ERK/MAPK Regulates the Kv4.2 Potassium Channel
by Direct Phosphorylation of the Pore-forming Subunit

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Running Title – ERK phosphorylation of Kv4.2

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ABSTRACT

Kv4.2 is the primary pore-forming subunit encoding A-type currents in many neurons throughout the nervous system, and also contributes to the I\textsubscript{to} of cardiac myocytes. A-type currents in the dendrites of hippocampal CA1 pyramidal neurons are regulated by activation of ERK/MAPK, and Kv4.2 is the likely pore-forming subunit of that current. We have previously shown that Kv4.2 is directly phosphorylated at 3 sites by ERK/MAPK (Threonine 602, Threonine 607 and Serine 616). In this study we determined whether direct phosphorylation of Kv4.2 by ERK/MAPK is responsible for the regulation of the A-type current observed in neurons. We made site-directed mutants, changing the phosphosite serine (S) or threonine (T) to aspartate (D) to mimic phosphorylation. We found that the T607D mutation mimicked the electrophysiological changes elicited by ERK/MAPK activation in neurons: a rightward shift of the activation curve and an overall reduction in current compared to wildtype. Surprisingly, the S616D mutation caused the opposite effect, a leftward shift in the activation voltage. KChIP3 ancillary subunit coexpression with Kv4.2 was necessary for the 607D effect, as the 607D mutant when expressed in the absence of KChIP3 was not different from wildtype Kv4.2. These data suggest that direct phosphorylation of Kv4.2 at T607 is involved in the dynamic regulation of the channel function by ERK/MAPK and an interaction of the primary subunit with KChIP is also necessary for this effect. Overall these studies provide new insights into the structure/function relationships for MAP kinase regulation of membrane ion channels.
INTRODUCTION

Many studies have shown that the extracellular-signal regulated kinase (ERK) is important for regulation of neuronal function, particularly playing a role in the regulation of synaptic plasticity and long-term memory formation (3, 13, 14, 30, 46, 48). Considerable evidence is accumulating that ERK activation plays a role in the regulation of postsynaptic excitability, specifically operating in the context of synaptic plasticity (40, 46, 48). One potential mechanism of this regulation by ERK is indirect, through long-term modulation of cell properties through the control of gene transcription and regulation of channel gene expression (9). Another possible mechanism for ERK to modulate neuronal excitability is through direct regulation of membrane ion channels that regulate the membrane potential and thereby intrinsic membrane properties.

In our recent studies we have focused on regulation of ion channels by ERK because modulation of excitability may be a critical factor that ultimately controls the induction of long-lasting changes in synaptic strength. One possible direct target of ERK is the K\(^+\) channel, Kv4.2, which encodes a transient A-type K\(^+\) current that is present in the dendrites of CA1 pyramidal neurons. Since these currents are at high density in dendrites where the neurons receive synaptic input, rapid voltage-dependent activation of these channels can limit the peak amplitude of back-propagating action potentials, as well as modulate incoming synaptic information. Thus, these currents can exert profound effects on hippocampal network communication (23). Recent studies indicate that ERK activation can regulate the dendritic A-type K\(^+\) currents (15, 47, 51). For example, the amplitude of A-type K\(^+\) currents in neurons is decreased by PKA and PKC activation, an effect that is mediated by ERK
activation (21, 22, 25, 47, 51). This ERK modulation of A-type current amplitude can enhance the peak amplitude of back-propagating action potentials in the dendrites (22, 51) and thereby enhance the depolarization seen at synapses. Via this mechanism ERK can ultimately, albeit indirectly, control voltage-dependent NMDA receptor activation.

There is now good evidence that Kv4.2 is the channel-forming primary subunit that underlies this transient A-type current characterized in pyramidal neurons of the hippocampus, as well as in other brain areas and in cardiac myocytes (17-19, 39, 41, 50). Studies show that Kv4.2 is localized to the pyramidal cell dendrites (29, 37, 42). Furthermore, physiological studies demonstrate that the pharmacological and kinetic properties of Kv4.2-encoded currents in expression systems are similar to the transient outward currents in dendrites (8, 23, 34, 36). In addition, Kv4.2-encoded currents become even more similar in their characteristics compared to the endogenous A-type current when Kv4.2 is coexpressed with other interacting subunits such as KChIPs (2, 24, 33) as well as DPPX (27, 31). Finally, preliminary studies indicate that Kv4.2 knock-out animals show a greatly reduced dendritic A-type current in pyramidal cell dendrites (50).

In prior studies we have shown that the cytoplasmic carboxy-terminus of Kv4.2 is phosphorylated by the ERK subtype of the mitogen activated protein kinases (MAPKs) (1). In these earlier studies we identified three ERK/MAPK phosphorylation sites at amino acid residues T602, T607 and S616. In the present studies we tested the hypothesis that ERK regulates the Kv4.2 A-type potassium channel via direct phosphorylation of the pore-forming alpha subunit at the three sites previously mapped. We used a site-directed mutagenesis approach to determine if mimicking phosphorylation of Kv4.2 at these sites alters channel functional properties, biophysical characteristics, and cell-surface expression.
Overall, our results support the hypothesis that ERK regulates Kv4.2 channels by direct phosphorylation. Our mutagenesis and functional studies indicate that introduction of a negative charge at serine 607 in the C-terminus of Kv4.2 affects the voltage-dependence of channel activation and the rate of recovery from inactivation. These effects are reminiscent of the effects of ERK activation on A-current in intact neurons. This regulation of the outward K\textsuperscript{+} current is due to changes in gating kinetics of the current as ERK phosphorylation has no effect on protein expression or surface membrane localization. These results provide a bridge between prior biochemical results obtained \textit{in vitro} (1) and functional studies performed in the dendrites of neurons and indicate that direct ERK phosphorylation of Kv4.2 channel is a primary mechanism for the ERK-mediated decrease in A-type currents previously reported (21, 22, 47, 51).

\section*{METHODS}

\textit{Functional expression in Xenopus oocytes}

Oocytes were harvested as described previously (33). After approximately 24 hours, oocytes were injected with 3-10 ng of DNA Kv4.2 (wildtype or mutants) + KChIP3 in 1:1 ratio or Kv4.2 (wildtype or mutants) + GFP (1:1) using a Nanoject microinjector (Drummond Scientific Co.) into the nucleus of Stage V- Stage VI oocytes. Currents were recorded after 2 days under two-electrode voltage-clamp using an Axoclamp 2A amplifier (Axon Instruments) at room temperature. Microelectrodes were pulled from filamented glass (1.5 mm x 0.86 mm – A-M Systems) filled with 3M KCl. The current electrode had resistance of 0.30-0.50 M\textOmega, while the voltage electrode ranged from 0.3-1.0 M\textOmega. Currents were leak subtracted on line using P/4 leak subtraction. Data was digitized at 2 kHz and stored to
computer using a Digidata 1200. Current protocols used to obtain data include: 1) activation - hyperpolarization to -110 mV then depolarization to +40 mV for 400-800 msec and repeated in -5 or -10 mV step intervals. 2) Inactivation – depolarization to 0 mV then hyperpolarization to -110 mV for 650 msec, changing this step by +5mV intervals, then depolarization to 0 mV. 3) Recovery from inactivation for oocytes expressing Kv4.2 alone a two pulse protocol was used, which included a 500 ms depolarization to 0 mV followed by a hyperpolarization to -110 mV of varying durations (5 msec with subsequently longer hyperpolarizations increasing in increments of 100 ms) for a final hyperpolarization of 705 msec, then depolarized to 0 mV. Kv4.2+KChIP3 recovered from inactivation more quickly than Kv4.2 alone, so the initial hyperpolarization pulse was 2 milliseconds increasing in increments of 10 milliseconds for a final duration of hyperpolarization of 192 milliseconds.

The chamber was continuously perfused at a rate of 3-6 ml/min with ND-96 (in mM): 96 NaCl; 2 KCl; 1.8 CaCl₂; 1 MgCl₂; and 5 HEPES pH 7.4 with NaOH. Oocytes expressing mutant DNA and wildtype (control) DNA were always recorded on the same day, and were recorded in at least 3 batches of oocytes. The data from oocytes expressing wildtype DNA from different days were not different, therefore all wildtype data was combined. Data was analyzed using Clampfit, Origin, and Prism programs. Inactivation time constants were fit in Clampex with the Simplex method. Peak currents were obtained and conductance was determined using a reversal potential (V_{rev}) of -95 mV according to the equation, G = I_p/(V_c-V_{rev}). Where I_p is the peak of the current at a given voltage command (V_c). Activation and inactivation curves were fit with Boltzmann sigmoidal curve with the equation of G/G_{max} = 1/{1+exp ((X-V_{1/2})/Slope)}. Where X is equal to the test potential (V_m). The mean ± SEM V_{1/2} (voltage at which half the currents are activated) was determined from the Boltzmann fit.
and compared among the mutants and WT using a One-Way Anova and post-hoc Bonferroni test.

**DNA preparation and Site-directed Mutagenesis**

The original Kv4.2 and KChIP3 cDNA were provided by Dr. Paul Pfaffinger. Both constructs are in a CMV vector. Point mutations were made using the site-directed mutagenesis kit (Stratagene). The primers (upper sequence) used include T602A-5’-gcattccagacctccagtaaccacccag-3’, T602D – 5’-gcattccagatctccagtaacc-3’, T607A – 5’-cctccagtaacgccccagaag-3’, T607D-5’-cctccagtaacgccccagaag-3’, S616A-5’-gacagcccaggattctgattccggga-3’, S616D-5’-gacagcccaggattctgattccggga-3’. Mutations were confirmed by restriction enzyme digest and DNA sequencing. In most cases, the entire Kv4.2 region was sequenced to ensure that no other mutations existed.

**Expression in COS-7 cells**

The FuGene 6 Transfection Reagent (Roche) was used for COS-7 cells transfections with plasmid DNAs of wildtype Kv4.2 or mutant DNA and KCHIP3 (1:1 ratio, 2.0 µg of total DNA). Transfected cells were grown on 35-mm plates to a $2 \times 10^5$ cell density. Approximately 24 hours after transfection, the cells were harvested by scraping in 1 ml homogenization buffer (HB) containing (in mM) 20 Tris pH 7.5; 1 EGTA; 1 EDTA; 4 PNPP; 1 Na$_3$VO$_4$; 100 PMSF; 1 Na$_4$P$_2$O$_7$; protease inhibitor cocktail added, 1:100 (Sigma) and centrifuged at 10,000 rpm for 5 minutes at 4°C. The cell pellet was resuspended in 500µl HB, sonicated and centrifuged (60,000 rpm for 20 minutes at 4°C), and the supernatant was removed. The membrane pellet was resuspended in 5% SDS/HB with 100 mM dithiothreitol
(DTT), and protease inhibitor cocktail (1:100 Sigma). Sample buffer was then added, and the samples were loaded on a SDS-PAGE gel (10%) for Western blotting (see below).

**Surface Biotinylation**

Transfected COS cells were rinsed twice with cold PBS (pH 7.2), followed by a rinse with cold PBS pH 8.0 with 1mM CaCl₂ and 1mM MgCl₂. The cells were then incubated with 200 µl of 0.5 mg/ml EZ-link sulfo-NHS-LC-biotin – Pierce (in PBS pH 8) at 4°C for 30 minutes. The cells were washed with cold PBS twice and incubated with 100mM glycine and PBS at 4°C for 30 minutes to quench the biotinylation reaction. The cells were washed 3 times in PBS. Cells were lysed with 250 µl RIPA buffer containing in mM: 150 NaCl; 10 Tris pH 7.2; 5 EDTA; 0.1% SDS; 1% Triton; 1% deoxycholate with phosphatase and protease inhibitor cocktail added in each well and constant shaking for 1 hour at 4°C. Lysates were then centrifuged at 20,000 g for 30 min at room temperature. The supernatant (150 µl) was incubated in a 1:1 slurry of beads (Ultralink Neutravidin - Pierce) for 2 hours at room temp with RIPA buffer. The pellet was eluted with 50µl Laemmli loading buffer and boiled at 100°C for 3 min and the samples were loaded on a SDS-PAGE gel. The biotinylated Kv4.2 protein was normalized to the total Kv4.2 assayed from the total cell lysate and then normalized to wildtype surface expression.

**Western blotting**

Membrane proteins were loaded and run on a 10% acrylamide gel to resolve Kv4.2. Gels were then blotted electrophoretically to Immobilon membrane paper using a transfer tank maintained at 4°C, with typical parameters being an overnight transfer at a constant current of
250 mA (transfer buffer: 192 mM glycine, 25 mM Tris, 20% methanol, pH 8.3). Immobilon membranes were blocked for 1 hr at room temperature in either TTBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) + 5% powdered milk, 3% BSA, (+ 0.05% microcystin for phospho-antibodies) for Kv4.2 antibodies or TTBS + 3%BSA for the ERK antibodies. Primary antibody concentrations were as follows: 1:500 Kv4.2 polyclonal antibody from Chemicon (Temecula, CA), 1:500 304 phospho-Kv4.2 antibody).

**Antibody detection.** Immobilon membranes were incubated sequentially with primary antibody and a biotin-labeled goat anti-rabbit IgG secondary antiserum (Cell Signalling 1:20,000) and then developed using enhanced chemiluminescence (Amersham Biosciences). Blots were washed extensively in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 after incubations with primary and secondary antibodies (typically, four washes of 10 min each).

**Alkaline phosphatase treatment**

Entire mouse hippocampus or COS-7 cells transfected with Kv4.2 + KChIP3, were homogenized in 500 µl of homogenization buffer (HB) containing in mM –20 Tris pH 7.5; 1 EGTA; 1 EDTA; 1 Na₄P₂O₇; 4 PNPP; 1 Na₃VO₄; 100 PMSF; microcystin (1:100, protease inhibitor cocktail added). Hippocampi (or COS cells) to be treated with alkaline phosphatase were homogenized in buffer without phosphatase inhibitors (PNPP (Sigma), Na₃VO₄; microcystin). Homogenate was treated with 1 or 3 units alkaline phosphatase (2 units for COS cells) and incubated for 15 minutes at 30°C. After treatment, homogenates were centrifuged at 1000 x g at 4°C, for 5 minutes. Supernatant was then centrifuged at 60,000
rpm at 4° C, for 20 minutes. Pellet was resuspended in 5%SDS/HB with 200mM dithiothreotol (DTT) and protease inhibitor cocktail, PMSF and microcystin. Protein was normalized using Biorad DC protein assay kit and 4X sample buffer containing in mM – 3 Tris pH 6.8; 200 DTT and 40% glycerol, 8% SDS and 0.08 mg/ml bromphenol blue added.

Protein expression and purification

The WT and AAA Kv4.2 C-terminal proteins were expressed in *E. coli* as glutathione S-transferase (GST) fusion proteins using methods modified from Hakes and Dixon (20). Plasmids containing the Kv4.2 C-terminal cDNAs were constructed using the GST-fusion vector pGEX-KN (20). A single colony of BL21(DE3)-pLysS cells transformed with the protein plasmid was grown in Luria broth (LB: 170 mM NaCl, pH 7.5, 1% tryptone, 0.5% yeast extract) containing 20 µg/ml carbenicillin and then used to seed a 500 ml culture. After growing to an optical density of 0.6-0.8 (A₆₀₀) the culture was centrifuged (Beckman Model J2-21M, 1000 g, 15 min, 4°C). The cell pellet was resuspended in 500 ml LB with carbenicillin. The bacteria was induced by incubation at room temperature with 200 µM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 hr and harvested by centrifugation.

The cells were resuspended and incubated in Tris buffer 1 (50mM Tris-HCl, pH 8.0, 2mM EDTA, 10 mg/ml pepstatin, 10 µg/ml leupeptin, 100 µM PMSF) containing 10 mM β-mercaptoethanol and 100 µg/ml of lysozyme (Sigma) for 15 min at 30°C. Following solubilization with 1.5% *N*-laurylsarcosine, the lysate is incubated with 20 µg/ml Dnase I (Boehringer Mannheim) and 10 mM MgCl₂. The lysate is then centrifuged (Sorvall RT 6000B, 1000 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 hr at 4°C. The beads were washed 3 times with Tris buffer 1 by repeated centrifugation (Sorvall, 100 g, 5 min, 4°C). After the final wash, the bead preparation was resuspended in Tris buffer 2 (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM Na₄P₂O₇, 10 µg/ml aprotinin, 10 µg/ml leupeptin.

Protein phosphorylation

WT and AAA GST fusion proteins were phosphorylated with activated ERK2 for 0.5-1 hour at 37ºC according to the manufacturers’ directions (Stratagene). The reaction included: 1.0µCi/µl reaction of [γ-³²P]ATP in the presence of activated ERK2, HEPES buffer (in mM: 25 HEPES, 0.5 EDTA, 0.5 EGTA, 1 Na₄P₂O₇, 10 µg/ml aprotinin, 10 µg/ml leupeptin), 10 mM MgCl₂, and 100 µM ATP. The reaction was stopped with sample buffer [in mM: 30 Tris-HCl, pH 6.8, 200 dithiothreitol, 40% glycerol, 8% sodium dodecyl sulfate (SDS), 0.04 mg/ml bromophenol blue], and the samples were boiled for 5 min. The fusion proteins were separated using 10% SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining. The phosphopeptides were identified by autoradiography.

RESULTS

We previously identified three candidate ERK phosphorylation sites in the C-terminal domain of Kv4.2 through in vitro studies (Figure 1) (1). Two other ERK consensus sequences exist within the Kv4.2 C-terminal sequence, but these sites were not phosphorylated in our initial studies. We have also developed a phospho-specific antibody
that recognizes full-length Kv4.2 when it is phosphorylated at all three ERK phosphorylation sites (1). Using this affinity-purified antiserum we observed that basal levels of phosphorylation of Kv4.2 exist in mouse hippocampal tissue (Figure 1C) and COS-7 cells (Figure 1D), confirming that these sites are indeed sites of phosphorylation in the intact Kv4.2 channel in both COS-7 cells and hippocampus.

Given an ERK-mediated decrease in the A-type current in hippocampal slices (47, 51), we hypothesized that phosphorylation of Kv4.2 at these three phosphorylation sites (T602, T607 and S616) would cause a decrease in Kv4.2 channel function. To determine whether direct phosphorylation of Kv4.2 at the ERK sites is responsible for ERK modulation of Kv4.2 function, we chose to construct site-mutants of each phosphorylation site to study the effects of phosphorylation at each individual site, as direct ERK activation in cells is difficult. We used aspartate (D) site-mutants of the 3 ERK phosphorylation sites to mimic Kv4.2 phosphorylation. As controls we utilized both the wild-type channel and mutants that had the phosphorylation sites mutated to an alanine (A). For both the biophysical and protein surface expression studies, all mutants and wildtype Kv4.2 constructs were co-expressed with KChIP3 (unless otherwise indicated – figure 5), a modulatory subunit that we and others have shown affects the biophysical properties and expression levels of Kv4.2. The co-expression of Kv4.2 + KChIP3 in expression systems such as oocytes and COS-7 cells better approximates the functional properties of the native channels (2, 24, 31, 33).

**Mutation of all three sites to aspartate mimics the effect seen in neurons**

In our first experiment we tested the effects of mimicking ERK phosphorylation of Kv4.2 by mutating the T602, T607 and S616 amino acid side-chains to aspartate (D). A
wide variety of previous studies, including studies of Kv4.2, have indicated that D mutations can mimic the effects of serine or threonine phosphorylation (6, 11, 45). We found that indeed, mutation of all three sites to an aspartate (triple D mutant - T602D T607D S616D) decreased the transient outward K⁺ current (Figure 2). Specifically, the triple D mutant displayed a more depolarized activation voltage compared to wildtype (Figure 2B). The activation curve of the triple D mutant was shifted 17 mV to the right toward more depolarized membrane potentials. The V₁/₂ for the triple D mutant was -7 ± 2 mV, which was significantly different from WT (-24 ± 3 mV; p<0.01) and the triple A mutant (-26 ± 3 mV; p < 0.01). The inactivation curve (at +0 mV) was fit with a single exponential and determined that the time constant of inactivation of the triple D mutant (35 ± 5 ms) was significantly different (p<0.05) from WT (57 ± 5 ms). Steady-state inactivation of the triple D mutant was not different from WT (Table 1). While there was a trend toward a slower rate of recovery from inactivation for the triple D mutant, it was not significantly different from wildtype (Table 1).

To investigate the contribution of each individual phosphorylation site to the regulation of the transient outward K⁺ current, we investigated the functional consequences of single aspartate mutants to mimic ERK phosphorylation at each individual phosphorylation site. We first investigated the effects of mutation of S616 to an aspartate.

**Mutation of serine 616 to aspartate does not mimic the effect seen in neurons**

Surprisingly, mutation of the S616 site to aspartate caused an effect contrary to the effect of the triple D mutant. Mutation of S616D shifted the activation voltage to more hyperpolarized voltages compared to WT (Figure 3B). The activation curve of the S616D
mutant was shifted 11 mV to the left toward more hyperpolarized membrane potentials. The mean $V_{1/2}$ for the S616D mutant was $-35 \pm 2$ mV, compared to WT ($-24 \pm 3$ mV). Interestingly, the effect of the S616D mutant is opposite the effect of ERK on A-currents in hippocampal dendrites. This finding suggests that phosphorylation at this site does not mediate the effects of ERK on A-currents in the dendrites of hippocampal pyramidal neurons. The data with the 616D mutant suggest that phosphorylation at T602 and T607, the remaining two sites, might cause an effect similar to that seen in response to ERK activation in the dendritic A-current in intact cells and similar to the triple D site mutants as well. We therefore determined if mutation of both T602 and T607 to D caused channel inhibition as we anticipated based on neuronal recordings of A-currents and the effect of the Triple D mutant in the oocyte expression system.

**Threonine 602D,607D mimics the effect seen in neurons**

Consistent with this idea, the T602D,T607D double mutant channel exhibited a shift in the voltage-dependence of activation to more depolarized potentials. The T602D,T607D double mutant showed a 14 mV shift in the voltage-dependence of activation (Figure 3B). The mean $V_{1/2}$ of the T602D,T607D mutant (-10 ± 2 mV) was significantly different (p<0.01) from WT (-24 ± 3 mV). The double mutant also displayed a slower recovery from inactivation (19 ± 5, n = 6) that was significantly different than wildtype (Table 1; p<0.05). Similar to the triple D mutation, the inactivation was fit with a single exponential and determined that the time constant of inactivation of the T602D,T607D mutant (29 ± 6 ms) was significantly different from WT (57 ± 5 ms; p<0.01).
The T602D, T607D double mutant channel was quantitatively similar to the triple D mutant channel as the values for $V_{1/2}$ and recovery from inactivation were not significantly different from the triple D ($p > 0.05$). This suggests that the effects of the aspartate mutation at T602 and T607 are responsible for the channel inhibition that we observed in the triple D mutant channel.

Overall our data suggest that there are different effects of phosphorylation of Kv4.2 by ERK at the S616 site versus the T602 and T607 sites. Mutation of T602 and T607 to an aspartate has an overall effect of less current (shift of activation and slower recovery from inactivation), whereas the effect of the aspartate mutation at 616 has the opposite effect, with insertion of a negative charge causing more current and faster recovery from inactivation. It is important to note that the phosphorylation of T602 and T607 mimic the effect of ERK activation on the dendritic A-current in intact cells. Moreover, taken together our data suggest that if all three sites are phosphorylated (mimicked in the triple D mutant), the inhibitory influence dominates over the potentiating effect. That is, we predict that in the native channels, the 602/607 site effect will predominate over the S616 effect if all the sites are phosphorylated simultaneously.

**Mutation of threonine 602 to aspartate has no effect**

We next sought to determine the effects of mutation of the 602 and 607 sites individually. Mutation of T602 alone to an aspartate (T602D) or to an alanine (T602A) had no significant effect on Kv4.2 current (data not shown). There was no apparent shift in activation voltage of Kv4.2 current, steady-state inactivation, or recovery from inactivation.
(Table 1). These data suggest that modification of the 602 site is not sufficient to mimic dual modification of the 602 plus 607 sites.

**Mutation of threonine 607 to aspartate mimics the effect in neurons**

In contrast, mutation of T607 to aspartate caused a pronounced shift toward depolarized potentials in the voltage-dependence of activation of Kv4.2 currents. Thus, mimicking phosphorylation of T607 causes the current to activate at more depolarized potentials (Figure 3A,B). The mutation of T607 to alanine is an important control that suggests that any mutation of this site does not simply cause a conformational change in the protein structure. The activation curve of the T607D mutant also showed an 18 mV shift (-6 ± 3 mV, n = 12) toward more depolarized membrane potentials that was significantly different compared to WT (p<0.001; -24 ± 3 mV) and T607A (p<0.01; -26 ± 3 mV). Similar to what we observed with the triple D and T602D,T607D mutants, mutation of T607 to aspartate significantly (p < 0.01) slowed the rate of recovery from inactivation (20 ± 3 ms, n = 10 vs WT - 10 ± 1 ms, n =25, Figure 4). Also similar to the triple D and T602D T607D mutations, the shift in the activation curve may have been caused by an increase in the rate of inactivation. The inactivation was fit with a single exponential and determined that the time constant of inactivation of the T607D mutant (28 ± 3 ms) was significantly different (p<0.01) from WT vs 57 ± 5 ms.

Mutation of T607 to an aspartate has a similar effect on the outward K⁺ current as both the triple D mutation and the T602D,T607D double mutation. Specifically, the V$_{1/2}$ and the recovery from inactivation of the T607D mutant channel were not significantly different from the triple D or the double mutant channel. Importantly, these changes are similar to the
effects of ERK activation on the A-type current in hippocampal dendrites. A parsimonious interpretation of our data is that ERK phosphorylation of T607 \textit{in vivo} is a mechanism by which ERK inhibits A-currents formed by Kv4.2 in neurons. This is suggested by the shift of the activation voltage of the T607D mutant to more depolarized potentials and the decrease in the rate of recovery from inactivation in the T602D and T607D mutants. As we also observed a decrease in the rate of recovery from inactivation in the T602D mutant, phosphorylation of this site may also contribute to channel inhibition as well.

\textbf{T607D Effect Requires KChIP Coexpression}

We have previously reported that the functional effects of PKA phosphorylation of Kv4.2 require coexpression with KChIP3 (Schrader et al 2002). Since phosphorylation of the T607 site is particularly relevant to the A-currents formed by Kv4.2, we examined the functional effects of the T607D and T607A mutants expressed in the absence of KChIP3 coexpression. In the absence of coexpression with KChIP3 the \( V_{1/2} \) of the T607 mutants (alanine or aspartate) did not show any significant differences compared to wildtype Kv4.2 also expressed in the absence of KChIP3 (Table 1). Interestingly, while expression of KChIP3 with the WT and T607A channels causes a leftward shift in the activation voltage, only a small shift of the activation voltage is revealed with the T607D mutation (Figure 5). In addition no effect was seen on the rate of recovery from inactivation for either mutation (Table 1). Therefore it appears that coexpression of KChIP3 is necessary for the functional effects of ERK phosphorylation at the C-terminal ERK phosphorylation site (T607). These data are reminiscent of the necessity of KChIP3 for PKA regulation of Kv4.2 as well and
may suggest a conserved functional role for KChIPs in conferring phosphorylation-dependent regulation on Kv4.2 currents.

Since KChIP3 coexpression with Kv4.2 is necessary for the functional effects of ERK phosphorylation, the possibility exists that KChIP coexpression may be necessary for phosphorylation of Kv4.2 by ERK. To consider this possibility, we used our antibody directed at Kv4.2 phosphorylated by ERK to determine if Kv4.2 phosphorylation by ERK was affected by KChIP3 coexpression (Figure 6A&B). COS cells were transfected with Kv4.2 + control (GFP) or Kv4.2 + KChIP3. Since KChIP3 increases expression of Kv4.2, we determined the ratio of recognition of the antibodies of Kv4.2 alone to Kv4.2 + KChIP3. The ratio of total Kv4.2 antibody recognition of Kv4.2 to Kv4.2+KChIP3 (0.66 ± 0.03, n = 3) was not significantly different (p= 0.94) from the ratio of the phospho-Kv4.2 antibody recognition (0.65 ± 0.09; n = 3). This data suggests that KChIP3 is not necessary for ERK phosphorylation of Kv4.2.

One caveat to the interpretation of our results is that there are two other minimal ERK consensus sites (simply a serine or threonine followed by a proline) in the C-terminal of Kv4.2, at S516 and T567. While these sites could potentially be phosphorylated by ERK, we did not observe any phosphorylation of these sites in our initial characterization of ERK phosphorylation of the Kv4.2 C-terminus (Adams et al 2000). Therefore, it is unlikely that they are phosphorylated in the full-length protein. In an additional control experiment (data not shown), we made a GST-fusion protein construct of the WT C-terminal and triple A mutant C-terminal (T602A, T607A, S616A) and performed an in vitro phosphorylation with activated ERK. As expected the WT C-terminal fusion protein was phosphorylated by
ERK/MAPK. Phosphorylation of the triple A mutant C-terminal was greatly reduced (24 ± 5% of control; n=6), normalized to protein concentration) suggesting that blocking phosphorylation at the T602, T607 and S616 sites greatly reduces ERK phosphorylation of the C-terminal. These data thereby serve as an independent confirmation of our previous direct sequencing studies and indicate using the site-directed mutagenesis approach that these sites are indeed phosphorylated by ERK/MAPK. The residual amount of phosphorylation suggests that the S516 or T567 sites may be phosphorylated in vitro. However, we also cannot rule out the possibility that mutation of the three sites to alanine may alter protein structure and reveal a previously unphosphorylated site.

Protein expression and surface localization

Finally, to determine whether ERK phosphorylation alters protein expression and/or channel surface localization, we expressed the ERK site mutants in the COS-7 cell expression system. This heterologous expression system is more suitable for these studies as levels of protein expression can be highly variable in oocytes. Moreover, we have previously observed phosphorylation-dependent and KChIP-dependent alterations in channel trafficking in COS cells (38, 45). We found no statistically significant effect on total protein expression of any of the site mutants (Table 1). As COS-7 cells have significant basal ERK activity and Kv4.2 phosphorylation (see Figure 1), these data suggest that ERK phosphorylation of Kv4.2 is not a major determinant of overall Kv4.2 channel expression in these cells.

We also found no significant effect of phosphorylation of the Kv4.2 channel on surface expression (Figure 6C). The surface Kv4.2 protein (biotinylated and precipitated with
avidin beads) was normalized to total Kv4.2 protein (from cell homogenates). The triple A (101 ± 23% of WT, n=4) or triple D (137 ± 32% of WT, n = 5) mutants were not significantly different from wildtype surface expression (n=5), although there was a trend toward increased surface expression for the Triple D mutant. These data suggest that ERK phosphorylation of Kv4.2 has no effect on protein expression or surface localization. This observation is consistent with the effects of ERK activation on dendritic A-type current data, as the effects on the A-type current are more rapid than would be expected for changes in total protein or altered protein trafficking (21, 22, 47, 51). Overall, these observations are consistent with our model that the gating effects described above account for the effects of ERK activation in neurons, and that direct phosphorylation of the Kv4.2 pore-forming subunit by ERK causes a decrease in the A-type current of dendrites.

DISCUSSION

We show here that mimicking ERK phosphorylation with aspartate mutants at the T607 site causes a decrease in the Kv4.2-mediated outward K⁺ current. This effect occurs through an increase in the voltage required to activate the channel and a slower recovery from inactivation compared to wildtype. Before we began these studies, four relevant observations had been established in the literature. First, that ERK modulates A-currents in pyramidal neuron dendrites (47, 51). Second, that Kv4.2 is a likely candidate molecule encoding dendritic A-currents (32, 35, 43, 44, 50). Third, that ERK is capable of directly phosphorylating the Kv4.2 C-terminal domain in vitro (1) (see also Figure 1C). Finally, that these candidate ERK sites are capable of being phosphorylated in the intact channel in vivo (Figure 1C and (1)). The present studies provide a direct bridge from these earlier in vitro
Based on our findings, we hypothesize that ERK modulates the A-type current by direct regulation of the Kv4.2 alpha subunit. Our findings support this hypothesis by demonstrating that mimicking ERK phosphorylation of the Kv4.2 alpha subunit is capable of regulating channel biophysical and functional properties. Specifically, we found that mimicking phosphorylation of T607 significantly decreases the current.

Interestingly, the three ERK phosphorylation sites do not appear to have a universal effect. Specifically, introduction of a negative charge at the S616 site causes an effect opposite to that of the T602 and T607 sites. The S616D mutation causes a leftward shift of the activation curve and a faster recovery from inactivation, while the S616A mutation slows recovery from inactivation. This effect of the alanine mutation at S616 has 2 possible explanations: 1) mutation of S616 causes structural changes that affect the Kv4.2 current – specifically altering inactivation kinetics (see below); or 2) S616, and not T602 or T607, is endogenously phosphorylated in the oocyte, and the S616A mutant blocks this effect. Considering the first possible explanation, a structural change in the protein could have results unrelated to phosphorylation at the S616 residue, thus causing an effect independent of phosphorylation. In the second explanation, endogenous phosphorylation in the oocyte could be the result of phosphorylation by ERK or another MAPK. Indeed, activation of ERK vs other MAPKs has been shown to have antagonistic effects (reviewed in (10, 28)).

Finally, while we did not find an effect on surface expression with our mutants in COS cells, we cannot rule out that these sites may participate in targeting the Kv4 subunits to subcellular domains within a specific cell type.
Mechanism of effects

The functional changes that occur upon direct ERK phosphorylation of Kv4.2 appear to be dependent upon its interaction with KChIP. For example, we saw no effect on channel properties with mutation of T607 to an aspartate or to an alanine in the absence of KChIP. In addition, we observed an effect of mimicking ERK phosphorylation on the activation voltage as well as recovery from inactivation, two kinetic characteristics that have been shown to be modified by KChIP interaction (4, 5, 16). This suggests that KChIP coexpression is necessary for functional effects as we and others have previously observed for other phosphorylation sites or other biophysical modulation (24, 33). We cannot rule out the possibility that interactions with other accessory subunits, such as the dipeptidyl peptidase-like proteins (26, 27) may also play an important role in the regulation of the current by phosphorylation.

Functional implications

The present studies implicate the Kv4.2 voltage-dependent potassium channel as a direct target of ERK. In the context of neuronal development and differentiation as well as disease processes, direct ERK/MAPK phosphorylation of Kv4 channel subunits presents a possible mechanism for the acute regulation of $K^+$ currents by growth factors as has been previously reported (12, 49).

In addition, we and others recently have implicated MAPKs in long-term neuronal plasticity and memory formation, and have hypothesized that MAPKs regulate voltage-dependent ion channels in neurons as part of a coordinated mechanism for triggering lasting change in neurons. Previous studies have shown that the dendritic A-current is modulated by
ERK activation (21, 22, 47, 51). Moreover, in an animal model of temporal lobe epilepsy, dendritic A-type currents are reduced along with Kv4.2 protein as well as mRNA. Interestingly, an increase in ERK phosphorylation of the remaining Kv4.2 channels is observed, and kinase inhibition can partially reverse the hyperexcitable effects of decreased A-current (7), suggesting dual regulation of Kv4.2 at a transcriptional as well as posttranslational level. Our analysis shows that the gating properties of Kv4.2 can be dynamically modulated by direct ERK phosphorylation. This modification of Kv4.2 by ERK/MAPK decreases A-current amplitude and increases the amplitude of back-propagating action potentials in neuronal dendrites. These changes may allow greater depolarization at the synapse and increase the likelihood for the induction of plasticity or, in the case of temporal lobe epilepsy, hyperexcitability. The current studies specifically extend this model by suggesting that the effects of ERK on Kv4.2 function are direct, involving ERK phosphorylation of T607 in the C-terminal cytoplasmic domain of Kv4.2.

Acknowledgements

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Figure Legends

Figure 1. The K⁺ channel primary subunit Kv4.2 is phosphorylated at 3 sites by ERK/MAPK. A. Schematic diagram of Kv4.2 with 6 transmembrane spanning domains and N- and C-termini. KChIP is shown interacting with the N-terminal of Kv4.2. B. The amino acid sequence of the Kv4.2 subunit. The three ERK sites that we previously reported (T602, T607 and S616) are represented by black asterisks on the schematic diagram and bold print in the amino acid sequence. Two other ERK/MAPK consensus sites exist within the Kv4.2 sequence, S516 and T567 represented by gray asterisks in A. C. Basal ERK phosphorylation of Kv4.2 in hippocampus. Upper - A phospho-specific antibody directed against all 3 mapped phosphorylation sites was generated. This antibody recognizes a specific band at the level of Kv4.2 (approximately 69kD) in membrane preparations from the hippocampus and is phosphospecific. Hippocampal homogenates were treated with increasing concentrations of alkaline phosphatase to dephosphorylate the protein (see methods). The immunoreactivity of the phospho-Kv4.2 antibody decreases with increasing alkaline phosphatase concentrations. Immunoreactivity is absent at 3 units alkaline phosphatase. D. Basal ERK phosphorylation of Kv4.2 in COS cells. COS cells were transfected with Kv4.2 and KChIP3, or no DNA as a control (untransfected). Cell homogenates were treated with 2 units of alkaline phosphatase. Alkaline phosphatase decreased immunoreactivity of a specific band by the phospho-Kv4.2 antibody (upper).

Figure 2. Mutation of all three sites to an aspartate to mimic phosphorylation (Triple D mutant) causes a shift in the activation voltage compared to wildtype and triple A
**mutation.** The WT, triple A and triple D mutants were expressed with KChIP3 and compared to WT + KChIP3. **A.** Example outward K\(^+\) current traces from a wildtype (black) and triple D mutant (red) recorded on the same day from the same batch of oocytes. (Right) - The maximal outward current from the triple D mutant is scaled up to show the change in activation at lower membrane potentials. **B.** Example outward K\(^+\) current traces from a wildtype (black) and triple A mutant (red) recorded on the same day from the same batch of oocytes. (Right) - The maximal outward current from the WT is scaled up to the amplitude of the triple A to show that there is no effect on activation. The voltage protocol used to obtain the traces is shown below the raw data. The traces are truncated– similar to the figure in A. **C.** Activation curve showing the normalized conductance versus test voltage of the WT vs Triple D and Triple A mutants. The activation curve of the triple D mutant is shifted toward more depolarized membrane potentials, while the activation curve of the triple A is not different from WT. The slopes of the activation curve for WT (18 ± 0.7) was not significantly different from the Triple D mutant (18 ± 1) or triple A (18 ± 1). Inset - Bar graph showing the mean ± SEM V\(_{1/2}\) (acquired from a Boltzman fit of the curve) of the WT, triple A and triple D mutants. The mean V\(_{1/2}\) of triple D (-7 ± 2 mV) was significantly different (**, p<0.01, One Way Anova and post-hoc Bonferroni) from WT (-24 ± 3 mV) and triple A mutant (-26 ± 3 mV).

**Figure 3.** **Mutation of T607D mimics the effect of the triple D mutation.** **A.** Example of outward K\(^+\) current recorded the same day from oocytes expressing the T607D mutant (red) and WT (black) channels. The maximal outward current from the T607D mutant is scaled up to show the change in activation at lower membrane potentials. **B.** Activation curve showing the normalized conductance vs test voltage of the WT, T607D, T602D,T607D and S616D mutants.
The activation curves of the T607D and T602D,T607D mutants are shifted to the right, while the curve of the S616D mutant is shifted to the left. The slopes of the activation curves were not significantly different WT (18 ± 0.7); T602D,T607D (18 ± 1); T607D (16 ± 0.2) and S616D (17 ± 1). 

C. Bar graph showing the mean ± SEM V_{1/2} (acquired from a Boltzman fit of the curve) of the WT (-24 ± 3 mV); T602D,T607D (DD) (-10 ± 2 mV); T607D (-6 ± 3 mV); T607A (-26 ± 3 mV); S616D (-35 ± 2 mV); and S616A (-25 ± 3 mV) mutants. The mean V_{1/2} of T607D and T602DT607D was significantly different (***, p<0.001, **, p < 0.01, respectively; One Way Anova with post-hoc Bonferroni test) from WT.

Figure 4. The T607D mutant recovered from inactivation slower than wildtype. A. Current traces (not leak subtracted – capacitance transients are truncated) recorded from the T607D + KChIP3 mutant and wildtype Kv4.2 + KChIP3 from the same batch of oocytes on the same day. The T607D mutant reaches maximal current slower than the wildtype. B. Peak current after each hyperpolarized recovery pulse were measured and normalized to the maximal current. Mean ± SEM normalized data were plotted vs the recovery time. That curve was fit with a single exponential. The T607D mutant recovered from inactivation with a t_{1/2} of 17 ± 3 ms, n = 6. This is significantly different from WT (n = 26, 10 ± 1 ms) and T607A (9 ± 1 ms, n = 13).

Figure 5. Coexpression of KChIP3 with the T607 mutant is necessary to manifest effects on activation voltage and recovery from inactivation. The T607D and T607A mutants and WT were expressed alone (without KChIP3 – with control GFP) and compared to + KChIP3 expression. A. Conductance-voltage curve for WT + KChIP3 (squares), and WT-KChIP3 (open
triangles).  **B.** Conductance-voltage curve for T607A + KChIP3 (squares) and T607A-KChIP3 (open triangles).  **C.** Conductance-voltage curve for T607D + KChIP3 (squares) and T607D-KChIP3 (open triangles).  While + KChIP3 coexpression caused a leftward shift of the activation voltage for the WT and T607A constructs, there was no shift for the T607D mutant, illustrating the activation curve of T607D + KChIP3 is similar to T607D alone. The mean $V_{1/2}$ of the T607A, T607D and WT mutants of Kv4.2 expressed in the absence of KChIP3 were not significantly different ($p>0.05$; One Way Anova and post-hoc Boneferroni test).

**Figure 6.** KChIP3 coexpression does not effect ERK phosphorylation of Kv4.2 and phosphorylation of Kv4.2 does not alter surface expression.  **A.** COS cells were transfected with Kv4.2 alone + control (GFP) or Kv4.2 + KChIP3. Cell membrane preparations were run on an SDS gel and western blots were probed with Total Kv4.2 (upper – TKv4.2) or Phospho-Kv4.2 (lower – PKv4.2).  **B.** Bar graph showing the ratio of antibody recognition for Kv4.2 expressed alone to Kv4.2 + KChIP3. The ratio of antibody recognition was not different for the total Kv4.2 vs the phosphorylated Kv4.2 antibody.  **C.** The triple D mutant channel or triple A mutant channel construct does not show altered surface expression compared to WT.  **A.** COS cells were transfected with WT + KChIP3, triple D + KChIP3 or triple A + KChIP3. The surface proteins were isolated by biotinylation with an avidin pulldown. The biotinylated proteins (surface pool, S) and total cell homogenates (T) were run on an SDS gel and western blots with the total Kv4.2 antibody were done. The surface protein from each of the mutants was normalized to the total protein expression from that mutant. That amount was then compared as a percentage to the wildtype surface expression.  **B.** Quantification reveals that there is no
difference in surface expression between WT (100%), triple D (137 ± 32%) and triple A mutants (101 ± 32%; p>0.05, One Way Anova).

REFERENCES


**Figure 1 Schrader et al**

### A

![Diagram](image)

### B

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- **Phospho Kv4.2**

**Legend:**

- KChIP
- S616 T607 T602

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Figure 2 Schrader et al

A

WT

Triple D 20 ms

Scaled

B

Triple A

WT

2 nA 50 ms

WT

C

0 -10 -20 -30

V_{1/2} (mV)

**

-30

Wildtype

Triple D

triple A

-75 -25 25

Test potential (mV)

G/G_{max}
Figure 3 Schrader et al

A

Wildtype
T607D

B

Test potential (mV)
G/G_{max}

Wildtype
T607D
T602D T607D
S616D

C

V_{1/2} (mV)

WT DD T607D T607A S616D S616A
Figure 4 Schrader et al

A

T607D

Wildtype

B

$\frac{I}{I_{\text{max}}}$

0 50 100 150 200 250

Time (ms)

WT

T607D

T607A
Figure 5 Schrader et al

A. WT

B. T607A

C. T607D

Test potential (mV)

G/G_max

WT - KChIP
WT + KChIP
T607A - KChIP
T607A + KChIP
T607D - KChIP
T607D + KChIP
Figure 6 Schrader et al

A

$\text{Kv4.2}$

$\text{Kv4.2+KChIP3}$

$69 \text{ kDa}$

T-Kv4.2 Ab

$69 \text{ kDa}$

P-Kv4.2 Ab

B

WT / WT+KChIP

0.00

0.25

0.50

0.75

1.00

T-KV4.2 P-KV4.2

Surface biotinylation

C

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$\% \text{Wildtype}$

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100

200

AAA

DDD
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