FEBS Letters 547 (2003) 162–164 FEBS 27442

Differential association of the auxiliary subunit Kvβ2 with Kv1.4 and Kv4.3 K^+ channels

Lin Wang[a,1], Koichi Takimoto[b], Edwin S. Levitan[a,*]

[a] *Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA 15261, USA*
[b] *Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA 15261, USA*

Received 8 May 2003; revised 9 June 2003; accepted 12 June 2003

First published online 23 June 2003

Edited by Maurice Montal

Abstract Kvβ2 subunits associate with Kv1 and Kv4 K^+ channels, but the basis of preferential association is not understood. For example, detergent resistance suggests stronger auxiliary subunit association with Kv4.2 than with Kv1.2, but Kvβ2 preferentially localizes with the latter channels in brain. Here we examine the interaction of Kvβ2 with two native binding partners in brain: Kv4.3 and Kv1.4. We show that the auxiliary subunit binds more efficiently to Kv1.4 than to Kv4.3 in mammalian cells. However, preexisting Kvβ2 complexes with Kv1.4 and Kv4.3 have similar detergent sensitivity. Thus, preferential steady state binding may reflect a difference in initial association rather than stability. We also find that that the cytoplasmic C-terminus of Kv4.3 inhibits Kvβ2 association. Apparently, a region proximal to the Kv4.3 pore contributes to the inefficient auxiliary subunit interaction that produces preferential binding of Kvβ2 to Kv1 channels.

Key words: Potassium channel; Ancillary subunit; Subunit assembly; Channel biogenesis; T1 domain

1. Introduction

Auxiliary cytoplasmic subunits affect expression of voltage gated K^+ channels. The first such auxiliary subunits were the Kvβ proteins that were identified as a component of the dendrotoxin receptor [1]. These subunits are known to associate with the pore forming Kv1 subunits early in biogenesis of the channels in the endoplasmic reticulum [2]. This association is based on direct binding to a region of the N-terminus of Kv1 channels called the T1 domain [3–5]. This results in altered gating and enhanced surface expression [6,7].

Recently, it was discovered that Kvβ subunits also associate with Kv4 family channels. This was originally detected in biochemical and electrophysiological studies of Kvβ2 and Kv4.2 in heterologous expression systems [8,9]. Subsequently, Kvβ2 was found to be associated with native brain Kv4.3 channels [10]. This interaction does not alter gating, but total Kv4.3 channel protein and activity are enhanced by the auxiliary subunit. In contrast, Kvβ3 can alter both expression and gating of Kv4.3 channels [11].

Previous experiments generated two unexpected results. First, because Kv1 and Kv4 subunits are related, it was expected that the basis of Kvβ association should be identical. However, studies of chimeric channels showed that the C-terminus of Kv4.3 affects this association [10], while the N-terminal T1 domain of Kv1 channels is sufficient to explain Kv1 interaction [3–5]. Second, the auxiliary subunit mostly colocalizes with brain Kv1 channels [12], and only associates with a fraction of brain Kv4.3 protein [10], but binding to Kv4.2 is more detergent resistant than complexes with Kv1.2 [8]. To explore these issues, we have compared the interaction of Kvβ2 with Kv1.4 and Kv4.3. These subunits are native binding partners in brain that do not require the auxiliary subunit for expression. Because the latter association does not produce a gating effect, a biochemical approach was used to measure subunit–subunit binding. Our results indicate that the detergent resistances of Kvβ2 association with Kv4.3 and Kv1.4 are comparable. However, expression in intact cells shows that association with Kv1.4 is preferred. This can be attributed in part to an inhibitory role for the C-terminus of Kv4.3.

2. Materials and methods

2.1. Construction of myc tagged Kv channels

Six myc epitope tags were removed from pCS2+MT vector and inserted before the start codons of the rat Kv1.4 cDNA and the rat Kv4.3 long isoform cDNA [13] in the pRC-CMV vector. The C-terminal truncation constructs ΔC62, ΔC179, and ΔC225 were created by inserting stop codons by site directed mutagenesis.

2.2. Cell culture and transfection

HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. cDNAs were transiently transfected by calcium phosphate–DNA coprecipitation.

2.3. Protein–protein interaction assay by coimmunoprecipitation

Forty-eight hours after transfection, cells were harvested in cold 1×phosphate buffered saline (PBS) and lysed in 20 mM Tris–HCl, 0.15 M NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM iodoacetamide and 0.2 mM phenylmethylsulfonyl fluoride, pH 7.4. After centrifugation at 10 000×*g* for 10 min, 1 μl of supernatant from each sample was mixed with protein assay dye solution (Bio-Rad) to measure the total protein concentration at 595 nm wavelength by spectrophotometer. Then 300 μg of total protein from each sample was incubated with protein G beads (Sigma) prebound with a monoclonal anti-β2 antibody (kindly provided by J.S. Trimmer) at 1:50 for the coimmunoprecipitation, and precipitated material of total extract was applied to immunoblot. After denaturation, all the samples were mixed with 2×sodium dodecyl sulfate (SDS) sample buffer and denatured at 100°C for 5 min. All the denatured samples were analyzed by 7.5% SDS–polyacrylamide gel electrophoresis followed by a membrane

*Corresponding author. Fax: (1)-412-648 1945.
E-mail address: levitan@server.pharm.pitt.edu (E.S. Levitan).

[1] Present address: Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA.

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00705-1

transfer. The transferred nitrocellulose membrane was then blocked in 5% non-fat dry milk in 1×PBST (PBS with 0.1% Tween 20) overnight at 4°C followed by an incubation with a polyclonal rabbit anti-myc primary antibody (Research Diagnostics) at 1:400 at 4°C for 4–6 h. Then the membrane was washed in 1×PBST followed by incubation with a horseradish peroxidase conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) at 1:3000 at room temperature for 1 h. Then the membrane was washed in 1×PBST and incubated with chemiluminescent solution (Perkin-Elmer) at room temperature for 1 min. The protein signals were detected on X-ray films (BioMax, Kodak). Finally, to detect Kvβ2 gene expression, the nitrocellulose membrane was first stripped in Restore Western Blot Stripping buffer (Pierce) at room temperature for 15 min to eliminate the bound primary and secondary antibodies. Then the membrane was blocked again in 5% non-fat dry milk in 1×PBST as described above and incubated with a polyclonal rabbit anti-β2 antibody (Quality Controlled Biochemicals) at 1:400 at 4°C for 4–6 h. Secondary antibody incubation and protein detection are as described earlier.

3. Results

Previous SDS resistance studies suggested that Kvβ2 association with Kv4.2 is stronger than with Kv1.2 [8]. To date, Kvβ2 association with native brain Kv4.2 has not been shown. Furthermore, Kv1.2 surface expression in the absence of auxiliary subunits is inefficient. Therefore, we compared Kvβ2 association with two known binding partners in brain that express well on their own: Kv1.4 and Kv4.3. By using epitope tagged Kvα subunits, we ensured that signals from the two distinct subunits expressed in HEK293 cells could be compared directly. Each construct was expressed with Kvβ2, or all three subunits were expressed together to allow direct competition for the auxiliary subunit in the same cells. Transfection conditions were adjusted so that Kv1.4 and Kv4.3 protein levels were similar (Fig. 1A) and so that Kvβ2 was also present (Fig. 1B). Immunoprecipitation with anti-Kvβ2 antibodies revealed that association was more efficient with Kv1.4 than Kv4.3 whether the subunits were expressed separately or together (Fig. 1C). Hence, Kvβ2 prefers to associate with the Kv1 subunit rather than the Kv4 subunit in mammalian cells.

Because this result seems to contradict the conclusions based on Kv1.2 and Kv4.2 detergent resistance experiments, we examined the SDS resistance of Kvβ2–Kv4.3 and Kvβ2–Kv1.4 complexes. Kvβ2 is efficiently immunoprecipitated in the detergent concentration range used here ([8]; data not shown). However, the higher levels of SDS inhibit coprecipitation of the Kvα subunits. Indeed, the detergent sensitivities of auxiliary subunit association with Kv1.4 (Fig. 2A) and Kv4.3 (Fig. 2B) are similar. This result is consistent with the conclusion that Kvβ2 complexes with Kv1.4 and Kv4.3 are comparably strong once formed, even though the generation of the Kv1.4 complex is preferred. Of course, other measurements, such as dependence on ionic strength, might reveal that Kvβ2 association is not identical between Kv1 and Kv4 subunits. Nevertheless, this result shows that the conclusion of much stronger association with Kv4.2 than Kv1.2 cannot be generalized to Kv4.3 and Kv1.4.

The comparable detergent resistance is surprising when one considers that the C-terminus of Kv4.3 affects binding to Kvβ2 [10], while the N-terminus of Kv1 subunits mediates auxiliary subunit association [3–5]. To identify the relevant region of the Kv4.3 C-terminus, epitope tagged Kv4.3 and

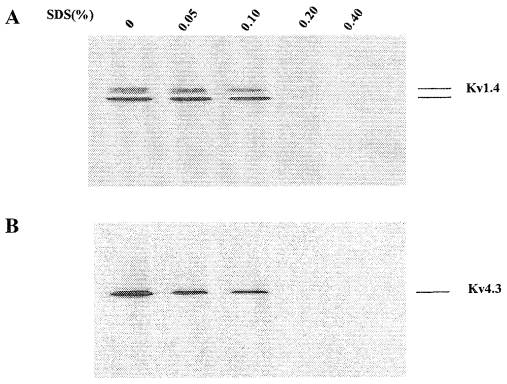

Fig. 2. Detergent resistance of Kvβ2 complexes with Kv1.4 and Kv4.3. Epitope tagged Kv1.4 (A) and Kv4.3 (B) were cotransfected with Kvβ2. Immunoprecipitation of the auxiliary subunit was performed in 0, 0.05%, 0.1%, 0.2% and 0.4% SDS. Note that associations of Kv1.4 and Kv4.3 show similar detergent sensitivity.

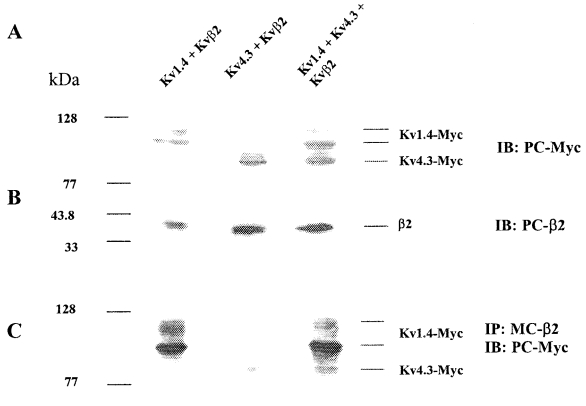

Fig. 1. Kvβ2 interacts with Kv1.4 more efficiently than with Kv4.3. Kvβ2 was expressed with Kv1.4, Kv4.3 and with the two pore forming subunits together to allow competition for the auxiliary subunit. A: Immunoblot (IB) showing epitope tagged Kvα subunits expressed separately and together. B: Immunoblot showing Kvβ2 expression in each of the three conditions. C: Immunoblot showing Kv1.4 and Kv4.3 proteins immunoprecipitated (IP) with Kvβ2.

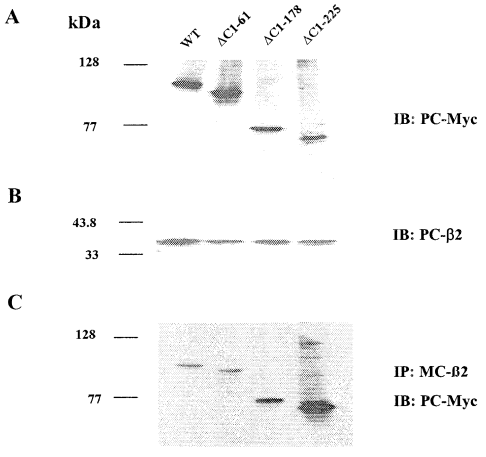

Fig. 3. A region of the Kv4.3 C-terminal cytoplasmic region inhibits association with Kvβ2. A: Immunoblot (IB) of epitope tagged full length and truncated Kv4.3. B: Immunoblot of coexpressed Kvβ2. C: Immunoblot showing Kv4.3 constructs immunoprecipitated (IP) with Kvβ2. Note that the largest deletion leads to increased association.

three C-terminal truncations, ΔC62, ΔC179 and ΔC225, were expressed with Kvβ2 in HEK293 cells. Cell lysates were either used for immunoprecipitation with anti-Kvβ2 antibody for assaying protein–protein interaction or for immunoblot for measuring expression of Kvα and Kvβ subunit protein. Immunoprecipitation showed that the two larger deletions associate more with Kvβ2 than the full length Kv4.3 channel (Fig. 3). Hence, the C-terminus of Kv4.3 affects association with the auxiliary subunit, but this interaction is inhibitory. This effect is particularly striking for the ΔC225 construct indicating that the region close to the pore reduces association with Kvβ2.

4. Discussion

This study was motivated by two conundrums posed by previous results. First, initial detergent resistance experiments suggested that association of Kvβ2 with Kv1 channels is weaker than with Kv4 channels [8], but the auxiliary subunit is found colocalized with Kv1 subunits in brain [12], and only immunoprecipitates a fraction of brain Kv4.3 protein [10]. Second, although Kv1 and Kv4 subunits are closely related, the binding of Kvβ2 in the former case is to an N-terminal region called the T1 domain [3–5], while the Kv4.3 C-terminus affects interaction with the auxiliary subunit [10]. To address these issues, we quantified immunoprecipitation of Kvβ2 with epitope tagged Kv4.3 and Kv1.4 channels. This approach was favored over electrophysiology because the auxiliary subunit does not alter gating of Kv4.3 channels [10]. Furthermore, epitope tagged Kvα subunits were used so that levels of each protein could be directly compared with a single antibody. These two particular subunits were chosen because they each can be expressed without ancillary proteins, and because both associate with Kvβ2 in vivo.

Our first result is that Kvβ2 associates with Kv1.4 more efficiently than with Kv4.3 in living cells. Thus, colocalization with Kv1 subunits in brain neurons that also express Kv4 subunits is accounted for by this preferential binding. We also showed that this preferential association is not reflected in the SDS resistance of the complexes formed by Kvβ2. We suggest that steady state association of the auxiliary subunit reflects competition between Kv4.3 and Kv1.4 in channel biogenesis. It is known that Kvβ2 associates with *Shaker* and mammalian Kv1 subunits in the endoplasmic reticulum [2,7]. Perhaps, this initial association, which might be cotranslational for Kv1 subunits, is not as efficient for Kv4 channels. On the other hand, detergent resistance may be a measure of strength of association of preformed complexes. Thus, it is possible that mature complexes have similar detergent resistance, even though formation of certain complexes (i.e. Kvβ2–Kv1.4) is favored during channel synthesis.

Our experiments also clarify the role of the Kv4 C-terminus in Kvβ2 binding. Specifically, deletion analysis shows that the C-terminus of Kv4.3 is not required for association with the auxiliary subunit. Hence, the direct binding interactions that mediate Kvβ2 association are likely to be similar for these two related channel subfamilies. Additionally, our results reveal that the C-terminus of Kv4.3 inhibits Kvβ2 association. In particular, the region proximal to the pore tends to prevent formation of Kvβ2–Kv4.3 complexes. Intrasubunit crosslinking studies of *Shaker* channels suggest that this region interacts with the N-terminus of the neighboring Kvα subunit in the complex [14]. Perhaps, such an interaction obstructs Kvβ2 binding to the N-terminus of Kv4 channels. Preliminary results indicate that auxiliary subunit association to Kv1.4 is still favored over the C-terminally truncated version of Kv4.3 (unpublished results). Thus, the Kv4.3 C-terminus contributes to, but does not fully account for, the preferential binding of Kvβ2 to Kv1 channels over Kv4 channels.

Acknowledgements: We thank Dr. James Trimmer (SUNY Stony Brook) for anti-Kvβ2 antibody. This research was supported by NIH Grant R01 HL55312.

References

[1] Scott, V.E., Rettig, J., Parcej, D.N., Keen, J.N., Findlay, J.B., Pongs, O. and Dolly, J.O. (1994) Proc. Natl. Acad. Sci. USA 91, 1637–1641.
[2] Nagaya, N. and Papazian, D.M. (1997) J. Biol. Chem. 272, 3022–3027.
[3] Yu, W., Xu, J. and Li, M. (1996) Neuron 16, 441–453.
[4] Sewing, S., Roeper, J. and Pongs, O. (1996) Neuron 16, 455–463.
[5] Gulbis, J.M., Zhou, M., Mann, S. and MacKinnon, R. (2000) Science 289, 123–127.
[6] Rettig, J., Heinemann, S.H., Wunder, F., Lorra, C., Parcej, D.N., Dolly, J.O. and Pongs, O. (1994) Nature 369, 289–294.
[7] Shi, G., Nakahira, K., Hammond, S., Rhodes, K.J., Schechter, L.E. and Trimmer, J.S. (1996) Neuron 16, 843–852.
[8] Nakahira, K., Shi, G., Rhodes, K.J. and Trimmer, J.S. (1996) J. Biol. Chem. 271, 7084–7089.
[9] Perez-Garcia, M.T., Lopez-Lopez, J.R. and Gonzalez, C. (1999) J. Gen. Physiol. 113, 897–907.
[10] Yang, E.K., Alvira, M.R., Levitan, E.S. and Takimoto, K. (2001) J. Biol. Chem. 276, 4839–4844.
[11] Deschenes, I. and Tomaselli, G.F. (2002) FEBS Lett. 528, 183–188.
[12] Rhodes, K.J., Strassle, B.W., Monaghan, M.M., Bekele-Arcuri, Z., Matos, M.F. and Trimmer, J.S. (1997) J. Neurosci. 17, 8246–8258.
[13] Takimoto, K., Li, D., Hershman, K.M., Li, P., Jackson, E.K. and Levitan, E.S. (1997) Circ. Res. 81, 533–539.
[14] Schulteis, C.T., Nagaya, N. and Papazian, D.M. (1996) Biochemistry 35, 12133–12140.