Inactivation of a Voltage-dependent K⁺ Channel by β Subunit

MODULATION BY A PHOSPHORYLATION-DEPENDENT INTERACTION BETWEEN THE DISTAL C TERMINUS OF α SUBUNIT AND CYTOSKELETON

(J. Biol. Chem. 272, 14021–14024, 1997)

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Kv1.1/Kvβ1.1 (αβ) K⁺ channel expressed in Xenopus oocytes was shown to have a fast inactivating current component. The fraction of this component (extent of inactivation) is increased by microfilament disruption induced by cytochalasins or by phosphorylation of the α subunit at Ser-446, which impairs the interaction of the channel with microfilaments. The relevant sites of interaction on the channel molecules have not been identified. Using a phosphorylation-deficient mutant of α, S446A, to ensure maximal basal interaction of the channel with the cytoskeleton, we show that one relevant site is the end of the C terminus of α. Truncation of the last six amino acids resulted in αβ channels with an extent of inactivation up to 2.5-fold larger and its further enhancement by cytochalasins being reduced 2-fold. The wild-type channels exhibited strong inactivation, which could not be markedly increased either by cytochalasins or by the C-terminal mutations, indicating that the interaction of the wild-type channels with microfilaments was minimal to begin with, presumably because of extensive basal phosphorylation. Since the C-terminal end of Kv1.1 was shown to participate in channel clustering via an interaction with members of the PSD-95 family of proteins, we propose that a similar interaction with an endogenous protein takes place, contributing to channel connection to the oocyte cytoskeleton. This is the first report to assign a modulatory role to such an interaction: together with the state of phosphorylation of the channel, it regulates the extent of inactivation conferred by the β subunit.

In contrast with the well understood structural determinants within the core of voltage-gated K⁺ (Kv) channel proteins that underlie many functional properties (e.g. voltage-sensing regions, pore region; Refs. 1–5), much less is known about the role of extrinsic factors in determining Kv channel function. One such factor is the interaction of ion channels with the cytoskeleton, which could affect structural and functional organization of neuronal elements. Recently, a family of membrane-associated putative guanylate kinases were shown to bind directly to K⁺ channels of the Shaker (Kv1) family and to induce their clustering in COS7 cells (6, 7). This family includes PSD-95, an abundant synaptic protein found both pre- and postsynaptically, and SAP97, a protein found in axons of neurons as well as in non-neuronal cells (for reviews, see Refs. 8 and 9). These data together with the demonstration of colocalization of the channels with PSD-95 (6, 10) in the brain, particularly in nerve terminal plexuses of basket cells in the cerebellum, provide correlative evidence of a direct association in vivo. Apart from a probable role in clustering, it is not known whether the association with PSD-95 has a functional role of modulating the biophysical characteristics of the channel.

For several years now our interest has been the modulation by direct phosphorylation of the voltage-gated K⁺ channel Kv1.1 (originally cloned from rodent brain cDNA libraries; Refs. 11–14), which is expressed in Xenopus oocytes as a delayed rectifier type (15, 16). Recently it became clear that part of the diversity of Kv channels may arise from association of pore-forming K,1 subunits with auxiliary K,β subunits (for review, see Ref. 17) and that the functional consequence of the association between K,1.1 (α) and rat brain K,β1.1 (β) is the appearance of rapid inactivation of the current (18–20). Therefore, we studied also the Kv1.1/Kvβ1.1 (αβ) channel and its modulation by direct phosphorylation (21). We demonstrated that the inactivation of the αβ current is not complete, even under conditions where the α polypeptide is saturated with the β polypeptide, and has an inherent sustained component, indistinguishable from a pure α current. The extent of the inactivation (the fraction of the inactivating component) is increased either by phosphorylation of the α subunits at Ser-446 or by depolymerization of the microfilaments by cytochalasins; the latter effect occludes the former. To account for our findings we proposed a simple model that assumes the existence of two modes of the αβ channels, in one mode inactivation is conferred on the channels by the β, in the other mode no inactivation is observed despite the fact that α is physically associated with β. Interaction of the channels with microfilaments shifts the equilibrium between the two modes toward the noninactivating mode, and phosphorylation (which impairs the interaction of the channels with microfilaments by an unknown mechanism) shifts the equilibrium toward the inactivating mode. The α protein has at its very C terminus a TDV sequence (amino acids 493–495), which presumably mediates the coclustering of Kv1.1 channels with PSD-95 and SAP97 in COS7 cells (7). Thus, we assumed that this site might mediate the interaction of the αβ channels with the oocyte microfilaments; phosphorylation of Ser-446, which is also in the cytoplasmic C terminus of the α protein, seemed an attractive mechanism to modulate this interaction. Supportive of such a notion is the recent demonstration that the binding of the inwardly rectifying K⁺ channel Kir 2.3 to PSD-95 is regulated by phosphorylation of the

* This work was supported by grants from the United States-Israel Binational Science Foundation (to I. L.), the Israel Academy of Sciences (to I. L.), and the Israel Ministry of Sciences (to D. C. and I. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Vol. 272, No. 22, Issue of May 30, pp. 14021–14024, 1997
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Printed in U.S.A.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
channel; however, in this case the phosphorylation occurs at the binding site itself (22).

In this study we set out to test the site of interaction with the cytoskeleton by studying the effect of truncation of the last C-terminal amino acids of the α protein on the extent of inactivation of the phosphorylated and nonphosphorylated αβ channels and their susceptibility to microfilament depolymerization. The results are in agreement with a scenario where the very C terminus of the α subunit in αβ channels interacts with the microfilaments via a PSD-like protein; the interaction is phosphorylation-dependent and affects the extent of channel inactivation.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were from Sigma (Rishon Le-Zion, Israel) unless stated otherwise. Vanadate (sodium orthovanadate) and okadaic acid were from Alomone Laboratories (Jerusalem). [35S]Met/hione/cysteine mix and [γ-32P]ATP were from Amersham Corp. K,1.1 antisera was generated against a 23-amino acid peptide that corresponds to the N terminus of RCK1 ([SGENADEASAAPGHPQDGSYPRQ], as described (15).

DNA Constructs and mRNAs—K,1.1 cDNA was subcloned into a vector to yield a SupEx-RCK1 construct that confers high levels of expression in oocytes (15). All substitution mutants of K,1.1α were subcloned into the same vector. Oligonucleotide-mediated mutagenesis was performed using a mutagenesis kit (CLONTECH) according to the manufacturer’s instructions, using double-stranded DNA as templates and oligonucleotide primers (synthesized by General Biotechnology Inc., Rehovot, Israel) encoding the desired mutations. S446A was generated as described (16); K490cs and T493A were generated on either the WTαβ or S446A mutated molecules using the primers (underlined nucleotides encode the mutated residues): 5’-CTTATAACAGCTAGGTCCTGAGCCG-3’; 5’-GCAAAGCTTGGGCCATGTTGTTA-AAAAGC-3’, respectively. mRNAs were transcribed in vitro using T7 RNA polymerase.

Oocytes, Drug Treatments, and Electrophysiological Recording—Frogs (Xenopus laevis) were maintained and dissected, and their oocytes were prepared as described (23). Oocytes were injected with 2–5 ng/ml of plasmid DNA for two-electrode voltage clamp studies and 100–200 ng/ml K,1.1α mRNA for 200 μg/ml K,1.1β mRNA for macropatch and biochemical studies. The concentrations of the injected mutant RCK1 mRNAs deviated slightly from the above concentrations as they were adjusted to give similar current amplitudes with K,β,1.1. Injected oocytes were incubated at 22 °C for 1–3 days in ND96 solution (96 mM NaCl, 2 mM MgCl2, 1 mM CaCl2, 5 mM HEPES-Tris) (21) supplemented with 2.5 mM pyruvate, streptomycin, and 100 units/ml penicillin (NDE solution) and then assayed either electrophysiologically or biochemically. Cytochalasins were added 4–6 h before the electrophysiological assay.

Two electrode voltage clamp recordings were performed as described (16). To avoid possible errors introduced by series resistance, only currents up to 4 nA were recorded, and in a given experiment the amplitudes of WT and mutant currents were similar. Oocytes were placed in a 1-ml bath continuously perfused with ND96 solution. Currents were elicited by stepping the membrane potential from a holding potential of −80 mV to +50 mV for 70 ms. Net current was estimated by subtraction of the scaled leak current elicited by a voltage step to −90 mV.

Metabolic Labeling with [35S]Met/hione/cysteine ([35S]Met/Cys), Homogenization, and Immunoprecipitation—This was done essentially as described (16). Following injection, six to eight oocytes were incubated at 22 °C for 4 h in NDE solution, then for 3 days in NDE containing 0.2 mM/ml [35S]Met/Cys, and then homogenized in 150–300 μl of medium consisting of 20 mM Tris, pH 7.4, 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 50 μg/ml propionylsulfonyl fluoride, 1 mM iodoacetamide, 1 μM pepstatin, 1 μM 1,10-phenanthroline supplemented with protein phosphatase inhibitors: 50 mM okadaic acid, 0.5 mM vanadate, and 50 mM KF. Debris was removed by centrifugation at 1000 × g for 10 min at 4 °C. After addition of Triton X-100 to a final concentration of 4%, followed by centrifugation for 15 min at 4 °C, antiserum was added to the supernatant for 16 h at 4 °C. The antibody-antigen complex was incubated for 1 h at 4 °C with protein A-Sepharose and then pelleted by centrifugation for 1 min at 8000 × g. Immunoprecipitates were washed four times with immunowash buffer (150 mM NaCl, 6 mM EDTA, 50 mM Tris, pH 7.5, 0.1% Triton X-100); the final wash contained no Triton X-100.

Quantification of Labeling Intensities and Generation of Digitized PhosphoImager Scans—Gels were dried and placed in a PhosphorImager (Molecular Dynamics) cassette for about 1 day. Using the software ImageQuant, a digitized scan was derived, and relative intensities of protein bands were estimated quantitatively by the software ImageQuant as described (16).

Statistical Analysis—Data are presented as means ± S.E.; N denotes the number of oocytes assayed, n denotes the number of oocytes assayed. The Student’s t test was used to calculate the statistical significance of differences between two populations.

RESULTS

The mRNAs of Kv1.1 (α) and Kvβ1.1 (β) were co-injected at a β-to-α mRNA ratio of 100, which ensures saturation of α polypeptides with β. As described before (21), the resulting outward current assayed by the two-electrode voltage clamp technique had a fast inactivating component (Ii) and a non-inactivating sustained component (Is) remaining at the end of a depolarizing pulse to +50 mV. The apparent extent of inactivation (inactivating fraction) was defined as Ii/Ip (Ip = peak current). Previously, we showed that the extent of inactivation of the αs446Aβ channel (in which Ser-446 of the α subunit was substituted with alanine; S446A) is significantly smaller (by about 70%) than that of the WT (αWTβ) channel and related it to the fact that the mutation, unlike the wild-type, channel is not phosphorylated in its basal state in the oocyte (21). In this study the average extent of inactivation of αWTβ current was 0.57 ± 0.02 (mean ± S.E.; N = 3 oocyte batches, n = 41 oocytes) and that of αs446Aβ currents was 0.12 ± 0.025 (N = 6 oocyte batches, n = 78 oocytes).

Truncation of the very end of the C terminus of S446A α polypeptide by deletion of the last six amino acids (substituting Lys-490 with stop codon: K490sc), or substitution of Thr-493 with Ala (T493A), increased by 155 and 65% the extent of inactivation of the respective αs446Aβ channels (see Fig. 1, A and B, open bars). The same mutations in the WT α polypeptide did not increase the extent of inactivation of the corresponding αβ channels (Fig. 2, A and B, open bars). Previously, we showed that treatment of oocytes with 40 μM of the microfilament-disrupting agent dihydrocytochalasin B (DHCb) increases the extent of inactivation, the effect being severalfold larger in the αs446Aβ channels than in the αWTβ channels. If, as we believed, the above increases in the extent of inactivation of the αs446Aβ channels in which the C terminus of the α subunit was truncated are due to some impairment of the interaction of the channels with the cytoskeleton, one would expect that these effects will be occluded by the DHCb treatment. Indeed, in oocytes of the same frogs the inactivations of αs446Aβ and each one of its C-terminal mutants were practically the same after DHCb treatment (Fig. 1B, hatched bars). Thus, whereas the effect of DHCb was about 400% on αs446Aβ, it was more than 2-fold smaller (about 185%) on either one of the mutants. The effects of DHCb on the αWTβ channels (about 10%) and on its C-terminal mutations were much smaller (Fig. 2B, hatched bars).

One possibility to account for the larger extent of inactivation of the αs446Aβ mutants (as compared with αs446Aβ itself) could be that the β-binding capacities of the mutant polypeptides were larger. This possibility was excluded by the concomitant biochemical analysis. SDS-PAGE analysis of [35S]Met/Cys-labeled α and β polypeptides coprecipitated by anti-α antibody showed that the β-binding capacity of all the αs446Aβ polypeptide species is similar, namely, the same amount of β was coprecipitated per equal amounts of any of the α polypeptides (Fig. 3). This result is in accord with our previous data.
Fig. 1. The extent of inactivation of the \( \alpha_{S446A} \beta \) channel is increased by two separate mutations in the very C terminus of the \( \alpha \) subunit and is occluded by DHCB treatment. A, two superimposed representative current traces (normalized to peak current), assayed by two-electrode voltage clamp from oocytes of the same frog expressing \( \alpha_{S446A} \beta \) channels with and without the mutation K490c in the very C terminus of \( \alpha \). B, normalized extent of inactivation of \( \alpha_{S446A} \beta \) and of its two \( \alpha \) subunit mutants (as indicated): basal (open bars) and following DHCB treatment (hatched bars). Oocytes were incubated with or without 40 \( \mu \)M DHCB for 4–6 h prior to two-electrode voltage clamp assay. Numbers above bars indicate the number of frogs assayed; numbers in parentheses indicate the number of cells assayed. A total of six frogs were assayed. Error bars are S.E. *, \( p < 0.0001 \).

Showing that under the conditions of such an experiment the \( \beta \)-to-\( \alpha \) ratio of the coprecipitated polypeptides was the maximal possible, when >90% of \( \alpha \) was saturated with \( \beta \) (21). The same SDS-PAGE analysis (Fig. 3) shows that, as demonstrated before (16), the S446A \( \alpha \) polypeptides migrate as a 54-kDa polypeptide, which is the nonphosphorylated form, whereas the WT \( \alpha \) polypeptide migrates as a doublet of 54- and 57-kDa polypeptides, the latter being the phosphorylated form. It should be noted that the WT \( \alpha \) polypeptide was basally phosphorylated to a large extent, as is evident from the relative intensities of the 57- and 54-kDa bands.

Discussion

Our preceding study (21) demonstrated that the inactivation of the Kv1.1/Kv1.2 (\( \alpha \beta \)) channel is hampered by interaction of the channel with microfilaments and that this interaction can be disrupted to a significant extent by phosphorylation of a single C-terminal residue (Ser-446) of the \( \alpha \) subunit. Thus, in the present study, the \( \alpha_{S446A} \beta \) channel having the S446A \( \alpha \) subunit, which, unlike the WT, is not basally phosphorylated in the oocyte, had a substantially weaker inactivation (~10% of total current) than the \( \alpha_{WT} \beta \) channel (~60%). Consequently, \( \alpha_{S446A} \beta \) displayed a much stronger increase (by ~90%) in inactivation than \( \alpha_{WT} \beta \) (~10%) upon treatment with DHCB, which disrupts the microfilaments (21). Therefore, to look for the interaction between the \( \alpha \beta \) channel and the cytoskeleton, rather than using the wild-type channel, we utilized the \( \alpha_{S446A} \beta \) mutant in which the channel-cytoskeleton interaction is functionally intact and which responds to any reduction in the strength of this interaction by a marked change in inactivation kinetics.

The interaction of the \( \alpha \beta \) channel with microfilaments could occur at sites on both the \( \alpha \) and the \( \beta \) polypeptides. A highly probable candidate was the consensus TDV sequence at the end of the C terminus, recognized by the PDZ domain of the Dlg/PSD-95 family of proteins (for reviews, see Refs. 8 and 24), thus providing cytoskeletal interaction (9) and inducing clustering of Kv1.1 channels in COS7 cells (7). To test this hypothesis, we studied the effects of truncation of the very C terminus of \( \alpha \) subunits, and of a point mutation in the TDV sequence, on the extent of inactivation, both basal and following DHCB treatment, of the \( \alpha_{S446A} \beta \) channels. One would expect that if the interaction between the channel and the cytoskeleton was disrupted by the mutations, the extent of inactivation would increase and the enhancement of inactivation by DHCB would be weakened. Indeed, the two different C-terminal mutations in the S446A \( \alpha \) subunit (a deletion of the last six amino acids and a replacement of the crucial Thr-493 with alanine) significantly increased the basal extent of inactivation of \( \alpha_{S446A} \beta \) and reduced the magnitude of the inactivation enhancement by DHCB. As expected, under the conditions of these experiments, in which the \( \alpha_{WT} \beta \) channels were essentially detached from the cytoskeleton in their basal state in the oocyte (as indicated by the large basal inactivation and small DHCB effect), the same

Fig. 2. The effect of mutations in the very C terminus of the \( \alpha \) subunit of \( \alpha_{WT} \beta \) channel on the extent of inactivation, both basal and after DHCB treatment. A, two superimposed representative current traces (normalized to peak current), assayed by two-electrode voltage clamp from oocytes of the same frog as Fig. 1A, expressing \( \alpha_{WT} \beta \) channels with and without the mutation K490c in the very C terminus of \( \alpha \). B, normalized extent of inactivation of \( \alpha_{WT} \beta \) and of its two \( \alpha \) subunit mutants (as indicated): basal (open bars) and following DHCB treatment (hatched bars). Oocytes were incubated with or without 40 \( \mu \)M DHCB for 4–6 h prior to two-electrode voltage clamp assay. Numbers above bars indicate the number of frogs assayed; numbers in parentheses indicate the number of cells assayed. A total of three frogs (out of the six frogs assayed for the experiments of Fig. 1B) were assayed. *, \( p < 0.004 \).
C-terminal mutations of the WT α subunit did not increase the inactivation of the Δαβ channels. This is also in accord with the large extent of basal phosphorylation in the oocyte used in these experiments (Fig. 3). In conclusion, the data presented in this report corroborate the hypothesis that the very C terminus of α interacts with the microfilaments.

The interaction of the very C terminus of the α subunit with the oocyte’s cytoskeleton is via an endogenous protein in the oocyte, possibly resembling protein members of the dlg/PSD-95 family. However, truncation of all last six amino acids in the C-terminal tail was more effective than substitution of Thr-493 in the TDV sequence with alanine, a substitution that was shown to abolish the interaction of a related α protein, Kv1.4, with PSD-95 (6). Thus, it seems that the TDV motif in Kv1.1 is not the only determinant of α-subunit binding to the postulated endogenous protein. In view of the demonstration that the interaction of Kv1.1 with PSD-95 was much weaker than that of Kv1.4 in COS7 cells, that Kv1.1 is missing a glutamate that precedes the TDV sequence in Kv1.4 and was shown to be critical for the Kv1.4 interaction with PSD-95 (7), it seems plausible that Kv1.1 interacts in vivo with a protein having a somewhat different recognition sequence, yet to be identified. An alternative explanation to the stronger interaction of Kv1.4 with PSD-95 could be that the basal phosphorylation of this channel is weaker than that of Kv1.1 in the cells tested.

It should be pointed out that the DHCB effect occluded the effects caused by truncations of the C terminus of α in Δαβ channels, much as it occluded (21) the effect of Ser-446 phosphorylation of α in Δαβ channels. This suggests that disruption of microfilaments by DHCB causes maximal enhancement of inactivation extent of the αβ channel experimentally achievable, probably by a massive disruption of interactions between the channels and the microfilaments. However, the fact that the effect of DHCB was still prominent, though significantly reduced, in the C terminus-truncated mutants suggests that there are additional sites of interaction with the microfilaments on the αβ channel molecules that modulate the inactivation.

The present study correlates the extent of inactivation of the Kv1.1/Δβ1.1 channel with its interactions with PSD-95-like proteins. This is the first report that assigns a modulatory role to such interactions. Such a functional interaction might be physiologically relevant at synaptic sites in different brain areas where PSD-95 family members were shown to be concentrated and colocalized with Shaker-type K⁺ channels including Kv1.1 (6).

Acknowledgment—We thank Dr. N. Dascal for helpful discussions.

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