Localization of A-type K\(^+\) Channel Subunit Kv4.2 in Rat Brain

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Abstract

Kv4.2, a voltage-gated K\(^+\) (Kv) channel subunit, has been suggested to be the key component of the subthreshold A-type K\(^+\) currents (I\(_{SA}\)) recorded from the specific subcellular compartments of certain CNS neurons. To correlate Kv4.2 localization with the I\(_{SA}\) detected, immunohistochemistry will be useful. Although the Kv4.2 immunostaining pattern in the hippocampus and cerebellum has been reported, the Kv4.2 antibody used was not specific. Furthermore, Kv4.2 localization in other brain regions remains unclear. In this report, we first demonstrated the specificity of a new Kv4.2 antibody, and then used it to examine Kv4.2 localization throughout adult rat brain by immunohistochemistry. At the cellular level, Kv4.2 was found in neurons but not glia. At the subcellular level, Kv4.2 was localized in the somatodendritic compartment of most neurons examined. Nevertheless, our preliminary data indicated that Kv4.2 might be also present in the axon/terminal compartment. At the functional level, our data indicates that Kv4.2 localization and I\(_{SA}\) correlate quite well in some CNS neurons, supporting that Kv4.2 is the key component of some I\(_{SA}\) recorded in vivo.

Key Words: voltage-gated potassium channel, Kv4.2, subthreshold A-type K\(^+\) currents, immunohistochemistry, subcellular localization, immunoprecipitation

Introduction

A-type K\(^+\) currents (I\(_{SA}\)) are activated transiently and inactivated rapidly even during membrane still in depolarizing state. I\(_{SA}\) can be activated over a very wide range of membrane potentials (-80 to -10 mV). Subthreshold A-type K\(^+\) current (I\(_{SA}\)), a type of I\(_{SA}\), has been specified because it can be activated below the threshold of action potential (-55 mV). Since I\(_{SA}\) delays the time required for membrane depolarization to reach the threshold for action potential firing, it can regulate the frequency of action potentials (1). Native I\(_{SA}\) have been recorded from the somatodendritic compartment of many CNS neurons, such as the cerebellar granule cells (2), hippocampal CA1 and CA3 pyramidal neurons (3,4), thalamic relay neurons (5), and a type of pyramidal neurons in the layer V of cerebral cortex (6). In addition, I\(_{SA}\) have been also recorded from the axon/terminal compartment, such as the nerve terminals of posterior pituitary (7,8), and the axons of hippocampal CA3 pyramidal cells (9).

So far only the voltage-gated K\(^+\) (Kv) channel Kv4 members (Kv4.1, Kv4.2, Kv4.3) have the ability to evoke I\(_{SA}\) in heterologous expression systems (10). In Xenopus oocytes, Kv4.2 subunit alone is significantly activated between -40 mV to -50 mV (1,11). When Kv4.2 is co-expressed with the unidentified factor(s) encoded in low molecular weight brain mRNAs or the Kv channel-interacting protein (KChIP), its activation V\(_{1/2}\)
shifts to more negative membrane potentials by 10-30 mV(1,10), similar to the $i_{\text{Na}}$ recorded in vivo (2-9).

To correlate Kv4.2 localization with $i_{\text{Na}}$ detected in the specific subcellular compartments of those CNS neurons, immunohistochemistry will be a useful approach. Although the immunohistochemical localization of Kv4.2 has been reported (12-15), the Kv4.2 antibody seems non-specific. Comparing the immunogenic peptide sequence of that Kv4.2 antibody (Kv4.2C: residues 484-502, CLEKTTNHEFVDEQVEES) (12-15) and the corresponding Kv4.2 peptide sequence (residues 482-500, CLEKTNHEFIDEQMFEQDN) (16), only two amino acids at the C-terminal are not similar. It is possible that the Kv4.2 antibody used previously could also recognize Kv4.3. Furthermore, only immunostaining in the hippocampus and cerebellum has been addressed (12-15, 17), while other brain regions remain unclear. In this report, we first demonstrated the Kv4.2 antibody used here is specific to Kv4.2, but not the Kv4.2C antibody. Then, by immunohistochemistry, we examined the localization of Kv4.2 throughout the whole adult rat brain.

Materials and Methods

Antibodies and Antigenic Peptides

Anti-Kv4.2 and anti-Kv4.3 antibodies, as well as their antigenic peptides, were purchased from Alomone Labs (Jerusalem, Israel). The sequence of the Kv4.2 antigenic peptide is (C)SNQI$q$OSDEPAFVSKS, corresponding to residues 454-469 of rat Kv4.2 polypeptide (11), with an additional N-terminal cysteine. The sequence of the Kv4.3 antigenic peptide is (Y) NEALELGTFEEDEH[Norleucine]GK, corresponding to residues 451-467 of rat Kv4.3 polypeptide (16), with an additional N-terminal tyrosine and methionine replaced with norleucine. According to the manufacturer, the Kv4.2 or Kv4.3 polyclonal antibody was raised in rabbits against the antigenic peptide described above, and purified by affinity column chromatography immobilized with the same antigenic peptide. Another Kv4.2 antibody, Kv4.2C, has been described (12).

In Vitro Transcription/Translation

Rat Kv4.2 cDNA was a gift from Dr. Lily Jan (University of California at San Francisco). Plasmids containing rat Kv4.2 cDNA (11) or rat Kv4.3 cDNA (16) were linearized with NotI and Apal, and transcribed in vitro with GppG capping using T7 and T3 RNA polymerases, respectively (Ambion, Austin, TX). In the presence of [35S]-methionine (Pharmacia, Uppsala, Sweden), 1 μg of transcript was translated in vitro using a rabbit reticulocyte lysate system (Promega, Madison, WI).

Immunoprecipitation

Aliquots of the protein mixture synthesized by in vitro transcription/translation were diluted with cold protein buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.5% NP-40) and preabsorbed with normal rabbit sera. For immunoprecipitation, primary antibody (1:100 dilution) was added for 1 hour, followed by
Table 1. Relative Intensity of Kv4.2 Immunoreactivity in Selected Regions of Adult Rat Brain

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Basal ganglia</th>
<th>Brain region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>Caudate putamen</td>
<td>S. oriens</td>
</tr>
<tr>
<td>Piriform cortex</td>
<td>Globus pallidus</td>
<td>Pyramidal cell layer</td>
</tr>
<tr>
<td>Perirhinal cortex</td>
<td>Lateral septum</td>
<td>S. radiatum</td>
</tr>
<tr>
<td>Temporal cortex</td>
<td>Epithalamus</td>
<td>S. lacunosum mol.</td>
</tr>
<tr>
<td>Hippocampal formation</td>
<td>Medial habenula</td>
<td>CA1</td>
</tr>
</tbody>
</table>

CA1
- S. oriens ++
- Pyramidal cell layer ++
- S. radiatum ++
- S. lacunosum mol. ++

CA3
- S. oriens ++
- Pyramidal cell layer ++
- S. lucidum ++
- S. radiatum ++
- S. lacunosum mol. ++

Dentate gyrus
- Granule cell layer +
- Molecular layer +
- Hilus +
- Entorhinal cortex +
- Paraubiculum +
- Presubiculum +

Olfactory bulb
- Glomeral layer +
- External plexiform layer ++
- Mitral cell layer +
- Internal plexiform layer ++
- Granule cell layer +

Anterior olfactory n. +

Cerebellum
- Molecular layer -
- Purkinje cell layer -
- Granule cell layer +++
- Deep n. -

Hypothalamus
- Suprachiasmatic n. +/-
- Lateral mammillary n. ++

Intensity was rated in an arbitrary scale according to the darkness of immunostaining. Intensity: +++ the strongest; ++ strong; + moderate; +/ very low but clearly above background; - background level. Abbreviations: n., nucleus; S., stratum.

protein A- Sepharose (Pharmacia, Uppsala, Sweden) for 45 minutes, with gentle mixing at 4 °C. Competition analysis was performed by pre-incubating antigenic peptides with primary antibody for 1 hour before addition to the immunoprecipitation reactions, at a final concentration of 10 μg/ml for each peptide. Immunoprecipitates were washed with cold protein buffer for 5 times, boiled in SDS sample buffer in the presence of β-mercaptoethanol, and separated by SDS/polyacrylamide gel electrophoresis. Labeled polypeptide bands were visualized by exposing dried gels to x-ray films.

Animals

The experiments were carried out on Sprague-Dawley rats provided by the Animal Center, National Yang-Ming University. Adult male rats (275-300 g) were used. Rats were housed and handled according to the guidelines of Animal Center (National Yang-Ming University).

Immunohistochemistry

Animals were injected intraperitoneally (i.p.) with 2000 units/kg heparin to prevent blood clotting. After
10 minutes, animals were anesthetized with sodium pentobarbital (120 mg/kg, i.p.), and perfused transcardially with normal saline first and then 4% paraformaldehyde in PBS. The brains were removed, dehydrated in 30% sucrose, and cut with a cryostat into 50 μm for free floating sections, or 16 μm for sections attached onto slides. After washing in Tris-buffer saline (TBS, 250 mM Tris [pH 7.5], 8.5% NaCl), sections were treated with 0.2% hydrogen peroxide for 10 minutes for the floating sections, or 20 minutes for the sections on slides. Non-specific binding was blocked by 3% normal goat serum plus 2% BSA in TBS containing 0.3% Triton X-100 for 1.5 hours. Primary antibody was applied at 1:200 dilution for the floating sections, or a 1:100 dilution for the sections on slides, with gentle shaking at room temperature overnight. Sections were washed with TBS, and incubated with biotinylated goat anti-rabbit secondary antibody (1:1000 dilution) and avidin-biotin-horseradish peroxidase.
apparatus. By the insertion of a glass micropipette through a hole on rat skull, 100 ml of 10 mg/ml kainic acid was injected very slowly into the ventromedial/ventroposterior thalamic areas (AP +6.7 mm, ML +1.8 mm, DV +6.8 mm) (18, 20, 21). Animals recovered gradually after the skin was sutured. Eight days after lesion, animals were processed for immunohistochemistry as described above.

Results

Specificity of Two Kv4.2 Antibodies

Using rat brain plasma membrane proteins for immunoblots, the Kv4.2 antibody (Alomone Labs) could recognize a protein band ~70 kDa, which could be competed away by preincubation with excess Kv4.2 antigenic peptide (see Catalog of Alomone Labs, 1999 Spring). Since Kv4.2 and Kv4.3 polypeptides are similar in molecular weight (72 and 69 kDa, respectively), it is not enough to confirm the specificity of Kv4.2 antibody simply by immunoblot. Therefore, to test whether the Kv4.2 antibody can also recognize Kv4.3 polypeptide, we synthesized both Kv4.2 and Kv4.3 polypeptides by in vitro transcription/translation. $[^35]S$-methionine labeled Kv4.2 gene product could be immunoprecipitated by the Kv4.2 antibody, which was blocked by preincubation with excess Kv4.2 antigenic peptide (Fig. 1, upper panel, lanes 1-4). Most significantly, Kv4.3 gene product was not immunoprecipitated by the Kv4.2 antibody (Fig. 1, upper panel, lanes 5-6). In contrast, when applying the Kv4.2C antibody (12-15), 40%-60% of the Kv4.3 gene product (bands on x-ray films estimated by densitometry) was immunoprecipitated (Fig. 1, lower panel). These data demonstrates that the Kv4.2 antibody (Alomone Labs) is specific to Kv4.2 polypeptide, while the Kv4.2C antibody used in previous reports can also recognize Kv4.3 polypeptide and is therefore nonspecific.

General Distribution of K4.2 in Rat Brain

We used the Kv4.2 antibody (Alomone Labs) for immunohistochemistry. Kv4.2 immunostaining was widely distributed in adult rat brain (Figs. 2A-G, 3A-D), and the characteristic pattern could be completely abolished by preincubation with excess Kv4.2 immunogenic peptide (Fig. 2H). The strongest immunoreactivity was detected in the hippocampus.
cerebellar granular layer, olfactory bulb, and habenular nucleus (Figs. 2A-G, 3A-D). The relative intensity in various brain regions is summarized in Table 1. When compared with the Kv4.2 C immunostaining pattern reported previously (12), a major difference appeared in the thalamus. Immunostaining in the thalamus was generally low, except the reticular thalamic nuclei, dorsal lateral geniculate, and paraventricular thalamic nuclei (Figs. 2B, C; Table 1). For comparison, we also did immunohistochemistry with the Kv4.2 C antibody in adjacent sections. Indeed, strong immunostaining was detected in almost all thalami (Fig. 2I). We also found that Kv4.2 C staining was present in the hypothalamus (Fig. 2I). Coincidently, heavy Kv4.3 immunoreactivity was detected in the thalamus and hypothalamus where strong Kv4.2 C staining was located (Tsaur et al., unpublished observation), indicating that the Kv4.2 C antibody could also recognize Kv4.3. In addition to immunoprecipitation described above, immunostaining presented here also demonstrates that the Kv4.2 C antibody is not specific.

Localization of Kv4.2 protein in other selected brain regions is analyzed below.

**Hippocampus**

The most characteristic pattern of Kv4.2 staining was in the hippocampal CA1 region (Figs. 2B-D, F, G; Fig. 4B). Although the Kv4.2 mRNA signal appeared equally abundant in the CA1 pyramidal cells and dentate granule cells (Fig. 4A), it could be higher in the CA1 pyramidal cells, because signal is more easily trapped in the highly compacted granule cells than the loosely
stratum radiatum, where the basal and apical dendrites of CA1 pyramidal cells arborize, respectively. Moderate staining was detected in the stratum lacunosum moleculare. Nevertheless, their somata or proximal dendrites located within the pyramidal cell layer showed only background level of staining. Similar but weaker expression pattern was found in the CA3 region, except that staining was also absent from the stratum lucidum (Fig. 4B). In the dentate gyrus, immunostaining was concentrated in the molecular layer where the dendrites of granule cells arborize, but absent from their somata and proximal dendrites located in the granule cell layer (Figs. 4B, C). Surprisingly, we found a stronger immunoreactivity in a narrow zone in the molecular layer near the hilus, which was composed of discontinuous varicosities (Figs. 4B, C). These might be the nerve terminals of axons projecting from the supramammillary area of hypothalamus (22). However, since the immunoreactivity in the molecular layer was quite strong, sometimes this zone could not be observed clearly. This may be the reason why it has not been addressed in previous reports.

Cerebellum

Strong Kv4.2 immunoreactivity was detected only in the granule cell layer, but not in the Purkinje cell layer or molecular layer (Fig. 5A). Within the granule cell layer, staining was concentrated in the glomeruli and lightly at the boundaries of granule cell somata (Fig. 5B), indicating that more Kv4.2 proteins are localized on the dendrites than somata of granule cells. A recent report indicated that Kv4.2 mRNA displayed a decreasing rostral to caudal gradient in the cerebellar granule cell layer (23). Nevertheless, we did not observe this difference in the distribution of Kv4.2 mRNA in an earlier study (19). At the protein level, since previous reports did not analyze Kv4.2 expression in various lobules of vermis and hemispheres (12, 15), it was unknown whether Kv4.2 protein also displayed a rostral to caudal gradient. To verify this controversy, we further examined the intensity of Kv4.2 immunostaining in different cerebellar lobules. Our results indicated that Kv4.2 staining was distributed in the granule cell layer evenly throughout the cerebellum, including the ten lobules of vermis, and the lobules in both hemispheres, such as the simplex lobule, ansiform lobules and paraflocculus (Figs. 2E, 5A). This finding demonstrates that Kv4.2 protein is expressed in the somatodendritic compartment of granule cells evenly throughout the whole cerebellum.

Fig. 7  Localization of Kv4.2 in the olfactory bulb. (A) Immunostaining pattern of olfactory bulb in horizontal sections. Strong staining was distributed in the external plexiform layer (EPL) and the internal plexiform layer (IPL, indicated by a white triangle). (B) A higher magnification shows staining in different layers of olfactory bulb. Short lines on the right side indicate boundaries between layers. Abbreviations: AO, anterior olfactory nucleus; GL, glomerular layer; Grl, granule cell layer; ML, mitral cell layer. Scale bar: A, 750 μm; B, 75 μm.
Cerebral Cortex

An intense zone-like staining was detected in the middle of most cerebral cortex (Fig. 2), except the piriform cortex (Fig. 2A). Taking the parietal cortex as an example, under higher magnification (Fig. 6A), immunoreactivity was concentrated on the basal dendrites and somata of pyramidal neurons in layer V, as well as their apical dendrites extending to layer I. Since layer V is next to layer IV, to confirm that Kv4.2 is not present on the thalamocortical projections located in layer IV, unilateral lesion in the ventroposterior and ventromedial thalamus by kainic acid was made. Axons from these two thalamic regions terminate in the layer IV of parietal cortex and occipital cortex. We found that staining in the layer IV of parietal cortex and occipital cortex was not affected (Fig. 6B), demonstrating that Kv4.2 was not localized on the thalamocortical projections. In the retrosplenial cortex, due to compression of layers I-V (Figs. 2B; 6D), staining on these layers became more intense than other cerebral cortex (Fig. 6E), especially the patch-like immunoreactivity in layer I (Fig. 6E). In addition to layer V neurons, a weaker staining was detected in layer VI neurons, in their somata, basal dendrites, and apical dendrites extending to layer I (Figs. 6A, E).

Two different patterns were observed in the perirhinal cortex and the temporal cortex area 2 (Te2), although they are two parts of cerebral cortex link together anatomically. Te2 showed strong staining in layer V (except at the junction right next to perirhinal cortex), a pattern similar to parietal cortex (Fig. 6C). Oppositely, expression of Kv4.2 in the perirhinal cortex was not the same as the rest of cerebral cortex. Staining was strong in layers I-IV but absent from layer V in the perirhinal cortex, similar to that in the entorhinal cortex, a part of hippocampal formation (Fig. 6C).

Olfactory Bulb

Heavy Kv4.2 staining was detected on fibers throughout the entire external plexiform layer (EPL), and many cell bodies scattered in the granule cell layer (Figs. 7A, B). Since the olfactory granule cells lack axons, we reasoned that Kv4.2 protein was localized on the somata of superficial granule cells (G_{s}), deep granule cells (G_{d}) and intermediate granule cells (G_{i}), as well as their dendrites ramifying in the superficial EPL, deep EPL and all levels of the EPL, respectively. Consistently, Kv4.2 mRNA was abundantly expressed in the granule cell layer of olfactory bulb (23). Another strong staining was found in a thin zone at the junction of the mitral cell layer and the internal plexiform layer (Fig. 7A). There are many interneurons located at this junction, such as Cajal cells and horizontal cells (24). However, due to the heavy staining on the granule cell dendrites passing through this region, it has been hard to tell whether Kv4.2 is localized on these interneurons. Although staining is weak in the glomerular layer, many dot-like cells scattered outside this layer showed significant staining (Fig. 7B). These cells are likely to be olfactory sensory neurons.

Summary

At the cellular level, Kv4.2 was localized in neurons but not glial cells in adult rat brain. At the subcellular level, Kv4.2 was mainly detected in the somatodendritic compartment of most CNS neurons examined. Nevertheless, Kv4.2 staining was also found in the axon/terminal compartment, namely, in the terminal field of hippocampal molecular layer and the internal plexiform layer of olfactory bulb. At the regional level, expression of Kv4.2 protein in the cerebellar granule cells is evenly throughout the granule cell layer in the whole cerebellum.

Discussion

In this report, by immunoprecipitation and immunohistochemistry, we first demonstrated that the Kv4.2C antibody used in previous studies was not specific, because it could also recognize Kv4.3. Then, combining in situ hybridization and immunohistochemistry, as well as a lesion approach, a comprehensive picture of Kv4.2 localization in adult rat brain has been provided. New insights have been raised from the re-examination of hippocampus and cerebellum. The localization of Kv4.2 in the cerebral cortex and olfactory bulb has also been analyzed carefully. These data are useful in the identification whether Kv4.2 is responsible for the native I_{KS} recorded from the specific subcellular compartment of some CNS neurons. Most importantly, information provided here should be helpful in elucidating the role of Kv4.2 in the CNS. The significance of Kv4.2 expression in the specific subcellular compartment of some CNS neurons is discussed below.

In the hippocampus, in the CA1/CA3 pyramidal neurons and dentate granule cells, we found that tremendous amount of Kv4.2 protein was expressed in their distal dendrites, but only in background levels on the somata and proximal dendrites. However, Kv4.2 staining was found on the somata of these hippocampal
Deep in the olfactory bulb, Kv4.2 was abundantly expressed in the dendrites of all types of GABAergic granule cells (types I, II, and III). In the EPL, the dendrites of granule cells receive excitatory input from the secondary dendrites of mitral and tufted cells. Excitation of these granule cell interneurons then make negative feedback to the secondary dendrites of mitral and tufted cells, and results in prolonged inhibition of mitral and tufted cells. Consistently, a type of $I_A$ (not $I_{SA}$) recorded from the somatodendritic compartment of olfactory granule cells, has been found to be important in regulating the inputs from mitral and tufted cells (31). Based on the intense immunoreactivity in the granule cells presented here, Kv4.2 is probably the channel molecule responsible for this $I_A$.

Finally, it has been demonstrated that Kv4.2 is localized only in the somatodendritic compartment at the subcellular level (12-15). Here, our preliminary evidence suggests that Kv4.2 is also present in the axon/terminal compartment. Since only Kv4 subunits can evoke $I_{SA}$, our finding could be supported by the electrophysiological experiments reported previously, that is, $I_{SA}$s have been recorded from the axon/terminal compartment (7-9). However, to provide a direct evidence for presynaptic localization, further analysis using electronic microscopy will be required.

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