A VOLTAGE-GATED POTASSIUM CHANNEL, Kv3.1b, IS EXPRESSED BY A SUBPOPULATION OF LARGE PYRAMIDAL NEURONS IN LAYER 5 OF THE MACAQUE MONKEY CORTEX

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Abstract—In the cerebral cortex, the voltage-gated potassium channel, Kv3.1b, a splicing variant of Kv3.1, has been associated with fast-firing interneurons. Here, we report strong expression of Kv3.1b-protein and mRNA in both Betz and Meynert pyramidal cells of the monkey, as shown by immunohistochemistry and in situ hybridization. Strong expression also occurs in large pyramidal neurons in layer 5 of several cortical areas. In addition, most of these Betz and layer 5 pyramids, and about 10% of the labeled Meynert cells weakly co-expressed the calcium binding protein parvalbumin. Electron microscopy shows that the expression of Kv3.1b is localized to the somal and proximal dendritic cytoplasmic membrane, as expected for a channel protein. These results suggest that some large pyramidal neurons may constitute a functional subpopulation, with a distinctive distribution of voltage-gated potassium channels capable of influencing their repetitive firing properties. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: Betz cell, fast firing neurons, fast-rhythmic burst firing neurons, infragranular layers, Meynert cell, parvalbumin.

Cortical pyramidal cells are not a homogeneous class, but rather can be distinguished on the basis of firing properties, connectivity, and morphology (reviewed in DeFelipe and Farinas, 1992; Douglas et al., 2004). In the primate, two types of giant pyramidal cells, Betz cells of primary motor cortex and Meynert cells of primary visual cortex, have attracted particular attention (reviewed in Sherwood et al., 2003). In addition to their large somatic volume, these cells have pronounced basal dendritic arborizations and thick myelinated axons, and exhibit intense immunostaining for nonphosphorylated neurofilament protein (Chan-Palay et al., 1974; Scheibel and Scheibel, 1978; Meyer, 1987; Hof and Morrison, 1990; Hof et al., 2000; Rivara et al., 2003). They stain strongly for the metabolic enzyme cytochrome oxidase (Carroll and Wong-Riley, 1984; Matelli et al., 1985; Payne and Peters, 1989), an indicator of high metabolic activity, and are selectively vulnerable to several neurological diseases (Hof and Morrison, 1990; Pringle et al., 1992).

Recently, Betz cells in primates have been shown to be positive for markers more usually associated with fast spiking GABAergic interneurons in rodent neocortex. That is, Betz cells express the calcium-binding protein, parvalbumin (PV; Preuss and Kaas, 1996), and are surrounded by Wisteria floribunda agglutinin-labeled perineuronal nets (PN; Bertolotto et al., 1991; Hausen et al., 1996; Hartig et al., 1999). In the cortex of rats and mice, these features are found only in GABAergic interneurons, especially in fast spiking interneurons (Kawaguchi and Kubota, 1997; Hartig et al., 1999). For Meynert cells in primate visual cortex, there is some suggestion that these also are surrounded by PNs, as visualized by Cat-301 antibody (Hendry et al., 1988; DeYoe et al., 1990). There have so far been no reports in monkey that these cells are positive for PV (but see Leuba and Saini, 1997, concerning human visual cortex). In mouse and rat, PV-ir interneurons surrounded by PNs express the mRNA and protein of the voltage-gated potassium channel, Kv3.1b, one of the two splicing variants of Kv3.1 (Weiser et al., 1995; Sekirnjak et al., 1997). In these neurons, Kv3.1b can repolarize action potentials quickly without affecting the threshold for action potential generation, by means of its high activation voltage and fast deactivation rates (reviewed in Rudy et al., 1999; Rudy and McBain, 2001). A recent light microscopic study on cortical neurons immunopositive for Kv3.1b has reported that some large layer 5 pyramidal neurons in monkey primary motor cortex (possibly Betz, but not identified as such) express Kv3.1b protein (Hartig et al., 1999).

Here, we report strong expression of Kv3.1b-protein and mRNA in both Betz and Meynert cells, as well as in large pyramidal neurons in layer 5 of several cortical areas of the monkey. This is demonstrated by light and electron immunohistochemical methods and by in situ hybridization. The expression of PV was also investigated.

EXPERIMENTAL PROCEDURES

Perfusion and tissue preparation

Six adult macaque monkeys (Macaca mulatta and Macaca fascata) were used in this study. All experimental protocols were approved by the Experimental Animal Committee of the RIKEN.
Institute, and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23; revised 1996). Every effort was made to minimize the number of animals used and any pain or discomfort. Animals were anesthetized with ketamine (11 mg/kg, i.m.) and Nembutal (overdose, 75 mg/kg, i.p.), and were perfused transcardially, in sequence, with saline containing 0.5% sodium nitrite, 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3) for 30 min, and chilled 0.1 M PB with 10%, 20%, and 30% sucrose. For one animal used for electron microscopic (EM) analysis, we added 0.1% glutaraldehyde to the fixative solution, and omitted the postfixation wash with 30% sucrose in 0.1 M PB. The brains were removed from the skull, and were immersed into 30% sucrose in 0.1 M PB for light microscopy (LM), and into 0.1 M PB for EM. After the brains sank, the blocks for LM were cut serially in the coronal plane by frozen microtomy (50 μm thickness). Blocks for in situ hybridization and for EM were trimmed from motor cortex and V1. Sections for in situ hybridization (35 μm thickness) were cut by frozen microtomy, and those for EM, by vibratome (VT 1000S; Leica Microsystems, Nussloch, Germany).

### Immunoperoxidase staining for Kv3.1b or PV for LM

Sections were incubated for 1 h in 0.1 M PB saline (PBS; pH 7.3) containing 0.5% Triton X-100 and 5% normal goat serum (PBS-TG) at room temperature, and then 40–48 h at 4 °C with PBS-TG containing rabbit polyclonal anti-Kv3.1b antibody (Chemicon; 1:500) and mouse monoclonal anti-PV antibody (Swant, Bellinzona, Switzerland; 1:50,000). After rinsing, the sections were placed in PBS-TG containing biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA, USA; 1:200) or mouse monoclonal anti-PV antibody (Swant, Bellinzona, Switzerland; 1:50,000). After rinsing, the sections were treated in 2 l RNase buffer (PBS; pH 7.3) for 30 min, and chilled 0.1 M PB with 10%, 20%, and 30% sucrose. For one animal used for electron microscopic (EM) analysis, we added 0.1% glutaraldehyde to the fixative solution, and omitted the postfixation wash with 30% sucrose in 0.1 M PB. The brains were removed from the skull, and were immersed into 30% sucrose in 0.1 M PB for light microscopy (LM), and into 0.1 M PB for EM. After the brains sank, the blocks for LM were cut serially in the coronal plane by frozen microtomy (50 μm thickness). Blocks for in situ hybridization and for EM were trimmed from motor cortex and V1. Sections for in situ hybridization (35 μm thickness) were cut by frozen microtomy, and those for EM, by vibratome (VT 1000S; Leica Microsystems, Nussloch, Germany).

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### In situ hybridization

PCR primers for Kv3.1b (CCTGCTTCCCTTATCAACC and GTCAARTGAGCAGTACTG) were designed based on the rat cDNA sequence of Kv3.1b (Genbank No. NM_012856) and the corresponding human genome sequence. The amplified region is unique to Kv3.1b and not shared by other splicing variants. The DNA fragments were produced by RT-PCR from human monocyte cDNA. PCR fragments were ligated into the pBluescript II (KS vector). The plasmids were extracted and linearized by Asp718 or XhoI before being used for the template of antisense or sense probes. The digoxigenin (DIG)-dUTP labeling kit (Roche, Basel, Switzerland) was used for in vitro transcription.

### Immunoperoxidase staining for Kv3.1b or PV for LM

Sections were incubated for 1 h with PBS-TG at room temperature, and then 40–48 h at 4 °C with PBS-TG containing both rabbit polyclonal anti-Kv3.1b antibody (Chemicon; 1:500) and mouse monoclonal anti-PV antibody (Swant; 1:5000). Finally, the sections were incubated for 1.5 h in PBS-TG containing Alexa Fluor 488-conjugated anti-rabbit IgG goat polyclonal antibody (Molecular Probes, Eugene, OR, USA; 1:200), and Alexa Fluor 594-conjugated anti-mouse IgG goat polyclonal antibody (Molecular Probes; 1:200). Fluorescent photomicrographs were obtained with a Zeiss LSM 5 Pascal confocal microscope (Jena, Germany); and images labeled with different fluorochromes were merged with software of this confocal system.

For controls, one of the primary antibodies was omitted. No immunofluorescence was detected for the antibody under these circumstances.

### Immunoperoxidase staining for Kv3.1b for EM

The procedures were basically as described above, except that Triton X-100 was omitted in all solutions, and for the DAB step, nickel was also omitted. To improve antibody penetration, sections were incubated in 20% sucrose for 2 h, and then were freeze-thawed with liquid nitrogen. Sections were osmicated, dehydrated (including treatment with 1% uranyl acetate in 70% ethanol) and flat-embedded in resin (Araldite M; TAAB, Aldermaston, UK). Ultrathin sections were collected on formvar-coated, single-slot grids, and examined with an electron microscope (JEM 1200-EX, JEOL, Tokyo, Japan). To confirm the identification of Betz and Meynert cells, we inspected adjacent semithin sections, where the large Kv3.1b-ir somata could be clearly visualized.

### Nomenclature

Cortical areas were identified by reference to sulcal landmarks, by comparison with published maps, and by architectonic analysis of selected histological sections stained for cell bodies (Brodmann, 1909; von Bonin and Bailey, 1947).

### Image processing

LM photographs were taken on digital cameras (Axioskop2 and Axiocam, Carl Zeiss Vision, Munchen-Hallbergmoos, Germany). EM photographs were also taken as digital images by using imaging plates (Fuji Photo Film, Minamiashigara, Japan). Size, brightness, and contrast were modified using Photoshop software (Adobe Systems, San Jose, CA, USA).

### RESULTS

Inspection of cortical areas after immunohistochemistry for Kv3.1b protein revealed abundant non-pyramidal shaped neurons through layers 2–6, with some in layer 1 as well. Their distribution, quantity, and shape together suggest that these are GABAergic interneurons, consistent with previous reports in rodents (Weiser et al., 1995; Sekimjik et al., 1997). In addition, several pyramidal cell populations were strongly Kv3.1b-ir.

In layer 5 of primary motor cortex, large pyramidal neurons exhibited Kv3.1b-ir soma in the soma, in proximal basal dendrites and up to 200 μm in proximal apical dendrites (Fig. 1A). These pyramidal neurons had distinctive triangular somata, which measured 25–50 μm at their base, and basal dendrites which could frequently be detected as exiting from the midpoint as well as the base of the soma (Figs. 1D and 3A). From their large size, laminar location, and basal dendrites which could frequently be detected as exiting from the midpoint as well as the base of the soma (Figs. 1D and 3A). From their large size, laminar location, and dendrite morphology, these neurons were identified as giant Betz cells (see Rivara et al., 2003). Comparison with adjacent Nissl-stained sections revealed that most of the Betz cells were strongly Kv3.1b-ir. Some pyramidal neu-
rons in the deeper part of layer 3 were Kv3.1b-ir as well, but at a weaker level (Fig. 1D inset). Kv3.1b-ir seemed to occur both within cytoplasm and on the cell membrane surface.

In area V1, large Kv3.1b-ir pyramidal neurons were again obvious, now at the border of layers 5/6 (Fig. 1B, E). On the basis of their location, soma size, and scattered distribution, these could be identified as giant Meynert cells. Compared with Betz cells, their Kv3.1b-ir was weaker; and basal and apical dendrites were immunopositive only in their more proximal portions. In area V1, Kv3.1b-ir pyramidal neurons could also be found in layer 4B (Fig. 1E inset). These were more weakly stained than Meynert cells in the deeper layers, usually smaller, and were even more sparsely distributed.

Large layer 5 pyramidal neurons strongly stained by Kv3.1b antibody were found in several other areas; namely, premotor cortex, especially dorsally, parietal association area PE (Fig. 1C, F), and, more scattered, areas PG and both banks of the intraparietal sulcus. For these cells, the soma size was as large as Betz cells (25–50 μm at the base), and the level of staining was as strong, but not as widespread, as in the motor cortex.
their distribution was much sparser. In primary somatosensory and secondary visual cortex, a few large pyramidal neurons in layer 5 were Kv3.1b-ir, but only weakly so. In all these areas, as in the primary motor cortex, layer 3 contained a small number of weakly Kv3.1b-ir pyramidal neurons in its deeper part (Fig. 1F inset).

To establish mRNA expression of Kv3.1b, in situ hybridization was carried out on tissue from primary motor and visual cortices. In both areas, small neurons expressing mRNA were diffusely distributed, and appeared to correspond to Kv3.1-ir interneurons, as reported in rodents. In addition, large neurons, comparable in size and laminar location to Betz and Meynert cells, strongly expressed mRNA of Kv3.1b (Fig. 1A, B). Some pyramidal neurons in layer 3 in primary motor cortex and in layer 4B in primary visual cortex expressed mRNA of Kv3.1b, but only weakly.

We further examined sections stained for PV peroxidase immunohistochemistry. Consistent with previous observations (Preuss and Kaas, 1996), many, but not all, Betz cells expressed PV. Expression level was less than for PV-positive interneurons (Fig. 2A). We found PV-ir pyramidal neurons in layer 5 of all the same areas that have strongly Kv3.1b positive pyramidal neurons, including V1 (Fig. 2B) and area PE (Fig. 2C). In all cases, the PV-ir population seemed less numerous than the Kv3.1b-ir pyramidal neurons, and thus may be a subset of these.

Double immunohistochemistry for Kv3.1b and PV supported the interpretation of subpopulations. Of the Kv3.1b strongly positive layer 5 pyramidal neurons described above, most of Betz cells and those in other cortical areas were also weakly PV-ir (Fig. 3A–F), but in area V1, only about 10% of the Kv3.1b positive Meynert cells were also PV-ir. Weakly Kv3.1b positive pyramidal neurons in layer 3 (or layer 4B in area V1) seemed PV negative (Fig. 3G–I for area 4). Kv3.1b-ir non-pyramidal shaped neurons were PV positive, as expected from findings in rodent cortex and in support of our identification of these as interneurons (Fig. 3).

Finally, immuno-electron microscopic analysis of Betz and Meynert cells was carried out to ascertain the cellular localization of the Kv3.1b reaction product. This revealed abundant DAB reaction product close to or on the somal and/or dendritic membranes (Fig. 4). Kv3.1b-ir often appeared in the cytoplasm and associated with the rough endoplasmic reticulum. In addition, Kv3.1b-ir boutons were apparent around the labeled cells. These made symmetrical synaptic contacts, and presumably originated from GABAergic interneurons (Fig. 4C). No Kv3.1b-ir boutons were found forming asymmetrical synapses on these cells.

Fig. 3. Confocal images of sections double-stained for Kv3.1b and PV. (A–C) A strongly Kv3.1b-positive Betz cell (arrow) in layer 5 of area 4 is weakly PV-positive. (D–F) A strongly Kv3.1b-positive Meynert cell (arrow) in visual cortex is weakly PV-positive. (G–I) Two weakly Kv3.1b-positive pyramidal neurons (arrows) in layer 3 of area 4 are PV-negative. Arrowheads in all figures point to interneurons that are double-labeled for Kv3.1b and PV. Note also that, in all figures, Kv3.1b-positive pyramidal neurons are surrounded by PV-positive basket-like terminations. Scale bar=50 μm.
DISCUSSION

Our results show that Kv3.1b, both in its protein and mRNA, is strongly expressed in a subpopulation of large infragranular pyramidal neurons in the monkey cortex. EM study of Betz and Meynert cells further determines that the Kv3.1b protein is preferentially associated with the somal and proximal dendritic membranes, as expected for a channel protein. The strong expression of Kv3.1b in large infragranular pyramidal neurons raises the possibility that this group of cells has distinct physiological characteristics.

Several properties of Betz and Meynert cells would be consistent with high firing rates, as has been proposed for Kv3.1b expressing non-pyramidal cells in rodent (Perney et al., 1992; Massengill et al., 1997; Martina et al., 1998; Gan and Kaczmarek, 1998). For example, these large pyramidal cells stain densely for the metabolic enzyme cytochrome oxidase (Carroll and Wong-Riley, 1984; Matelli et al., 1985; Payne and Peters, 1989), and may be assumed to have high metabolic activity. In addition, extracellular recordings in behaving and anesthetized monkeys show that these cells can fire at frequencies $\geq 100$ Hz (Evarts, 1965; Movshon and Newsome, 1996). Finally, another characteristic of most Betz

![Fig. 4. Electron micrographs of sections processed for Kv3.1b immunoreactivity. (A) Kv3.1b-ir is localized close to the external membrane (arrowheads) of a Betz cell. Small arrows point to Kv3.1-ir in the cytoplasm. (B) Enlarged image from boxed region of A. (C) A Kv3.1b-ir terminal, presumably from interneuron, making a symmetric synaptic contact (thick arrow) on the Betz soma. Arrowheads point to Kv3.1b-ir reaction product localized close to the external cell membrane. (D) Kv3.1b-ir is localized close to the external membrane (arrowheads) of apical dendrites of Betz cells. Small arrow points to Kv3.1-ir in the cytoplasm. B, bouton; D, dendrite; S, soma. Scale bar = 1 $\mu$m in A; 300 nm in B, C; 1.5 $\mu$m in D.]
cells and some Meynert cells is that they also contain PV (Preuss and Kaas, 1996; this study) and are surrounded by PNs (Hendry et al., 1988; DeYoe et al., 1990; Hartig et al., 1999). In rodents, these two markers are associated with fast spiking interneurons (Kawaguchi and Kubota, 1997; Hartig et al., 1999), and are thought to act as buffers for excessive intracellular calcium (by PV) and extracellular cations (by PNs).

Subcategories of pyramidal neurons have been defined on the basis of dendritic morphologies and projectional target (reviewed in DeFelipe and Farinas, 1992; Lund et al., 1994; Douglas et al., 2004). In rodents, layer 5 pyramidal neurons have frequently been subdivided as burst firing and regular spiking (Connors et al., 1982; McCormick et al., 1985; Chagnac-Amitai et al., 1990; Mason and Larkman, 1990), but neither subtype expresses Kv3.1b protein (Perney et al., 1992; Weiser et al., 1995; Sekirnjak et al., 1997). In cats and ferrets, however, fast-rhythmic burst firing neurons (FRB) have been identified, and the suggestion has been made that their high-frequency repetitive firing is related to the presence of voltage-gated potassium currents of the Kv3 subfamily (Steriade, 2004). The issue of subcategories is further complicated by the possibility that firing characteristics, which have been used as a major classification criterion, might not be fixed, but rather change so that the same neuron can exhibit several discharge patterns that are otherwise associated to different subtypes (reviewed in Steriade, 2004).

In comparison with FRB cells, several roles may be proposed for the large Kv3.1b-expressing pyramidal cells: in network oscillations, mode switching, or synaptic plasticity. They may be preferentially epileptogenic (see Steriade, 2004). Aside from the association with fast firing rate, the Kv3.1b protein is known to be part of several neuromodulatory cycles (Gan and Kaczmarek, 1998; Rudy and McBain, 2001). Perhaps significantly, monkey Betz cells strongly express the $\beta$-subtype of protein kinase C (PKC; Tominaga et al., 1993). PKC, a calcium-activated and phospholipid-dependent protein kinase, inhibits Kv3.1b current (Kanemasa et al., 1995). The area and laminar specific location of these Kv3.1b-expressing pyramidal cells might be a clue as to their functional role. That is, some trend can be noted toward dorsal, and not ventral cortical areas, although this is not entirely consistent (i.e. no Kv3.1b-ir pyramidal were found in area MT). In the context of connectivity properties, both Betz and Meynert cells are known to have widespread intrinsic axon collaterals (Yamashita and Arikuni, 2001; Rockland and Knutson, 2001; Li et al., 2003); and both commonly have extensive collateral branches to multiple targets (Ugolini and Kuppers, 1986; Vogt Weisenhorn et al., 1995). This could indicate a special role in coordinating spatially distributed populations. Voltage-dependent fast oscillations might relate to different vigilance states, again spatially distributed populations. Voltage-dependent fast oscillations might relate to different vigilance states, again spatially distributed populations. Voltage-dependent fast oscillations might relate to different vigilance states, again spatially distributed populations. Voltage-dependent fast oscillations might relate to different vigilance states, again spatially distributed populations. Voltage-dependent fast oscillations might relate to different vigilance states, again spatially distributed populations. Voltage-dependent fast oscillations might relate to different vigilance states, again spatially distributed populations. Voltage-dependent fast oscillations might relate to different vigilance states, again spatially distributed populations. Voltage-dependent fast oscillations might relate to different vigilance states, again spatially distributed populations. Voltage-dependent fast oscillations might relate to different vigilance states, again spatially distributed populations. Voltage-dependen

Less is known concerning the collateralization or other properties of this group.

Further investigations are needed in monkeys to elucidate the firing and membrane properties of these large pyramidal neurons with strong Kv3.1b expression. Extended work might address whether the same cell types express channel subunits or auxiliary subunits which have properties similar to Kv3.1b (e.g. Kv3.1a and Kv3.2) or which can form heteromeric channels with Kv3.1b (e.g. other Kv3 families and MinK-related peptide 2; Baranauskas et al., 2003; reviewed in Rudy and McBain, 2001). A reasonable conclusion, however, is that the expression of Kv3.1b indicates a distinct specialization in this particular subpopulation of pyramidal neurons in the monkey cerebral cortex.

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