material, and the top two-thirds of the supernatant used for further experiments. To prepare membrane extracts from the GP, the nucleus was dissected from slices prepared as described for the preparation of dissociated neurons (see following text) and proteins solubilized as described in the preceding text.

To prepare immunoblots, 50 μg of membrane protein was mixed 1:1 with a sample buffer [10% (vol) glycerol, 5% (vol) b-mercaptoethanol; 60 mM Tris-HCl pH 6.8; 0.001% (weight) bromophenol blue and 3% SDS], heated for 3 min at 80°C, and electrophoresed in a 8% SDS polyacrylamide gel (Harlow and Lane 1988). The electrophoresed proteins were transferred onto a nitrocellulose filter (Bio-Rad Laboratories) and a krypton/argon mixed gas laser. Images were collected digitally and transferred to a graphics program (Adobe Photoshop 4.0). After brightness and contrast adjustments, the image files were printed on a Tektronix printer (Phaser 440).

Immunoprecipitation

Before immunoprecipitation, 300 μl of solubilized membranes (~400 μg protein) in 1% Triton X-100 in (in mM) 50 Tris, 150 NaCl, 1 EDTA, and 1 EGTA, pH 7.4, were preclarred for 30 min at 4°C with protein A-Sepharose beads (Sigma Chemicals). After removing the beads, the extracts were incubated for 4 h at 4°C with Kv3.2 antibodies at a 1:10 dilution or Kv3.1b antibodies at 1:2000, followed by incubation with horseradish peroxidase-linked anti-rabbit secondary antibodies (Promega). Bound antibodies were detected using chemilluminscence (Pierce).

Electrophysiological analysis

Electrophysiological recordings used voltage clamp under the whole cell configuration of the patch-clamp technique (Hamill et al. 1981). The same extracellular and intracellular solutions were used for recordings with CHO cells and pallidal neurons. Extracellular solution (NES) was supplemented with 1 μM tetrodotoxin (TTX, Alomone, Jerusalem, Israel) and 200 μM of CdCl₂ (Sigma Chemical) to block voltage-dependent sodium and calcium currents, respectively. By inhibiting calcium influx, CdCl₂ also limited the activation of calcium-activated membrane conductances. The setup was voltage controlled using a Digidata 1322A controller (Axon Instruments, Foster City, CA). To generate voltage clamp

Dissociation of neurons from the GP

Young Sprague-Dawley rats 10–16 days of age were used. The brain was quickly removed and submerged in ice-cold normal extracellular solution (NES) containing (in mM) 130 NaCl, 3 KCl, 2 MgCl₂, 1 NaHCO₃, 0.5 NaH₂PO₄, 5 HEPES, 2 CaCl₂, and 5 glucose, pH 7.4. The solution was gassed with 95% O₂-5% CO₂ for 15 min before starting the dissociation and then continuously during the procedure. Once the cerebellum had been removed, the brain hemispheres were separated along the midline and cut in the parasagittal plane with a vibratome (Campden Instruments, London, UK) in 400 μm slices. The slices were collected and maintained in ice-cold NES solution. The GP was identified by visual inspection under a stereomicroscope (see Fig. 7). The GP from four to seven slices was dissected out and subjected to enzymatic digestion for 25–35 min (depending on the age of the animal) at 37°C in NES with Pronase (Sigma Chemicals, St. Louis, MO). The tissue then was washed three times in NES without calcium and triturated mechanically by means of glass Pasteur pipettes with tips of decreasing diameters in a final volume of 2 ml. An aliquot (~500 μl) of the cell suspension was seeded in a recording chamber (RC-13, Warner Instruments, Hamden, CT) and mounted on the plate of an inverted microscope for electrophysiological recording. The cells were perfused continuously (~1 ml/min) with NES at room temperature.
protocols and for data acquisition and analysis, we used the pClamp software (Axon Instruments). Large neurons with short processes (<40 μm) were selected for recording. Series resistance was estimated from the time constant of the capacity transient and ranged between 5 and 10 MΩ in the cells used for analysis. The series resistance was compensated (70–80%) and monitored throughout the course of the experiment. If the series resistance could not be compensated or if it changed by >20%, the cell was discarded. Input capacitance of the cells included in this study ranged between 15 and 33 pF and the time constant of the capacity current transient between 0.18 and 0.31 ms. Drugs were applied locally by means of a puffer pipette with a relatively wide tip (10 μm), made of a pulled borosilicate glass capillary and placed with a second micromanipulator to a distance of 100–200 μm of the cell. The application was made either by gravity or pressure pulses (10 psi) controlled with a picospritzer device (General Valve).

Single-cell reverse transcriptase-polymerase chain reaction (single-cell RT-PCR)

The presence of Kv3 mRNAs in dissociated GP cells was determined by single-cell RT-PCR. This was performed as described by Vega-Saenz de Miera et al. (1997) with the following modifications: the solutions were prepared in double-distilled RNase-free water from Sigma. The content of individual cells was collected from the recording chamber using gentle suction with a wide tip glass micropipette (Gibco-BRL), 4.4 μl of a solution containing 150 mM KCl, 30 mM Tris-HCl, pH 8.3, and 10 U of Promega RNase inhibitor. The tip of the pipette was broken inside a 500-mL of the cell. The tubes were placed in dry ice-ethanol bath to freeze the contents and then stored at −70°C until use.

For reverse transcription, 17 μl of a solution containing 1.18 mM dNTP, 3.82 mM MgCl2, 50 μM of Moloney Murine Leukemia Virus RNase H minus reverse transcriptase (Gibco-BRL), 4.4 μM random hexamer primer (Pharmacia), 20 U/ml of RNase inhibitor, 30 mM KCl, and 6 mM Tris-HCl pH 8.3, were added to the Eppendorf tube containing the neuron’s contents. Mineral oil (50 μl) were laid on top of the aqueous solution and the tubes incubated successively at 25°C for 5 min, 37°C for 15 min, and 42°C for 15 min. The tubes then were heated at 94°C for 5 min and then cooled to 0°C until used for PCR amplification.

For PCR amplification, 75 μl of a solution containing 50 mM KCl, 0.85 mM MgCl2, 1.7 μM of the sense and antisense external primers, and 10 mM Tris-HCl, pH 8.3 were added to the tubes containing the reverse-transcribed DNA. The tubes were heated for 5 min at 94°C. While the tubes were at 94°C, 5 μl of a solution containing 2.5 units of Perkin Elmer Taq polimerase, 50 mM KCl, and 10 mM Tris-HCl pH 8.3 were introduced through the oil. The tubes then were subjected to 35 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C, for 1 min followed by one final incubation at 72°C for 7 min. One microliter of a 1:1 dilution of the previous reaction was used as template for the second PCR reaction in a new PCR tube. 94 μl of a solution containing 2.63 mM MgCl2, 210 μM dNTPs, 50 mM KCl, 10 mM Tris-HCl pH 8.3, and 160 mM of the specific primers were added to these tubes. Oil (50 μl) was laid on top and the tubes heated and Taq polimerase added as described in the preceding text. The tubes were subjected to 35 cycles of PCR amplification (1 min each of denaturation, annealing and extension at 94, 55, and 72°C, respectively) followed by a last extension period at 72°C for 7 min. The PCR products were analyzed by electrophoresis in 2% agarose gels.

The external degenerate primers, designed to amplify all Kv3 sequences, from *Drosophila* were used, but most in the first PCR reaction, had the following sequences: sense primer: CTCT GAA TTC TTT TTG TG (T/G)T (A/G)A (A/G) ACN CA; antisense primer: CTC- GAATTC GGA (A/G)TA (A/G)TA CAT N(C/G)C (G/A)AA (G/A)TT. The sequence for the specific primers was: Kv3.1, sense primer: CGC TTC AAC CCC ATC GTG AAC (position 1801–1821 in Accession No. M68880); antisense primer: GTG TGT GTG TCC GCT GCC GCT (2290–2310). The size of the expected product is 532bp. Kv3.2, sense primer CC AGC GCT GTT CTC CAG TAT (882–901 in No. M34052); antisense primer: C AA GGG GAT GAT TTT GAA CTT (1358–1337). The size of the expected product is 477 bp. Kv3.4, sense primer: CCT GAT ACG TTT GAC TTT GTC (1312–1333 in No. X62841), antisense primer: ATT GCC CCG TGG GTC AGA T (1629–1647). The size of the expected product is 336 bp.

RESULTS

Expression of Kv3.1 and Kv3.2 proteins in the rat GP

Kv3 mRNAs are not expressed at significant levels before birth (Goldman-Wohl et al. 1994; Perney et al. 1992; Vega-Saenz de Miera et al. 1994; reviewed in Rudy et al. 1999). Immunoblots using antibodies against Kv3.1 (Weiser et al. 1995) and Kv3.2 (Chow et al. 1999) were used to study the postnatal developmental expression of Kv3.1 and Kv3.2 proteins in membrane extracts from the GP (Fig. 1B). Membranes were prepared from GP dissected in the same fashion as the tissue used to prepare dissociated neurons (Fig. 7D). The levels of both proteins increased significantly after postnatal days 6–8, although it appears that the expression of Kv3.1 develops somewhat faster than the expression of Kv3.2. Maximum levels of Kv3.1 protein were seen around p15 (Fig. 1B, lanes 1–4), whereas for Kv3.2, maximum levels were not achieved until p20 (Fig. 1B, lanes 5–7). Both proteins were not detectable earlier than p6–7. Similar results were obtained in three separate experiments with each antibody.

The bands observed with Kv3.1 or Kv3.2 antibodies are not seen when the immunoblots are reacted with antibodies preincubated with an excess of the Kv3.1 (Weiser et al. 1995) or Kv3.2 peptides (Fig. 1A, lane 2) used to prepare the antiserum. Also, no bands are detected in immunoblots treated with the preimmune sera derived from the rabbit used to raise the Kv3.2 antibodies (Fig. 1A, lane 3). Moreover, the Kv3.2 protein is absent in membranes derived from Kv3.2 −/− mice (Fig. 1A, lane 5) but is present in membranes from Kv3.1 −/− mice (Fig. 1A, lane 4), whereas the reverse is seen with Kv3.1 antibodies (Fig. 1A, lanes 6 and 7).

Immunohistochemistry was used to study the cellular and subcellular localization of Kv3.1 and Kv3.2 proteins in the rat GP (Fig. 2). Antibodies against Kv3.1 produced strong staining of the somatic membrane and the cytoplasm immediately beneath the membrane of neurons located throughout the GP (Fig. 2A). Fewer stained cells were seen in the most lateral border of the nucleus. The same section shown in Fig. 2A was stained with antibodies against parvalbumin (Fig. 2B). Most neurons positive for Kv3.1 also are stained for parvalbumin and vice versa. As in other neurons expressing Kv3.1, there is little staining of the dendrites of pallidal neurons. There was a faint staining of the pallidal neuropile (Fig. 2A). Higher magnification images from another experiment confirmed that Kv3.1b proteins and parvalbumin are expressed in the same pallidal neurons (Fig. 2, C–E), although parvalbumin staining tends to occupy most of the cytoplasm, whereas Kv3.1b staining is stronger in the proximity of the membrane. Antibodies against Kv3.2 also were expressed in parvalbumin-containing neurons (Fig. 2, F–H), although Kv3.2 antibodies produced a...
somewhat stronger staining of the neuropile than the antibodies against Kv3.1. Background staining is observed when the sections are treated with antibodies preincubated with the corresponding immunogenic peptide (data not shown). Kv3.1 and Kv3.2 antibodies also stain the GP in mouse (Fig. 3, A and D).

Kv3.1 or Kv3.2 staining is absent in tissue derived from the corresponding knockout mice (Fig. 3), confirming the specificity of both antibodies for immunohistochemistry.

The immunohistochemical data (Fig. 2) suggest that both Kv3.1 and Kv3.2 are co-expressed in the same pallidal neurons, the projecting, parvalbumin-containing neurons, which are the main neuronal population in the GP (Hontanilla et al. 1994; Kita 1994; Rajakumar et al. 1994a,b; Riedel et al. 1998). Kv3 proteins form heteromultimeric channels in vitro with other Kv3 proteins but not with proteins of other subfamilies (K. McCormack et al. 1990; Vega-Saenz de Miera et al. 1994; Weiser et al. 1994). This is similar to what has been observed with other Kv proteins (Coetzee et al. 1999). Because both Kv3.1 and Kv3.2 proteins are expressed in the same pallidal neurons, it is likely that both proteins are part of the same heteromeric channels. To test this hypothesis, we used immunoprecipitation assays from pallidal membranes solubilized without nondenaturing detergents (Sheng et al. 1993; Wang et al. 1993). As shown in Fig. 1C, antibodies against Kv3.1 or Kv3.2 proteins immunoprecipitate both Kv3.1 and Kv3.2 proteins. Thus immunoblots with Kv3.1 antibodies stain Kv3.1 proteins immunoprecipitated with Kv3.1 (Fig. 1C, lane 1) or Kv3.2 antibodies (Fig. 1C, lane 2); and immunoblots with Kv3.2 antibodies identify Kv3.2 proteins in Kv3.1 (Fig. 1C, lane 6) and Kv3.2 (Fig. 1C, lane 5) immunoprecipitates. No channel protein is detected when the immunoprecipitation is done with antibodies preincubated with the corresponding immunogenic peptide (Fig. 1C, lanes 3 and 4 and 7 and 8). The coimmunoprecipitation studies demonstrate that heteromeric complexes

**FIG. 1.** Expression of Kv3.1 and Kv3.2 proteins in the rat globus pallidus (GP). A: immunoblots of rat pallidal membrane extracts treated with Kv3.2 antibodies (lane 1), with Kv3.2 antibodies pretreated with Kv3.2 peptides (lane 2), with preimmune sera (lane 3), and immunoblots of pallidal membrane extracts obtained from Kv3.1 −/− mice (Ho et al. 1997) (lanes 4 and 6) or Kv3.2 −/− mice (Lau et al. 1998) (lanes 5 and 7) treated with Kv3.2 antibodies (lanes 4 and 5) or Kv3.1 antibodies (lanes 6 and 7). B: Kv3.1 proteins detected in immunoblots of rat brain membrane extracts obtained from postnatal day 8 rats (lane 1), postnatal day 11 (lane 2), postnatal day 15 (lane 3), and postnatal day 20 animals (lane 4); and Kv3.2 proteins detected in immunoblots of rat brain membrane extracts obtained from postnatal day 15 rats (lane 5), postnatal day 20 (lane 6), and adult (11 wk, lane 7). C: immunoblots of Kv3.1 proteins immunoprecipitated from GP membrane extracts from 2-wk-old rats with anti-Kv3.1b antibodies (lane 1), anti-Kv3.2 antibodies (lane 2), anti-Kv3.1b antibodies pretreated with Kv3.1b peptide (lane 3), and anti-Kv3.2 antibodies pretreated with Kv3.2 peptides (lane 4). Immunoblots of Kv3.2 proteins immunoprecipitated from the same membrane extracts as in lanes 1–4 with anti-Kv3.2 antibodies (lane 5), anti-Kv3.1b antibodies (lane 6), anti-Kv3.2 antibodies pretreated with Kv3.2 peptides (lane 7), and anti-Kv3.1b antibodies pretreated with Kv3.1b peptide (lane 8).
of both types of channel proteins exist in pallidal membranes. Taken together the data suggest that parvalbumin-containing projecting pallidal neurons have heteromeric channels containing both Kv3.1 and Kv3.2 subunits.

Kv3.1 and Kv3.2 currents in transfected CHO cells

To compare the properties of the currents recorded from CHO cells transfected with Kv3.1 or Kv3.2 cDNAs with putative Kv3 currents in freshly dissociated pallidal neurons, all cells were recorded with identical intra- and extracellular solutions. CHO cells transfected with cDNAs encoding Kv3.2a or Kv3.1b proteins had large delayed rectifier-type voltage-dependent K⁺ currents (Fig. 4) that resembled the currents observed in Xenopus oocytes injected with Kv3.2a or Kv3.1b cRNAs (reviewed in Vega-Saenz de Miera et al. 1994). Both Kv3.2a or Kv3.1b currents (see Fig. 4, A and C, respectively) start activating when the membrane is depolarized to potentials more positive than −10 mV and rise relatively fast (as compared with other voltage-gated K⁺ channels) (see Coetzee et al. 1999), with a similar time course, to a maximum level that is maintained for the duration of the pulses used in this experiment. A slow inactivation becomes evident with pulses of longer duration (data not shown). Untransfected CHO cells had negligible outward currents under the same pulse protocols (<100 pA for the largest depolarizations).

Preliminary studies with pallidal neurons showed that a large proportion of the outward current in these cells could be suppressed by holding the membrane at depolarized potentials. This could be a useful strategy to eliminate a fraction of the potassium currents if Kv3 currents are not affected by such treatment. We therefore tested the effect of varying the holding potential on Kv3.1 and Kv3.2 currents expressed in isolation. Figure 4 compares the currents produced by a series of voltage steps applied from a holding potential of −80 or −40 mV in the Kv3.1- and Kv3.2-transfected CHO cells. As seen in these representative examples, the currents generated by the depolarizing test pulses are very similar in magnitude and kinetics whether the cell is held at −80 or −40 mV. Changing the holding potential to −40 mV suppressed Kv3.1 and Kv3.2 currents by only 9.3 ± 0.1% (mean ± SE; n = 4) and 9.8 ± 0.6% (n = 4), respectively, with no effect on current kinetics.

Kv3.1 and Kv3.2 currents in CHO cells also have a similar voltage dependence, as observed in Xenopus oocytes (reviewed in Vega-Saenz de Miera et al. 1994). Figure 4, E and F, shows the normalized conductance (G/Gₘₐₓ) as a function of voltage, obtained from several cells expressing Kv3.2 or Kv3.1 currents, respectively. The data were fitted to Boltzmann functions...
of the form $G/G_{\text{max}} = 1/[1 - \exp(V_m - V_{1/2})/k]$. From these fits, we derived a midpoint of activation of $V_{1/2} = 12.1 \pm 1.26$ mV ($n = 6$) for Kv3.2 currents and $18.1 \pm 1.01$ mV ($n = 6$) for Kv3.1 and a steepness parameter $k$ of $8.4 \pm 0.25$ mV for Kv3.2 and $11.0 \pm 0.2$ mV for Kv3.1. Kv3 currents are unusual in requiring very depolarized potentials to start activating. However, the midpoints of the conductance-voltage relationships of Kv3 channels are not that different from those of other voltage-dependent K$^+$ currents, reflecting a steep voltage dependence. This distinguishes mammalian Kv3 currents from the currents expressed by the *Drosophila* Shaw protein, which also starts activating at high voltages but has a very weak voltage dependence, producing a midpoint of activation above $+70$ mV (Johnstone et al. 1997; Smith-Maxwell et al. 1998).

Another unusual feature of Kv3 currents is the fast rate of deactivation on repolarization, first described for Kv3.1 currents expressed in NIH-3T3 and L929 cells by Grissmer et al. (1994). These authors found that Kv3.1 currents deactivated ~10 times faster than several other cloned mammalian voltage-gated K$^+$ channels when compared at the same membrane potentials. Since then, many new voltage-gated channels have been identified in mammals; however, only one of them, Kv1.7, a nonneuronal member of the Kv1 family, deactivates fast (closing rates are ~3 times the deactivation rates of Kv3.1 channels) (Coetzee et al. 1999). Kv3.1 currents also deactivate extremely fast in CHO cells under our recording conditions (Fig. 5). Kv3.2 currents deactivate at rates somewhat slower than Kv3.1 but still significantly faster than K$^+$ currents from channels of other subfamilies (Fig. 5).

At present there are no specific blockers for Kv3.1 and Kv3.2 channels. However, in *Xenopus* oocytes all Kv3 currents are blocked by low concentrations of TEA or 4-aminopyridine (4-AP) (reviewed in Vega-Saenz de Miera et al. 1994). Kv3.1 and Kv3.2 currents in CHO cells, under our recording conditions, were also very sensitive to these channel blockers. TEA dose-response curves for Kv3.1 and Kv3.2 currents are shown in Fig. 6. From these curves, we derived IC$_{50}$s of 0.28 mM ($n = 4$) for Kv3.2 and 0.38 mM ($n = 4$) for Kv3.1 currents. We also have confirmed that as in other experimental systems (Grissmer et al. 1994; Vega-Saenz de Miera et al. 1994), Kv3.1 and Kv3.2 currents in CHO cells are not affected by dendrotoxin or charybdotoxin at concentrations of $\leq 1 \mu$M (data not

**FIG. 3.** Immunolocalization of Kv3.1 and Kv3.2 proteins in mouse GP. Sections through the GP obtained from wild-type (A and D), Kv3.2 knockout (B and E), and Kv3.1 knockout (C and F) mice were immunostained with Kv3.1b (A–C) and Kv3.2 (D–F) antibodies. Insets: dark field images of the area shown in the main panels. Scale: 150 μM.
shown). The effects of 4-AP on these currents were not examined in detail. Preliminary experiments discarded 4-AP as a useful tool to distinguish native currents carried by channels of the Kv3 subfamily in pallidal neurons because the drug blocks, also at low concentrations, a component of the outward current that also is blocked by dendrotoxin.

The currents obtained when both Kv3.1 and Kv3.2 proteins are co-expressed in Xenopus oocytes (Vega-Saenz de Miera et al. 1994; Weiser et al. 1994) or CHO cells (data not shown) are similar to those obtained in cells expressing only one of the two subunits. This is not surprising given the similarities between Kv3.1 and Kv3.2 currents and is consistent with the observation that heteromultimeric Kv channels have properties intermediate between those of the corresponding homomultimeric channels (Christie et al. 1990; Isacoff et al. 1990; K. McCormack et al. 1990; Ruppersberg et al. 1990; Weiser et al. 1994). The properties of Kv3.1 and Kv3.2 currents in CHO cells are summarized in Table 1.

**K^+ currents in acutely dissociated neurons from the rat GP**

As a product of the enzymatic dissociation of the GP, we typically found two morphological subtypes of neurons (Fig. 7), similar to those observed in previous studies of dissociated pallidal neurons (Stefani et al. 1992, 1995; Surmeier et al. 1994). We also found astrocytes and a population of small cells (data not shown). All our records were obtained from the two

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**Figures**

**Fig. 4.** Kv3.1 and Kv3.2 currents in transfected Chinese hamster ovary (CHO) cells. A and C: families of K^+ currents recorded from a holding potential of -80 mV in a Kv3.2 (A)- and a Kv3.1-transfected (C) cell. B and D: K^+ currents recorded from the same cells from a holding potential of -40 mV. In all cases, depolarizing pulses were applied from -30 to +40 mV in 10-mV increments. E and F: plots of normalized conductance (G/G_{max}) as a function of voltage obtained from current records from several Kv3.2 (E)- and Kv3.1-transfected (F) cells, (n = 4). Conductance (G = IV - V_0) values were computed from current data using a potassium equilibrium potential (V_K) of -80 mV, which was the average reversal potential in these cells and is close to the expected K^+ equilibrium potential of -90 mV.

**Fig. 5.** Deactivation kinetics of Kv3.1 and Kv3.2 channels in transfected CHO cells. A and B: records of tail currents obtained by repolarizing the cell to a series of membrane potentials from -110 to -30 mV in 10-mV increments after a 220-ms depolarization to +40 mV. C: time constants of deactivation as a function of voltage from several Kv3.1 (○) or Kv3.2 (●) transfected CHO cells obtained from single exponential fits to the tail currents.

**Fig. 6.** TEA dose-response curve of Kv3.1 and Kv3.2 currents in CHO cells transfected with Kv3.1 and Kv3.2 transcripts. Plotted is the fraction of the currents remaining (I_{TEA}/I_c) at +40 mV as a function of the concentration of TEA in the bath. IC_{50} obtained by fitting the experimental data to the Sigmoid Logistic Function [1/(1 + exp(-x))] for Kv3.1 and Kv3.2 currents was 0.38 (- - -) and 0.28 mM (—), respectively (n = 4).
main types shown in Fig. 7. Most neurons (type A, Fig. 7, A
and B) had bipolar-fusiform or triangular shape and were
similar in size and morphological appearance to pallidal
GABAergic projecting neurons (Park et al. 1982; Surmeier et
al. 1994). The second type, much less frequently encountered
(type B, Fig. 7 C), consisted of multipolar cells that were
distinguished mainly by having somas significantly larger than
those of type A cells. These cells may correspond to the large
cholinergic neurons that lie along the medial border of the GP
(Surmeier et al. 1994), but no evidence of this was obtained in
this study.

It was also possible to group the dissociated GP neurons
according to the amount and kinetics of the transient currents
observed when the cells were depolarized from a holding
potential of −80 mV. Figure 8 shows records from three
different neurons at two holding potentials. The cell shown in
A has very little low-voltage activating A-type current, whereas
this current is large in the cells shown in C and E. In addition,

<table>
<thead>
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<th>Activation</th>
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<tr>
<td>$V_{on}$, mV</td>
<td>$V_{1/2}$, mV</td>
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<tr>
<td>Kv3.1</td>
<td>−20 to −10</td>
</tr>
<tr>
<td>Kv3.2</td>
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<tr>
<td>Kv3.4</td>
<td>−20 to −10</td>
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<tr>
<td>$I_{GP-TEA}$ (A)</td>
<td>−20 to −10</td>
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<td>$I_{GP-TEA}$ (B)</td>
<td>−20 to −10</td>
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All measurements are reported at room temperature. References: 1, this work; 2, Rudy et al. (1999). $V_{on}$, minimum voltage at which there is significant activation of the current; $V_{1/2}$, membrane potential at which the conductance is half maximal; $k$, slope of normalized $G-V$ curve; $t_{on}$, time for the current to rise from 10 to 90% of its final value for noninactivating currents and time to peak for inactivating currents, both at $+40$ mV; $t_{off}$, time constant of deactivation at $−40$ mV; Inactivation $t$, time constant of inactivation at $+40$ mV; NA, not applicable.
the cell shown in E has a substantial amount of a slowly inactivating A-type current similar to the $I_{5A}$ described in pallidal neurons by Stefani et al. (1995). The records in Fig. 8 also show that a holding potential of $-40\text{ mV}$ inactivates not only the transient currents but also a substantial portion of the sustained current. We did not observe a clear correlation between the phenotype of the currents at a holding potential of $-80\text{ mV}$ and the cell’s morphology.

The experiments described next, aimed at searching for Kv3.1- and Kv3.2-like currents in dissociated pallidal neurons, asked whether in these cells there is a component of the potassium current whose kinetics, voltage dependence, and pharmacology resembles those of the currents recorded under the same experimental conditions in CHO cells expressing Kv3.1 or Kv3.2 proteins. The results will be presented in two parts each comprising results obtained from one of two distinct subpopulations of GP neurons, distinguishable by their morphology and electrophysiological characteristics: type A and type B cells. As indicated in METHODS, identical intra- and extracellular solutions to those used to record currents in CHO cells were used in pallidal neuron recordings.

**Type A GP neurons**

This group was composed of cells with fusiform/bipolar and triangular/multipolar somata (such as those shown in Fig. 7, A and B). Typical records obtained from a cell with these morphological characteristics are shown in Fig. 9. Figure 9A shows a family of currents obtained during depolarizing pulses from a holding potential of $-40\text{ mV}$, and Fig. 9B illustrates the currents obtained in the same cell after the application of 1 mM TEA to the external solution. The studies with Kv3.1 and Kv3.2 currents in heterologous expression systems indicate that such a concentration of TEA should eliminate an important fraction ($\sim 80\%$) of the current generated by Kv3 channels. A holding potential of $-40\text{ mV}$ was used to reduce the components of the total $K^+$ current and facilitate the isolation of Kv3-like currents, which are not significantly affected by holding the membrane at this potential (see Fig. 4).

The currents recorded under control conditions ($I_{5PC}$) begin to activate at about $-20\text{ mV}$ and have characteristics of delayed rectifying currents. The TEA-resistant current component ($I_{5P, R}$) also possesses characteristics of a sustained current of the delayed rectifier-type, although its activation kinetics is slower than that of $I_{5PC}$. Figure 9C shows the current component sensitive to TEA ($I_{5P, TEA}$), obtained by digital subtraction of the current traces shown in B from the corresponding traces in A. In type A cells, $I_{5P, TEA}$ is also a sustained current of the delayed rectifier type that represents in this cell $\sim 50\%$ of the total current from a holding potential ($V_H$) of $-40\text{ mV}$. Typically the activation kinetics of $I_{5P, TEA}$ was faster.

**FIG. 8.** Outward currents from acutely dissociated pallidal neurons. A, C, and E: currents in 3 representative GP neurons expressing different proportions of low-voltage-activating transient, A-type currents. Depolarizing pulses from $-30\text{ to } +40\text{ mV}$ were applied from a holding potential of $-80\text{ mV}$ in 10-mV increments. B, D, and F: records obtained from the same cells (A, C, and E, respectively) when depolarized to the same voltages from a holding potential of $-40\text{ mV}$. Note that in addition to the transient current, a fraction of the sustained current is also inactivated by holding the membrane at $-40\text{ mV}$.

**FIG. 9.** Voltage-dependent $K^+$ currents obtained from a typical type A pallidal neuron. A–C: currents obtained from a GP neuron held at $-40\text{ mV}$ during depolarizing pulses from $-30\text{ to } +40\text{ mV}$ in 10-mV increments, before (A) and after (B) the application of 1 mM TEA. Traces in C show the TEA-sensitive component obtained by digital subtraction of the traces in B from those in A. Inset: transition from $+40\text{ to } -40\text{ mV}$ with the tail current sampled at 30 $\mu$A/point. D: plot of relative conductance ($G/G_{\text{max}}$) of the TEA-sensitive current from several type-A pallidal neurons ($n = 6$). $\cdots$, fit to a Boltzmann function.

of the data to a Boltzmann equation with a $V_{R}$ and 2.72 here were reproducibly observed.

40 mV, with a mean of 54%), the kinetic features illustrated $I_{GP}$, TEA. This component of the current starts activating between 20 and 40 mV. The continuous line represents the fit of the data to a Boltzmann equation with a $V_{1/2}$ and $k$ of 16.94 mV and 10.59 mV$^{-1}$, respectively ($n = 6$). These data show that the component of the outward current from type A cells that is blocked by 1 mM TEA resembles in voltage dependence as well as in activation and deactivation kinetics the currents carried by Kv3.1 and Kv3.2 channels.

To explore this conclusion further, we compared more closely the kinetics of activation and deactivation of $I_{GP}$, TEA with the kinetics of Kv3.1 and Kv3.2 currents in CHO cells. Figure 10 shows current records obtained with identical pulse protocols in CHO cells transfected with Kv3.1 and Kv3.2 cDNAs (A and B, respectively) and $I_{GP}$, TEA in a type A GP neuron (C). In D–G, we have scaled and superimposed the first 150 ms of the current traces in A–C, at four different voltages. It is clear from this comparison that the TEA-sensitive component of the $K^+$ current in type A GP neurons has activation kinetics that closely resembles the activation kinetics of Kv3.1 and Kv3.2 currents in CHO cells.

$I_{GP}$, TEA also resembles Kv3.1 and Kv3.2 currents in deactivation kinetics. Figure 11 examines the kinetics of the tail current recorded under control conditions in a type A GP neuron. The tail current that results from repolarizing the membrane potential from +40 to −40 mV (Fig. 11A) was best fitted by the sum of two exponentials, suggesting that the deactivation process of this current includes two components with fast and slow time constants ($\tau_1 = 1.78$ ms and $\tau_2 = 21.8$ ms, respectively). In contrast, the tail current of the TEA-insensitive component (Fig. 11B) and the TEA-sensitive component (Fig. 11C) can be fitted to a single exponential with time constants of 28.8 and 2.27 ms, respectively. These two values resemble the time constants of the two components seen in the total current. The time constant of deactivation of the TEA-sensitive current from a number of experiments ($n = 4$) is plotted against the repolarizing membrane potential in Fig. 11D. $I_{GP}$, TEA deactivates very fast, at rates similar (and roughly intermediary) to those of Kv3.1 and Kv3.2 currents in CHO cells. A summary of the comparison of the properties of $I_{GP}$, TEA in type A GP neurons with the properties of Kv3.1 and Kv3.2 currents is shown in Table 1.

**Type B GP neurons**

The characteristics of the currents obtained from GP neurons described earlier were typical of the majority of the cells studied. However, we found that in a small group of cells, 1 mM TEA blocked a fast inactivating current. Many of these cells (type B) had a distinct morphology characterized by large multipolar somas with about four to five dendrites (such as the cell illustrated in Fig. 7C). Typical records from one of these cells are shown in Fig. 12. Depolarizing pulses from a holding potential of −40 mV produced currents of the delayed rectifier type similar to those seen in type A cells (Fig. 12A), although the kinetics of activation of these currents was faster than that of the currents recorded in type A GP neurons (rise time between 10 and 90% at +40 mV of 14.0 ± 1.2 ms, $n = 3$ for type B cells and 25 ± 5 ms, $n = 6$ for type A cells). The predominant time constant of deactivation of the currents in these cells is slow ($\tau = 25.86 ± 1.17$ ms after a pulse to +40 mV; $n = 4$). Application of 1 mM TEA inhibited 10–15% of the current (Fig. 12B). The TEA-resistant current had slower activation kinetics than the control (10–90% rise time: 22.4 ± 2.5 ms, $n = 3$). The TEA-sensitive component ($I_{GP}$, TEA), obtained by digital subtraction (Fig. 12C), was composed predominantly of a current that activates very rapidly starting at voltages more positive than −10 mV (time to peak at +40 mV of 3.6 ± 0.7 ms, $n = 4$) and presented marked and fast inactivation that could be fitted to a single exponential function. Figure 12E plots the time constant of inactivation of the transient current versus the potential during the pulse for this cell. Clearly the rate of inactivation is dependent on the voltage, becoming faster as the depolarization increases.

The TEA-sensitive component in these cells also includes a
sustained component. The ratio of sustained to transient component varied from cell to cell. It is small in the cell illustrated in Fig. 12 but represented up to $\sim 40\%$ of the current in some type B neurons. The TEA-sensitive current in these cells started to activate at very depolarized potentials ($\sim -10$ mV) as was the case of the $I_{\text{GP, TEA}}$ in type A cells and Kv3.1 and Kv3.2 in CHO cells. The normalized peak conductance ($G/G_{\text{max}}$) as a function of voltage for the fast transient component of several type B neurons is shown in Fig. 12D. The experimental data were fit to a Boltzmann function with a $V_{1/2}$ of 15.47 mV $\pm$ 0.5 and 14.34 $\pm$ 1.0 mV$^{-1}$, respectively ($n = 4$).

The transient currents recorded from type B neurons of the GP resemble the currents expressed by Kv3.4 proteins (Table 1). These are proteins of the Kv3 subfamily that express fast activating and inactivating currents resembling Kv3.1 and Kv3.2 in voltage dependence and pharmacology (Rudy et al. 1991b, 1999; Schröter et al. 1991; Vega-Saenz de Miera et al. 1994). This result was surprising at first because in situ hybridization studies reported that Kv3.4 was only expressed at very low levels in a scattered pattern in the GP (Weiser et al. 1991b, 1999; Schroter et al. 1991; Vega-Saenz de Miera et al. 1994). This result was surprising at first because in situ hybridization studies reported that Kv3.4 was only expressed at very low levels in a scattered pattern in the GP (Weiser et al. 1994).

Because there are no antibodies available against Kv3.4 proteins, we used single-cell RT-PCR to investigate whether type B cells in the GP contain Kv3.4 transcripts. Single-stranded cDNA synthesized from the mRNA obtained from the cytoplasm of several freshly dissociated type A and type B GP neurons was used as template for two rounds of PCR amplification. In the first round, we used a pair of primers designed to amplify the products of all Kv3 genes. This was followed by amplification using internal primers designed to amplify specifically the products of Kv3.1, Kv3.2, and Kv3.4 genes (see details in Methods). The amplified products obtained when cDNA for each Kv3 gene was used as template are shown in Fig. 13A. Each product has a different molecular weight facilitating the identification of the transcript. Figure 13, B–D, shows the amplified bands obtained with several type A and type B cells. These studies showed that Kv3.2 transcripts are found in most type A (81%; $n = 17$) and some type B cells (64%; $n = 8$), Kv3.1 is found mainly in type A cells (85%), and Kv3.4 only in type B cells.

**Discussion**

**Key properties of Kv3.1 and Kv3.2 currents expressed in mammalian cell lines**

Both Kv3.1 and Kv3.2 cDNAs result in the expression of similar currents in CHO cells that resemble the currents expressed in *Xenopus* oocytes (reviewed in Vega-Saenz de Miera et al. 1994). These currents have several properties (see Table 1) that distinguish them from those of other delayed rectifier $K^+$ channels known (Coetzee et al. 1999). One property is an activation voltage range that is more positive than that of other heterologously expressed voltage-gated $K^+$ channels, besides those of the Kv3 subfamily. The channels with the nearest activation voltage (Kv2.1 and Kv2.2) start activating at 10–20 mV more negative potentials. Although Kv3 channel opening starts at high potentials (more positive than $-10$ mV), the probability of channel opening increases steeply with voltage, and $>80\%$ of the channels are opened at $\pm 30$ mV. The currents deactivate very fast, at rates that are $\geq 7$–10 times faster (when compared at the same voltage) than those of other known mammalian voltage-gated $K^+$ channels, except for Kv1.7 a nonneuronal member of the Kv1 subfamily (deactivation rates are 2–3 times slower than Kv3.1 or Kv3.2) (Coetzee et al. 1999; Grissmer et al. 1994). The rate of rise of the currents is relatively fast; faster than many other voltage-gated $K^+$ channels (e.g., Kv2.x; Kv1.2) but slower than that of other voltage-gated $K^+$ channels such as several Kv1 channels like Kv1.1 and Kv1.5 (Coetzee et al. 1999). In contrast to other...
delayed rectifiers, Kv3.1 and Kv3.2 currents are not significantly inactivated by depolarizing prepulses (see Fig. 4) and do not show cumulative inactivation (Grissmer et al. 1994; unpublished observations). These distinctive properties are likely to endow neurons with special electrophysiological properties (see following text).

Kv3.1–Kv3.2 channels in pallidal neurons

The studies described here provide strong evidence that in pallidal neurons, Kv3.1–Kv3.2 proteins in heteromultimeric complexes form K⁺ channels mediating a high-voltage-activating component of the delayed rectifier current that closely resembles the currents expressed by these proteins in heterologous expression systems. The immunohistochemical studies demonstrated that there is expression of both Kv3.1 and Kv3.2 proteins in the GP and that both proteins are colocalized in the same cell type, the triangular and bipolar parvalbumin-containing (PV⁺) neurons, which constitute the major neuronal population in the rodent GP (see following text). Moreover, anti-

bodies against Kv3.1 or Kv3.2 proteins coprecipitate both subunits, strongly suggesting that the proteins exist in heteromeric complexes. It remains to be shown, however, that the functional channels are heteromultimeric. This will be a difficult task unless major, not yet detected, differences between homomultimeric and heteromultimeric channels are discovered. We also have demonstrated that in acutely dissociated pallidal neurons having triangular or bipolar shapes (type A), shown to express Kv3.1 and Kv3.2 transcripts by single cell RT-PCR, a component of the current not inactivated when the cell is held at −40 mV and blocked by 1 mM TEA, has properties that closely resemble those of Kv3.1 and Kv3.2 channels in CHO cells (see Table 1).

The concentration of TEA used to isolate the current in pallidal neurons (1 mM) blocks >80% of Kv3.1 and Kv3.2 currents in heterologous expression systems. This concentration of TEA produces significant inhibition of only a few other known K⁺ channels. These include the large conductance Ca²⁺-activated K⁺ channels containing proteins of the slo family (K⁺d 80–330 µM) and Kv1.1 channels (K⁺d ≈ 0.5 mM) (Coetzee et al. 1999). These channel types are unlikely to contribute to the current isolated from the pallidal neurons in these studies. In our experiments, the activation of Ca²⁺-activated K⁺ channels was suppressed by using Cd²⁺ in the extracellular solution and by using BAPTA in the intracellular solution. In the pallidal area Kv1.1 proteins apparently are expressed somatically only in the ventral pallidum (Wang et al. 1994). Moreover, Kv1.1 channels also are blocked by dendro-
toxin ($K_d \sim 10–20 \text{nM}$) (Coetzee et al. 1999). In pallidal neurons, this toxin blocked a small inactivating current component ($\sim10–15\%$ of total outward current) resembling a $D$ current (Wu and Barish 1992), which was suppressed significantly by holding the cell at $-40 \text{mV}$. Dendrotoxin also blocks other channels of the Kv1 subfamily that are not highly sensitive to TEA (Coetzee et al. 1999) and may mediate the $D$-like current in pallidal neurons.

This study confirms and extends the observations of Du et al. (1996), who showed that hippocampal interneurons expressing Kv3.1 proteins had a current that showed resemblance to Kv3.1 currents in heterologus expression systems. Also the $l$-type current in $T$ lymphocytes was shown to be very similar to Kv3.1 currents in Xenopus oocytes when recordings in the two preparations were obtained with the same solutions (Grissmer et al. 1992). Kv3.1-like currents also have been described in auditory neurons (Wang et al. 1998).

It appears from these results that the properties of native channels containing Kv3.1 and Kv3.2 proteins are not significantly affected by factors such as associated subunits or post-translational modifications as might be the case for other cloned subunits, at least in the cells studied until now. It is therefore valid to ask why were Kv3.1- and Kv3.2-like currents not described in the many neuronal populations expressing these proteins prior to the cloning studies? Most likely the Kv3 channel-mediated current was buried in other components of the $\text{K}^+$ current. This emphasizes the suggestion made in the introduction that experimental conditions and methods to isolate individual components of the $\text{K}^+$ current tailored to search for specific current components are required before it is possible to determine whether native currents resemble those in heterologous expression systems. In the specific case of pallidal neurons, the Kv3.1–Kv3.2 current isolated in our study was most likely buried in the delayed rectifier component ($I_k$) isolated with 10 mM TEA by Steffani et al. (1992, 1995).

**Physiological significance**

The GP in rodents consist of a main neuronal mass homologous to the external segment of the GP in primates and often is referred to simply as the GP. A smaller nuclear group situated at a certain distance and embedded among the fiber bundles of the internal capsule usually called entopeduncular nucleus (not included in most of our dissociations) is thought to correspond to the internal segment of the GP in primates (Heimer et al. 1995). The GP proper contains several neuronal populations, the majority of which are medium to large neurons (20–40 $\mu\text{m}$ in length along their longer axis) with a fusiform (bipolar) or triangular shape. There are also small neurons (12–16 $\mu\text{m}$ in length), which may correspond to the small dissociated cells that were excluded from the present study, and a few scattered large multipolar cells located mainly in the medial border in rat, which may correspond to cholinergic neurons (Difiglia et al. 1982; Fox et al. 1974; Heimer et al. 1995; Iawhori and Mizuno 1981; Kita 1992, 1994; Kita and Kitai 1994; Millhouse 1986; Morizumi and Hattori 1992; Park et al. 1982; Tkatch et al. 1998). Most pallidal neurons, including the triangular and fusiform cells, are GABAergic. Many of these cells stain for parvalbumin, which labels about two-thirds of projecting pallidal neurons (Hontanilla et al. 1994; Kita 1994; Rajakumar et al. 1994a,b; Riedel et al. 1998). According to our immunohistochemical studies, Kv3.1 and Kv3.2 are present in the PV+ neurons (Fig. 2). Furthermore the morphology of the majority of the dissociated cells identified as type A in this study corresponds to the morphology of the PV+ cells in situ. Together with the results from the single cell RT-PCR, this suggests that the neurons expressing Kv3.1- and Kv3.2-like currents correspond to the projecting GABAergic, PV+, neurons. The ventral pallidum, which may have contaminated some of our dissociations, contains similar GABAergic and cholinergic neurons (Heimer et al. 1995).

The Kv3.1–Kv3.2 component of the delayed rectifier current in pallidal neurons represents a significant component of the total $\text{K}^+$ current ($\sim50\%$ of the current when the cell was depolarized from a holding potential of $-40 \text{mV}$) and is therefore likely to contribute to the firing properties of these cells. Because Kv3.1–Kv3.2 channels are not opened until the membrane potential is depolarized beyond $-10 \text{mV}$, it has been suggested that these channels are activated late in the action potential and, when present in sufficient amounts, influence action potential repolarization. Thus Kv3.1–Kv3.2 channels would help dictate action potential duration without competing much with the $\text{Na}^+$ current in generating the rising phase of an action potential and influencing firing threshold, in contrast to $\text{K}^+$ channels that are activated earlier during a spike (Lenz et al. 1994; Moreno et al. 1995; Rudy et al. 1999; Sekirnjack et al. 1997; Vega-Saenz de Miera et al. 1994; Wang et al. 1998; Weiser et al. 1995). These arguments, supported by computer modeling (A. Erisir, D. Lau, B. Rudy, and C. S. Leonard, unpublished data), suggest that high-voltage-activating $\text{K}^+$ channels would modulate firing properties more selectively than $\text{K}^+$ channels that activate at negative voltages.

Many of the neuronal populations expressing Kv3.1 and Kv3.2 channels are present in the PV+ neurons (Erisir et al. 1998; Martina et al. 1998; Massengill et al. 1997; Perney et al. 1992; Rudy et al. 1992; Sekirnjack et al. 1997; Wang et al. 1998; Weiser et al. 1994, 1995), such as fast-spiking interneurons in the cortex and the hippocampus. Kv3.1–Kv3.2 channels may help maintain high firing rates by keeping action potentials short, reducing $\text{Na}^+$ channel inactivation, and facilitating fast recovery of $\text{Na}^+$ channels from inactivation by hyperpolarizing the cell following the spike. Their fast deactivation on repolarization will quickly eliminate the increase in $\text{K}^+$ conductance, and therefore these channels will contribute little to increasing the refractory period. $\text{K}^+$ channels that are open at lower potentials or do not deactivate as fast could repolarize the spike but at the same time also limit firing frequency by contributing to the refractory period. In fact pharmacological treatments that suppress Kv3 currents impair fast spiking in neocortical neurons, but blockade of other $\text{K}^+$ currents actually increases firing frequency (Erisir et al. 1998).

Although there has not been a study combining immunohistochemistry and electrophysiology of pallidal neurons, it is likely that the PV+ pallidal neurons correspond to the repetitive firing group of cells recorded in an in vivo intracellular study in rats by Kita and Kitai (1991) because they both represent the largest cell population and they have similar morphologies (Kita 1994; Kita and Kitai 1991, 1994). These cells, which probably correspond to the type II neurons observed in intracellular recordings from guinea pig slices (Nambu and Llinas 1994, 1997), show fast repetitive firing ($\leq200 \text{Hz}$) with weak accommodation when depolarized.
It is possible that the role of Kv3.1–Kv3.2 channels in PV+ pallidal neurons is similar to their proposed role in cortical interneurons to facilitate sustained high firing rates. The firing frequency of the repetitive firing cells in the GP is not as high or sustained as that of fast-spiking cortical neurons. Analysis of the currents in the latter cells shows that they have a significantly higher proportion of Kv3-like currents than pallidal neurons and lack subthreshold-activating A-type currents (A. Erisir, D. Lau, B. Rudy, C. S. Leonard, unpublished data). These differences in channel composition may explain the differences in spike frequency adaptation of the two cell types. The resting potential of pallidal neurons will change the contribution of Kv3 currents to the total current, it is therefore also possible that the firing frequency or adaptation of PV+ pallidal neurons will depend on the resting potential.

Expression and role of channels containing Kv3.4 proteins in a small subpopulation of pallidal neurons

The most surprising result of this study was the finding of fast, transient, high-voltage-activating, TEA-sensitive currents in a small subpopulation of the dissociated cells. The currents resemble those expressed by Kv3.4 subunits in heterologous expression systems (Table 1) (see also Vega-Saenz de Miera et al. 1994). The hypothesis that these transient currents in GP neurons are mediated by channels containing Kv3.4 proteins is supported by the findings from single-cell RT-PCR, which showed that Kv3.4 transcripts are present only in the cells having the high-voltage activating transient current. We did not expect to find Kv3.4 channels in pallidal neurons because Weiser et al. (1994) reported very weak expression of Kv3.4 transcripts in the GP (in situ hybridization signals for these mRNAs were reported as “weakly above background”). However, Weiser et al. (1994) cautioned in their paper that low expression of Kv3.4 transcripts in neurons expressing other Kv3 proteins could be of physiological significance because Kv3.4 subunits can form heteromultimeric channels with other Kv3 proteins resulting in a large amplification of the transient current. Type B pallidal neurons might be an example of the situation predicted in this paper.

Nevertheless relative to the other outward currents, the Kv3.4-like current in type B pallidal neurons contributes such a small proportion of the total current that one could be tempted to suggest it could play little role in the excitability of these cells. However, although the Kv3.4-like current contributes little current, it produces a large effect on the rise time of the total current. The currents remaining after 1 mM TEA are not very different in magnitude from the original current; however, they are clearly much slower (see Fig. 12). This suggests a new role for Kv3.4-like currents: to accelerate the rate of rise of the repolarizing currents. Further studies of type B pallidal neurons or other cells expressing Kv3.4 channels will allow future tests of this hypothesis.

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