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Localization of Kv1.3 channels in presynaptic terminals of brainstem auditory neurons

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Abstract

Elimination of the Kv1.3 voltage-dependent potassium channel gene produces striking changes in the function of the olfactory bulb, raising the possibility that this channel also influences other sensory systems. We have examined the cellular and subcellular localization of Kv1.3 in the Medial Nucleus of the Trapezoid Body (MNTB) in the auditory brainstem, a nucleus in which neurons fire at high rates with high temporal precision. A clear gradient of Kv1.3 immunostaining along the lateral to medial tonotopic axis of the MNTB was detected. Highest levels were found in the lateral region of the MNTB, which corresponds to neurons that respond selectively to low frequency auditory stimuli. Previous studies have demonstrated that MNTB neurons and their afferent inputs from the cochlear nucleus express three other members of the Kv1 family, Kv1.1, Kv1.2 and Kv1.6. Nevertheless, confocal microscopy of MNTB sections co-immunostained for Kv1.3 with these subunits revealed that the distribution of Kv1.3 differed significantly from other Kv1 family subunits. In particular, no axonal staining of Kv1.3 was detected and most prominent labeling was in structures surrounding the somata of the principal neurons, suggesting specific localization to the large calyx of Held presynaptic endings that envelop the principal cells. The presence of Kv1.3 in presynaptic terminals was confirmed by co-immunolocalization with the synaptic markers synaptophysin, syntaxin, and synaptotagmin and by immunogold electron microscopy. Kv1.3 immunogold particles in the terminals were arrayed along the plasma membrane and on internal vesicular structures. To confirm these patterns of staining, we carried out immunolabeling on sections from Kv1.3^{-/-} mice. No immunoreactivity could be detected in Kv1.3^{-/-} mice either at the light level or in immunogold experiments. The finding of a tonotopic gradient in presynaptic terminals suggests that Kv1.3 may regulate neurotransmitter release differentially in neurons that respond to different frequencies of sound.

Keywords

voltage-dependent potassium channels; medial nucleus of the trapezoid body; calyx of Held

Introduction

Potassium channels represent a large and diverse group of proteins that have a variety of physiological functions. Recent findings have also demonstrated that, in addition to their role in regulating the transmembrane flux of ions, some potassium channels may participate directly in cellular signaling pathways, for example by providing a voltage sensor that can regulate the rate of enzyme reactions (Hegle et al., 2006; Kaczmarek, 2006). Of the several families of potassium channels, the Kv family of voltage-dependent potassium channels is the most numerous and diverse in its members, and is comprised of 12 subfamilies. Members of the first subfamily (Kv1) are typically either delayed rectifier or A-current channels that inactivate relatively slowly. Such channels shape the waveform of the action potential and regulate the rate of neuronal firing.

Kv1.3, one of seven members of the Kv1 subfamily, has a number of unusual characteristics that differentiate it from other channels in this subfamily. Repeated depolarization of Kv1.3 channels produces a use-dependent inactivation that can suppress current flow through these channels for periods of a minute or more following repeated stimulation (Marom and Levitan, 1994). It has been proposed that Kv1.3 functions as more than a channel protein that simply transduces changes in membrane potential into alteration in potassium flux. In non-neuronal cells, the Kv1.3 channel forms a tight physical link with β -integrins, and the activity of the channel has been found to regulate integrin signaling and cell proliferation as well as the killing of neurons by microglia (Artym and Petty, 2002; Fordyce et al., 2005; Levite et al., 2000; Pardo, 2004).

Elimination of Kv1.3 expression in mice by gene targeting leads to several interesting phenotypes, including metabolic changes and a change in the mechanisms by which lymphocytes regulate their membrane potential (Koni et al., 2003; Xu et al., 2003; Xu et al., 2004). The loss of Kv1.3 has a particularly striking effect on olfactory function (Fadool et al., 2004). Kv1.3 is expressed at high levels in mitral cells of the olfactory bulb. Deletion of Kv1.3 alters the firing pattern of these cells, alters the levels of multiple signaling molecules, increases the number of synaptic glomeruli in the olfactory bulb and produces a 1,000–10,000 fold lower threshold for detection of odors and an increased ability to discriminate between odorants (Biju et al., 2008; Fadool et al., 2004).

The characteristics of voltage-dependent potassium channels, and the relative expression levels of different channels, has been found to be particularly important for the function of auditory brainstem neurons, where a high degree of accuracy of timing of action potentials is required for the detection and interpretation of auditory stimuli (Kaczmarek et al., 2005). The medial nucleus of the trapezoid body (MNTB), which relays auditory information relevant to sound localization, has been widely used for studies of the role of potassium channels in auditory function (Brew and Forsythe, 1995; Dodson et al., 2002; Dodson et al., 2003; Ishikawa et al., 2003; Kaczmarek et al., 2005; Li et al., 2001). In large part, this is because of the very large presynaptic terminals (calyces of Held) that synapse onto the somata of the principal neurons of the MNTB, allowing investigators to characterize potassium currents in both the presynaptic and postsynaptic elements of the MNTB (Forsythe, 1994). It has been established that members of the Kv1 family that are sensitive to the blocking agent dendrotoxin (Kv1.1, Kv1.2 and Kv1.6) are present in axonal membranes of the presynaptic terminals and in the axons of the MNTB principal neurons (Dodson et al., 2002; Grigg et al., 2000). Their presence in the terminals is required to prevent aberrant presynaptic action potentials following depolarization of the terminals (Dodson et al., 2003; Dodson and Forsythe, 2004), while postsynaptically their expression in axonal membrane contributes to the fact that each excitatory postsynaptic potential evokes only a single action potential (Brew and Forsythe, 1995; Wang et al., 1998). Because of the

striking role of Kv1.3 channels in olfactory processing, we have now examined the possibility that Kv1.3 may play a role in auditory function. We have determined that Kv1.3 is present in the MNTB, that its expression levels follow a tonotopic distribution and that it is localized primarily to the presynaptic calyces of Held.

Materials and Methods

Animals

All experimental protocols involving animals were approved by the Yale University Animal Use and Care Committee and experiments were carried out on either 2 month old C56BL/6J wild-type mice or Kv1.3^{-/-} mice in the same C56BL/6J background (Fadool et al., 2004; Koni et al., 2003). The genotype of all animals was confirmed using the polymerase chain reaction (PCR) on extracts of tail DNA. For wild type mice the primers used for PCR were KCNA-F (5-GTACTTTGACCCACTCCGCAATGA-3) and KCNA-R (5-GCAGAAGATGACAATGGAGATGAG-3) (342 bp product). For Kv1.3^{-/-} mice the primers used were Neo-F (AGGATCTCCTGTCATCTCACCTT) and Neo-R (CCTCAGAAGAACTCGTCAAGAAG) (499 bp product). The PCR conditions were as follows: 94°C 30 min. 60°C 30 min, 72 °C 40 min; 32 cycles.

Immunohistochemistry—Adult mice were anesthetized with sodium pentobarbital (60 mg/kg,i.p.) and perfused through the left ventricle with a PBS solution(100 mM Na₂HPO₄/NaH₂PO₄, pH 7.4, and 150 mM NaCl) containing 0.5% NaNO₂ and 1000 U heparin, followed by a 0.1M phosphate buffer(PB) solution (100 mM Na₂HPO₄/NaH₂PO₄, pH 7.2) containing 4% paraformaldehyde. Brains were removed, post fixed in 4% paraformaldehyde for 2 h at 4°C, rinsed twice with PB, and then placed either in 30% sucrose at 4°C for 24 h or immediately sliced coronally or sagittally on a vibratome at 40 µm. Brains not sectioned were embedded in O.C.T. compound (Tissue-Tek, Torrance, CA), rapidly frozen in acetone containing dry ice, and stored at -80°C until cryostat sectioning. Slices were collected in multi-well plates containing PBS and were then post fixed in 4% paraformaldehyde for 5 min, followed by two washes with PBS (for 5–10 min on orbital shaker). Slices were then permeabilized with a PBS solution containing 1% bovine serum albumin (BSA), 5% normal donkey serum, 0.2% glycine, 0.2% lysine, and 0.2% Triton X-100 (blocking solution) for 1 hour at room temperature or 4°C overnight and were rinsed twice with PBS containing 1% BSA alone. Triton X-100 was omitted when the rabbit anti Kv1.3 (Alomone) antibody was used alone. At this point, slices were processed for immunofluorescence. Details of antibodies used in this study are presented in table 1.

Diaminobenzidine (DAB) Staining—The free-floating sections were washed with PBS three times for 10 min each, incubated with 1% H₂O₂ for 10 min, washed three times with PBS and then incubated with anti rabbit Kv1.3 antibody (Alomone Labs) in blocking solution for 48 hours at 4°C under constant agitation. The tissue was washed twice with PBS containing 1% BSA, followed by incubation with biotinylated donkey anti-rabbit antibody at room temperature for 1 hour under constant agitation. The sections were again washed and then reacted with an avidin-biotin peroxidase complex (Vectastain; Vector Laboratories Inc.), and the immunoreactivity was visualized using nickel-enhanced diaminobenzidine (DAB). In brief, 10 mg of DAB (Sigma, St. Louis, MO) was dissolved in 18.4 ml PB, followed by the addition of 200 µl of 0.4% NH₄Cl, 200 µl of 20% β-D-glucose (Sigma) and 800 µl of 1% (NH₄)₂Ni(SO₄)₂ · 6H₂O (Sigma). The solution was then filtered by a 0.45 µm syringe filter, followed by the addition of 20 µl glucose oxidase (Sigma). This solution was then added to the sections, and the reaction was allowed to proceed for 10 minutes. The reaction was terminated by adding fresh phosphate buffer (0.1M), and this was then followed by two rinses with PB. Stained sections were mounted on gelatin-coated slides,

dehydrated through graded ethanols followed by xylene, and cover slipped with Permount (Fisher Scientific, St. Louis, MO). Control experiments using previously published techniques (Bhattacharjee et al., 2002) included using the secondary antibody alone, or the primary antibody blocked with excess Kv1.3 (211–224) peptide.

Fluorescent Staining—After the post fixed, permeabilized and blocking preparation, free-floating slice sections were processed for either single labeling with FITC-labeled anti Kv1.3 or double labeling with rabbit anti-Kv1.3 (Alomone labs) and mouse anti-Kv 1.1, 1.2 and 1.6 (NeuroMab). After 48 hours of incubation at 4°C in primary antibody, sections were washed three times for 10 min each with 1X PBS. The fluorescent secondary antibodies used in double-labeling experiments were donkey anti rabbit Alexa 488 (Invitrogen) for Kv1.3 staining and donkey anti- mouse Cy3 (Jackson Immuno Research) for Kv 1.1, 1.2 and 1.6 staining. In another set of experiments, sections were incubated with rabbit Kv1.3 (Alomone labs) or with mouse anti-Kv1.3 (NeuroMab) and with mouse anti-syntaxin (Sigma), mouse anti-synaptophysin (Sigma), mouse anti-synaptotagmin (Sigma) and rabbit anti PSD-95 (Cell Signaling). After 48 hours of incubation at 4°C in primary antibody, sections were washed three times for 10 min each. Fluorescent secondary antibodies were used to identify the double labeling; Mouse/Rabbit Donkey anti Alexa 488 (Invitrogen) for Kv1.3 staining and Donkey anti mouse/rabbit Cy3 (Jackson Immuno Research) for syntaxin, synaptophysin, PSD 95 and synaptotagmin for 1 hour at room temperature. Sections were mounted on gelatin-coated slides, air dried, and cover slipped with 2.5% DL -2-amino-5-polyvinyl alcohol/1, 4-diazabicyclo (2.2.2) octane (PVA-DABCO). Control sections were processed through similar immunohistological procedures, except that primary or secondary antibodies were omitted. In all cases, the omission of primary or secondary antibodies resulted in the lack of specific labeling, confirming the specificity of immunocytochemical analysis (Chen et al., 2009; Santi et al., 2006).

Image acquisition and analysis

Confocal Microscopy—Sections were examined with a Zeiss laser scanning microscope (LSM 150 META) coupled to a computer with Zeiss image acquisition and analysis Zeiss image analysis software. Images were acquired in the multi-track mode, because this function permits several tracks to be defined as one configuration for the scan procedure and this does not allow fluorescence to bleed into the other channel. Images were acquired using a and a C-Apochromat 40x/1.2W corr (water) or 63x/1.2W corr (water) objective (water correction) for brain slices, and the optical thickness of the slices was constant for both tracks. Alexa Fluor 488 has excitation/emission of 496/519, and Cy3 has excitation/emission of 550/570. Double-immunofluorescence images were also displayed as dual-color merged images. The Zeiss C-Apochromat objective (C for confocal) was developed to match the requirements of diffraction limited optics, and combine high numerical apertures and perfect chromatic correction. The C-Apochromat series water immersion objectives are corrected for an extended range of wavelengths.

Light Microscopy—For light microscopy we used Zeiss Axiophot microscope with AxioCam HRc and AxioVision Software. We used Adobe Photoshop (Adobe Systems, San Jose, CA) to sharpen images, adjust brightness and contrast level, and compose final plates.

Electron Microscopy—Adult mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused through the left ventricle with saline nitrite (4.5 g of NaCl and 0.5g of NaNO₂ in 500 ml of distilled water), followed by 4% of paraformaldehyde and 0.1% glutaraldehyde in PBS. After the perfusions animals were kept on ice for 30 min and brains were excised and post fixed for two hours with 4% paraformaldehyde and 0.1% glutaraldehyde in 1X PBS.

Image acquisition and analysis for Electron Microscopy

After fixation tissue samples were rinsed three times in PBS and cryo-protected in 2.3 M sucrose at 4°C overnight (Tokuyasu, 1973). Specimens were mounted on aluminum supportors and rapid frozen in liquid nitrogen and stored in liquid nitrogen for further use. Cryo-ultrathin sections were performed on a Leica Ultracut Cryo Microtome type UC-6 with a FC-6 cryo-attachment and collected on form var coated nickel grids. Immunogold labeling of cryo-ultrathin sections was performed using a standard protocol (Slot and Geuze, 2007). Briefly, for double immunogold labeling, primary polyclonal antibody, anti-rabbit synaptophysin and anti-mouse monoclonal Kv1.3, were applied sequentially and individually developed in the sequence the primary antibodies were applied, using 5 and 10 nm colloidal gold conjugated affinity purified secondary antibodies, donkey anti-rabbit and donkey anti-mouse, respectively (Jackson ImmunoResearch). Finally, sections were negative stained with a coat of methylcellulose-uranylacetate, dried overnight at room temperature and imaged on a FEI TECNAI Biotwin electron microscope, equipped with a Morada digital camera and Olympus imaging software.

Co-Immunoprecipitation of Kv1.3 and other Kv1 subunits from mouse brains

Adult mouse brains were taken as described previously (Chen et al., 2009) and then homogenized and briefly sonicated in a cold buffer containing (mM): 50 NaCl, 50 KCl, and 50 Tris-HCl, pH7.4. The membrane fractions were isolated, and protein samples were solubilized in the same buffer containing 1% triton X-100. Co-Immunoprecipitation of Kv1.3 and other Kv1 subunits from brain tissues was done with the ProFound Co-Immunoprecipitation Kit (Pierce). Briefly, rabbit anti-Kv1.3 antibodies were coupled onto agarose beads on a column. After incubation, the extracted membrane protein samples were passed through this column, and the channels or channel complexes containing Kv1.3 subunits were eluted. Kv1.3 and other Kv1 subunits (Kv1.1, Kv1.2, and Kv1.6) proteins were detected by standard western blotting analysis with mouse monoclonal anti-Kv1.1, anti-Kv1.2, anti-Kv1.3, and anti-Kv1.6 antibodies (all from NeuroMab).

Digital image quantification—Quantification of the tonotopic gradient was carried out by a blinded investigator using a modified approach to densitometric methods that have been described previously (Li et al., 2001; von Hehn et al., 2004). The technique is illustrated in a supplementary figure (Fig. S5) showing images of a representative section at each stage in the densitometric analysis. Using ImageJ software, images were converted to 8-bit and a threshold of 140 a.u. (on a scale of 0–255) was set for cell identification. Cells were outlined using a particle analysis algorithm (Minimum area = 200 pixels) and images were carefully examined by a blinded investigator to exclude artifacts. Following cell identification, images were divided into five equal zones and cells were sorted into according to their location. In some cases, contiguous regions of immunoreactivity represented more than one cell. In order to control for differences in the size and density of Kv1.3-labeled cells between zones, the integrated optical density in each zone (total sum of grey values for all pixels) was divided by the total number of pixels-above-threshold (PAT) in that zone. No distinction was made between nuclear and membrane immunoreactivity, nor was it possible to distinguish between pre- and post-synaptic immunoreactivity. A total of five sagittal and five coronal sections from ten separate animals were quantified. To facilitate comparisons across sections, optical density measurements were normalized to the darkest cell in the each section.

Antibody Characterization

1. Anti Kv 1.3 (extracellular)—Anti Kv 1.3 (extracellular) antibody was obtained from Alomone labs. Control experiments with antigen-preadsorbed antibody resulted in complete

prevention of staining. We used this antibody for light and confocal microscopy experiments.

2. Synaptophysin—Monoclonal anti-synaptophysin antibody (clone SVP-38) was obtained from Sigma Chemical Company and, in western blots of rat brain samples, recognizes a single band at 38 kDa (Jahn et al., 1985).

3. Synaptotagmin—Anti-synaptotagmin antibody was obtained from Chemicon and, on western blots, recognizes a single band at 65 kDa in rat brain samples (Matthew et al., 1981).

4. Syntaxin—Monoclonal anti-syntaxin antibody was obtained from Sigma Chemical Company and recognizes a single band at 35 kDa on western blots of rat brain samples (Bennett et al., 1992).

5. Synaptophysin1—Polyclonal anti-synaptophysin 1 antibody was obtained from Synaptic Systems and recognizes a single band at 35 kDa on western blots of rat brain samples (Wimmer et al., 2006).

6. Synaptophysin—This antibody was made in the laboratories of Drs. Paul Greengard and Reinhard Jan, and was a gift from Dr. Pietro De Camilli. It recognizes a single band at 38 kDa on western blots of rat brain samples (De Camilli et al., 1988; Jahn et al., 1985).

7. Kv 1.1—Anti-Kv1.1 antibody was obtained from NeuroMab. It recognizes a single band corresponding to 86 kDa on immunoblots of rat brain membranes or of COS cells transfected with Kv1.1 (Vacher et al., 2007).

8. Kv 1.2—Anti-Kv1.2 antibody was obtained from NeuroMab. Kv1.2 has a calculated MW of 56.7 kDa but may run at 66–88 kDa depending on the degree of post translational modification. On immunoblots of rat brain neuronal membrane preparations, a single band corresponding to 66 kDa was detected (Chi and Nicol, 2007).

9. Kv 1.3—Anti-Kv1.3 antibody was obtained from NeuroMab. On western blots of rat post-ganglionic sympathetic neurons, it detected a band close to 70 kDa, the predicted weight for Kv1.3 (Doczi et al., 2008). We used this antibody for Electron Microscopy.

10. Kv 1.6—Anti-Kv 1.6 antibody was also obtained from NeuroMab. On western blots of COS cell lysates transfected with rat Kv1.6, it recognizes single 60 kDa band, the predicted weight for Kv1.6.

11. PSD 95—Anti-PSD 95 antibody was obtained from Cell Signaling. This antibody recognizes a single band of 95 kDa on western blots of lysates of rat primary cerebellar granule neuronal cultures (Cao et al., 2005).

Results

Immunostaining for the Kv1.3 potassium channel subunit in the MNTB

We carried out immunohistochemical staining of coronal and sagittal sections of adult (2 month old) mouse brain using a rabbit anti-Kv1.3 antibody that is directed against an extracellular epitope located between the first and second transmembrane domains (S1 and S2). We localized this channel subunit using both diaminobenzidine and immunofluorescence labeling techniques. Kv1.3 immunoreactivity was found in the

cerebellum, olfactory bulb, hippocampus, and in the cerebral cortex, particularly in frontal cortex, consistent with previous *in situ* hybridization and immunolabeling studies demonstrating the presence of Kv1.3 mRNA and protein in neurons within these areas (Beckh and Pongs, 1990; Kues and Wunder, 1992; Veh et al., 1995).

In coronal sections of the brainstem, prominent Kv1.3 immunoreactivity was found bilaterally in the medial nuclei of the trapezoid body (MNTB), a nucleus that plays a key role in circuits that detect the localization of sounds in the azimuth (Fig. 1A,C). Lower levels of Kv1.3 were also detected in several other auditory brainstem nuclei (Table 2). To test for specificity of the staining, immunolocalization was carried out using sections of brainstem from mice in which the Kv1.3 gene had been deleted (Fadool et al., 2004; Koni et al., 2003). These sections were processed simultaneously with, and in the same incubation wells as those from the wild-type mice. No sections from Kv1.3^{-/-} animals contained any specific staining (Fig. 1B,D).

Higher magnification of the sections from wild-type mice immunostained using DAB revealed that Kv1.3 immunoreactivity is confined to the principal neurons of the MNTB (Fig. 1E, F). A low level of immunostaining was detected throughout the somata of these cells. At the periphery of the somata, much stronger staining was observed. Typically this peripheral staining was highly punctate. Such a pattern of staining is consistent with localization of Kv1.3 channels either to the plasma membrane of the somata of the principal neurons or to the large calycyl presynaptic terminals that envelop much of the somata. Little or no axonal Kv1.3 immunoreactivity could be detected.

Tonotopic gradient of Kv1.3 expression in the MNTB

The MNTB is organized tonotopically such that neurons that respond preferentially to low frequency sounds are located near the lateral edge of this nucleus, while those with characteristic frequencies that are at the high frequency end of the auditory spectrum are located in the medial region (Sonntag et al., 2009). In all coronal sections that were examined the intensity of Kv1.3 immunoreactivity in neurons within lateral regions of the MNTB appeared substantially enhanced over that in neurons in the medial aspect of the nucleus (Fig 1E, F).

Examination of Kv1.3-immunostained sagittal sections through the MNTB revealed that a strong gradient of Kv1.3 also exists along the anterior to posterior axis, with highest levels found at the anterior pole of the nucleus. (Fig. 2A). To quantify the anterior-posterior and lateral-medial gradients, we carried out a densitometric analysis of cell staining. Coronal sections from five different animals were divided into five equal zones from the lateral to medial ends of the MNTB (Fig. 2B). Similarly, sagittal sections from another five animals were subdivided into five equal zones from the anterior to posterior ends of the nucleus (Fig. 2C). The mean optical density of all cells in each segment was measured and normalized to the darkest cell in the entire section. A comparison of the staining of all MNTB neurons in each of the five zones revealed a steep gradient in the levels of Kv1.3 immunoreactivity within individual cells along the tonotopic lateral to medial axis in coronal sections (Fig. 2D). A similar gradient of Kv1.3 immunoreactivity was measured along the anterior-posterior axis in sagittal sections (Fig. 2E). Adjusted *r*-square values for the five coronal sections were 0.82366, 0.89029, 0.6897, 0.50057, and 0.5221; for sagittal sections, adjusted *r*-square values were 0.47824, 0.77737, 0.48172, 0.45734, and 0.7861.

Kv1.3 does not co-localize with other Kv channel subunits in MNTB neurons

Voltage-dependent potassium channels are either homo- or hetero-tetramers of Kv subunits, and biochemical studies in brain have demonstrated that the Kv1.3 subunit can form

heteromeric channels with other Kv1 subfamily subunits that contain Kv1.1, Kv1.2, Kv1.4 (Coleman et al., 1999; Koch et al., 1997; Wang et al., 1999). Among the subunits demonstrated to form heteromers with Kv1.3 *in vivo* are Kv1.1, Kv1.2 and Kv1.6, which are abundantly expressed in MNTB neurons and comprise the major low-threshold potassium currents recorded in the principal neurons and in the presynaptic calyces of Held (Dodson et al., 2002; Dodson et al., 2003; Dodson and Forsythe, 2004). By contrast, other Kv1 subunits, such as Kv1.4 and Kv1.5, appear to be absent from the MNTB (Dodson et al., 2002). Using Western analysis on extracts of entire brainstem, we have confirmed that Kv1.1, Kv1.2 and Kv1.6 can be co-immunoprecipitated with Kv1.3 (Supplementary Fig. S1), suggesting that Kv1.3 could exist as a component of these heteromeric channels in the MNTB. To test this possibility, we carried out double immunolabeling to examine the co-localization of Kv1.3 with Kv1.1, Kv1.2 or Kv1.6.

Diffuse immunoreactivity for Kv1.1, Kv1.2 and Kv1.6 was detected in the cytoplasm of MNTB neurons with no obvious additional staining at the plasma membrane (Fig. 3A, B, C center panels, red). For Kv1.1 and Kv1.2 clear staining was also observed in axonal tracts. This is consistent with previous studies showing that Kv1.1 and Kv1.2 are not expressed in the plasma membrane of the somata or the presynaptic terminals but are expressed selectively in the axons of both MNTB neurons and their presynaptic inputs (Dodson et al., 2002; Dodson et al., 2003). In contrast, little or no Kv1.3 staining was detected in axons (Fig. 3A,B,C., left panels, green). As was found with diaminobenzidine staining, Kv1.3 was confined to punctate rings surrounding the somata, together with a lower level of diffuse cytoplasmic staining. In images of Kv1.3 immunofluorescence merged with those of each of the other three Kv family channels, it is clear that the areas of major regions of peripheral Kv1.3 staining are devoid of Kv1.1, Kv1.2 or Kv1.6. These findings are consistent with the selective expression of Kv1.3 in the postsynaptic plasma membrane and/or the presynaptic terminals, regions from which Kv1.1, Kv1.2 and Kv1.6 are excluded (Dodson et al., 2002; Dodson et al., 2003).

Kv1.3 co-localizes with presynaptic markers syntaxin, synaptophysin and synaptotagmin

To determine whether Kv1.3 is expressed in the presynaptic terminals that envelop the somata of MNTB principal neurons, we carried out double labeling experiments to examine whether Kv1.3 is co-localized with three markers of presynaptic terminals: i) syntaxin, an integral membrane protein of the presynaptic plasma membrane, ii) synaptophysin, a synaptic vesicle protein and iii) synaptotagmin, an integral membrane protein of synaptic vesicles that interacts with syntaxin. In contrast to Kv1.3, very little diffuse postsynaptic staining was detected in the somata of MNTB neurons for any of these three proteins (Fig. 4, 2nd column). In both low-power and high power merged confocal images of combined Kv1.3/synaptic marker staining, however, very clear co-localization was observed in the peripheral regions surrounding the somata (Fig. 4, columns 1–4), as evidenced by the strong orange and yellow signals in these regions. In most cases the strongest overlapping signals (yellow) were located at the inner face of this peripheral ring. These findings are consistent with the hypothesis that Kv1.3 is preferentially expressed in the presynaptic calyces of Held.

Immunogold electron-microscopic localization of Kv1.3 in the MNTB

Immunogold electron-microscopic localization of Kv1.3 in MNTB was first carried out using 10 nm gold particles to localize channels subunits (Fig. 5). Three wild-type animals were used for these experiments and a minimum of 12 MNTB sections were analyzed from each animal. In wild-type animals, gold particles were primarily localized to presynaptic structures, identified by the presence of numerous synaptic vesicles. Arrays of Kv1.3 gold particles were found aligned along the plasma membrane of the terminals (black arrows in Fig. 5A, B). A high proportion of Kv1.3 particles were also localized in clusters on

intracellular organelles within the cytoplasm of the terminals (white arrows in Fig. 5A, B). Significantly fewer Kv1.3 particles were detected in the cytoplasm of the postsynaptic MNTB neurons (black asterisk in Fig. 5A). Particles were occasionally also detected within mitochondria (white asterisk in Fig. 5A). Previous studies have indicated that Kv1.3 may also be localized to the inner mitochondrial membrane of T-lymphocytes (Szabo et al., 2008; Szabo et al., 2005).

To control for the specificity of Kv1.3 immunolabeling at the EM level, we repeated these Kv1.3 immunogold labeling experiments using sections of MNTB from three Kv1.3^{-/-} animals (Fig. 5C,D). No labeling of any structures were detected in Kv1.3^{-/-} tissue although in rare sections, occasional 10 nm gold particles could be found in random locations (Fig. 5C arrow).

Immunogold electron-microscopic localization of Kv 1.3 to presynaptic terminals

To confirm that the structures to which Kv1.3 immunogold particles are selectively localized in the electron-microscopic images are indeed presynaptic terminals, we carried out double immunogold particle labeling to compare the distribution of the presynaptic marker synaptophysin with that of Kv1.3. As for the single label experiments, three wild-type animals were used for these experiments and a minimum of 12 MNTB sections was analyzed from each animal. In these double-label experiments, Kv1.3 channels were localized using smaller 5 nm particles, while synaptophysin was labeled using 10 nm gold particles. The small and large particles could readily be differentiated at high levels of magnification (Fig. 6A, B). Lower power views confirmed that the structures rich in 5 nm Kv1.3 particles are presynaptic terminals, which are also strongly labeled with the 10 nm synaptophysin particles (Fig. 6C, D, E, F). Although a lower level of both Kv1.3 and synaptophysin particles could sometimes be found outside of morphologically defined terminals (see Fig. 6D), the synaptophysin-rich terminals were mostly associated with clusters of Kv1.3 particles, either aligned along the plasma membrane or on internal organelles. Conversely, in no case were higher densities of Kv1.3 particles found in structures that did not also contain high synaptophysin labeling.

As a final test for the specificity of the Kv1.3 immunogold labeling, we compared Kv1.3 immunogold localization in the MNTB of wild type and Kv1.3^{-/-} animals. As for the light level localization studies, sections from wild-type and Kv1.3^{-/-} animals were prepared in parallel. In this series of experiments we omitted glutaraldehyde from the post-fixation step, which allows greater access of the anti-Kv1.3 antibody. In the resultant images from wild-type animals, this provided a higher density of Kv1.3 immunogold particles in the synaptophysin-labeled presynaptic terminals (Fig. 6G, H). In contrast, in the sections from Kv1.3^{-/-} animals, the presynaptic terminals were clearly labeled by the 10 nm synaptophysin gold particles but only occasional smaller gold particles could be seen (Fig. 6I, J). These smaller particles may represent either background staining or variance in the size of the nominally 10 nm synaptophysin particles. These experiments confirm that a high density of Kv1.3 potassium channel subunits is found in the plasma membrane and internal organelles of presynaptic terminals in the MNTB.

Discussion

The MNTB is a component of the binaural pathway that computes the direction of a sound source by comparing differences in the timing and intensity of incoming auditory stimuli. The principal cells of the MNTB receive excitatory inputs from the globular bushy cells of the contralateral anteroventral cochlear nucleus (AVCN) and give rise to axons that provide inhibitory inputs to the medial superior olive (MSO) and lateral superior olive (LSO). A unique morphological feature in this sound localization pathway is the very large calyx of

Held synapse, which arises from the incoming axons of globular bushy cells in the AVCN and engulfs the somata of the MNTB principal neurons (von Gersdorff and Borst, 2002). Our present findings indicate that the Kv1.3 potassium channel subunit is expressed in this presynaptic terminal.

The calyx of Held is one of a number of morphological and biochemical specializations within the MNTB that allow this nucleus to relay accurate information on the timing and intensity of auditory inputs. The firing pattern of MNTB neurons is shaped by several classes of potassium channels that are expressed in the presynaptic cells and in the MNTB principal neurons (Frick et al., 2003; Hardman and Forsythe, 2009; Kaczmarek et al., 2005). Kv3 family channels, which are located in the plasma membrane of the terminals and the somata of the principal neurons, ensure that both presynaptic and postsynaptic elements are capable of generating the very high firing rates (up to ~800 Hz) that are characteristic of this pathway (Taschenberger et al., 2002). In contrast, the Kv1 family channels Kv1.1, Kv1.2 and Kv1.6, which are located selectively in the plasma membrane of the presynaptic and postsynaptic axons and in the initial segment of the MNTB neurons ensure that each incoming action potential that invades the calyx triggers only a single precisely-timed action potential in the MNTB neurons (Brew and Forsythe, 1995; Ishikawa et al., 2003).

Our confocal images of immunocytochemical staining of the MNTB with antibodies against Kv1.1, Kv1.2 and Kv1.6 closely match those previously described for these channel subunits (Dodson et al., 2002; Dodson et al., 2003). Kv1.1 and 1.2 are localized mainly to axonal segments and diffuse staining for all three is present within the cytoplasm of the principal neurons. The latter may represent newly synthesized Kv1 family subunits in the process of being trafficked to distal sites. We also observed similar diffuse cytoplasmic staining for Kv1.3, and found that this is absent in MNTB neurons of Kv1.3^{-/-} animals. It has previously been shown that heteromeric channels that contain Kv1.3 together with each of these other Kv1 family subunits exist in the nervous system (Coleman et al., 1999; Koch et al., 1997; Wang et al., 1999), and we have confirmed such interactions by co-immunoprecipitation from extracts of mouse brainstem. Thus we cannot exclude the possibility that the diffuse cytoplasmic staining represents such Kv1.3-containing heteromeric channels.

In contrast to Kv1.1, Kv1.2 and Kv1.6, strong immunoreactivity for Kv1.3 was also found in a peri-cellular ring surrounding the MNTB principal neurons. Our studies, both at the light and EM microscopic levels, indicate that this peri-cellular staining reflects the presence of Kv1.3 in presynaptic terminals, from which Kv1.1, Kv1.2 and Kv1.6 are excluded (Dodson et al., 2002; Dodson et al., 2003). Because heteromer formation in voltage-dependent potassium channels is believed to be limited to channel subunits from the same family, and because no other Kv1 family subunits have been found to be expressed in the MNTB (Dodson et al., 2002), it is likely that Kv1.3 within the terminals exists as a homomeric channel. We cannot, however, exclude the possibility that Kv1.3 exists in a complex with as yet uncharacterized channel subunits.

In general, many ion channels are expressed in presynaptic terminals, where their activity provides for fine-tuning of neurotransmitter release (Meir et al., 1999). Thus Kv1.3 channels in the plasma membrane of the presynaptic terminals within the MNTB may regulate the amount or timing of glutamate release in response to incoming action potentials. Pharmacological manipulation of Kv3 family channels, which like Kv1.3 are localized to the terminal itself, has been found to produce major changes in the amount of neurotransmitter release, the rate of synaptic depression, and the rate of recovery of the readily releasable pool of neurotransmitter. Homomeric Kv1.3 channels are selectively inhibited by the channel blocker margatoxin at picomolar concentrations (Koch et al., 1997). This blocker

has been found to lower the threshold for action potential generation in the calyx of Held, allowing for the generation of repetitive firing in response to prolonged depolarizations (Ishikawa et al., 2003). At the higher (nanomolar) concentrations used in these experiments, however, margatoxin is not selective and its action was attributed to its blockade of Kv1.2-containing channels.

In contrast to calcium channels, the physiological conditions under which modulation of presynaptic potassium regulates transmitter release from the calyx of Held are not well characterized (Kushmerick et al., 2004; Takahashi et al., 1996). Our EM localization of Kv1.3 in the terminals indicates that, although some Kv1.3 subunits are localized at the plasma membrane, a very significant proportion of this channel is located on internal organelles that could represent channels that are either destined for insertion into the plasma membrane or recently retrieved by endocytosis. This raises the possibility that, as has been found for other ion channels (Zhang et al., 2008), channel activity at release sites is modulated by rapid recruitment and retrieval to and from an intracellular pool of channels. This allows the number of channels in the plasma membrane to change rapidly in response to cycles of exocytosis/endocytosis that are triggered by second messenger pathways.

Our findings indicate that Kv1.3 is not expressed uniformly across the MNTB. There exists a strong gradient of Kv1.3 protein expression that increases along the medial-to-lateral and along the posterior-anterior axes of the nucleus. Gradients of expression of ion channels along the tonotopic medial-to-lateral axis have been described for other channels in the MNTB, such as the high-threshold Kv3.1b channel (Brew and Forsythe, 2005; Li et al., 2001; von Hehn et al., 2004) as well as for the low-threshold Kv1.1 currents in the MNTB (Brew and Forsythe, 2005) and the LSO, a target of MNTB axons (Barnes-Davies et al., 2004). The direction of the Kv1.3 expression gradient along the tonotopic axis within the MNTB is opposite to that of both Kv3.1b and to the low-threshold currents ascribed to Kv1.1, such that highest levels of Kv1.3 are found in those cells that are most sensitive to low frequency sounds and that are located at the lateral aspect of the MNTB. Kv1.3 differs in its kinetic properties from other Kv1 family members that are expressed in the MNTB in that it undergoes a slow cumulative use-dependent inactivation during repeated depolarizing pulses (Marom and Levitan, 1994). Thus these kinetic properties could play a role in regulating neurotransmitter release specifically in low-frequency neurons. Nevertheless the physiological significance of the Kv1.3 gradient along either the medial-lateral axis is not yet known. It should be noted that, in addition to potassium channels, other components that regulate the physiological functions of this circuit, including neurotransmitter receptors and signaling molecules, have been found to be expressed along tonotopic gradients. (Person et al., 2004; Sanes and Constantine-Paton, 1983; Sanes et al., 1987).

Our findings also indicate that there exists a strong gradient of Kv1.3 levels along the posterior-anterior axis of the MNTB. In contrast to the medial-lateral axis, no tonotopic organization of neuronal responses has been found along this axis (Sonntag et al., 2009). It is very possible that responses along the posterior-anterior represent some other, aspect of auditory information that has yet to be defined. Nevertheless, this finding also raises the possibility that the contribution of Kv1.3 channels to the excitability of the presynaptic terminals could be relatively minor and secondary to a cellular signaling function. Recent findings have shown that many ion channels have “non-conducting” functions in addition to their role in shaping firing patterns (Kaczmarek, 2006). Gating of the Kv1.3 channel has been found to have cellular effects that include the regulation of integrin signaling, which may result from a direct biochemical interaction between the Kv1.3 channel and β -integrins (Artym and Petty, 2002; Fordyce et al., 2005; Levite et al., 2000; Pardo, 2004). By analogy with these findings in non-neuronal cells, a major role of Kv1.3 in presynaptic terminals

could be to act as a voltage sensor that regulates activity-dependent development of the terminal and modulates its biochemical properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Immunolocalization of Kv1.3 potassium channels subunits in the MNTB using diaminobenzidine labeling. A. Low power view of part of a coronal section of brainstem, showing bilateral immunostaining of MNTBs in a wild-type (wt) mouse (white arrows). B. Image of a coronal section of brainstem from a Kv1.3^{-/-} mouse, immunostained for Kv1.3 in parallel with and in the same wells as the wild type section in A. C. Coronal section view of a single Kv1.3-immunostained MNTB from a wild-type mouse. D. Kv1.3-immunostained section as in C, but from a Kv1.3^{-/-} animal. E and F. Higher power images of Kv1.3-immunostained principal neurons within the MNTB of wild-type mice. Immunostaining is shown for neurons in the lateral (E) and medial (F) aspects of the same MBTB. Black arrows point to example of highly punctate staining at the periphery of the cell.

Figure 2.

Gradients of Kv1.3 immunoreactivity in sagittal and coronal sections of the MNTB. A. Sagittal section of MNTB immunostained for Kv 1.3. (scale bar, 20 µm). B and C. Coronal (B) and sagittal (C) sections were divided into 5 equal zones for quantification of immunoreactivity. In coronal sections (B), highest levels of immunoreactivity are observed in neurons in lateral regions of the nucleus. D and E. Quantification of Kv1.3 gradients. Five individual sections from five separate animals were used to quantify the lateral-to-medial gradient in the coronal plane (E) or the anterior-to-posterior gradient in the sagittal plane (D). The optical density of Kv1.3 immunoreactivity in each zone was measured and normalized to the darkest cell in each section. Error bars represent the standard error of mean optical density (O.D.) of regions above threshold in each zone.

Figure 3.

Comparison of localization patterns of Kv1.3 in MNTB with Kv1.1, Kv1.2 and Kv1.6. Top row (A–C) shows double immunofluorescence labeling of Kv1.3 with Kv1.1. A and B show Kv1.3 and Kv1.1 immunolabeling and C shows the merged fluorescence images. Panels D–F, as for A–C but for co-labeling of Kv1.3 with Kv1.2. Panels G–I, as for A–C but for co-labeling of Kv1.3 with Kv1.6. Rabbit anti-Kv 1.3 primary immunoreactivity was localized using Alexa 488 (left panels) and mouse anti-Kv 1.1, -Kv1.2 and -Kv1.3 primary antibodies were localized using Cy 3-labeled secondary antibodies (center panels). Merged images are shown in right column. White arrows in center panels of A and B indicate staining of axonal tracts by anti-Kv1.1 and anti-Kv1.2 antibodies. Scale bar: 20 μ m. A magenta-green copy of this figure is available as supplementary figure S2.

Figure 4.

Co-localization of Kv 1.3 with syntaxin, synaptophysin and synaptotagmin. Double immunofluorescence labeling of Kv1.3 with syntaxin (*top panels A–E*), synaptophysin (*center panels F–J*) and synaptotagmin (*bottom panels K–O*). Kv 1.3 immunoreactivity was labeled with Alexa 488, (green, *panels A, F and K*), Syntaxin, Synaptophysin and Synaptotagmin were labeled with Cy3 (red, *panels B, G and L respectively*). The third column (*panels C, H and M*) shows co-localization in merged images. The scale bars of the first three columns are 20 μm . The last two columns (*panels D, E, I, J, N and O*) show enlarged parts of the respective merged sections (Scale Bar 5 μm). A magenta-green copy of this figure is available as a supplementary figure S3.

Figure 5.

Electron microscopic immunogold labeling of Kv 1.3 in the MNTB of Wild-type and Kv 1.3 $-/-$ mice. A and B. Examples of sections stained for Kv1.3 using 10 nm gold particles. Particles are localized primarily to pre-synaptic structures, where they are found in association with either the plasma membrane (black arrows) or intracellular organelles (white arrows). Parts of the plasma membrane have been traced with a white dotted line (PM). A lower number of particles can sometimes be detected postsynaptically in the cytoplasm (black asterisk in A) or in mitochondria (white arrow in A). C and D. No immunogold particles could be detected in sections from Kv 1.3 $-/-$ animals except for rare background staining (thin black arrow in C).

Figure 6.

Electron microscopic co-localization of Kv1.3 with synaptophysin in wild-type and Kv1.3^{-/-} animals. Double immunogold labeling was carried out using large (10 nm) gold particles to detect synaptophysin and smaller (5nm) particles to localize Kv 1.3. A and B. High power images showing the co-existence of small and large particles in MNTB of wild-type animals. The smaller Kv1.3 particles are labeled with red arrows. Scale bar, 20 nm. C and E. Lower power views that include the particles shown in panels A and B respectively (yellow boxes). Scale bars 100 nm. D and F. Graphical depiction of the location of small (red) and large (green) particles in images C and E, respectively. Both large and small particles are confined to the same presynaptic structure. G and I. Lower power views of Kv1.3 and synaptophysin gold particle localization using wild-type (G) and Kv1.3^{-/-} animals. A higher ration of anti-Kv1.3 to anti-synaptophysin antibody was used for the experiments in G and I. Scale bar, 100 nm. H and J. Graphical depiction of the location of small (red) and large (green) particles in images G and I, respectively. A high density of small Kv1.3 particles is associated with the synaptophysin-labeled terminal in wild-type animals, but only very occasionally can small particles be detected in images from Kv1.3^{-/-} animals. A magenta-green copy of this figure is available as supplementary figure 4.

Table 1

Protein	Species	Catalog No.	Lot No.	Immunogen	Source	Dilution
Kv 1.3 (extracellular)	Rb	APC -101	AN-02, AN-06	Peptide KDYPASTSQDSFEA(C) corresponding to residues211–224 of human Kv1.3 (Accession P22001)	Alomone labs	1:100
Kv 1.3 (extracellular)	Rb	APC -101F	AN-01	Peptide KDYPASTSQDSFEA(C) corresponding to residues211–224 of human Kv1.3 (Accession P22001)	Alomone labs	1: 100
Synaptophysin	Ms	S5768 clone SVP-38	na	A synaptosome preparation from rat retina was used as immunogen	Sigma	1: 200
Synaptotagmin	Ms	MAB 5202	na	Rat brain synaptic junctional protein complexes	Chemicon	1: 500
Syntaxin	Ms	S0664 clone HPC-1	064K4789	A synaptosomal plasma -membrane fraction from adult hippocampus	Sigma	1: 200
Synaptophysin 1	Rb	101 002	na	Synthetic peptide GPOGAPTSFSNQM (aa 301–313 in human synaptophysin 1) coupled to key-hole limpet hemocyanin via an added N-terminal cysteine residue	Synaptic Systems	1: 500
Synaptophysin	Rb	G 95	na	It was generated by immunization with p38 isolated from synaptic vesicles fractions of rat brain	Gift from P. D. Camille P. Greengard and R. Jahn	1: 500
Kv1.1	Ms	75-007 clone K20/78	440-4HK-42	Synthetic peptide amino acids 458–476 C terminus of rat Kv 1.1 (EEDMNNISIAHYRQANIRGTG; accession number P10499)	NeuroMab	1: 100
Kv 1.2	Ms	75-008 clone K14/16	413-7RR-30	Antibody produced against bacterially-expressed GST-fusion protein corresponding to amino acids 428–499 of rat heart Kv 1.2 Epitope mapped to within amino acids 463–480	NeuroMab	1: 100
Kv 1.3	Ms	75-009 cloneL23/27	413-5RR-07	C-terminus) of rat Kv 1.3 (GGMNHSAFPQTPFKTGNYSY ATC, accession number P15384)	NeuroMab	1: 100
Kv1.6	Ms	75-012 cloneK19/36	413-5RR-51A	Synthetic peptide 507–525 (RERRSSYLPTPHRAYAEKR; cytoplasmic C-terminus) of rat Kv 1.6 (accession number P17659)	NeuroMab	1: 100
PSD 95	Rb	2507	1	Synthetic peptide (KLH-coupled) toaa310–336 of the human PSD 95 protein.	Cell Signaling	1:200

Ms: Mouse, Rb: Rabbit, na: not available

Table 2

Kv 1.3 immunoreactivity in auditory brainstem nuclei. The number of + signs indicates relative density of Kv1.3 expression within neurons of the indicated nuclei.

Area	Kv1.3 immunostaining
Medial Nucleus of Trapezoid body (MNTB)	++++
Lateral Superior Olive (LSO)	++
Dorsal Cochlear nucleus	++
Granular layer, cochlear nucleus	++
Ventral Cochlear nucleus	++
Lateral lemniscal nuclei	++
Dorsal periolivary region (DPO)	+
Superior periolivary nuclei (SPO)	+
Medial superior olive (MSO)	+