Cdk-mediated phosphorylation of the Kvβ2 auxiliary subunit regulates Kv1 channel axonal targeting

Hélène Vacher,1,3,4 Jae-Won Yang,1 Oscar Cerda,1 Amapola Autillo-Touati,3,4 Bénédicte Dargent,3,4 and James S. Trimmer1,2

1Department of Neurobiology, Physiology, and Behavior, College of Biological Sciences, and 2Department of Physiology and Membrane Biology, School of Medicine, University of California, Davis, CA 95616.
3Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche 641, Marseille 13916, France
4Université de la Méditerranée, Institut Fédératif de Recherche 11, Marseille 13916, France

Kv channels are concentrated at specific sites in the axonal membrane, where they regulate neuronal excitability. Establishing these distributions requires regulated dissociation of Kv1 channels from the neuronal trafficking machinery and their subsequent insertion into the axonal membrane. We find that the auxiliary Kvβ2 subunit of Kv1 channels purified from brain is phosphorylated on serine residues 9 and 31, and that cyclin-dependent kinase (Cdk)-mediated phosphorylation at these sites negatively regulates the interaction of Kvβ2 with the microtubule plus end–tracking protein EB1. Endogenous Cdks, EB1, and Kvβ2 phosphorylated at serine 31 are colocalized in the axons of cultured hippocampal neurons, with enrichment at the axon initial segment (AIS). Acute inhibition of Cdk activity leads to intracellular accumulation of EB1, Kvβ2, and Kv1 channel subunits within the AIS. These studies reveal a new regulatory mechanism for the targeting of Kv1 complexes to the axonal membrane through the reversible Cdk phosphorylation-dependent binding of Kvβ2 to EB1.

Introduction

Voltage-gated potassium channels of the Kv1 subfamily play an important role in regulating the initiation and the shape of the axonal action potential, as well as synaptic efficiency (Bean, 2007; Clark et al., 2009; Johnston et al., 2010). In addition, mutations in genes encoding Kv1 channel subunits have been implicated in the etiology of several neuronal excitability disorders and diseases (Adelman et al., 1995; Kullmann and Hanna, 2002; Jen et al., 2007). Importantly, Kv1 channel complexes show intricate axonal localizations with regard to their subunit composition and discrete subdomain distributions. For example, in neocortical layer 2/3 pyramidal neurons, in pyramidal neurons in hippocampal CA1, and in retinal ganglion cells, Kv1.1 and Kv1.2 subunits are highly clustered at the distal end of the axon initial segment (AIS) (Inda et al., 2006; Van Wart et al., 2007; Goldberg et al., 2008; Lorincz and Nusser, 2008). In the case of myelinated nerve fibers, Kv1 channels are restricted beneath the myelin sheath and flanking each node of Ranvier at sites termed juxtaparanodes (Wang et al., 1993; Rhodes et al., 1995; Rasband, 2004). However, the mechanisms underlying the precise assembly of high density populations of Kv1 channels in distinct axonal membrane subdomains remain elusive.

Kv1 channels function as supramolecular protein complexes, composed of four pore-forming and voltage-sensing principal, or α, subunits, with four cytoplasmic auxiliary Kvβ subunits (Rhodes et al., 1996; Pongs et al., 1999; Long et al., 2005). These Kv1α (Kv1.1–1.8) and Kvβ (Kvβ1, Kvβ2) subunits can heteromultimerize to yield biophysically and pharmacologically distinct channel complexes (Ruppersberg et al., 1990; Rettig et al., 1994; Xu et al., 1998). Auxiliary Kvβ subunits are each ~300 amino acids in length and contain a unique N-terminal domain followed by a common conserved core (over 85% amino acid identity; Trimmer, 1998). Studies of Kv1 channel biosynthesis have shown that Kv1α and Kvβ subunits coassemble in the ER and remain together as a stable complex (Shi et al., 1996; Shi et al., 2000).

Correspondence to Helene Vacher: helene.vacher@univmed.fr
J.-W. Yang’s present address is Medical University of Vienna, Institute of Pharmacology, A-1090 Vienna, Austria.
Abbreviations used in this paper: +TIP, microtubule plus end–tracking protein; AIS, axon initial segment; Ank-G, ankyrin-G; APC, adenomatous polyposis coli; API, axonal polarity index; CLASP, CIP-associated protein; DIV, days in vitro; LCMS/MS, liquid chromatography tandem mass spectrometry; MT, microtubule; phosphosite, phosphorylation site; WT, wild type.

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Results

Identification of in vivo phosphosites on brain Kvβ2

To identify in vivo Kvβ2 phosphosites, we undertook an unbiased liquid chromatography tandem mass spectrometry (LC-MS/MS)–based analysis of Kvβ2 subunits immunopurified from mammalian brain. To specifically isolate the population of brain Kvβ2 associated with Kv1 channels, we used an anti-Kv1.2 antibody (Ab) to coinmunopurify Kv1/Kvβ2 complexes from detergent extracts of a crude rat brain membrane (RBM) fraction. We then size-fractionated the components of the purified complexes by SDS-PAGE. The Coomassie blue–stained band representing the putative Kvβ2 subunit pool of Mw = 40 kD was excised and subjected to in-gel trypsin digestion. Peptides were fractionated and identified using LC-MS/MS. A Mascot database search of identified mass spectra resulted in 437 matched peptides and 79% overall coverage of the Kvβ2 primary sequence.

Figure 1. In vivo phosphosites on mammalian brain Kvβ2. (A) Identification of phosphosites on rat brain Kvβ2 using LC-MS/MS. A doubly charged, singly phosphorylated peptide at m/z 647.8, derived from Kvβ2 purified from rat brain, was fragmented to produce this MS/MS spectrum (aa 1–12). The phosphosite was unambiguously assigned to Ser9 because of mass assignments from β-eliminated y9, y10, y11, y12, b9, and b11; fragment ions with neutral loss of phosphoric acid H3PO4. (B) Deduced amino acid sequence of rat Kvβ2. Phosphorylated serine residues, identified by MS, are in red. The sequence coverage is indicated in bold.
impact of S9 and S31 Kvβ2 mutations on Kv1 channel axonal targeting in neurons

Previous studies showed that Kvβ auxiliary subunits and Kv1 subunits coassemble before the resultant αβ4 channel complexes exit the ER (Shi et al., 1996; Nagaya and Papazian, 1997) and that Kvβ2 association is crucial for efficient cell surface trafficking of Kv1.2 (Shi et al., 1996; Campomanes et al., 2002). To determine if any of the identified Kvβ2 phosphorylation sites are involved in cell surface trafficking of Kv1.2, we replaced the phosphorylated Ser residues with Ala residues. We first looked at the effect of these phosphosite mutations on the cell surface expression of Kv1.2 using immunostaining with an ectodomain-directed anti-Kv1.2 Ab (Kv1.2e). Coexpression of Kv1.2 with the S9A, S31A, and S9A/S31A mutants in COS-1 cells led to a decrease of the number of cells exhibiting cell surface immunostaining for Kv1.2, when compared with coexpression with wild-type (WT) Kvβ2, or with the S20A and S112A mutants (Fig. 2 A). We also observed a significant reduction in Kv1.2 ionic currents in whole-cell patch-clamp recordings of HEK293 cells expressing either S31A (Fig. 2 B) or S9A (not depicted), when compared with cells expressing WT Kvβ2 (Fig. 2 B) or S20A (not depicted). The macroscopic voltage-dependent activation and inactivation gating characteristics of Kv1.2 were not detectably different in cells coexpressing mutant and WT Kvβ2. Together, these results indicate that phosphorylation at S9 and S31 is involved in regulating cell surface expression levels of Kv1.2, presumably due to effects on intracellular trafficking.

In hippocampal neurons in culture, Kvβ2 mediates the polarized targeting of Kv1 channel complexes to axons (Gu et al., 2003). We next asked whether mutating Kvβ2 phosphosites would affect the polarized expression of Kv1.2 in axons. Rat hippocampal neurons in culture were cotransfected at 7 days in vitro (DIV), a time before the expression of endogenous Kv1α subunits and Kvβ2 (Gu et al., 2006), with Kv1.2 and WT or mutant isoforms of Kvβ2, and the localization of cell surface Kv1.2 determined 2–3 d later. Intact neurons were immunostained with the external Kv1.2e Ab to detect surface Kv1.2, and then permeabilized and immunostained to determine the localization of the overall population of WT and mutant cytoplasmic Kvβ2 subunits (Fig. 2 C). To quantify the polarity of the expression of cell surface Kv1.2, we determined the surface axonal polarity index (API), defined as the ratio of average fluorescence intensity for major axonal to dendritic branches (Gu et al., 2003). As previously shown (Gu et al., 2003), cell surface Kv1.2 exhibited a nonpolarized surface distribution when expressed in the absence of Kvβ2 (Fig. 2 D; API = 0.74 ± 0.17, n = 13), and a highly polarized axonal surface distribution when coexpressed with Kvβ2 (Fig. 2 D; API = 1.90 ± 0.35, n = 17; significantly [P < 0.05] different than with no Kvβ2). In contrast, when Kv1.2 was coexpressed with either the S9A or S31A mutant, it exhibited a nonpolarized surface distribution similar to that observed in the absence of Kvβ2 (Fig. 2 D; API = 0.73 ± 0.21, n = 14; and API = 0.73 ± 0.23, n = 14, respectively, not significantly different than with no Kvβ2). Moreover, the expression of S9A/S31A in mature neurons (e.g., endogenously expressing Kv1 channels and Kvβ2) also led to a decrease in the axonal distribution of endogenous Kv1.2 (Fig. 2 D). Together, these results indicate that Kvβ2 S9 and S31 are crucial to both the intracellular trafficking of Kv1.2 to the cell surface and the axonal localization of cell surface Kv1.2.

Although none of the identified phosphosites are located in the region of Kvβ2 that serves as the primary mediator of its interaction with Kv1.2 (Gulbis et al., 2000; Long et al., 2005), we nonetheless determined experimentally whether mutations at these sites could impact Kv1.2 trafficking by simply disrupting the interaction of Kvβ2 with Kv1.2. We performed reciprocal coimmunoprecipitation experiments from coexpressing COS-1 cells, and found that both WT and mutant Kvβ2 subunits were associated with Kv1.2 (Fig. 2 E), and vice versa (not depicted). Anti-Kvβ2 Abs did not directly immunoprecipitate Kv1.2 (Fig. 2 E), and anti-Kv1.2 Abs did not immunoprecipitate Kvβ2 (not depicted). These results suggest that the negative impact of the S9A and S31A mutations on the intracellular trafficking and axonal localization of cell surface Kv1.2 are due to events subsequent to Kv1.2/Kvβ2 assembly.

Kvβ2 S9 and S31 are key residues in modulating the interaction of Kvβ2 with EB1

A previous study showed that axonal targeting of Kv1.2 is dependent on the direct interaction of Kvβ2 with EB1, and that Kvβ2 associates with the EB1 C terminus via interactions requiring intact Kvβ2 N-terminal (aa 1–90) and C-terminal (aa 338–367) domains (Gu et al., 2006). The model that arose from these studies is that association of Kvβ2 with EB1 enables the recruitment of Kv1–Kvβ2 complexes to MTs, allowing for the transport of these complexes to the axon (Gu et al., 2006). To determine whether the disruption of Kvβ2-mediated Kv1.2 axonal compartmentalization by the S9A and S31A mutations was due to a perturbation of interaction with EB1, we first examined the recruitment of these Kvβ2 phosphosite mutants along

Table I. LC-MS/MS identification of in vivo phosphosites on Kvβ2 purified from brain

<table>
<thead>
<tr>
<th>Phosphorylation site</th>
<th>Rat brain</th>
<th>Native</th>
<th>Mouse brain</th>
<th>Human HC</th>
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<tbody>
<tr>
<td>pS9</td>
<td>+</td>
<td>–</td>
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<tr>
<td>pS20</td>
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<td>pS31</td>
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<td>pS112</td>
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HC, hippocampus; +, identified phosphorylation site; –, phosphorylation site not identified.
Figure 2. **Mutating Kvβ2 N-terminal phosphosites impacts Kv1.2 cell surface expression.** (A) Intact COS-1 cells cotransfected with rat Kv1.2 and WT Kvβ2, or Kvβ2 mutants (S9A, S20A, S31A, S112A, and S9A/S31A) were double immunostained with external Kv1.2e Ab, followed by permeabilization and immunostaining with K14/16 mAb. A surface expression efficiency index was determined as the percentage of Kv1.2-expressing (K14/16-positive) cells that exhibited Kv1.2e surface immunostaining (Kv1.2 + Kvβ2 = 49.2 ± 2.7%; Kv1.2 + S9A = 40.7 ± 1.2%; Kv1.2 + S31A = 37.8 ± 1.8%; and Kv1.2 + S9A/S31A = 28.7 ± 12.0%). Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test and statistical significance was considered at: *, P < 0.05; and ***, P < 0.001 (n = 6 experiments of 100 Kv1.2-positive cells counted per experiment). (B) Whole-cell patch-clamp recordings from HEK293 cells expressing rat Kv1.2 alone (squares), or Kv1.2 together with WT Kvβ2 (circles), or the Kvβ2 S31A mutant (triangles). The cells were held at −80 mV and step depolarized to +40 mV for 200 ms in +10-mV increments. Peak current amplitudes at each test potential were divided by the cell capacitance to obtain the current densities. Mean ± SE of current densities obtained (Kv1.2, n = 14; Kv1.2 + Kvβ2, n = 5; Kv1.2 + S31A, n = 5) were plotted against each test potential. (C) Cultured hippocampal neurons (7 DIV) were cotransfected with Kv1.2 and either WT Kvβ2 or Kvβ2 S31A. 2 d after transfection, intact neurons were immunostained with Kv1.2e Ab, and then permeabilized and immunostained with anti-Kvβ2 and anti-MAP2 Abs. White arrows indicate the axon. Bar, 50 μm. (D) Surface axonal polarity index was determined by quantifying the surface immunofluorescence intensity profiles of the axon versus three dendritic branches using NIH Neuron/J. (E) Coimmunoprecipitation assays from heterologous cells coexpressing Kvβ2 mutants and Kv1.2 channels. Input into and products of immunoprecipitation reactions performed with anti-Kvβ2 mAb K25/73 on lysates from COS-1 cells coexpressing Kv1.2 and WT Kvβ2 or Kvβ2 mutants (S9A, S20A, or S31A), and immunoblotted for Kv1.2 using K14/16. Asterisk indicates the mouse IgG band. Input and IP lanes are not normalized.
Our results show that dephosphorylation of Kv2 pull-down assays with GST-EB1C (see previous paragraph). with alkaline phosphatase, and then subjected the extracts to digestion with EB1. We subjected cell extracts containing Kv2 phosphorylated in heterologous cells; see Fig. S1) to digestion with EB1. We subjected cell extracts containing Kv2 whether Kv2 phosphorylation could modulate its interaction to EB1, we next determined phosphosites altered Kv2 mutagenesis results suggested that mutating Kv2 in phosphorylation state of these EB1 binding partners. As our interaction of EB1 with APC (Honnappa et al., 2005) and CLASP2 (Watanabe et al., 2009) is regulated through changes in silico analyses revealed a match for both S9 (pSPAR) and S31 (pSPKR) phosphosites with the consensus motif [(S/T)PX(R/K)] for Cdk phosphorylation, specifically that catalyzed by Cdk2 and Cdk5. To examine the possibility that Cdk-mediated phosphorylation regulated Kv2–EB1 interaction, we cotransfected Kv2 in COS-1 cells with active (Cdk2-HA) or dominant-negative (D146N, Cdk2-DN) versions of Cdk2. GST-EB1C pull-down assays were then performed on the extracts obtained from these cells. The presence of Cdk2-HA abrogated the binding of Kv2 to EB1, an effect that was not observed with Cdk2-DN (Fig. 4 B). We next used a phosphospecific Ab specific for Kv2 phosphorylated at S31 (“Kv2P”; Fig. S1, B and C) to probe blots of bacterially expressed Kv2 that had been phosphorylated in vitro by either Cdk2/cyclin A or Cdk5/p35 purified complexes. Phosphospecific Kv2P immunoreactivity against Kv2 was detected in the reactions performed with either Cdk2/cyclin A or Cdk5/p35 (Fig. 4 C), showing that Kv2 could be directly phosphorylated by these Cdks. Together, these results show that Kv2–EB1 interaction is regulated by Cdk-mediated phosphorylation of Kv2 at the S31 phosphosite, and perhaps by phosphorylation at other sites (e.g., S9) as well.

MTs in the presence of EB1. As previously shown (Nakahira et al., 1998; Campomanes et al., 2002), we observed that Kv2 expressed in COS-1 cells is present uniformly throughout the cytoplasm, whereas EB1-EGFP is mainly found along MTs (Skube et al., 2010; unpublished data). However, coexpression of EB1-EGFP promoted the recruitment of Kv2 to MTs in 57.0 ± 4.0% (n = 500 cells, three independent experiments) of cotransfected cells (Fig. 3). In contrast, the EB1-dependent MT recruitment of the S9A (40.2 ± 4.3%), S31A (41.6 ± 2.0%), and S9A/S31A (30.0 ± 3.0%) mutants was significantly decreased versus that observed for WT Kv2 (n = 500 cells, three independent experiments; Fig. 3). To address whether these effects were due to differences in EB1 binding, we performed GST pull-down assays, similar to those used previously to demonstrate Kv2–EB1 interaction (Gu et al., 2006), using bacterially expressed GST-EB1C (C-terminal domain, 165–268) and Kv2 expressed in COS-1 cells. As shown in Fig. 3 C, the S9A and S31A mutants were deficient in EB1C binding relative to WT Kv2. These findings suggest that S9 and S31 are involved in regulating the association of Kv2 with EB1, and the EB1-mediated association of Kv2-containing channels with MTs.

Cdk phosphorylation negatively regulates the interaction between Kv2 and EB1 The interaction of EB1 with APC (Honnappa et al., 2005) and CLASP2 (Watanabe et al., 2009) is regulated through changes in phosphorylation state of these EB1 binding partners. As our mutagenesis results suggested that mutating Kv2 S9 and S31 phosphosites altered Kv2 binding to EB1, we next determined whether Kv2 phosphorylation could modulate its interaction with EB1. We subjected cell extracts containing Kv2 (which is phosphorylated in heterologous cells; see Fig. S1) to digestion with alkaline phosphatase, and then subjected the extracts to pull-down assays with GST-EB1C (see previous paragraph). Our results show that dephosphorylation of Kv2 greatly enhances its binding to EB1 (Fig. 4 A), suggesting that phosphorylation of Kv2 also negatively regulates its binding to EB1. To determine which protein kinases could regulate Kv2–EB1 interaction, we used consensus site algorithms to analyze the sequences surrounding S9 and S31 phosphosites. These in silico analyses revealed a match for both S9 (pSPAR) and S31 (pSPKR) phosphosites with the consensus motif [(S/T)PX(R/K)] for Cdk phosphorylation, specifically that catalyzed by Cdk2 and Cdk5. To examine the possibility that Cdk-mediated phosphorylation regulated Kv2–EB1 interaction, we cotransfected Kv2 in COS-1 cells with active (Cdk2-HA) or dominant-negative (D146N, Cdk2-DN) versions of Cdk2. GST-EB1C pull-down assays were then performed on the extracts obtained from these cells. The presence of Cdk2-HA abrogated the binding of Kv2 to EB1, an effect that was not observed with Cdk2-DN (Fig. 4 B). We next used a phosphospecific Ab specific for Kv2 phosphorylated at S31 (“Kv2P”; Fig. S1, B and C) to probe blots of bacterially expressed Kv2 that had been phosphorylated in vitro by either Cdk2/cyclin A or Cdk5/p35 purified complexes. Phosphospecific Kv2P immunoreactivity against Kv2 was detected in the reactions performed with either Cdk2/cyclin A or Cdk5/p35 (Fig. 4 C), showing that Kv2 could be directly phosphorylated by these Cdks. Together, these results show that Kv2–EB1 interaction is regulated by Cdk-mediated phosphorylation of Kv2 at the S31 phosphosite, and perhaps by phosphorylation at other sites (e.g., S9) as well.

Cdk inhibition increases Kv2 recruitment to MTs and consequently decreases Kv1 surface expression To better understand the impact of Cdk5 on Kv2 properties, we first tested the impact of the pharmacological inhibition of Cdk5 on the recruitment of Kv2 to MTs. We treated COS-1 cells coexpressing Kv2 and EB1 for 24 h with roscovitine, an inhibitor of Cdk kinases (Cdk1, Cdk2, and Cdk5; Bach et al., 2005).
The inhibition of Cdk5 dramatically increased the recruitment of Kvβ2 to MTs (Fig. 4 D). Importantly, inhibition of Cdk5 did not affect the MT recruitment of the S9A/S31A mutant, demonstrating that the effects are mediated through Cdk phosphorylation of S9 and S31. We next looked at the effect of Cdk inhibition on the cell surface expression of Kv1.2. Roscovitine treatment of COS-1 cells coexpressing Kv1.2 and Kvβ2 led to a decrease of the number of cells exhibiting cell surface immunostaining for Kv1.2 (Fig. 4 E). Together, these results suggest that Cdk-mediated phosphorylation of Kvβ2 releases Kv1–Kvβ2 complexes from MTs, allowing for their expression in the plasma membrane, by disrupting Kvβ2 interaction with EB1.

Cdk5 and phosphorylated Kvβ2 colocalize with neuronal Kv1 complexes in vitro and in vivo

In brain, Kv1 channel complexes are found predominantly localized to axons, where they show discrete cell type–dependent localization within subdomains of the axonal membrane (Wang et al., 1993; Rhodes et al., 1997; Lorincz and Nusser, 2008; Ogawa et al., 2008). Given the role of Cdk5 in mediating Kvβ2–EB1 association revealed in the studies presented in the previous paragraph, we first examined the localization of Kvβ2 phosphorylated at S31 using the phosphospecific Kvβ2P Ab to immunostain rat hippocampal neurons in culture. As the developmental expression of Kv1 complexes in cultured hippocampal neurons begins after 2 weeks of culture (Gu et al., 2006), we used hippocampal neurons at 3 weeks in culture (21 DIV) for these experiments. In these neurons, we observed Kvβ2P immunostaining in the axon, with a high concentration at the AIS (identified by ankyrin-G [Ank-G] immunostaining), and colocalizing extensively with the overall pools of Kvβ2 (Fig. 4 A) and Kv1.2 (not depicted). Note that Kvβ2P also exhibited additional nuclear immunostaining that appeared to be nonspecific as it did not correspond to immunostaining for Kvβ2. Although Cdk2 immunostaining was barely distributed throughout these cultured neurons, double immunostaining for Cdk2 and Kvβ2 revealed these proteins colocalized in the axon, and specifically at the AIS (Fig. 5 A). Similarly, immunostaining for Cdk5, which was also broadly expressed in both somatodendritic and axonal compartments, exhibited a prominent colocalization with Kvβ2 at the AIS (Fig. 5 A). To extend these results obtained in cultured neurons, we next used immunostaining to examine the in vivo distribution of Cdk5 in myelinated axons, where Kv1 channels are highly enriched at the juxtaparanode (Rashband, 2004). The localization of Cdk5 in axons was assessed on sciatic nerve sections, which have been used previously to define the juxtaparanodal localization of Kv1α subunits (Mi et al., 1995; Rashband et al., 1998) and Kvβ2 (Vabnick et al., 1999). In adult mouse sciatic nerve, Cdk2 immunostaining colocalized with that for Kv1.2 at the juxtaparanode, and was also present at the node of Ranvier (Fig. 5 B). Cdk5 immunostaining

Figure 4. Cdk5 regulates the interaction between Kvβ2 and EB1. (A) Effect of Kvβ2 phosphorylation on its interaction with EB1. Input and products of GST pull-down reactions performed with GST-EB1c on control and alkaline phosphatase–treated Kvβ2 lysates. GST was used as a negative control. The gel was blotted with anti-Kvβ2 mAb K25/73. (B) Role of Cdk2 in regulating Kvβ2–EB1 interaction. Input and products of GST-EB1c pull-down reactions performed on HEK293 lysates expressing Kvβ2 and either Cdk2-HA or Cdk2-DN, and blotted with an anti-Kvβ2 mAb K25/73. The control lane contains a GST-EB1c pull-down performed from HEK293 lysates. GST was used as a negative control. (C) Immunoblots of bacterially expressed Kvβ2 and Kvα2 alone. (D) Effect of Cdk2 inhibition on the recruitment of Kvβ2 to MTs. COS-1 cells were cotransfected with EB1-EGFP and WT Kvβ2, or Kvβ2 S9A/S31A (ratio 1:1). After the transfection (i.e., subsequent to Kv1.2/Kvβ2 expression and assembly), cells were treated with 20 µM roscovitine for 24 h. MT recruitment was quantified by dividing the number of cells with MT-like Kvβ2 immunostaining by the total number of cells coexpressing Kvβ2 and EB1; 500 cells were counted from three independent experiments. ***, P < 0.001. (E) Effect of Cdk inhibition on Kv1.2 surface expression. COS-1 cells were cotransfected with WT Kv1.2 and Kvβ2 (ratio 1:4). After the transfection, cells were treated with 20 µM of roscovitine for 24 h. Intact COS-1 cells were double immunostained with external Kv1.2e Ab, and then after permeabilization with cytoplasmic anti-Kv1.2 K14/16 and anti-Kvβ2 K25/73 mAbs. A surface expression efficiency index was determined as the percentage of Kv1.2-expressing (K14/16-positive) cells with Kv1.2e surface immunostaining. Statistical significance was considered at **, P < 0.01. (n = 3 independent experiments of 100 Kv1.2-positive cells counted per experiment).
neurons at 20 DIV for 24 h with roscovitine. Because Kv1 subunits, Kvβ2, Cdk2, Cdk5, and EB1 (Fig. S3) are all concentrated at the AIS, we analyzed the effects of Cdk inhibition on their AIS localization. Immunofluorescence staining intensity was quantified by taking the ratio of average fluorescence intensity for the AIS, as defined by immunostaining for Ank-G, relative to that on dendrites. In roscovitine-treated neurons, the immunostaining for Kvβ2, EB1, Kv1.1, and Kv1.2 at the AIS was increased by an average of 30% compared with control (untreated) neurons (n = 50, three independent experiments; Fig. 6, A and B). The increase in Kvβ2 and Kv1.2 immunostaining after roscovitine treatment was also observed in proximal (adjacent to the AIS) and distal axonal domains (Fig. S4). However, the level of PSD-93, a scaffolding protein critical to anchoring of Kv1 channels at the AIS (Ogawa et al., 2008), was also found enriched at the juxtaparanode in sciatic nerve axons, where it colocalizes with Kv1.2. Cdk5 immunostaining is enriched at the juxtaparanode, and is present at paranode and at the node of Ranvier. CASPR immunostaining marks the paranodal compartment. Bar, 10 µm.

Cdk inhibitors modulate the endogenous localization of Kvβ2, EB1, and Kv1 channels

Finally, we tested the impact of pharmacological inhibition of Cdk5 on the Axonal localization of endogenous neuronal EB1, Kvβ2, and Kv1α subunits. We treated cultured hippocampal neurons at 20 DIV for 24 h with roscovitine. Because Kv1α subunits, Kvβ2, Cdk2, Cdk5, and EB1 (Fig. S3) are all concentrated at the AIS, we analyzed the effects of Cdk inhibition on their AIS localization. Immunofluorescence staining intensity was quantified by taking the ratio of average fluorescence intensity for the AIS, as defined by immunostaining for Ank-G, relative to that on dendrites. In roscovitine-treated neurons, the immunostaining for Kvβ2, EB1, Kv1.1, and Kv1.2 at the AIS was increased by an average of 30% compared with control (untreated) neurons (n = 50, three independent experiments; Fig. 6, A and B). The increase in Kvβ2 and Kv1.2 immunostaining after roscovitine treatment was also observed in proximal (adjacent to the AIS) and distal axonal domains (Fig. S4). However, the level of PSD-93, a scaffolding protein critical to anchoring of Kv1 channels at the AIS (Ogawa et al., 2008), was also found enriched at the juxtaparanode in sciatic nerve axons, where it colocalizes with Kv1.2. Cdk5 immunostaining is enriched at the juxtaparanode, and is present at paranode and at the node of Ranvier. CASPR immunostaining marks the paranodal compartment. Bar, 10 µm.
accumulation at these sites remain elusive, but presumably involve reversible protein–protein interactions between component subunits of the Kv1 channel complex and constituents of the neuronal trafficking machinery. In this study, we reveal a novel mechanism regulating axonal targeting of Kv1 channels via the Cdk-mediated phosphorylation of the Kvβ2 auxiliary subunit. Our data suggest a model whereby phosphorylation of Kvβ2 disrupts its binding to EB1, which consequently releases the Kv1–Kvβ2-containing vesicles from their association with EB1 and axonal MTs. We first identified in vivo phosphosites on Kvβ2 purified from mammalian brain using a phospho-proteomic approach. We demonstrated that mutation of two of the identified phosphosites, at S9 and S31, impacts Kvβ2–EB1 interaction, and that Cdk-mediated Kvβ2 phosphorylation negatively regulates this interaction. Furthermore, we showed that Cdk2 and Cdk5 directly phosphorylate Kvβ2 in vitro, and that inhibition of Cdks in heterologous cells leads to an increase of Kvβ2 recruitment on MTs and a decrease of Kv1 surface expression. We found that endogenous Cdk2, Cdk5, phosphorylated Kvβ2, and EB1 in cultured hippocampal neurons are all present in axons, and that inhibition of Cdks in heterologous cells leads to an increase of Kvβ2 recruitment on MTs and a decrease of Kv1 surface expression. We found that endogenous Cdk2, Cdk5, phosphorylated Kvβ2, and EB1 in cultured hippocampal neurons are present in axons, and that all are enriched at the AIS. Cdk2 and Cdk5 also colocalize with Kv1 channels at the juxtaparanode of sciatic nerves in vivo. Finally, acute inhibition of Cdks in cultured hippocampal neurons leads to an increase in the levels of intracellular populations of axonal Kvβ2, EB1, and Kv1 channels without affecting the levels of either the surface population of Kv1 channels or the Kv1 channel anchoring protein PSD-93.

Discussion

It has become clear that Kv1 channels present at the axon, especially at the AIS (Clark et al., 2009) and the juxtaparanode (Rasband, 2004), play a crucial role in controlling spike threshold, shape, and repetitive firing (Johnston et al., 2010). As such, these channels have become an attractive target for therapeutics aimed at restoring function in patients with peripheral demyelinating disorders (Judge et al., 2006). However, the molecular mechanisms responsible for their precise, high density accumulation at these sites remain elusive, but presumably involve reversible protein–protein interactions between component subunits of the Kv1 channel complex and constituents of the neuronal trafficking machinery. In this study, we reveal a novel mechanism regulating axonal targeting of Kv1 channels via the Cdk-mediated phosphorylation of the Kvβ2 auxiliary subunit. Our data suggest a model whereby phosphorylation of Kvβ2 disrupts its binding to EB1, which consequently releases the Kv1–Kvβ2-containing vesicles from their association with EB1 and axonal MTs. We first identified in vivo phosphosites on Kvβ2 purified from mammalian brain using a phospho-proteomic approach. We demonstrated that mutation of two of the identified phosphosites, at S9 and S31, impacts Kvβ2–EB1 interaction, and that Cdk-mediated Kvβ2 phosphorylation negatively regulates this interaction. Furthermore, we showed that Cdk2 and Cdk5 directly phosphorylate Kvβ2 in vitro, and that inhibition of Cdks in heterologous cells leads to an increase of Kvβ2 recruitment on MTs and a decrease of Kv1 surface expression. We found that endogenous Cdk2, Cdk5, phosphorylated Kvβ2, and EB1 in cultured hippocampal neurons are all present in axons, and that all are enriched at the AIS. Cdk2 and Cdk5 also colocalize with Kv1 channels at the juxtaparanode of sciatic nerves in vivo. Finally, acute inhibition of Cdks in cultured hippocampal neurons leads to an increase in the levels of intracellular populations of axonal Kvβ2, EB1, and Kv1 channels without affecting the levels of either the surface population of Kv1 channels or the Kv1 channel anchoring protein PSD-93.
Together, our findings reveal a new regulatory mechanism for
the targeting of Kv1 complexes to the axonal membrane through
the reversible phosphorylation-dependent binding of Kvβ2 auxili-
ary subunits to EB1.

Here, we show that two Ser residues, S9 and S31, regulate the
interaction of Kvβ2 with EB1, in that phosphorylation or
mutation of either Ser residue disrupts Kvβ2–EB1 interaction.
The S9 and S31 sites (SPAR and SPRK) are quite similar to a
phosphosite (SPRK) that acts as a negative regulator of APC
binding to EB1 (Honnappa et al., 2005, 2009). These Kvβ2 phos-
phosphosites are located within the Kvβ2 N-terminal domain
that among Kvβ subunits is unique to Kvβ2, suggesting that,
among Kvβ family members, the reversible binding to EB1
may be specific to the highly expressed Kvβ2. Honnappa et al.
(2009) also identified a highly conserved “microtubule tip local-
ization signal” among EB1-binding partners, in the form of
a short peptide motif Ser-X-Ile-Pro (SXIP) that targets these part-
ners to growing MT ends in an EB1-dependent manner. Phos-
phorylation of +TIPs at regulatory sites distinct from but near
the SXIP EB1 binding motif negatively regulates the localiza-
tion of +TIPs to MT ends by decreasing their affinity for
binding to EB1 (Honnappa et al., 2005, 2009). However, phos-
phorylation within the SXIP motif itself has not been detected
(Honnappa et al., 2009). There exists a consensus SXIP motif
within Kvβ2 (SGIP, aa 257–260), within a segment that among
Kvβ subunits is also unique to Kvβ2, and that within the Kvβ2
crystal structure forms a surface loop between the β1 strand and
the αg helix (Gulbis et al., 1999, 2000). However, we found that
mutating either S257 or the entire SGIP motif to Ala did not
abrogate Kvβ2–EB1 interaction (Fig. S5). This suggests that
Kvβ2 possesses a mechanism for phosphorylation-dependent
interaction with EB1 that is similar to but distinct from other
EB1-binding proteins.

Moreover, although our results show a role for N-terminal
Kvβ2 phosphorylation acting as a negative regulator of
binding to EB1, they are distinct from those obtained for
phosphorylation-dependent regulation of EB1 binding by
CLASPs (Kumar et al., 2009; Watanabe et al., 2009). For example,
Ser to Ala mutations in CLASPs yield constitutive CLASP/EB1
binding that is refractory to phosphorylation-dependent regu-
lation. Similar mutations in Kvβ2 disrupt its binding to EB1,
as do Ser to Asp mutations at these sites. We note that there
are numerous cases where Ser to Ala mutations are disruptive
to other protein–protein interactions that are negatively regu-
lated by phosphorylation. For example, while phosphoryla-
tion of GluR2 glutamate receptor subunit at S880 negatively
regulates GluR2 binding to its partner GRIP (Matsuda et al.,
1999), mutation of S880 to either Ala (Osten et al., 2000) or
Glu (Chung et al., 2000) disrupts this interaction, suggesting
a similar requirement for an intact, unphosphorylated Ser for
binding. Similarly, Kir2.3 channel binding to the PSD-95
scaffolding protein is negatively regulated by phosphorylation
at Kir2.3 S440, and by mutation of this Ser to Ala, Asp, or Glu
(Cohen et al., 1996). The negative effects of phosphorylation,
and of S9 and S31 mutations on Kvβ2 binding to EB1, may
reflect a similar strict requirement for an unphosphorylated
Ser at these positions.

In neurons, Kvβ2 orchestrates forward trafficking (Shi
et al., 1996; Campomanes et al., 2002; Gu et al., 2003) and sub-
sequent axonal targeting (Gu et al., 2003) of Kv1 channels
through interactions with EB1 and the microtubule-based mo-
tors KIF3A (Gu et al., 2006). KIF5B has also been shown to be
required for efficient targeting of Kv1 channels to axons
(Rivera et al., 2007), but any potential Kvβ2–KIF5B interaction
has not yet been characterized. One model derived from these studies is
that Kvβ2 acts as an adaptor protein, linking Kv1-containing
vesicles to these motor proteins. As such, Kvβ2 interaction with
KIF3 (and possibly KIF5B), which likely occurs after Kv1-
containing vesicles exit the Golgi apparatus, allows these ves-
icles to be transported to the axon along MTs. Once KIF-driven
vesicles containing Kv1–Kvβ2 complexes reach the plus end of
MTs that are distributed distally along the axon, the Kvβ2 adap-
tor, and the associated Kv1-containing vesicles, can switch from
binding these motors to binding EB1. The newly established
Kvβ2–EB1 binding then allows the vesicles to either stay bound
to the MTs, by shifting between different MTs in the bundle,
or to be released to be locally inserted into the plasma membrane.
Here, we found that Cdk phosphorylation inhibits Kvβ2
interaction with EB1, and that pharmacological inhibition of Cdks
increases the recruitment of Kvβ2 to MTs in heterologous cells,
and the concentration of Kv1–Kvβ2 complexes associated with
EB1 in axons. Thus, it is tempting to propose that Cdks play a
key role in promoting the release of Kv1–Kvβ2-containing
vesicles from EB1. Such a scenario is consistent with previous
studies showing that the unloading and transport efficiencies of
other cargos are regulated by phosphorylation events (Sato-
Yoshitake et al., 1992; Morfini et al., 2002; Guillaud et al.,
2008). Therefore, the phosphorylation of Kvβ2 by Cdks would
act as a molecular switch that controls the release of Kv1-
containing vesicles. We showed that Cdk inhibition
decreases the Kv1 channel surface pool in heterologous cells and has no
significant effect on the Kv1 surface pool and its anchoring pro-
tein PSD-93 in neurons. However, the inhibition of Cdks in het-
erologous cells was done at the outset of Kv1–Kvβ2 expression,
contrast to the experiments in neurons, where these channel
complexes were already transported to and concentrated at the
plasma membrane. Moreover, previous studies showed that the
axonal Kv1 complexes anchored by PSD-93 at the plasma
membrane are highly stable (Ogawa et al., 2008), and that PSD-93
interacts with Kv1 complexes only when they are at the cell
surface. Thus, it is likely that 24 h of Cdk inhibition is not long
enough to induce a decrease of the concentration of either sur-
face Kv1 channels or PSD-93 at the AIS.

The reversible posttranslational modification of neuronal
protein binding partners is a key process allowing a specific and
dynamic network of interactions in response to neuronal activ-
ity. This process relies on the expression and activity of specific
sets of protein kinases and phosphatases in distinct subcellular
compartments. Here, we show that neuronal Cdks, which colocal-
ize in axons with Kv1 subunits, Kvβ2, and EB1, are implica-
ted in regulating Kv1 channel axonal compartmentalization.
Thus, it is likely that the localization of Cdks at/near sites of
high densities of axonal Kv1 channels spatially restricts where
the phosphorylation events that regulate Kvβ2–EB1 occur.
This ensures that Kv1 channels are localized at the correct subcellular locations, and prevents their ectopic expression at sites that could result in deranged neuronal excitability. This is similar to the role proposed for the CK2 protein kinase, which is also highly enriched at the AIS and at nodes of Ranvier, and which regulates the local interaction between Nav channels and the scaffolding protein Ank-G (Bréchét et al., 2008). In this case, CK2-mediated phosphorylation increases the affinity of the AIS-targeting motif for binding to Ank-G, ensuring that Nav channels are spatially restricted to sites (e.g., the AIS and nodes of Ranvier) that are enriched in Ank-G. The initiation of action potentials depends on the precise density of Nav and Kv channels at the AIS (Clark et al., 2009). Fine tuning of the expression levels and localization of these axonal ion channels, through feedback mechanisms involving signaling pathways using the CK2 and Cdk protein kinases, and competing protein phosphatases, provides a powerful mechanism to dynamically regulate the biophysical properties of the spike-generating machinery and neuronal excitability.

Materials and methods

Preparation of brain membrane fractions and cell lysates

A crude synaptosomal membrane fraction was prepared from freshly dissected adult rat hippocampal brain, or from human hippocampal tissue from anonymous donors (the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD) by homogenization in 0.3 M sucrose, 5 mM sodium phosphate, pH 7.4, 5 mM NaF, 1 mM EDTA, anti-protease tablet (Roche), and centrifugations as described previously (Trimmer, 1991). The pellet of the crude membranes was suspended in the homogenization buffer and protein was determined using the BCA (bicinchoninic acid protein assay) method (Thermo Fisher Scientific). Harvested HEK293 and COS-1 cells or crude synaptosomal membrane lysates were lysed in 1% Triton X-100 extraction buffer as described previously (Vacher et al., 2007). Cells were transiently transfected using the Lipofectamine 2000 (Invitrogen) or Polyfect (QIAGEN) reagents using the manufacturer’s protocols.

Immunopurification, in-gel digestion, and MS

For large-scale immunopurification, 1% Triton X-100 extracts of rat brain membranes (RBM; 25 mg), mouse brain membranes (MBM; 10 mg), or human hippocampal membranes (10 mg) were incubated with affinity-purified rabbit anti-Kv1.2C polyclonal Ab (Rhodes et al., 1995), followed by binding to protein A–agarose beads. In-gel digestion of the Kv2 band excised from a Coomassie blue-stained SDS gel was performed in 10 ng/ml trypsin as described previously (Park et al., 2006). An ultra-performance liquid chromatography system (nanoACQUITY; Waters) directly coupled with an ion trap mass spectrometer (LTQ-FT; Finnigan) was used for LC-MS/MS data acquisition. MS/MS spectra were interpreted through the QuikChange site-directed mutagenesis kit (Agilent Technologies).

Generation of the phosphospecific Ab Kv1.2P

A synthetic peptide phosphorylated at S31 (aa 26–37, STRYG[p]PKRKLQG) and the nonphosphorylated equivalent peptide were synthesized (Pi Proteomics). The phosphopeptide was conjugated to keyhole limpet hemocyanin (EMD) at a ratio of 1 mg of peptide/mg of carrier protein using sulfo-N-maleimidobenzoxy-NHS ester (Thermo Fisher Scientific), and injected into rabbits for the production of polyclonal antisera (PRFUL). For affinity purification, the phosphorylated and nonphosphorylated peptides were conjugated to SulfoLink coupling gel (Thermo Fisher Scientific) via synthetic N-terminal cysteine residues, and phosphospecific Abs were affinity purified by a two-step affinity purification procedure (Park et al., 2006). Phosphospecificity was verified by ELISA assay against phosphorylated and nonphosphorylated peptide coupled to BSA.

Animals

Wistar rats (for neuronal cultures) and Swiss mice (for sciatic nerve immuno-histochemistry) used follow the guidelines established by the European Animal Care and Use Committee (86/609/CEE). Mice were deeply anesthetized with pentobarbital (120 mg/kg b.w., i.p.).

Neuron culture, transfection, and inhibition of Cdk activity

Primary hippocampal neurons were prepared from hippocampi of E18 rats, as described previously (Goslin and Banker, 1989). In brief, dissociated neurons were plated onto poly-L-lysine-treated glass coverslips at a density of 2,500–7,500 cells/cm² and cultured over a monolayer of astrocytes. Cells were maintained in Neurobasal medium (Invitrogen) supplemented with B27 and glutamine. Neurons were transfected at 7 DIV or at 10 DIV using Lipofectamine 2000 (Invitrogen). The conditioned medium was supplemented with 10 µM D-2-amino-5-phosphonovaleric acid (Tocris Bioscience). Transfected cells were processed for immunofluorescence 2 d after transfection. Inhibition of Cdk activity was performed using roscovitine (EMD), a potent inhibitor of Cdk1, Cdk2, and Cdk5 (Meijer et al., 1997; Bach et al., 2005). Roscovitine was dissolved in DMSO and added to the culture medium of rat hippocampal neurons at a final concentration of 10 µM for 24 h. Controls included the same amount of DMSO alone.

Immunofluorescence staining

For surface immunofluorescence staining (Tiffany et al., 2000), cells were immunostained 48 h after transfection with ectodomain-directed rabbit polyclonal Kv1.2Ab (Shi et al., 1996); or, for immunostaining of endogenous neuronal Kv1.1, at 21 DIV with ectodomain-directed mouse Kv1.1e mAb (K36/15; UC Davis/NIH NeuroMab Facility) before detergent permeabilization to detect the cell surface pool. The total cellular pools of the respective proteins were detected by immunostaining with cytoplastically

Immunoprecipitations, immunoblotting, GST pull-downs, and alkaline phosphatase

Procedures for immunoprecipitation and immunoblot analysis were performed as reported previously (Park et al., 2006). Mouse anti-Kvβ2 mAb K25/73, generated against a C-terminal peptide corresponding to Kvβ2 amino acids 350–367 (Rhodes et al., 1995), was used for immunoblocks. For GST pull-downs, transfected cell extracts or purified bacterially expressed WT and mutant Kvβ2 isoforms were incubated 4 h to overnight at 4°C with GSTβ1C, GSTβ1B, or GST prebound to glutathione–Sepharose 4B (GE Healthcare). The beads were then washed five times with lysis buffer (Vacher et al., 2007) and eluted with reducing sample buffer (125 mM Tris-HCl, 4% SDS, 20%, glycerol, and 2% β-mercaptoethanol). Membrane preparations and cell lysates were incubated without or with 100 U/ml of alkaline phosphatase (Roche) as reported previously (Murakoshi et al., 1997).

In vitro phosphorylation assay

Bacterially expressed GST-Kvβ2 (Bekele-Arcuri et al., 1996) fusion protein (2.5 µg) was incubated with 100 ng of human recombinant Cdk complexes (Invitrogen), either Cdk2/cyclin A, or Cdk5/p35, and 2 mM ATP in kinase reaction buffer (20 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, and 1 mM DTT) in a final volume of 50 µl for 1.5 min at 30°C. The reaction was stopped by adding 50 µl of 2x reducing sample buffer.

Plasmids and generation of mutant Kvβ2 cDNAs

Plasmids for transfection were as follows: rat Kv1.2/RBG4, rat Kvβ2/ RB2 (Nakahira et al., 1996), rat β1A-EGFP (a gift from Michelle Piel, Lehigh University, Bethlehem, PA), Cdk2-HA (Addgene plasmid 1884), and Cdk2-DN (D146N, Addgene plasmid 1882, from Don Heuvel and Harlow, 1993). Mutagenesis of recombinant rat Kvβ2 cDNA in the pRBg4 or pGEX-6F vectors (Nakahira et al., 1996) was performed using the QuikChange site-directed mutagenesis kit (Agilent Technologies).

Generation of the phosphospecific Ab Kv1.2P

A synthetic peptide phosphorylated at S31 (aa 26–37, STRYG[p]PKRKLQG) and the nonphosphorylated equivalent peptide were synthesized (Pi Proteomics). The phosphopeptide was conjugated to keyhole limpet hemocyanin (EMD) at a ratio of 1 mg of peptide/mg of carrier protein using sulfo-N-maleimidobenzoxy-NHS ester (Thermo Fisher Scientific), and injected into rabbits for the production of polyclonal antisera (PRFUL). For affinity purification, the phosphorylated and nonphosphorylated peptides were conjugated to SulfoLink coupling gel (Thermo Fisher Scientific) via synthetic N-terminal cysteine residues, and phosphospecific Abs were affinity purified by a two-step affinity purification procedure (Park et al., 2006). Phosphospecificity was verified by ELISA assay against phosphorylated and nonphosphorylated peptide coupled to BSA.
directed mouse mAbs K14/16 (anti-Kv1.2) or K20/78 (anti-Kv1.1), all from the UC Davis/NIH NeuroMab Facility, or K25/73 (anti-Kvβ2) after detergent permeabilization. For immunostaining endogenous neuronal targets, rat hippocampal neurons were incubated for 1 h with these mAbs and mouse anti-Ank-G mAb (N106/36 or N106/65), or mouse anti-P-93 (N18/30) all from the UC Davis/NIH NeuroMab Facility; rat anti-

References


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