

Kv4.2 Phosphorylation by Cyclic AMP-dependent Protein Kinase*

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Recent evidence suggests that K⁺ channels composed of Kv4.2 α -subunits underlie a transient current in hippocampal CA1 neurons and ventricular myocytes, and activation of the cAMP second messenger cascade has been shown to modulate this transient current. We determined if Kv4.2 α -subunits were directly phosphorylated by cAMP-dependent protein kinase (PKA). The intracellular domains of the amino and carboxyl termini of Kv4.2 were expressed as glutathione S-transferase fusion protein constructs; we observed that both of these fusion proteins were substrates for PKA *in vitro*. By using phosphopeptide mapping and amino acid sequencing, we identified PKA phosphorylation sites on the amino- and carboxyl-terminal fusion proteins corresponding to Thr³⁸ and Ser⁵⁵², respectively, within the Kv4.2 sequence. Kinetic characterization of the PKA sites demonstrated phosphorylation kinetics comparable to Kempptide. To evaluate PKA site phosphorylation *in situ*, phospho-selective antisera for each of the sites were generated. By using COS-7 cells expressing an EGFP-Kv4.2 fusion protein, we observed that stimulation of the endogenous PKA cascade resulted in an increase in phosphorylation of Thr³⁸ and Ser⁵⁵² within Kv4.2 in the intact cell. We also observed modulation of PKA phosphorylation at these sites within Kv4.2 in hippocampal area CA1. These results provide insight into likely sites of regulation of Kv4.2 by PKA.

Voltage-dependent K⁺ channels play a significant role in regulating membrane excitability (1). In both hippocampal pyramidal neurons and ventricular myocytes, a voltage-dependent, A-type K⁺ channel expressing a transient current is present in high density (2–4). Hoffman *et al.* (5) recently reported dense localization of voltage-dependent, A-type K⁺ channels to the distal dendrites of hippocampal CA1 pyramidal neurons. The A-type K⁺ current in the distal dendrites was found to limit action potential initiation and back propagation into the dendrites and thereby regulate the magnitude of the excitatory post-synaptic potential in response to synaptic activity (5). Likewise in ventricular myocytes, the transient current plays a role in regulating action potential amplitude and in myocardial contractile force (6, 7). In summary, there is strong evidence that transient K⁺ channels in both the hippocampus and myocardium significantly contribute to the regulation of membrane excitability in these regions.

The identity of the K⁺ channel subunit(s) responsible for transient A-type currents in hippocampal pyramidal neurons and ventricular myocytes is not conclusively known; however, several lines of evidence support the hypothesis that this current is mediated, at least in part, by channels containing Kv4.2, the Shal-type, K⁺ channel subunit proteins. 1) Kv4.2, forms voltage-dependent, rapidly inactivating K⁺ channels and is selectively localized and abundantly expressed on the soma and dendrites of dentate gyrus and CA1 and CA3 hippocampal neurons (3, 8, 9). 2) Ultrastructural studies have demonstrated that Kv4.2 is localized to the subsynaptic compartment (10). 3) In myocardium, Kv4.2 is more abundant in ventricular *versus* atrial myocytes and is more abundant than other subfamily α -subunits known to express a transient current (3, 11). 4) In some mammalian species it has been shown that the transient current occurs in a gradient across the ventricular wall with expression being highest in the epicardial layers (12, 13). As well, it has been shown in some species that the expression of Kv4.2 also follows a gradient in the ventricular myocardium (11). Therefore, both the localization and biophysical properties of Kv4.2 are consistent with the idea that this channel subunit contributes to A-type currents in ventricular myocytes and in dendrites of hippocampal pyramidal neurons.

Protein phosphorylation plays a critical role in the regulation of ion channel function and membrane excitability (14). It is known that some of the voltage-dependent K⁺ channels are regulated by protein phosphorylation. Voltage-dependent K⁺ channels are thought to be homo- or heterotetramers of α -subunits with each subunit characterized by six membrane-spanning domains, a pore-forming region, and cytoplasmic amino- and carboxyl-terminal domains (15–17). The α -subunits are typically associated with β -, or ancillary, subunits; however, to date, the ancillary subunits that are associated with Kv4.2 α -subunits have not been identified (2, 18). The majority of cases reported so far have demonstrated kinase regulatory sites on the α -subunits of voltage-dependent K⁺ channels (19). Furthermore, the recognized kinase regulatory sites lie in the cytoplasmic domains of these channels.

PKA¹ activation is a powerful regulator of hippocampal neuron excitability (20). However, little is known about the downstream molecular targets of PKA that directly influence neuronal excitability. Hoffman and Johnston (21) have shown that the voltage-dependent activation of dendritic A-type channels is down-regulated by activation of PKA, strongly suggesting that these channels are a molecular target for PKA. Based on both the selective localization of Kv4.2 in hippocampal dendrites and the demonstration by Hoffman and Johnston (21)

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¹ The abbreviations used are: PKA, cAMP-dependent protein kinase; GST, glutathione S-transferase; HPLC, high pressure liquid chromatography; PKC, protein kinase C; CaMKII, calcium/calmodulin-dependent protein kinase II; DTT, dithiothreitol; MAPK, mitogen-activated protein kinase; EGFP, enhanced green fluorescent protein; PAGE, polyacrylamide gel electrophoresis.

that dendritic A-type currents are regulated by PKA activation, one appealing hypothesis is that PKA directly alters the properties of Kv4.2 by phosphorylation of the channel α -subunit.

Although there are no direct lines of evidence for PKA regulation of transient currents in ventricular myocytes, it is known that ventricular myocardial contractility is influenced by neurotransmitter systems such as the β -adrenergic catecholaminergic system which result in downstream activation of the cAMP cascade (22, 23). Furthermore, it has been shown that in myocytes, transient channels thought to be composed of Kv4 subfamily α -subunits are modulated by additional second messenger systems (24). These findings further support the idea that Kv4.2 may be a molecular target for second messenger cascades involved in regulation of myocardial excitability.

The current study tests the hypothesis that the transient K⁺ channel, Kv4.2, is a downstream target for the PKA second messenger cascade. We determined that recombinant GST fusion protein constructs of the amino and carboxyl termini of Kv4.2 are substrates for PKA. The phosphorylation sites on the GST fusion proteins were identified with phosphopeptide mapping and automated amino acid sequencing of the recombinant proteins following phosphorylation by PKA *in vitro*. Kinetic analysis revealed that these sites are attractive PKA substrates with K_m and V_{max} values that are comparable to those of a well established PKA substrate, Kemptide. The sequencing data for the PKA phosphorylation sites on Kv4.2 were then used to generate phospho-site-selective antibodies, by using synthetic phosphopeptides corresponding to the amino- and carboxyl-terminal phosphorylation sites as antigens. These antibodies were then used to quantitate changes in phosphorylation of Kv4.2 expressed in COS-7 cells. Activation of the PKA cascade in COS-7 cells expressing Kv4.2 results in an increase in immunoreactivity, indicating that the amino- and carboxyl-terminal sites are phosphorylated within Kv4.2 in the intact cell. In the final series of experiments the antibodies were used to demonstrate modulation of PKA phosphorylation of Kv4.2 in hippocampal area CA1.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The Kv4.2 amino- and carboxyl-terminal domain proteins were expressed in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins using methods modified from Hakes and Dixon (25). Plasmids containing the amino-terminal (amino acid residues 1–133) and carboxyl-terminal (residues 411–630) domain cDNAs were constructed using the GST fusion vector pGEX-KN (25). A single colony of BL21(DE3)-pLysS cells transformed with the amino- or carboxyl-terminal plasmid was grown in Luria broth (LB, 170 mM NaCl, pH 7.5, 1% tryptone, 0.5% yeast extract) containing 20 μ g/ml carbenicillin (Life Technologies, Inc.) and then used to seed a 500-ml culture. After growing to an optical density of 0.6–0.8 (A_{600}) the culture was centrifuged (Beckman model J2–21M, 1000 \times g, 15 min, 4 °C). The cell pellet was resuspended in 500 ml of LB with carbenicillin. The bacteria were induced by incubation at room temperature with 200 μ M isopropyl β -D-thiogalactopyranoside (Sigma) for 4 h and were harvested by centrifugation.

The methods for protein purification and solubilization were modified from those previously published (26, 27). The cells were resuspended and incubated in Tris buffer 1 (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 μ g/ml pepstatin, 10 μ g/ml leupeptin, 100 μ M phenylmethylsulfonyl fluoride) containing 10 mM β -mercaptoethanol and 100 μ g/ml lysozyme (Sigma) for 15 min at 30 °C. Following solubilization with 1.5% *N*-laurylsarcosine, the lysate was incubated with 20 μ g/ml DNase I (Roche Molecular Biochemicals) and 10 mM MgCl₂. The lysate was then centrifuged (Sorvall RT 6000B, 1000 \times g, 15 min, 4 °C) and adjusted to a 2% Triton X-100 concentration.

The GST fusion proteins were purified using glutathione affinity absorption. Glutathione-agarose beads were washed, resuspended in Tris buffer 1, and then incubated with the lysate for 1 h at 4 °C. The beads were washed 3 times with Tris buffer 1 by repeated centrifugation (Sorvall, 100 \times g, 5 min, 4 °C). After the final wash, the bead preparation was resuspended in Tris buffer 2 (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM Na₄P₂O₇, 10 μ g/ml aprotinin, 10

μ g/ml leupeptin). The recombinant proteins were left on the beads for subsequent experiments.

PKA Phosphorylation of Kv4.2 Amino- and Carboxyl-terminal GST Fusion Proteins—Kv4.2 amino- or carboxyl-terminal GST fusion proteins were incubated for 30 min at 37 °C in reaction mixtures (25 μ l) containing 70 ng of the catalytic subunit of PKA, Tris buffer 2, and ATP mix 1 (100 μ M ATP, 100 mM MgCl₂, and 10 μ Ci [γ -³²P]ATP). Reactions were stopped by boiling for 5 min with sample buffer (30 mM Tris-HCl, pH 6.8, 200 mM DTT, 40% glycerol, 8% SDS, 0.04 mg/ml bromophenol blue). The GST fusion proteins were separated by SDS-PAGE (12.5%) and visualized by Coomassie Blue staining. Phosphopeptides were identified by autoradiography. As a control parallel reactions were performed for GST with and without PKA and ATP mix 1. A time course of PKA phosphorylation of the amino- and carboxyl-terminal proteins was performed.

Phosphopeptide Mapping—PKA phosphorylation reactions were performed as described previously except for the following modifications: a preparative scale (reaction volume of 300–400 μ l) was used, specific activity was increased (20 μ Ci of γ -³²P/25- μ l reaction volume and 50 μ M ATP), and the incubation period was increased to 90 min based on the time course of PKA phosphorylation of the fusion proteins. The phosphorylated amino- or carboxyl-terminal GST fusion proteins were separated by SDS-PAGE (12.5%). Following Coomassie Blue staining of the gels, the bands corresponding to the amino- or carboxyl-terminal fusion proteins were excised, and an in-gel digestion with trypsin or Lys-C was performed as described previously with minor modifications (28). Following extraction from the gel, the peptides were separated using reverse phase HPLC (high pressure liquid chromatography) with absorption monitoring at 214, 254, and 280 nm. Counts/min (cpm) in each HPLC fraction was measured as Cerenkov radiation. Phosphopeptides identified as HPLC fractions containing high radioactivity were applied to Sequelon arylamine membranes (Millipore Corp.) essentially as described by the manufacturer. After drying, the membrane was rinsed two times sequentially with 10 ml of methanol, then with water, and finally with 5 ml of 10% trifluoroacetic acid, 50% acetonitrile in water. The membrane was air-dried, cut into pieces with a scalpel blade, and inserted in a BLOTT cartridge and sequenced in an Applied Biosystems model 477A Protein Sequencer with an in-line 120A PTH-Analyzer (Applied Biosystems, Foster City, CA) using optimized cycles. Instead of butyl chloride, 90% methanol containing phosphoric acid (15 μ l/100 ml) was used to extract the cleaved amino acids. After conversion, 50% of the sample was transferred to the HPLC for phenylthiohydantoin-derivative identification, and the other 50% was collected in the instrument fraction collector for determination of radioactivity by scintillation counting.

Kinetic Characterization of the Kv4.2 Amino- and Carboxyl-terminal PKA Sites—Peptides were synthesized in the Protein Chemistry Core Laboratory (Baylor College of Medicine) which contained the Kv4.2 amino- and carboxyl-terminal phosphorylation sites. The synthetic peptides (NT-(32–44)) and (CT-(546–558)) had a total of 14 amino acid residues corresponding to Kv4.2 channel residues 32–44 on the amino terminus and residues 546–558 on the carboxyl terminus, respectively. The phosphorylation site was located in the middle (amino acid number 7) of the peptide, and a cysteine residue was located at the carboxyl terminus (amino acid number 14) of each peptide (Figs. 7A and 8A).

In the kinase assays used for kinetic characterization of the PKA phosphorylation sites, the reaction mixture (50 μ l) contained Tris buffer 2, 70 ng of the catalytic subunit of PKA, and the NT-(32–44) or CT-(546–558) synthetic peptides. All of the kinase assays were started by the addition of the ATP mix 2 (100 μ M ATP, 100 mM MgCl₂, 5 μ Ci of [γ -³²P]ATP) and were incubated at 25 °C. The reactions were stopped by spotting duplicate 20- μ l aliquots of the reaction mixture onto Whatman 81 phosphocellulose filter papers and washing in 75 mM H₃PO₄. After a last wash in 99% methanol, the papers were dried, immersed in Aquasol-2, and counted. For each experimental condition, counts obtained from a reaction without substrate peptide were subtracted. The assays used to determine K_m and V_{max} were linear with respect to time and linear with added kinase (PKA), and less than 10% of the peptide substrate was converted to product. To obtain the concentration curve for each of the peptides, peptide concentrations ranging from 5 to 400 μ M were used. As a control parallel reactions were included using the PKA substrate Kemptide.

The activated catalytic domain of protein kinase C (PKC), catalytic domain of calcium/calmodulin-dependent protein kinase II (CaMKII), and activated mitogen-activated protein kinase (MAPK) were assayed for phosphorylation of the NT-(32–44) or CT-(546–558) substrates. Known substrates for each of the kinases were used as positive controls as follows: PKC, NG-(28–43), a synthetic peptide analogue of a frag-

ment of neurogranin (29); CaMKII, Autocamtide-2; and MAPK, myelin basic protein. The methods used for the kinase assays were as described above with the following modifications: the PKC reaction contained 0.1 μ g of PKC; the CaMKII reaction contained 2 μ g of CaMKII, reaction buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.5 mM DTT, 0.1 mM Na₂EDTA), and 2.4 mM calmodulin; and the MAPK reaction mixture had 0.5 μ g of activated MAPK and reaction buffer (25 mM Hepes, pH 7.5, 10 mM magnesium acetate, 50 μ M ATP).

Antibody Characterization—The synthetic peptides NT-(32–44) or CT-(546–558) containing the phosphorylated Thr³⁸ and Ser⁵⁵² PKA sites were coupled to keyhole limpet hemocyanin and injected into rabbits. The antisera were screened by Western blotting using the phosphorylated and unphosphorylated ovalbumin-coupled synthetic peptides and GST fusion proteins. The antisera were affinity purified against the NT-(32–44) or CT-(546–558) synthetic peptides using Hi-trap columns (Amersham Pharmacia Biotech).

The purified antisera were used to examine PKA recognition of the phosphorylation sites on the amino and carboxyl termini when Kv4.2 was expressed as an EGFP fusion protein in COS-7 cells. The FuGene 6 Transfection Reagent was used for COS-7 cells transfections with plasmid DNAs (0.1–2.0 μ g/ μ l), using cytomegalovirus promoters expressing EGFP (enhanced green fluorescent protein) (30), EGFP-Kv4.2 fusion protein, or Kv4.2 alone. The EGFP-Kv4.2 fusion protein was made by introduction of a synthetic *NotI* site in the C terminus of EGFP. The amino terminus of Kv4.2 was adapted by PCR to clone in-frame with EGFP at the *NotI* site just before the initiation Met codon. The EGFP-Kv4.2 expression construct resulted in considerably higher levels of Kv4.2 protein expression compared with the Kv4.2 non-fusion construct, for unknown reasons, and therefore was the construct of choice for our cellular phosphorylation studies.

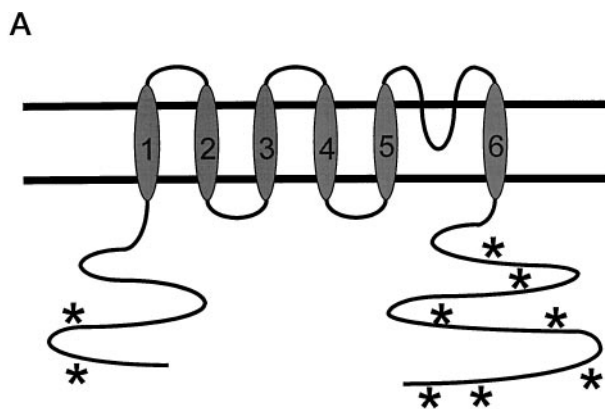
Transfected cells were grown on 35-mm plates to a 2×10^5 cell density. The cell cultures were then incubated for 10 min with phosphate-buffered saline (PBS) (pH 7.5, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄) containing either 50 μ M forskolin and 75 μ M RO126 in Me₂SO or Me₂SO alone (vehicle control). The cells were then harvested and centrifuged. The cell pellet was resuspended in 10% SDS with 100 mM DTT, 10 μ g/ml pepstatin, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 100 μ M phenylmethylsulfonyl fluoride. Sample buffer was then added, and the samples were loaded on a SDS-PAGE gel (10%) for Western blotting. Three antibodies were used for the Western blots as follows: the phospho-selective amino-terminal antibody, 2) the phospho-selective carboxyl-terminal antibody, and 3) the general carboxyl-terminal antibody (not phospho-selective). The third antibody was generated by injection of the carboxyl-terminal fusion protein construct of Kv4.2 and characterized in the laboratory of P. J. Pfaffinger. Immunoreactivity was measured using densitometry (NIH Image). Densitometry data were analyzed with a paired Student's *t* test.

In the final series of experiments the amino- and carboxyl-terminal phospho-selective antibodies were used to evaluate modulation of PKA phosphorylation of Kv4.2 in hippocampal area CA1. Transverse hippocampal slices were prepared using the methodology of Roberson *et al.* (31). After the slices had been maintained for 45–60 min in artificial cerebrospinal fluid (32 °C), 50 μ M forskolin and 75 μ M RO126 in Me₂SO or Me₂SO alone (vehicle control) was applied for 10 min. The methodology of Roberson *et al.* (31) was used for microdissection, storage, and sonication of the hippocampal CA1 subfields. The CA1 sonicates were centrifuged (Beckman, 100,000 $\times g$, 20 min, 4 °C), and the pellet was resuspended as described for the COS-7 cells except that 200 mM DTT was used in the 10% SDS solution and in the sample buffer. The CA1 membrane proteins were then used for Western blotting with the amino- and carboxyl-terminal phospho-selective antibodies. The entire sample from one CA1 subregion was loaded per lane.

Data Analysis—The GraphPad Prism software package was used for statistical analysis of the data. Error bars represent S.E.

Materials—The catalytic subunit of PKA, Kempide, myelin basic protein, and forskolin were obtained from Sigma. The catalytic subunit of PKC (rat brain) and Autocamtide-2 were obtained from Calbiochem. CaMKII (truncated, 1–325 amino acids) and calmodulin were obtained from New England Biolabs. Activated MAPK was obtained from Stratagene. Glutathione-agarose beads and [γ -³²P]ATP were obtained from Amersham Pharmacia Biotech. The FuGene 6 Transfection Reagent was obtained from Roche Molecular Biochemicals. The EGFP construct was obtained from CLONTECH. The anti-Kv4.2 antibody was obtained from Alomone Labs. This antibody was made against a peptide corresponding to Kv4.2 residues 454–469.

Synthetic Peptides—The NT-(32–44) or CT-(546–558) peptides were synthesized in the Protein Core Chemistry Laboratory at Baylor College of Medicine using an Applied Biosystems model 430A peptide



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1 MAAGVAAWLPPFARAAAIGWMPVASGPMPPAPPRQERKRTQDALIIVLNVSGT
51 RFQTWQDTLERYPDTLLLGSSERDFYHPETQQYFFDRDPDIFRHILNFYR
101 TGLLHYRHECISAYDEELAFFGLIPEIIGDCCYEYKDRRRENAERIQD
151 DADTDNTGESALPTMTARQVRWFRAFENPHTSTMALVFYVVTGFFIAVSVI
201 ANVVETVPCGSSPGHIKELPCGGERYAVAFCLDTACVMIFTVEYLLRLAA
251 APSRYRFRVRSVMSIIDVVAILPYYIGLVMTDNEDVSGAFVTLRVFRVRI
301 FKFSRHSQGLRILGYTLKSCASELGLFLFSLTMAIIIFATVMFYAEGSS
351 ASKFTSIPAAFWYITVMTTLGYGDMVPKTIAGKIFGSICSLSGLVLIAL
401 PVPVIVSNFSRIYHQNQRADKRRRAQKKARLARIRAAKSGSANAYMQSKRN
451 GLLSNQLQSSEDEPAFVSKSGSSFETQHHLLHLCLEKTTNHEFVDEQVFE
501 ESCMEVATVNRPSSHSPSLSSQGVTTSTCCSRRHKKSFRIPANVSGSHR
551 GSVQELSTIQIRCVERTPLSNRSSLNAKMECEVKLNCEQPYVTTAIISI
601 PTPVTTPEGDDRPESPEYSGGNIVRVSAL

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FIG. 1. The putative Shal-type K⁺ channel structure and Kv4.2 amino acid sequence. A, a schematic drawing of a mammalian potassium channel illustrates amino- and carboxyl-terminal cytoplasmic domains, six transmembrane domains, and extracellular domains. Regions within the cytoplasmic domains of the channel containing candidate PKA phosphorylation sites are marked by an *. B, inspection of the amino acid sequence of the Shal-type potassium channel, Kv4.2, reveals a number of candidate consensus PKA phosphorylation sequences (**bold**). There are two candidate PKA sites on the amino-terminal domain, one in the fifth transmembrane domain, two located extracellularly, and 7 on the carboxyl-terminal domain.

synthesizer and purified by reverse phase HPLC. Protein concentrations and amino acid compositions were determined by amino acid analysis.

Generation of Antisera—The NT-(32–44) or CT-(546–558) peptides were coupled to activated keyhole limpet hemocyanin. Albino New Zealand rabbits were injected with an initial inoculation of 100 μ g of antigen in Titremax adjuvant followed by four 50- μ g boosts according to the Cocalico Biological Labs protocol. Test bleeds were performed after the first 2 boosts.

RESULTS

Kv4.2 Amino- and Carboxyl-terminal Domains Are Substrates for PKA—Inspection of the amino acid sequence of Kv4.2 revealed a number of candidate PKA phosphorylation sites (Fig. 1). We deemed the most likely possibilities to be two sites within the amino-terminal and seven within the carboxyl-terminal cytoplasmic domains. These sites were of particular interest because previous studies have demonstrated Shaker-type K⁺ channel regulation by protein kinase phosphorylation of the cytoplasmic amino- and carboxyl-terminal regions (32) and also because these sites are in the putative intracellular domains accessible to protein kinases.

We first tested the hypothesis that the amino- and carboxyl-terminal cytoplasmic domains of Kv4.2 are PKA substrates. Recombinant protein constructs (GST fusion proteins) of the amino and carboxyl termini of Kv4.2 were phosphorylated with PKA *in vitro*. Reaction products were separated with SDS-PAGE. Bands corresponding to the amino- and carboxyl-terminal constructs were identified with Coomassie Blue staining

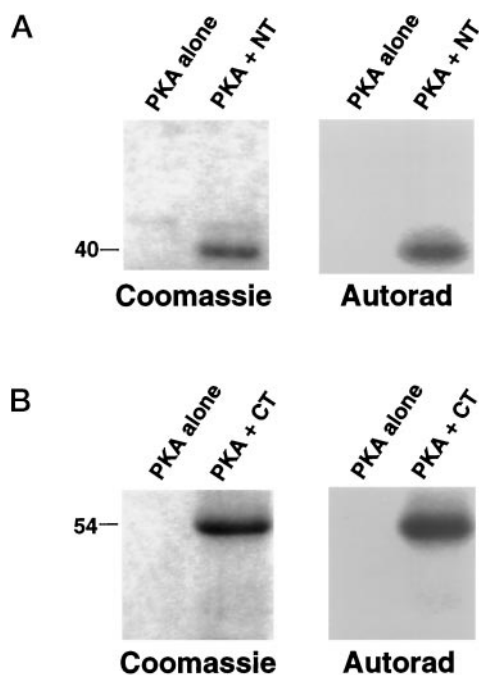


FIG. 2. Phosphorylation of Kv4.2 amino-terminal and carboxyl-terminal GST fusion proteins by PKA *in vitro*. Purified recombinant GST fusion proteins corresponding to the amino-terminal (NT) and carboxyl-terminal (CT) cytoplasmic domains of Kv4.2 were reacted with PKA *in vitro*. Reaction products were separated using SDS-PAGE. Coomassie Blue staining and autoradiography were performed to identify the phosphopeptides. *A*, the Coomassie Blue-stained gel (*Coomassie*) shows a 40-kDa peptide corresponding to the amino-terminal construct. The autoradiogram (*Autorad*) to the right demonstrates ^{32}P incorporation in this region, indicating phosphorylation of the amino-terminal construct. *B*, a 54-kDa peptide is seen on the Coomassie Blue-stained gel corresponding to the carboxyl-terminal construct. Autoradiography of the gel demonstrates ^{32}P incorporation in this region, indicating phosphorylation of the carboxyl-terminal construct.

(Fig. 2). Autoradiography revealed ^{32}P labeling in both the amino- and carboxyl-terminal recombinant proteins (Fig. 2) and confirmed that GST was not phosphorylated by PKA (not shown). Based on these findings, we conclude that both of the recombinant proteins corresponding to the amino- and carboxyl-terminal cytoplasmic domains of Kv4.2 are substrates for PKA.

Identification of the PKA Phosphorylation Sites on the Kv4.2 Amino- and Carboxyl-terminal Cytoplasmic Domains—Our next goal was to identify the PKA phosphorylation sites on the cytoplasmic domains of Kv4.2. The GST fusion proteins were utilized for these experiments because we could express large amounts of the proteins for use in phosphopeptide mapping and sequencing. First, the fusion proteins were incubated with PKA and ^{32}P *in vitro*, separated with SDS-PAGE, and then identified by Coomassie Blue staining. Bands corresponding to the amino- and carboxyl-terminal constructs were excised, eluted, and proteolyzed. We performed phosphopeptide mapping and amino acid sequencing of the phosphorylated Kv4.2 amino- and carboxyl-terminal fusion proteins.

A single PKA phosphorylation site was identified in the amino-terminal cytoplasmic domain. The phosphopeptide map for the amino-terminal construct demonstrated a single peak in radioactivity in HPLC fraction 48 (Fig. 3B) corresponding to a single HPLC absorbance peak (Fig. 3A). HPLC fraction 48 was sequenced using automated Edman degradation, and the amount of radioactivity released with each sequencing cycle was measured (Fig. 4B). This revealed a single phosphopeptide corresponding to Kv4.2 amino-terminal residues 36–51, with one phosphorylation site at Thr³⁸ (Fig. 4A).

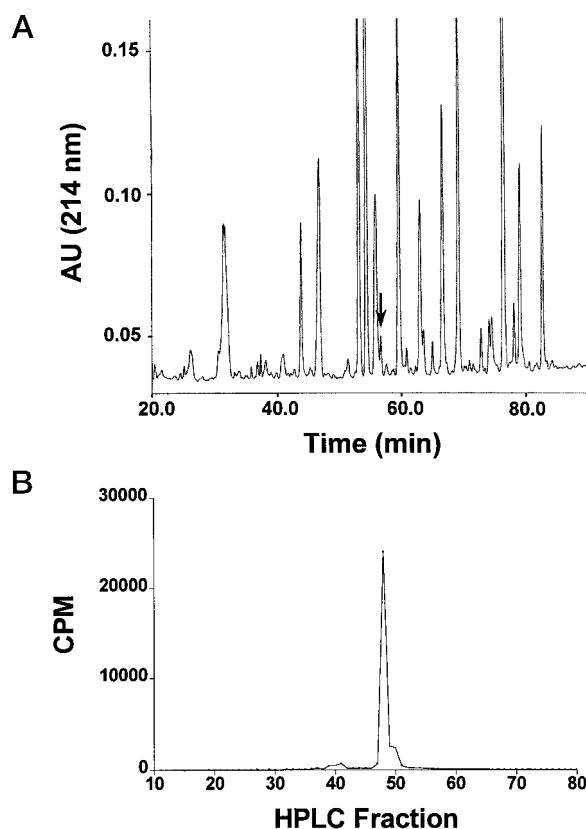


FIG. 3. Phosphopeptide mapping of the tryptic amino-terminal fragments. *A*, phosphopeptide mapping was performed following PKA phosphorylation *in vitro* of the recombinant protein representing the Kv4.2 amino-terminal cytoplasmic domain. The arrow indicates the absorbance peak corresponding to the HPLC fraction with the peak in radioactivity (fraction 48 shown below). In the HPLC trace, the y axis represents absorbance units (AU) at 214 nm and the x axis represents time in minutes of fraction collection. *B*, the radioactivity plot illustrates a single peak in radioactivity in HPLC fraction 48. In the radioactivity plot, ^{32}P counts/min (CPM) measured as Cerenkov radiation are represented on the y axis and the fraction numbers are represented on the x axis.

Confirmation of a single phosphorylation site within the Kv4.2 amino terminus was obtained by performing PKA phosphorylation experiments on recombinant proteins representing two aspects of the Kv4.2 amino terminus: the first representing the entire amino terminus (amino acids residues 1–133) and the second a truncated amino-terminal construct (amino acid residues 41–133) lacking the phosphorylation site identified by Edman degradation. The truncated construct demonstrated no ^{32}P labeling (not shown) narrowing the possibilities for candidate PKA phosphorylation sites to Thr³⁸ on the amino terminus. These studies together with the sequencing data suggest that we did not miss any PKA sites on the amino-terminal cytoplasmic domains of Kv4.2.

We found that there is also a single PKA phosphorylation site on the carboxyl-terminal cytoplasmic domain of Kv4.2. Phosphopeptide mapping of the carboxyl-terminal construct following ^{32}P labeling and trypsin digestion demonstrated a peak of radioactivity (Fig. 5B) that corresponded to a single HPLC absorbance peak (Fig. 5A). The HPLC fractions were pooled and sequenced, and the amount of radioactivity released with each sequencing cycle was measured (Fig. 6B). A single phosphopeptide was identified corresponding to residues 551–561 within the Kv4.2 sequence with phosphorylation of Ser⁵⁵² (Fig. 6A).

To ensure that we had not missed any PKA phosphorylation sites and to confirm the sequencing results from the carboxyl-

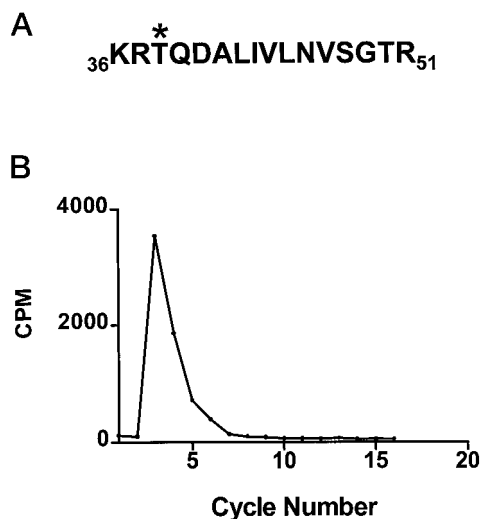


FIG. 4. Identification of the PKA phosphorylation site on the amino-terminal cytoplasmic domain of Kv4.2. *A*, the sequence of the phosphopeptide in HPLC fraction 48 that was determined by automated amino acid sequencing corresponded to amino acids 36–51 within the amino-terminal domain of Kv4.2. The phosphorylated amino acid, Thr³⁸, is marked by an asterisk. *B*, the radioactivity released with each sequencing cycle was measured. There is a peak in sequence cycle number 3 indicating that Thr³⁸ is the phosphorylated amino acid. The y axis represents the ³²P counts/min (CPM) and the x axis represents the sequencing cycle number.

terminal peptide, a second digest was performed using Lys-C. Phosphopeptide mapping following Lys-C digestion of the carboxyl-terminal construct resulted in peak radioactivity in 5 consecutive HPLC fractions (not shown). Amino acid sequencing of each of these fractions revealed a single phosphopeptide containing the phosphorylated amino acid residue corresponding to Ser⁵⁵² of Kv4.2. These results confirm our earlier findings of a single PKA phosphorylation site on the carboxyl-terminal cytoplasmic domain of Kv4.2.

Based on these phosphopeptide mapping and amino acid sequencing results, the PKA phosphorylation sites on the amino- and carboxyl-terminal cytoplasmic domains of Kv4.2 have been identified as Thr³⁸ and Ser⁵⁵². Each of the identified phosphorylated residues lies within a putative consensus PKA phosphorylation site within the Kv4.2 amino acid sequence.

Kv4.2 Amino- and Carboxyl-terminal Synthetic Peptides Are Efficacious Substrates for PKA—One caveat of this approach to the identification of PKA phosphorylation sites is that these sites may not be biologically relevant. One way to test this is to determine the efficiency with which these sites on the amino- and carboxyl termini of Kv4.2 are phosphorylated by PKA *in vitro*. In order to do this, we synthesized two peptides corresponding to residues 32–44 on the amino terminus (NT-(32–44)) and residues 546–558 on the carboxyl terminus (CT-(546–558)) (Figs. 7A and 8A, respectively). Kempptide, a well characterized PKA substrate, was used in parallel assays under the same conditions. The synthetic peptides and Kempptide were subjected to kinetic characterization. Kinetic characterization of Kempptide served as a standard for comparison to the results obtained with the synthetic peptides.

PKA phosphorylation of the NT-(32–44) synthetic peptide demonstrated substrate concentration dependence (Fig. 7B). Lineweaver-Burk analysis of the phosphorylation data using peptide concentrations ranging from 5 to 300 μ M resulted in a linear double-reciprocal plot (Fig. 7B, inset). However, at higher concentrations (400 μ M) the NT-(32–44) peptide exhibited some substrate inhibition. Consequently, the K_m and V_{max} from these data should be considered as estimates. The value for K_m was estimated to be 294 μ M, and the maximal velocity

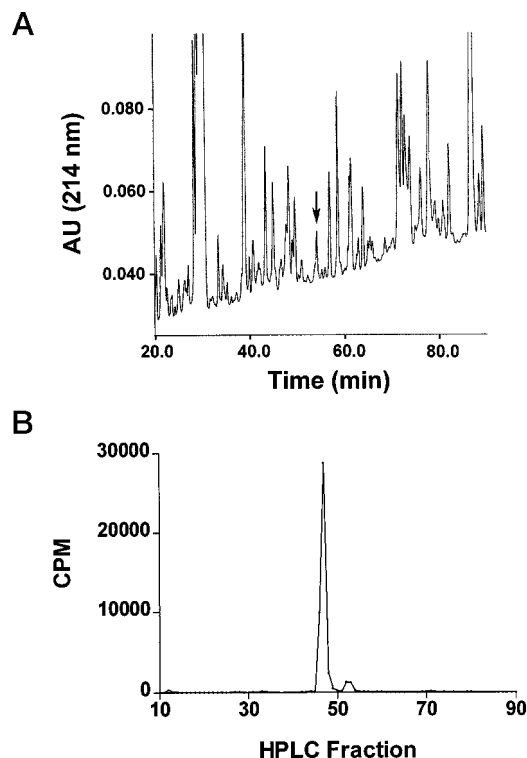


FIG. 5. Phosphopeptide mapping of the tryptic carboxyl-terminal fragments. *A*, the recombinant protein representing the Kv4.2 carboxyl-terminal cytoplasmic domain was phosphorylated with PKA *in vitro*. Peaks in radioactivity were present in three HPLC fractions (see below) that corresponded to a single absorbance peak (marked by an arrow). In the HPLC trace, the y axis represents absorbance units (AU) at 214 nm, and the x axis represents time in min of fraction collection. *B*, peaks in radioactivity were seen in HPLC fractions 46–48. These peaks corresponded to a single peak on the HPLC trace. In the radioactivity plot, the ³²P counts/min (CPM) measured as Cerenkov radiation are represented on the y axis, and the fraction numbers are represented on the x axis.

(V_{max}) of PKA phosphorylation of the amino-terminal peptide was estimated to be 7.7 μ mol/min/mg.

The CT-(546–558) synthetic peptide was also phosphorylated by PKA in a substrate concentration-dependent manner that was consistent with Michaelis-Menten kinetics (Fig. 8B). A linear double-reciprocal plot resulted from Lineweaver-Burk analysis of the phosphorylation data using substrate concentrations ranging from 5 to 400 μ M (Fig. 8B, inset). From these data, the apparent K_m value for the CT-(546–558) peptide is 133.7 μ M. The calculated V_{max} value for PKA phosphorylation of the CT-(546–558) peptide is 54.1 μ mol/min/mg. The K_m value for each of the synthetic peptides is in the range of those published and obtained in parallel kinase assays performed here with a well established PKA substrate, Kempptide ($K_m = 16 \mu$ M and $V_{max} = 20.2 \mu$ mol/min/mg, 30 °C) (33). These results suggest that both of the peptides are good substrates for PKA with the carboxyl-terminal peptide being more efficacious than the amino-terminal peptide. Whereas these data were obtained by phosphorylation of the sites *in vitro*, our findings suggest that there may be a preference for Ser⁵⁵² over Thr³⁸ when phosphorylated within the native channel *in vivo*.

In some cases multiple protein kinases have been shown to converge on a single phosphorylation site. We tested whether the synthetic peptides were substrates for PKC, CaMKII, and MAPK (Fig. 9). The NT-(32–44) and CT-(546–558) synthetic peptides do not have any proline residues suggesting that these peptides are not MAPK substrates, and the data obtained from MAPK incubation with the synthetic peptides support this conclusion. We have previously shown that PKC and CaMKII

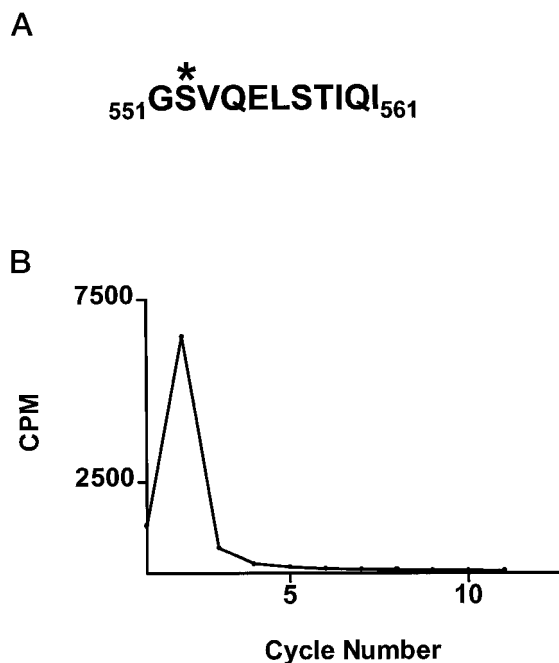


FIG. 6. Identification of the PKA phosphorylation site on the carboxyl-terminal cytoplasmic domain of Kv4.2. *A*, HPLC fractions 46–48 were pooled and then sequenced using automated amino acid sequencing. The phosphopeptide sequence that was obtained corresponded to amino acids 551–561 of the carboxyl-terminal domain of Kv4.2. The phosphorylated amino acid residue, Ser⁵⁵², is marked by an asterisk. *B*, the radioactivity released with each sequencing cycle was measured. The radioactivity peak in sequence cycle 2 indicates that Ser⁵⁵² is the phosphorylated amino acid. The y axis represents the ³²P counts/min (CPM), and the x axis represents the sequencing cycle number.

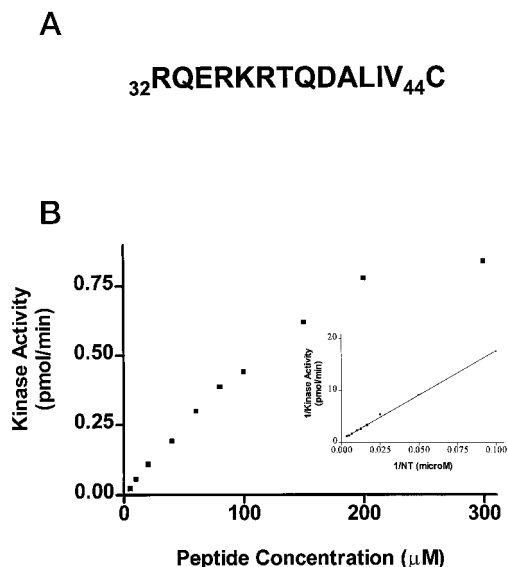


FIG. 7. Concentration curve for PKA phosphorylation of the Kv4.2 amino-terminal site. *A*, kinetic characterization was performed using a synthetic peptide (NT-(32–44)) containing the amino-terminal PKA phosphorylation site. *B*, the concentration curve for PKA phosphorylation of NT-(32–44) at concentrations ranging from 5 to 300 μ M is shown. Each point is the mean of two determinations assayed in duplicate. *Inset*, Lineweaver-Burk plot of the data.

phosphorylate the carboxyl terminus but not the amino terminus of Kv4.2 (34, 35). The results obtained here suggest that neither the CT nor the NT PKA sites are efficacious substrates for these kinases as there was only modest phosphorylation of the NT-(32–44) or CT-(546–558) synthetic peptides by PKC

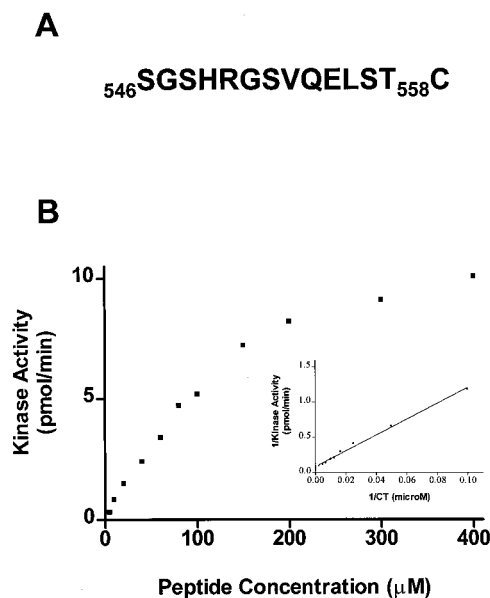


FIG. 8. Concentration curve for PKA phosphorylation of the Kv4.2 carboxyl-terminal domain. *A*, kinetic characterization of the carboxyl-terminal site was performed using a synthetic peptide (CT-(546–558)) containing the carboxyl-terminal PKA phosphorylation site. *B*, the concentration curve for PKA phosphorylation of CT-(546–558) at concentrations ranging from 5 to 400 μ M is shown. Each point is the mean of two determinations assayed in duplicate. *Inset*, Lineweaver-Burk plot of these data.

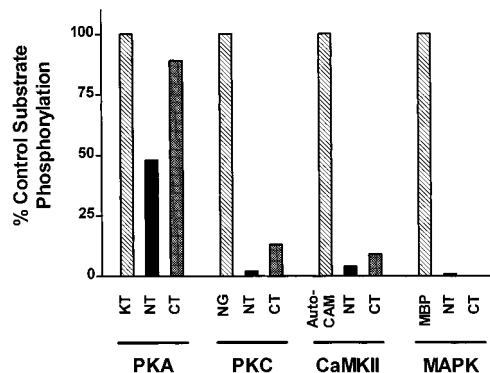


FIG. 9. PKC, CaMKII, and MAPK do not phosphorylate the amino- and carboxyl-terminal synthetic peptides. Saturating concentrations of the NT-(32–44) (300 μ M) and the CT-(546–558) (400 μ M) synthetic peptides were incubated with PKA, PKC, CaMKII, or MAPK and [γ -³²P]ATP *in vitro*. Parallel reactions were performed using known substrates (control substrate) for each of the kinases: Kemptide (KT) for PKA, NG-(28–43) (NG, neurogranin) for PKC, Autocamide-2 (Autocam) for CaMKII, and myelin basic protein (MBP) for MAPK. All reactions were performed in duplicate, and an average was taken. Phosphorylation of the NT-(32–44) and CT-(546–558) synthetic peptides is shown as a percentage of the control substrate phosphorylation for each kinase. Although there is minimal ³²P incorporation with PKC and CaMKII incubation with the synthetic peptides, it is less than 13% of that seen with neurogranin or Autocamide-2. Comparison to the ³²P incorporation in the NT-(32–44) and CT-(546–558) synthetic peptides by PKA demonstrates that these sites are much better substrates for PKA.

and CaMKII in comparison to known PKC or CaMKII substrates (Fig. 9).

Phospho-site-selective Antibodies for the Amino- and Carboxyl-terminal PKA Phosphorylation Sites on Kv4.2—As a final test of PKA phosphorylation at Thr³⁸ and Ser⁵⁵² on Kv4.2 we generated phospho-site-selective antibodies. The affinity purified antibodies were screened with Western blotting against the unphosphorylated and phosphorylated ovalbumin-coupled

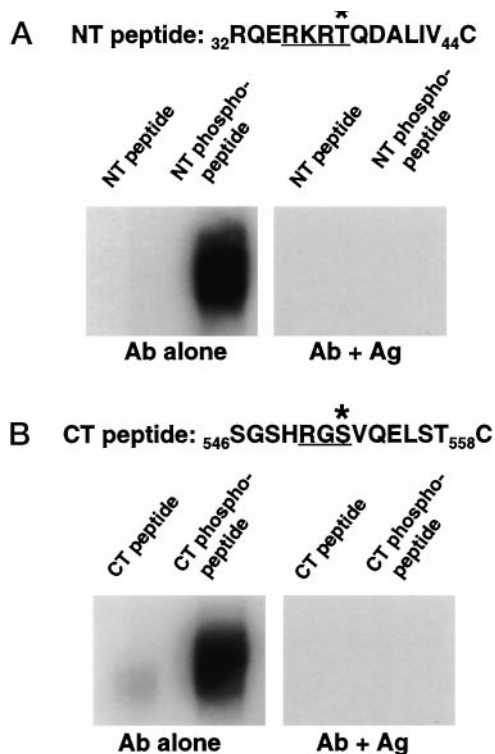


FIG. 10. Phospho-selectivity of the antibodies developed against the PKA phosphorylation sites identified on Kv4.2. Antisera were generated using the NT-(32–44) and CT-(546–558) synthetic peptides containing the phosphorylated amino- or carboxyl-terminal PKA phosphorylation sites and screened with Western blotting. **A**, Western blotting was performed using the unphospho- and phospho-NT-(32–44) synthetic (NT) peptides coupled to albumin. There is selective immunoreactivity of the amino-terminal antibody (Ab) for only the NT synthetic phosphopeptide (left panel). This immunoreactivity is blocked by preincubation with the NT phospho-peptide (Ag) (right panel). **B**, similar results were obtained from Western blotting of the ovalbumin-coupled unphospho- and phospho-CT-(546–558) synthetic (CT) peptides using the carboxyl-terminal antibody. The phospho-selectivity is blocked by preincubation with the phospho-CT peptide (Ag). From these findings, we conclude that both antibodies developed against the Kv4.2 amino- and carboxyl-terminal PKA phosphorylation sites are phospho-selective.

NT-(32–44) or CT-(546–558) synthetic peptides. There was selective immunoreactivity to only the phosphorylated synthetic peptides that were blocked by preincubation of the antibody with the antigen (Fig. 10). We next sought to determine whether the antisera recognized the PKA phosphorylation sites in the GST fusion proteins. Western blotting revealed that the antisera selectively recognized the phosphorylated amino- and carboxyl-terminal fusion protein constructs (Fig. 11). This immunoreactivity was blocked by preincubation with the antigen (not shown). These data confirm the sequencing results obtained with PKA phosphorylation of the fusion proteins. Furthermore, from these experiments we conclude that the amino- and carboxyl-terminal antibodies are phospho-selective.

Kv4.2 Phosphorylation by Endogenous PKA in the Intact Cell—The goal of this series of experiments was to investigate the phosphorylation of Kv4.2 by PKA in the intact cell. Kv4.2 was expressed as an EGFP fusion protein construct in COS-7 cells. Following expression of EGFP-Kv4.2 in COS-7 cells, green fluorescence was evident on both intracellular and plasma membranes. Forskolin was applied to the cell cultures for activation of the endogenous cAMP cascade. Examination of green fluorescence after forskolin stimulation revealed no obvious changes in EGFP-Kv4.2 protein levels or subcellular distribution. Western blotting using the phospho-selective amino-

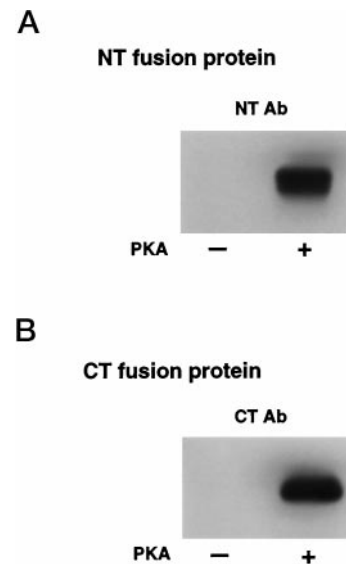


FIG. 11. Detection of the phosphorylated GST fusion proteins using the phospho-selective antibodies. The Kv4.2 amino- and carboxyl-terminal (NT and CT) recombinant GST fusion proteins were used to characterize further the phospho-selectivity of the antibodies (Ab). **A**, in this immunoblot, the NT antibody recognizes phosphorylated NT fusion protein (+PKA) but not the unphosphorylated fusion protein (–PKA). **B**, similar results were obtained using the CT antibody. The immunoreactivity shown in **A** and **B** is blocked by preincubation with the antigen (not shown). We conclude from these studies that each of the antibodies demonstrates selective immunoreactivity to the phosphorylated fusion protein construct.

and carboxyl-terminal antibodies and the non-phospho-selective carboxyl-terminal antibody was then performed (Figs. 12A and 13A, respectively). All of the antisera recognized the same band of immunoreactivity at the appropriate molecular weight for the EGFP-Kv4.2 fusion protein (97 kDa). Densitometric analysis demonstrated a significant increase in immunoreactivity with the amino- and carboxyl-terminal antibodies following forskolin stimulation (NT, $361.3 \pm 7.6\%$ of control; CT, $286.2 \pm 60.0\%$ of control, $n = 3$, $p < 0.05$) (Figs. 12B and 13B, respectively). There was no significant increase in immunoreactivity in parallel blots using the general carboxyl-terminal antibody following forskolin stimulation, indicating that this manipulation does not result in a change in protein expression (Figs. 12A and 13A). Thus we conclude that Thr³⁸ and Ser⁵⁵² within Kv4.2 are phosphorylated by endogenous PKA in the intact cell.

Modulation of PKA Phosphorylation of Kv4.2 in Hippocampal Area CA1—In the final series of experiments we evaluated the effects of forskolin stimulation on PKA phosphorylation of Kv4.2 in hippocampal area CA1 using the phospho-selective antibodies. Immunoblots probed with the amino-terminal antibody demonstrate very low basal phosphorylation of Kv4.2 at the amino-terminal PKA site compared with a significant increase in phosphorylation at this site following brief (10 min) forskolin stimulation ($440.5 \pm 15.6\%$ of control, $n = 3$, $p < 0.05$) (Fig. 14A). The immunoreactivity observed at 70 kDa (Fig. 14A) is blocked by preincubation of the antibody with the amino-terminal phosphopeptide against which the antibody was made (not shown). Immunoblots probed with the carboxyl-terminal antibody demonstrate minimal basal immunoreactivity that is significantly increased following forskolin stimulation ($333.4 \pm 47.2\%$ of control, $n = 3$, $p < 0.01$) (Fig. 14B). Preincubation with the carboxyl-terminal synthetic phosphopeptide also blocked this immunoreactivity (not shown). The 70-kDa band appears to be *bona fide* Kv4.2 as this protein is also recognized by a commercially available anti-Kv4.2 antibody (Alomone Labs)

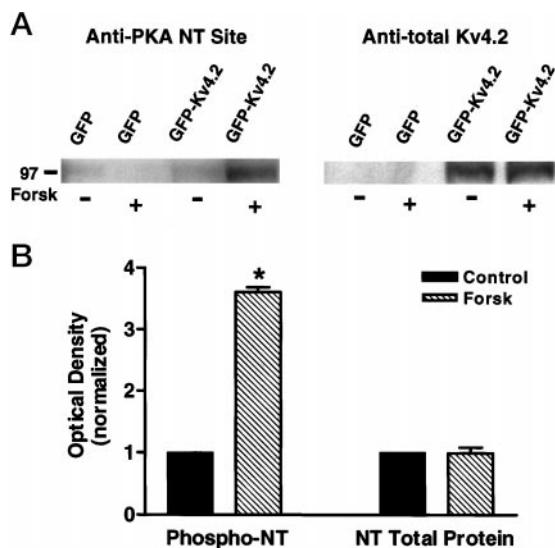


FIG. 12. Endogenous PKA recognizes the amino-terminal PKA phosphorylation site within Kv4.2 in the intact cell. COS-7 cells transiently expressing EGFP or EGFP-rKv4.2 were stimulated with 50 μ M forskolin to activate PKA. **A**, Western blotting was performed using two antibodies to Kv4.2 as follows: the phospho-selective antibody to the phosphorylated amino-terminal PKA site (*Anti-PKA NT Site*) (left panel) and a general antibody that recognizes the carboxyl terminus (*Anti-total Kv4.2*) (right panel). The immunoreactivity at 97 kDa that is seen with both antibodies corresponds to the EGFP-Kv4.2 fusion protein construct. By using the anti-PKA NT antibody, a significant increase in immunoreactivity is seen with forskolin (*Forsk*) stimulation compared with control (vehicle-treated) cultures (left panel). With the anti-total Kv4.2 antibody we were able to determine that there were good expression levels of the EGFP-Kv4.2 construct and that there was no significant change in protein expression levels with forskolin stimulation (right panel). **B**, the above change in immunoreactivity with forskolin stimulation compared with vehicle-treated cultures was evaluated using densitometry. There is a significant increase in immunoreactivity with forskolin stimulation using the anti-PKA NT antibody compared with control levels ($361.3 \pm 7.6\%$ of control, $n = 3$, $p < 0.05$). There is no significant change in immunoreactivity using the anti-total Kv4.2 antibody in the stimulated compared with control cultures ($99.4 \pm 8.9\%$ of control, $n = 3$). From these findings, we conclude that the amino-terminal phosphorylation site on Kv4.2 is recognized by endogenous PKA in intact cells.

and by several other anti-Kv4.2 antisera we have generated (not shown).

From these studies we conclude that, in the hippocampal CA1 subregion where Kv4.2 is expressed, there is modulation of PKA phosphorylation within the cytoplasmic domains of Kv4.2 on Thr³⁸ and Ser⁵⁵². These data provide candidate loci for the modulation of transient channels in CA1 of hippocampus by the cAMP cascade.

DISCUSSION

Our findings provide the initial basis for investigation of direct regulation of Kv4.2 by PKA phosphorylation. We determined that PKA phosphorylates the amino- and carboxyl-terminal cytoplasmic domains of Kv4.2 *in vitro*. We identified a single major phosphorylation site within each of these domains corresponding to Thr³⁸ and Ser⁵⁵² within the Kv4.2 amino acid sequence. These sequencing data were then used for generation of antisera that selectively recognize these sites. By using these phospho-selective antisera, we observed that the amino- and carboxyl-terminal cytoplasmic domains of Kv4.2 are phosphorylated by endogenous PKA in the intact cell. In the final series of experiments reported here, we used the phospho-selective antibodies to show modulation of PKA phosphorylation of the amino and carboxyl termini of Kv4.2 in hippocampal area CA1, where Kv4.2 is expressed and modulated by the cAMP cascade. The phospho-selective antisera for the PKA sites within Kv4.2

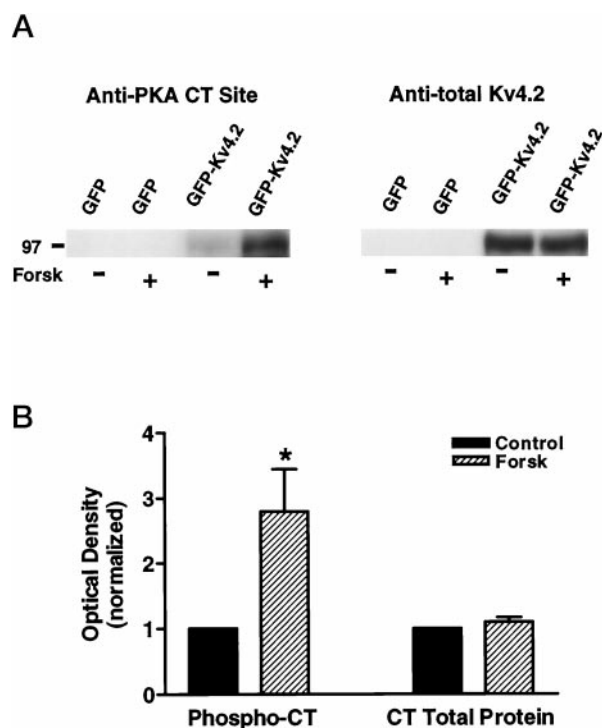


FIG. 13. Endogenous PKA recognizes the carboxyl-terminal PKA phosphorylation site on Kv4.2 in the intact cell. COS-7 cells transiently expressing EGFP or EGFP-rKv4.2 were stimulated with 50 μ M forskolin (*Forsk*) to activate PKA. **A**, Western blotting was performed using two antibodies to the carboxyl terminus as follows: the phospho-selective antibody to the phosphorylated carboxyl-terminal PKA site (*Anti-PKA CT Site*) (left panel) and an antibody that recognizes the carboxyl terminus of Kv4.2 (*Anti-total Kv4.2*) (right panel). The immunoreactivity at 97 kDa that is seen with both antibodies corresponds to the EGFP-Kv4.2 fusion protein construct. By using the anti-PKA CT antibody, a significant increase in immunoreactivity is seen with forskolin stimulation compared with control (vehicle-treated) cultures (left panel). By using the anti-total Kv4.2 antibody we saw good expression levels of the EGFP-Kv4.2 construct and no significant change in expression levels with forskolin stimulation (right panel). **B**, the increase in immunoreactivity with forskolin stimulation compared with vehicle-treated cultures was evaluated using densitometry. There is a significant increase in immunoreactivity using the anti-PKA CT antibody with forskolin stimulation compared with control levels ($286.2 \pm 60.0\%$ of control, $n = 3$, $p < 0.5$). There is no significant change in immunoreactivity using the anti-total Kv4.2 antibody in the stimulated compared with control conditions ($110 \pm 7.0\%$ of control, $n = 3$). Based on these studies, we conclude that the carboxyl-terminal phosphorylation site on Kv4.2 is recognized by endogenous PKA in intact cells.

provide a potentially powerful reagent for monitoring phosphorylation at specific PKA sites on the native channels expressed in ventricular myocardium and hippocampus following activation of the PKA cascade.

Voltage-dependent, transient K⁺ channels regulate membrane excitability in both hippocampal pyramidal neurons and ventricular myocytes (5, 6). The preponderance of evidence indicates Kv4.2 α -subunits contribute to forming these transient currents (3, 9). In hippocampal CA1 dendrites, voltage-dependent, transient K⁺ channels regulate both pyramidal neuron excitability and the magnitude of the post-synaptic excitatory post-synaptic potential (5). The electrophysiological properties of the transient K⁺ currents recorded in hippocampal CA1 dendrites are consistent with those of Kv4.2, and Kv4.2 is selectively localized to this region of hippocampus (3, 9). Of interest in the context of our findings is that the voltage-dependent activation of the transient currents recorded in dendrites is decreased by PKA or PKC activation (21). In ventricular myocardium, the transient current contributes to action

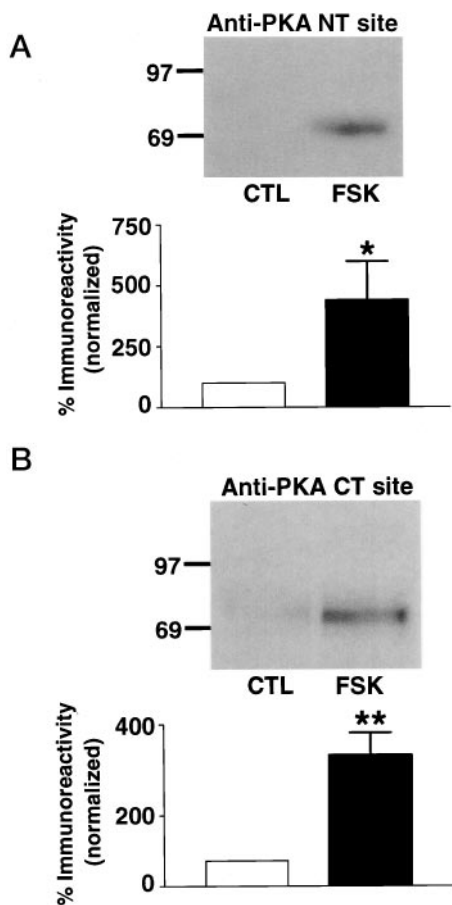


FIG. 14. Modulation of PKA phosphorylation of Kv4.2 in hippocampal area CA1. Representative Western blots of CA1 membrane proteins (1 CA1/lane) were probed with the Kv4.2 PKA site antibodies following brief stimulation with 50 μ M forskolin (*FSK*) to activate PKA versus Me₂SO vehicle (*CTL*). *A*, Western blotting with the phospho-selective antibody to the phosphorylated amino-terminal PKA site (*Anti-PKA NT Site*) is shown in the upper panel. Densitometry of the 70-kDa band corresponding to Kv4.2 shows that there is a significant increase in immunoreactivity following brief (10 min) forskolin stimulation (*FSK*) compared with low levels of immunoreactivity under basal conditions (*CTL*) (forskolin = $440.5 \pm 15.6\%$ of control, $n = 3$, $p < 0.05$). The immunoreactivity observed at 70 kDa (*A*) is blocked by preincubation of *Anti-PKA NT Site* with the amino-terminal phospho-peptide against which the antibody was raised (not shown). *B*, Western blotting using the phospho-selective antibody to the phosphorylated carboxyl-terminal PKA site (*Anti-PKA CT Site*) is shown in the upper panel. Densitometry of the 70-kDa band reveals a significant increase in immunoreactivity with forskolin stimulation (*FSK*) compared with basal conditions (*CTL*) ($333.4 \pm 47.2\%$ of control, $n = 3$, $p < 0.01$) (*B*). Preincubation with the carboxyl-terminal synthetic phosphopeptide blocked this immunoreactivity (not shown). The 70-kDa band is also recognized by a commercially available anti-Kv4.2 antibody (Alomone Labs) and by several other anti-Kv4.2 antisera we have generated (not shown). From these data we conclude that, in the hippocampal CA1 subregion, there is modulation of PKA phosphorylation within the cytoplasmic domains of Kv4.2 on Thr³⁸ and Ser⁵⁵².

potential duration and myocardial contractility, and it is thought to be involved in cardiac pacemaker actions (6). Studies have shown that in rat ventricular myocardium, Kv4.2 is one of the K⁺ channel α -subunits that significantly contributes to the transient current recorded in this region of the heart (3, 11, 36). Furthermore, there is evidence suggesting that transient currents in ventricular myocytes are modulated by neurotransmitter systems that are coupled to second messenger cascades (22–24). Together these data support the hypothesis that during the regulation of hippocampal and myocardial excitability the activity of Kv4.2 is modulated by protein kinase activation.

Protein phosphorylation is known to be an important mechanism of regulation of K⁺ channel activity (37–41). There are well documented examples of PKA or PKC phosphorylation of K⁺ channel α -subunit domains that result in modulation of channel properties. Phosphorylation of K⁺ channel amino- or carboxyl-terminal cytoplasmic domains by Ser/Thr kinases has been shown to alter inactivation kinetics, change levels of channel expression at the cell membrane, and modulate current amplitude (32). These findings and the idea that the cytoplasmic domains would be accessible to cellular kinases make it likely that these regions are sites of protein kinase regulation of the Kv4.2 α -subunit. An additional important point is that in the K⁺ channel cytoplasmic domains a divergence in homology could allow selective protein kinase regulation of various channel subtypes. On the other hand, conservation of protein kinase regulatory sites implies preservation of a regulatory site across channel subtypes. The Kv4 proteins demonstrate a very high degree of sequence homology in the regions on the six transmembrane domains and less homology in the amino- and carboxyl-terminal cytoplasmic domains (42–45). Interestingly, a PKA site at Thr³⁸ is not conserved among the Kv4 subfamily members, whereas the site at Ser⁵⁵² is conserved.

We initially focused on the identification of the sites at which PKA phosphorylates Kv4.2; in addition, we have previously demonstrated that PKC and CaMKII also phosphorylate the carboxyl- but not the amino-terminal domain of Kv4.2 (35). The amino acids within the Kv4.2 sequence that are phosphorylated by PKC and CaMKII have not yet been identified. However, our findings suggest that PKA, PKC, and CaMKII do not converge on Ser⁵⁵² as evidenced by the fact that there is relatively low ³²P incorporation into this site by PKC or CaMKII compared with known substrates for these protein kinases or compared with PKA phosphorylation of the site. Amino acid sequencing experiments to identify the PKC and CaMKII sites are required to determine conclusively if there is any overlap in the sites where these protein kinases phosphorylate Kv4.2.

In the current studies we have identified a novel approach to ascertaining the cytoplasmic domain phosphorylation sites on K⁺ channels. Our experimental approach provides a paradigm by which to study kinase phosphorylation of other K⁺ channels. The methodology is applicable to any channel or membrane protein with substantial cytoplasmic domains such as the amino and carboxyl termini of other voltage-dependent K⁺ channels. This approach, *i.e.* express the carboxyl and amino termini in *E. coli*, identify the phosphorylation sites in the recombinant protein, and then generate phospho-selective antisera, should be adaptable to the study of most K⁺ channels. However, one caveat to our approach is the possibility of missing some sites in the native channel. There may be phosphorylation sites in parts of the native channel that are not present in the cytoplasmic amino- and carboxyl-terminal domains or that are not efficacious sites for phosphorylation *in vitro*.

Our methodology provides advantages over the traditional approach of using site-directed mutagenesis for the identification of protein kinase phosphorylation sites. Although insight into protein kinase regulation of ion channels may be obtained through site-directed mutagenesis, this approach may cause conformational changes and alterations in tetramerization of α -subunits, leading to problems with the identification of physiologic phosphorylation sites. In addition, site-directed mutagenesis does not allow for evaluation of the phosphorylation sites in the native channel *in vivo*. In contrast, our approach using phospho-selective antibodies provides a tool for the studying phosphorylation sites within the native channel *in vivo*. The phospho-selective antibodies can be used to ascertain phosphorylation of the sites within the native channel by en-

ogenous protein kinases. The antibodies can also be used to evaluate the conditions under which these sites are phosphorylated. In addition, the subcellular distribution of phosphorylation at these sites can be evaluated. In this manner, our approach provides a powerful tool for the evaluation of K⁺ channel phosphorylation under physiologic conditions that could significantly facilitate the understanding of protein kinase regulation of membrane excitability.

The PKA cascade is a major site of regulation of hippocampal and myocardial excitability. Our findings provide the first insight into a specific molecular target, Kv4.2, for PKA in the context of regulation of membrane excitability. Future work will allow determination of the role of Thr³⁸ and Ser⁵⁵² phosphorylation in the regulation of Kv4.2 channel function. The use of phospho-selective antisera for these sites will allow quantitation of these specific phosphorylation events *in vitro* and *in vivo*.

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