SEQUENTIAL PHOSPHORYLATION MEDIATES RECEPTOR- AND KINASE-INDUCED INHIBITION OF TREK-1 BACKGROUND POTASSIUM CHANNELS*

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Running Title: Receptor and Kinase Inhibition of TREK-1
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Background potassium channels determine membrane potential and input resistance and serve as prominent effectors for modulatory regulation of cellular excitability. TREK-1 is a two-pore-domain background K+ channel (KCNK2, K2P2.1) that is sensitive to a variety of physicochemical and humoral factors. In this work, we used a recombinant expression system to show that activation of Goq-coupled receptors leads to inhibition of TREK-1 channels via protein kinase C (PKC), and we identified a critical phosphorylation site in a key regulatory domain that mediates inhibition of the channel. In HEK 293 cells co-expressing TREK-1 and either the thyrotropin-releasing hormone receptor (TRHR1) or the Orexin receptor (Orx1R), agonist stimulation induced robust channel inhibition that was suppressed by a bisindolylmaleimide PKC inhibitor but not by a protein kinase A blocker (Rp-cAMP-S). Channel inhibition by agonists or by direct activators of PKC (phorbol dibutyrate) and PKA (forskolin) was disrupted not only by alanine or aspartate mutations at an identified PKA site (Ser-333) in the C terminus, but also at a more proximal regulatory site in the cytoplasmic C terminus (Ser-300); S333A and S300A mutations enhanced basal TREK-1 current whereas S333D and S300D substitutions mimicked phosphorylation and strongly diminished currents. When studied in combination, TREK-1 current density was enhanced in S300A/S333D but reduced in S300D/S333A mutant channels. Channel mutants were expressed and appropriately targeted to cell membranes. Together, these data support a sequential phosphorylation model in which receptor-induced kinase activation drives modification at Ser-333 that enables subsequent phosphorylation at Ser-300 to inhibit TREK-1 channel activity.

The negative membrane potential that is essential for electrical signaling in neurons is provided in large part by voltage- and time-independent ‘leak’ or background potassium (K+) channels (1). The KCNK gene family of background K+ channel subunits have two pore domains with four transmembrane segments (2-4), and many are expressed at high levels in the central nervous system (3,5). When studied in heterologous expression systems, recombinant KCNK channels have proven remarkable as targets for dynamic regulation by diverse modulatory mechanisms. Even among this well-modulated family of two-pore-domain background K+ channels, TREK-1 (KCNK2, K2P2.1) is exceptional in its sensitivity to an impressive array of physicochemical factors, neuromodulators and clinically useful drugs. TREK-1 channel activity is enhanced by intracellular acidification, temperature, osmolarity and membrane stretch (6-10). TREK-1 channels are also activated by polyunsaturated fatty acids (PUFA)1, lysophospholipids (11) and by inhalational anesthetics (12). Elegant recent work has verified the importance of this TREK-1 channel...
modulation in vivo; TREN-1 knockout mice have impaired PUFA-mediated ischemic neuroprotection and reduced sensitivity to hypnotic and immobilizing actions of volatile anesthetics (13).

TREK-1 channels are also up- and down-regulated by neurotransmitters and/or their receptor-associated metabotropic signaling mechanisms. For example, nitric oxide enhances TREK-1 activity by stimulating a cGMP-dependent protein kinase (PKG) mechanism that requires a consensus PKG site, Ser-351, in the distal C-terminus of TREK-1 (14). Conversely, activation of Gαs-coupled receptors causes inhibition of TREK-1 channels via a protein kinase A (PKA)-dependent process involving Ser-333, located near the identified PKG site in the cytoplasmic C-terminus of TREK-1 (14,15). It is also clear that TREK-1 is inhibited by Gaq-coupled receptors (2,16) although the molecular mechanism underlying channel inhibition is less certain in this case. As expected for Gaq-coupled receptors, it appears that phospholipase C (PLC) contributes to receptor-mediated inhibition of TREK-1 channels (16). However, studies investigating signaling mechanisms beyond PLC have produced conflicting data and alternative hypotheses. One idea holds that diacylglycerol (DAG) or phosphatidic acid (PA), downstream products of PLC, may directly inhibit TREK-1 channels (16,17; but see 18). Another proposes that PLC-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), a phospholipid activator of the channels, could cause TREK-1 inhibition (18; but see 16). Finally, a role for PLC-mediated activation of PKC, a mechanism commonly associated with Gaq-coupled receptors, is supported by the observation that PKC-activating phorbol esters can inhibit TREK-1 currents (18,19; but see 16).

In the present study, we revisited the possibility that inhibition of TREK-1 by Gaq-coupled receptors involves a PKC-dependent pathway. We find that phorbol esters robustly inhibit TREK-1 and, by using pharmacological intervention and mutagenesis of potential PKC phosphorylation sites, we show that PKC activity is critical for receptor-mediated channel inhibition. Our molecular analysis indicates a necessary, though not sufficient, role for Ser-333 in channel modulation, but also identifies a phosphorylation site at Ser-300 that is both required and sufficient for channel regulation by receptor stimulation, PKC and PKA. The data suggest a sequential phosphorylation model whereby Ser-333 modification enables phosphorylation at Ser-300, which then inhibits channel activity. The novel phosphorylation site at Ser-300 is contained within a proximal C terminal regulatory domain important for modulation of channel activity by stretch, pH and phospholipids (6,7,17) providing a mechanism for receptor-mediated inhibition of channel gating by diverse modulators.

MATERIALS AND METHODS

Cloning and mutagenesis of mouse TREK-1

The coding region of mTREK-1 was obtained by PCR from mouse brain cDNA using forward and reverse primers from the initiating methionine and the stop codon (based on the original published sequence of mTREK-1 (19); GenBank U73488.1). The original published sequence and our initial clone were truncated after Leu-370. We generated a fragment of the remaining 3' region of the coding sequence by PCR and ligated it to our initial clone to produce a full length mTREK-1 construct in pRK5 (mTREK-pRK5). Three differences (at A63T, K84A and E101K) are noted between our clone and the updated sequence deposited in GenBank (U73488.2); these may represent true polymorphisms since we obtained identical sequence from clones derived from a separate PCR reaction using a different reverse primer (located in the M2 region) and a different cDNA preparation. This mTREK-pRK5 cDNA is the basis for all work presented here.

A C-terminal truncation of mTREK-1 was created by introducing a stop codon after Glu-327 (T328STOP). Presumptive PKC phosphorylation...
sites were identified in mTREK-1 at Thr-195, Ser-202, Ser-300, Thr-303, Thr-328, Ser-333 and Ser-345 and mutated to alanine or aspartate, either alone or in combination. All mutations were introduced by QuickChange using Pfu Turbo DNA polymerase (Stratagene, La Jolla, California, USA) and the coding region of each construct was fully sequenced to confirm planned substitutions and absence of unintended mutations.

**Cell culture and transfection**

For most experiments, we used an HEK293 cell that stably expresses the thyrotropin-releasing hormone receptor (TRHR1), the so-called E2 cells (from Graeme Milligan (20)); for some studies, we generated an HEK293 cell that stably expresses the Orexin receptor (Orx1R), the cDNA for which was kindly provided by Masashi Yanagisawa (UT Southwestern). Cells were transfected transiently with mTREK-1 channel constructs along with the green fluorescent protein (GFP; pGreenLantern; Life Technologies) by using Lipofectamine (Invitrogen). One day following transfection, cells were plated onto glass coverslips and individual green fluorescent cells chosen for electrophysiological recording.

**Western blot of membrane-targeted TREK-1 channel constructs**

E2 cells were transfected with TREK-1 channel constructs in 6 cm² tissue culture dishes using LipofectAMINE 2000. After ~16 h, the cells were washed with PBS CM (PBS with 0.1 mM CaCl₂ and 1 mM MgCl₂) and incubated for 30 min at 4°C in biotinylation buffer (10 mM triethanolamine pH 9.0, 2 mM CaCl₂, 150 mM NaCl, 2 mg/ml NHS-SS-biotin (Pierce). Free biotin was quenched in PBS CM with 100 mM glycine for 15 min at 4°C and the cells were washed with PBS CM before lysis in buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.5, 1% Triton X-100 and a cocktail of proteases inhibitors [leupeptin (2 µg/ml), PMSF (100 µM), pepstatin (2 µg/ml), and aprotinin (2 µg/ml)]. The lysate was centrifuged (5 min at 14000 rpm in a microfuge) and the supernatant collected for protein assay (BCA, Pierce). To precipitate biotinylated TREK-1 channels, 1 mg of lysate protein was incubated overnight at 4°C with streptavidin-agarose beads (50 µl; Pierce); the beads were spun down (14000 rpm, 2 min), and washed twice with lysis buffer, twice in high-salt wash buffer (lysis buffer with 500 mM NaCl and 0.1% Triton X-100), and twice in no-salt wash buffer (10 mM Tris, pH 7.5). Biotinylated proteins were eluted from the beads by boiling in 2X sample loading buffer (50% glycerol, 5% β-mercaptoethanol, 2.3% SDS and 0.0625 M Tris/H₃PO₄). The beads were spun down at 14000 rpm for 4 min and the supernatant, along with crude lysate (20 µg total protein) was separated on 10% SDS-PAGE. Proteins were electrotransferred to nitrocellulose membranes and TREK-1 was detected using commercially available antisera (Alomone Labs; 1:200) by enhanced chemiluminescence, according to the manufacturer’s instructions (Amersham).

**Recording and data analysis**

Coverslips containing transfected cells were placed in a chamber on an inverted microscope (Nikon TE300) and visualized using DIC optics and fluorescence. Cells were maintained at room temperature (24°C) in a bath solution containing (in mM): 140 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 2 mM Hepes, and 10 glucose, with pH adjusted to 7.3. Recording pipettes were pulled from borosilicate glass (Warner Instruments) to a DC resistance of ~2-4 MΩ, coated with Sylgard 184 (Dow Corning Co.) and filled with a solution of (in mM): 120 KCH₃SO₃, 4 NaCl, 1 MgCl₂, 0.5 CaCl₂, 10 Hepes, 10 EGTA, 3 ATP, 0.3 GTP-Tris, pH 7.2. Drugs were applied by perfusion during the recording of TREK current in the following concentration: 200 nM TRH & 20 nM orexin-A (from American Peptide & Peptide International); 1 µM phorbol 12, 13, dibutyrate (PdBu; Sigma), 1 µM 4α-PdBu (Alexis Biochemicals), 1 nM or 1 µM 1,9-dideoxyforskolin (FSK; Alexis). Where indicated, cells were pre-incubated and recorded in solutions containing the PKC inhibitor bisindoylmaleimide (BIS, 1 µM; Calbiochem) or in a cocktail containing 1 µM BIS and a broad
spectrum kinase inhibitor, H7 (5 µM; Sigma). In order to block PKA activity, Rp-cAMP-S (1 mM; Sigma) was added to the pipette solution. To inhibit phosphatase activity, cells were incubated with 20 nM okadaic acid (OA; Calbiochem).

Recordings were obtained under whole cell voltage clamp using an Axopatch 200B amplifier and a Digidata 1322A analog-digital converter (Axon Instruments) with pCLAMP9 software (Axon Instruments). Series resistance was compensated by ~70% and a liquid junction potential (10 mV) was corrected offline. Depolarizing ramps from -130 to +20 mV (0.2 V/s) were applied every 5 s to generate current-voltage (I-V) curves. For analysis, holding current was determined at -60 mV and slope conductance was calculated from linear fits to ramp currents obtained between -30 and +5 mV; cell capacitance was taken from the compensation circuit of the amplifier and used to normalize currents to cell size (current density). Data are presented as mean ± SEM. Statistical analysis was by t-test or one-way analysis of variance (ANOVA), with differences considered significant if P<0.05.

RESULTS

We expressed TREK-1 in HEK293 cells, together with either of two Gαq-coupled receptors, TRHR1 and Orx1R, in order to determine mechanisms of modulation by this class of receptor. The general characteristics of TREK-1 currents and their modulation are presented in Fig. 1. A robust outwardly rectifying current was apparent immediately upon gaining whole cell access; TREK-1 channel activity was quantified as the slope conductance between -30 and 5 mV, which typically increased over the first ~2-5 minutes of recording at which point it reached a new plateau (Fig. 1, Insets). Following this ‘run-up’ of channel current, activation of the respective receptors by bath application of TRH (200 nM) or Orexin A (20 nM) caused strong inhibition of TREK-1; averaged data reveal that TRH inhibited 63 ± 4% (n=11) and OrxA inhibited 55 ± 6% (n=11) of the peak current (Fig. 2C).

PKC mediates receptor-activated TREK-1 inhibition

TRHR1 and Orx1R couple to activation of PLC and subsequent PKC activation (21,22) and PKC activation by phorbol esters has been associated with inhibition of TREK-1 (18,19). Therefore, we tested the possibility that receptor-mediated inhibition of TREK-1 involves PKC.

Cells expressing TREK-1 channels were exposed to the PKC-activating phorbol dibutyrate (PdBu, 1 µM). As depicted in Fig. 2, PdBu inhibited TREK-1 conductance (55 ± 3% inhibition, n=10) to approximately the same degree as either TRH or OrxA (~55-60% inhibition). The inactive analogue, 4α-PdBu (1 µM) was without effect on TREK conductance (data not shown) and the effect of PdBu was significantly attenuated when cells were incubated with bisindolylmaleimide (BIS, 1 µM), an inhibitor of PKC (Fig. 2A & 2C). These results indicate that direct PKC activation by PdBu can lead to TREK-1 channel inhibition. Importantly, receptor-mediated TREK-1 inhibition also appears to involve PKC since TRH and OrxA caused only ~30% inhibition of TREK-1 in cells incubated with BIS (1 µM). Indeed, BIS was approximately equally effective at interfering with inhibition of TREK-1 by Gαq-coupled receptors and PdBu (Fig. 2A & 2C).

It is known that activation of PKA can inhibit TREK-1 currents (14,15,23) and, given the possibility that receptors can couple promiscuously to different signaling pathways under conditions of over-expression, we tested if PKA also contributes to inhibition of TREK-1 by TRH (Fig. 2B). As expected, activation of PKA with forskolin (FSK, 1 nM) caused a strong inhibition of TREK-1 (70 ± 4% inhibition, n=8), an effect that was unaffected by BIS (Fig. 2C) but significantly reduced when a PKA inhibitor, Rp-cAMPS (1 mM), was included in the pipette solution. Importantly, inhibition of TREK-1 by TRH was not decreased by Rp-cAMPS (59 ± 4% inhibition, n=9; Fig. 2B & 2C). Together, these data indicate that receptor-mediated inhibition of
TREK channels by TRH and OrxA involves PKC, but is largely independent of PKA.

*Two C-terminal phosphorylation sites are required for TRH and kinase-mediated inhibition of TREK-1*

The cytoplasmic carboxy-terminal domain of TREK-1 is important for regulation of TREK-1 by multiple factors, including stretch, temperature, protons, lysophospholipids and neurotransmitters (6-11). In order to determine the contribution of the C-terminus to channel inhibition by TRH and OrxA we generated a TREK-1 channel construct with the carboxy terminal truncated after Glu-327 (see schematic in Fig. 3A). This truncation removed previously identified phosphorylation sites for PKA and PKG at Ser-333 and Ser-351 (14,15). As shown in Fig. 3B, neither OrxA nor TRH had any effect on currents recorded from cells expressing TREK-1ΔC channels. Likewise, PdBu did not inhibit the C-terminally truncated channels (Fig. 3B, Inset). Note, also, that current amplitude was strongly diminished in cells expressing TREK-1ΔC channels (see also Fig. 4B, 4C). These data indicate that cytoplasmic C-terminal domains beyond Glu-327 are important for basal TREK-1 channel activity and also for regulation of TREK-1 by Gαq-coupled receptors and activators of PKC.

We examined intracellular regions of TREK-1 for potential PKC phosphorylation sites, based on linear consensus sequences and possible helical motifs favored by PKC (24). As shown in Fig. 3A, five sites were identified in the C-terminus of TREK-1, two of them located immediately after TM4, the final transmembrane segment (Ser-300 & Thr-303), and three further into the C-terminus, beyond the truncation point of the Cterminally deleted channel (Thr-328, Ser-333 & Ser-345). A PKG site identified at Ser-351 is also indicated (14). Two additional putative PKC sites were identified in the M2-M3 cytoplasmic loop separating the two P-domains (Thr-195 and Ser-202). In order to determine if these sites are required for receptor and PKC inhibition of TREK-1, we substituted these serine and threonine residues with alanine and tested effects of PdBu and TRH on the mutated channels.

Mutating potential phosphorylation sites in the M2-M3 cytoplasmic loop (T195A/S202A) had no effect on either PdBu- or TRH-mediated inhibition of TREK-1; likewise, channel inhibition by PdBu and TRH was preserved in channels bearing an alanine substitution at either Thr-328 or Ser-345 (*data not shown*). By contrast, as shown in Fig. 3C & Fig. 3D, mutation of the previously identified PKA site at Ser-333 (15) attenuated TREK-1 inhibition by both TRH and PdBu, suggesting that it also functions as a PKC site. As expected, the S333A mutation disrupted channel inhibition by FSK (Fig. 3D).

Alanine-substitution at both potential phosphorylation sites in the proximal C terminus (S300A/T303A) also diminished channel inhibition by PdBu and TRH. Individual mutations at each of these two sites revealed that the critical residue was Ser-300 (Fig. 3C, lower), since inhibition by TRH and PdBu was largely unaffected in T303A channels but was reduced to ~10-15% in S300A mutants (Fig. 3D). Notably, this same S300A mutation also suppressed inhibition by FSK, suggesting that Ser-300 is a previously unrecognized site for channel phosphorylation and modulation by PKA. Overall, these data indicate that the key sites necessary for receptor- and kinase-mediated inhibition of TREK-1 are Ser-300 and Ser-333.

*Ser-300 is primarily responsible for TREK-1 channel inhibition by TRH, PKC & PKA*

Our observation that TREK-1 channel modulation is disrupted in either S300A or S333A mutants prompted us to test if one of these sites is preeminent in mediating inhibition of the channel. For this, we generated S300D and S333D mutants to mimic a phosphoserine at those positions, and we tested effects of those mutations on TREK-1 currents when introduced alone or in combination with an alanine substitution at the alternate site.

As with alanine substitutions at these sites, single S333D or S300D substitutions strongly attenuated receptor- and kinase-mediated channel
inhibition (Fig. 3D & Fig. 4). In addition, basal TREK-1 currents obtained from channels bearing S333D or S300D mutations were strongly suppressed, consistent with aspartate substitutions at these sites mimicking a phosphorylated (and inhibited) form of the channel (Fig. 4). Note that this is in contrast to effects of S333A or S300A mutations, which abrogated receptor, PKC and PKA inhibition of the channel but did so in the context of much enhanced basal current levels (Fig. 4A & 4C). These data argue that alanine substitution at either Ser-300 or Ser-333 blocks phosphorylation-dependent inhibition of TREK-1 channels whereas incorporation of aspartate at those positions mimics phosphorylation and occludes channel inhibition. Since all mutated channels were expressed and targeted to the membrane in transfected cells at levels similar to wild-type channels (Fig. 5), we conclude that decreased TREK-1 currents reflect a change in activity of appropriately expressed channels.

The effects on TREK-1 currents were distinctly different when alanine and aspartate substitutions were introduced simultaneously at either of these two critical sites – and results from these doubly-mutated constructs indicate that decreased channel activity is mediated at Ser-300 rather than Ser-333. Thus, currents from S300A/S333D mutants were significantly greater than from wild-type channels, even though they included the S333D mutation that by itself strongly diminished basal TREK-1 channel currents. On the other hand, currents from the TREK-1 channel mutants that combined S300D with S333A were extremely small and unaffected by TRH and PdBu (Fig. 3D & Fig. 4), consistent with a mimic and occlusion of phosphorylation-dependent inhibition. Again, surface expression of these doubly mutated constructs was comparable to that of wild-type channels (Fig. 5).

We also tested the effect of incorporating an S300A mutation into the TREK-1ΔC channel. As shown in Fig. 4B & 4C, mutating the phosphorylation site at Ser-300 in the context of the C terminal truncation significantly enhanced current amplitude (330 ± 83 pA/pF for TREK-1ΔC/S300A vs. 74 ± 7 pA/pF for TREK-1ΔC, P<0.0001, n≥7). As expected, these TREK-1ΔC/S300A mutated channels, like TREK-1ΔC, were unaffected by TRHR1 receptor activation (see Fig. 4B for representative cells; 6.5 ± 4.0% inhibition by TRH, n=5). So, results with TREK-1ΔC/S300A channels, in which the truncation removes Ser-333, are similar to those obtained with the combined S333D/S300A mutation.

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The observation that an S333A mutation interferes with agonist-, PKC- and PKA-dependent channel inhibition but an S333D mutant cannot mimic phosphorylation-dependent inhibition when paired with S300A supports a necessary, but not sufficient, role for Ser-333. The S300D mutation, on the other hand, is sufficient to induce strong channel inhibition even in the context of S333A, arguing that Ser-300 is primarily responsible for inhibition of TREK-1 channel activity by phosphorylation, even the PKA-mediated inhibition previously attributed to Ser-333. Together, these data suggest a sequential mechanism by which Ser-333 must be modified in order to allow the phosphorylation at Ser-300 that modulates channel activity (see Discussion).

Constitutive phosphorylation at Ser-300 inhibits basal TREK-1 activity and channel dephosphorylation contributes to current ‘run-up’

The sequential phosphorylation model suggests that the diminished TREK-1 currents we observed with the single S333D mutation reflects an additional constitutive phosphorylation of the channel at the Ser-300 site. We tested this possibility by recording TREK-1(S333D) currents from cells pre-incubated with the PKC inhibitor, BIS (Fig. 6A & 6B). Under these conditions, basal channel activity was recovered to control levels, and FSK-mediated inhibition was partially restored. Since currents recovered with a PKC blocker could subsequently be inhibited with FSK, these results also suggest that Ser-300 serves as both a PKA and PKC site, like Ser-333. By contrast, we found no effect of pretreatment with protein kinase inhibitors on TREK-1(S300D)
currents, using either BIS alone or an H7/BIS cocktail (Fig. 6B).

As described above (see Fig. 1), TREK-1 currents in cells expressing wild type channels invariably increased over the first few minutes after obtaining whole cell access. As illustrated in Fig. 4A for exemplar cells expressing selected constructs, and in averaged data of Fig. 6C, this characteristic ‘run-up’ of the current was not observed in any of the substituted TREK-1 constructs containing mutations that disrupted channel modulation by TRH, PdBu and FSK. This included the C-terminally deleted TREK-1, as well as the constructs with point mutations in the critical C terminal sites at Ser-300 and Ser-333 (Fig. 6C). Note again that the initial TREK-1 current amplitude was higher in cells expressing channels with S300A or S333A mutations and lower in S300D or S333D, by comparison to cells transfected with wild-type channels. This implies a basal level of phosphorylation of wild-type TREK-1 channels at these sites that decreases initial channel activity, and that progressive dephosphorylation after whole cell access accounts for the current ‘run-up’. Consistent with this idea, as shown in Fig. 6D, we found that basal TREK-1 activity as well as current ‘run-up’ was reduced in cells pre-treated with 20 nM okadaic acid to inhibit phosphatase activity.

**DISCUSSION**

Regulation of background K⁺ channels represents a prevalent mechanism for dynamic control of cellular excitability. TREK-1 background channels are subject to modulation by multiple factors, including inhibition by G protein-coupled receptors (2,14-16,18). It is clear from earlier studies that PKA-dependent phosphorylation mediates TREK-1 inhibition by Gαs-coupled receptors (14,15). The data we present indicate that a PKC-dependent mechanism contributes to TREK-1 inhibition by Gαq-coupled receptors. Thus, direct activation of PKC by PdBu caused strong inhibition of TREK-1 channels, and the TREK-1 inhibition by either PdBu or the Gαq-coupled TRHR1 and OrxR1 receptors was abrogated by a PKC blocker (BIS) but not a PKA blocker (Rp-cAMP-S). We identified two TREK-1 phosphorylation sites that are required for PKA-, PKC- and receptor-mediated inhibition: a previously described PKA site at Ser-333 and a novel site at Ser-300. Substitution at each of these sites with alanine and/or aspartate coordinately disrupted PKC- and receptor-mediated channel inhibition; S→A replacements blocked channel modulation in the context of increased basal current density whereas S→D mutations, mimicking phosphorylation, strongly decreased basal currents and occluded further channel modulation. Pairwise substitutions of these residues revealed that TREK-1(S300A/S333D) was associated with increased current density while currents from TREK-1(S300D/S333A) were strongly reduced in amplitude, suggesting that Ser-333 has an enabling role but that the site primarily responsible for channel inhibition is Ser-300. Thus, we propose that sequential phosphorylation – first at Ser-333 and then at Ser-300 – is necessary for inhibition of TREK-1 channel activity. Based on its proximity to recognized regulatory domains in the proximal C terminus of TREK-1, the identification of Ser-300 as a critical site for channel inhibition by receptor- and kinase-mediated signaling also suggests an important mechanistic link between phosphorylation and other forms of modulation of this channel.

*A mechanistic model for TREK-1 inhibition by phosphorylation*

The various forms of modulation impinging on TREK-1 channels have been subjected to extensive structure-function analysis, an effort that has yielded a satisfying mechanistic model for understanding regulation of these channels by protons, polyunsaturated fatty acids, phospholipids and stretch (6,17). In short, a positively charged, proximal cytoplasmic region that is located immediately C-terminal to the final transmembrane domain is proposed to interact electrostatically with anionic phospholipids in the membrane inner leaflet (including, but not limited
to PIP$_2$) (17); channel activity and the actions of positive modulators are enhanced when the channel-membrane interaction is relatively stabilized (e.g., by protonation and neutralization of Glu-306) whereas channel activity is inhibited and modulators are rendered less effective when this electrostatic interaction is disrupted (e.g., by shielding inner leaflet phospholipids with polyamines) (6,17).

As depicted in the schematic of Fig. 7, our data implicating a primary mediating role for Ser-300 in kinase-dependent inhibition of TREK-1 can be readily understood in the context of this model. Thus, phosphorylation of Ser-300 (or aspartate substitution) introduces an additional negative charge in the midst of this critical regulatory domain that disrupts channel-membrane interaction, strongly diminishing channel activity. It has likewise been suggested that PKC-mediated inhibition of inwardly-rectifying K$^+$ (Kir) channels proceeds by disrupting channel interactions with inner leaflet phospholipids since PKC preferentially modulated Kir channels with low PIP$_2$ affinity and because application of PIP$_2$ restored activity to PKC-inhibited channels (25,26). The additional site at Ser-333 that we and others have described (14,15) appears to be necessary, but not sufficient, for receptor- and kinase-dependent channel inhibition: modification of that site provides a conformational context that supports phosphorylation at Ser-300. This idea is supported: [1] by the large basal currents and loss of receptor- and kinase-modulation in S333A mutants, despite an intact site at Ser-300; [2] by recovery of current observed after treatment with kinase inhibitors in S333D mutant channels; and [3] by the high basal currents in doubly mutated S300A/S333D channels. Accordingly, we propose that phosphorylation at Ser-333, under control of receptor-activated protein kinases, leads to a channel conformation that supports subsequent phosphorylation at Ser-300 – and that this sequential action provides a link for transmitting receptor-stimulated kinase actions at the distal C-terminus to the proximal regulatory region in order to cause TREK-1 channel inhibition. Further validation of this model will require direct biochemical analysis of phosphate incorporation at these functionally identified, but still presumptive, phosphorylation sites.

Receptor- and kinase-mediated TREK-1 inhibition may overwhelm effects of other positive modulators of channel activity, such as intracellular acidification, temperature or membrane stretch. In this respect, activation of PKA completely suppresses TREK-1 activity, even when the channel is strongly activated by protons or by raised temperature (6,8). On the other hand, however, an E306A channel mutation that mimics pH-dependent neutralization of Glu-306 has high basal activity and is unaffected by activators of PKA (6). Perhaps Ser-300 (and/or Ser-333) are inaccessible in the highly-active E306A channels that are permanently neutralized at that residue, but protonation of Glu-306 in wild-type channels is neither irreversible nor incompatible with phosphorylation. In terms of stretch activation, TREK-1 channels become substantially less sensitive to activation by pressure in the presence of poly-lysine, which like phosphorylation at Ser-300, is predicted to disrupt channel-membrane electrostatic interactions (17). Together, these observations suggest that the phosphorylation status of the channel, specifically at Ser-300, provides a master switch for regulating basal activity of TREK-1 channels and their modulation.

Other mechanisms proposed for inhibition of TREK-1 by Gaq-coupled receptors

Our data support a primary role for PKC-mediated phosphorylation in TREK-1 channel inhibition by Gaq-coupled receptors. We demonstrate that the PKC activator PdBu causes robust inhibition of TREK-1, and also that a bisindolylmaleimide PKC blocker decreases both PdBu-mediated and Gaq-coupled receptor-mediated channel modulation. These results are in agreement with previous studies reporting inhibition of TREK-1 by phorbol esters (18,19). They are in conflict, however, with two recent papers claiming that Gaq-coupled receptor-
mediated inhibition of TREK channels (by mGluR1 glutamate and M1 muscarinic receptors) is unaffected by various kinase inhibitors (staurosporine, PKC19-36, or calphostin C; (16,18) in COS-7 cells and Xenopus oocytes. We currently have no explanation for these discrepant findings, but we would note the difficulties in interpreting negative results from those earlier pharmacological studies. For example, perhaps distinct PKC isozymes with different pharmacological sensitivities are prominent in the cell types studied. Importantly, we buttress our own positive pharmacological results with complementary mutagenesis studies, showing that mutations in consensus phosphorylation sites coordinately disrupt both PKC- and receptor-mediated inhibition of TREK-1 channels. These data, therefore, provide strong support for involvement of PKC as a downstream mediator in TREK-1 inhibition by Gαq-coupled receptors.

Alternative hypotheses to explain TREK-1 inhibition by Gαq-coupled receptors have been advanced but experimental tests of those ideas by different groups have yielded conflicting results. According to one scheme, activation of either PLC or PLD leads to increased levels of DAG and PA, which directly inhibit TREK-1 channels (16). This proposal followed from the observation that DAG and PA cause direct inhibition of arachidonic acid-stimulated TREK-1 channel activity in excised patches and that a DAG lipase inhibitor slows recovery from receptor-mediated TREK-1 inhibition (16). However, it was subsequently reported that DAG analogs had no effect on TREK-1 currents in excised patches (18) and PA actually increased TREK-1 patch currents when assayed in the absence of arachidonic acid (17). An alternative hypothesis posits that PLC-mediated hydrolysis and depletion of PIP2 could contribute to TREK-1 channel inhibition (18), as has now been proposed to explain modulation of many other channels (25,27). Indeed, it was recently found that PIP2 and other phospholipids can directly activate TREK-1 (17,18). However, contrasting results have been obtained in what has emerged as a definitive test of the PIP2 depletion hypothesis for receptor modulation of ion channels – in one report, current recovery following receptor-mediated TREK channel inhibition was delayed by blocking PIP2 replenishment with high concentrations of wortmannin (18), but recovery from inhibition was unaffected in another study (16). In any case, a mediating role for either DAG production or PIP2 depletion is difficult to reconcile with effects of our channel phosphorylation site mutations that completely disrupted receptor-mediated TREK-1 inhibition in the context of either greatly enhanced (SÆA) or diminished (SÆD) basal channel activity. Perhaps TREK-1 activity is not critically dependent on membrane PIP2 levels, per se, since the channel is activated by a number of other inner leaflet phospholipids that are present in relative abundance (e.g., phosphatidyserine, phosphatidylethanolamine; (17)).

In summary, TREK-1 background K⁺ channels are distributed throughout the CNS and it is now clear from analysis of knockout mice that TREK-1 channel activity and modulation is important for control of brain excitability, PUFA-mediated neuroprotection and anesthesia-induced immobilization and hypnosis (13). We demonstrate here that multiple phosphorylation sites are required for kinase-dependent modulation of TREK-1, with Ser-300 in a regulatory domain serving as the critical residue for mediating inhibition of channel activity. This mechanism provides a means by which receptor activation can exercise powerful control over TREK-1 activity and its modulation.
REFERENCES


FOOTNOTES

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1The abbreviations used are: ANOVA, analysis of variance; BIS, bisindoylmaleimide; DAG, diacylglycerol; DC, direct current; FSK, 1,9-dideoxyforskolin; GFP, green fluorescent protein; HEK 293, human embryonic kidney 293; OA; okadaic acid; Orx1R, Orexin receptor; OrxA, orexin-A; PA, phosphatidic acid; PCR, polymerase chain reaction; PdBu, phorbol 12, 13, dibutyrate; PIP2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PLC, phospholipase C; PUFA, polyunsaturated fatty acids; TRHR1, thyrotropin-releasing hormone receptor; TRH, thyrotropin-releasing hormone.

FIGURE LEGENDS

Figure 1. Inhibition of TREK-1 currents by TRH and OrxA. Whole cell currents were recorded from HEK293 cells stably expressing the TRHR1 (A) or the Orx1R (B) following transient transfection with TREK-1. Outwardly rectifying current-voltage (I-V) relationships characteristic of TREK-1 were obtained from depolarizing voltage ramp commands (-130 to +20 mV at 0.2 V/sec) immediately upon whole cell access (control), after a time-dependent increase in current (run-up) and after treatment with the appropriate agonist (TRH or OrxA; at 200 nM and 20 nM). Inset: Plots illustrate the change in conductance (measured as a linear slope between -30 and +5 mV) as a function of time following whole cell access for the same cells; note the time-dependent run-up to a peak, followed by receptor-mediated TREK-1 current inhibition. Ramp I-Vs were obtained at the indicated time points.

Figure 2. TRHR1- and Orx1R-mediated inhibition involves PKC, but not PKA. A. Effect of pharmacological activation and inhibition of PKC on TREK-1 currents and their modulation by TRH and OrxA. Left → Right: The PKC activator, phorbol dibutyrate (PdBu; 1 µM), inhibited TREK-1 currents. TREK-1 inhibition by PdBu was abrogated by pretreatment with 1 µM bisindoylmaleimide, a PKC inhibitor (PdBu + BIS). Pretreatment with 1 µM BIS also diminished TREK-1 inhibition by TRH (TRH + BIS) and by OrexinA (OrxA + BIS). B. Effect of pharmacological activation and inhibition of PKA on TREK-1 currents and their modulation by TRH. Left → Right: The PKA activator, forskolin (FSK; 1 nM), inhibited TREK-1 currents. TREK-1 inhibition by FSK was reduced by including 1 mM Rp-cAMP-S, a PKA inhibitor, in the pipette (FSK + Rp-cAMP-S). Internal Rp-cAMP-S did not decrease TREK-1 inhibition by TRH (TRH + Rp-cAMP-S). C. Averaged data (± SEM) illustrating the effect of PdBu, TRH, OrxA and FSK on TREK-1 conductance (% inhibition, relative to control). The PKC inhibitor BIS was equally effective at blocking TREK-1 inhibition by PdBu, TRH and OrxA; by contrast, although Rp-cAMP-S interfered with FSK-mediated TREK-1 inhibition, it did not block TRH-mediated channel inhibition. TREK-1 inhibition by FSK was not blocked by BIS. *, P<0.01 by ANOVA, n≥7 per group.
Figure 3. Inhibition of TREK-1 by TRH, PKC and PKA requires phosphorylation substrates at Ser-333 and Ser-300. A. Schematic of a two-pore-domain TREK-1 channel subunit indicating the position of the C-terminal truncation (E327) and depicting residues identified as potential PKC phosphorylation sites; the critical residues at Ser-300 and Ser-333 are in bold. B. Ramp currents from an E2 cell expressing the C-terminally truncated TREK-1ΔC construct were unaffected by TRH. Note the scale on the ordinate, reflecting the small currents typically obtained from this construct. Inset: Averaged data (±SEM) reveal that TREK-1ΔC currents are not inhibited by TRH, OrxA or PdBu (P<0.001 by ANOVA, all different from respective WT control; n≥8 per group); inhibition of wild-type (WT) TREK-1 by these compounds is shown for comparison (from Fig. 2C). C. Ramp currents from E2 cells expressing wild-type TREK-1 (top), TREK-1(S333A) (middle) and TREK-1(S300A) (bottom), under control conditions and during exposure to TRH (left) or to PdBu (right). Note that TRH and PdBu had minimal effect on either of the mutated TREK-1 channels. D. Averaged data (±SEM) illustrating inhibition by TRH (0.2 µM), PdBu (1 µM) and FSK (1 µM) of wild-type TREK-1 and channel constructs with alanine and/or aspartate substitutions at the indicated residues. All mutations that blocked inhibition of TREK-1 channels by PKC (i.e., by PdBu) also disrupted TRH-mediated inhibition; mutations at Ser-300 and Ser-333 also interfered with PKA- (i.e., FSK)-mediated channel inhibition. (P<0.05 by ANOVA, all except T303A are different from respective WT control; n≥4 per group).

Figure 4. Activity of doubly mutated TREK-1 channels supports a model of sequential phosphorylation for channel inhibition by transmitters and protein kinases. A. Time-series illustrating basal conductance and run-up in representative E2 cells transfected with TREK-1 phosphorylation site mutants. Conductance was relatively high in cells expressing S333A or the S300A channels and much reduced in cells transfected with S333D or the S300D channels. In cells expressing doubly-mutated channels, TREK-1 conductance was enhanced when S300A was paired with S333D, but remained diminished when S300D was paired with S333A. Note that TRH was without effect and run-up was absent in cells expressing these mutant channels. B. Basal conductance and run-up in cells transfected with TREK-1(ΔC) and TREK-1(ΔC)/S300A; channel conductance was increased in C terminally truncated channels bearing the S300A mutation. C. Averaged data (±SEM) quantifying current density (peak current, normalized to cell capacitance) in cells expressing the indicated TREK-1 constructs (P<0.01 by ANOVA, all point mutants except T303A are different from WT; n≥7 per group).

Figure 5. TREK-1 mutant channels are expressed and targeted to cell membrane. A. A doublet corresponding to TREK-1 (see arrow) is evident in Western blots from HEK293 cells transfected with mTREK-1 (+), but not in untransfected cells (-); no immunoreactive bands were apparent when the primary antibody was preadsorbed with antigenic peptide. B. HEK293 cells were transfected with the indicated constructs, membrane proteins were biotinylated and cell lysates were precipitated by using streptavidin-linked agarose beads. The level of immunoreactive TREK-1 was not different among cells transfected with wild-type and mutant channels in streptavidin precipitates (upper; each 1.0 mg total added protein) or in cell lysates (lower; each 20 µg total protein), indicating that phosphorylation site mutations did not affect surface expression of the channels. The TREK-1(ΔC) channel construct runs at a lower MW, as expected. Control streptavidin precipitates obtained from non-biotinylated cells were devoid of TREK-1 immunoreactivity (WT: no biotin). Data are representative of at least two independent experiments.
Figure 6. Basal TREK-1 activity and run-up reflect constitutive phosphorylation at Ser-300.
A. Representative I-V and time course (Inset) illustrating effect of FSK on TREK-1(S333D) currents recorded in the presence of BIS (and following pretreatment with 1 µM BIS for ≥5 h). B. Following preincubation with BIS, TREK-1(S333D) current density (upper; n=11) and channel inhibition by 1 µM FSK (lower; n=7) were restored close to levels seen in WT channels (*, P<0.01, ANOVA); by contrast, TREK-1(S300D) currents were unaffected by pre-incubation with either BIS (n=5) or a BIS/H7 cocktail (n=4; data from both groups are pooled as S300D/BIS). This implies a requirement for additional phosphorylation for inhibition of TREK-1(S333D), but not for TREK-1(S300D). C. Averaged data (± SEM) quantifying ‘run-up’ (the ratio of steady state to initial conductance, Gss/Gi) in cells expressing the indicated TREK-1 constructs. Run-up was evident in wild-type channels but was absent in channels containing phosphorylation site mutations that blocked receptor- and kinase-mediated inhibition (P<0.001 by ANOVA, all except T303A are different from WT; n≥7 per group). D. Inhibition of phosphatase activity with okadaic acid (20 nM for ≥3 h) decreased TREK-1 conductance (left) and substantially diminished current run-up (right), indicating that constitutive phosphorylation inhibits basal channel activity and de-phosphorylation following whole cell access contributes to current run-up. (*, P<0.05, unpaired t-test; both n≥8).

Figure 7. A working model for TREK-1 inhibition by phosphorylation. Our data indicate that C-terminal truncation or PKA-/PKC-mediated phosphorylation (or aspartate substitution) at Ser-333 induce a change in channel conformation that allows subsequent modification at Ser-300. In addition, the proximity of Ser-300 to a proposed master regulatory region in the TREK-1 C terminus (17), which includes multiple positively charged residues, suggests a mechanism whereby phosphorylation of Ser-300 disrupts interaction of this regulatory region with the plasma membrane to inhibit channel activity.
Figure 1
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Figure 2
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Figure 3
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Figure 4
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A

S333A

S333D

S300A/S333D

S300D/S333A

B

TREK-1(ΔC)

ΔC/S300A

C

current density (pA/pF)

WT

ΔC

ΔC/S300A

S333A

S333D

S300A

S300D

S300A/S333D

S300D/S333A

T303A
Figure 5
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A

TREK-1 immunoblots

- + +

α-TREK-1 + peptide

B

α-TREK-1: avidin IP

WT S300A S333A S333D S300A-S333D ΔC WT: no biotin

α-TREK-1: lysate

Lysates

Lysates
Figure 6
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S333 phosphorylation or aspartate substitution

C terminal truncation

S300 phosphorylation or aspartate substitution

Figure 7
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Sequential phosphorylation mediates receptor-and kinase-induced inhibition of TREK-1 background potassium channels
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