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RESEARCH PAPER

PKC-dependent activation of human $K_{2P}18.1$ K^+ channels

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BACKGROUND AND PURPOSE

Two-pore-domain K^+ channels (K_{2P}) mediate K^+ background currents that modulate the membrane potential of excitable cells. $K_{2P}18.1$ (TWIK-related spinal cord K^+ channel) provides hyperpolarizing background currents in neurons. Recently, a dominant-negative loss-of-function mutation in $K_{2P}18.1$ has been implicated in migraine, and activation of $K_{2P}18.1$ channels was proposed as a therapeutic strategy. Here we elucidated the molecular mechanisms underlying PKC-dependent activation of $K_{2P}18.1$ currents.

EXPERIMENTAL APPROACH

Human $K_{2P}18.1$ channels were heterologously expressed in *Xenopus* laevis oocytes, and currents were recorded with the two-electrode voltage clamp technique.

KEY RESULTS

Stimulation of PKC using phorbol 12-myristate-13-acetate (PMA) activated the $hK_{2P}18.1$ current by 3.1-fold in a concentration-dependent fashion. The inactive analogue 4α-PMA had no effect on channel activity. The specific PKC inhibitors bisindolylmaleimide I, Ro-32-0432 and chelerythrine reduced PMA-induced channel activation indicating that PKC is involved in this effect of PMA. Selective activation of conventional PKC isoforms with thymeleatoxin (100 nM) did not reproduce $K_{2P}18.1$ channel activation. Current activation by PMA was not affected by pretreatment with CsA (calcineurin inhibitor) or KT 5720 (PKA inhibitor), ruling out a significant contribution of calcineurin or cross-talk with PKA to the PKC-dependent $hK_{2P}18.1$ activation. Finally, mutation of putative PKC phosphorylation sites did not prevent PMA-induced $K_{2P}18.1$ channel activation.

CONCLUSIONS AND IMPLICATIONS

We demonstrated that activation of $hK_{2P}18.1$ (TRESK) by PMA is mediated by PKC stimulation. Hence, PKC-mediated activation of $K_{2P}18.1$ background currents may serve as a novel molecular target for migraine treatment.

Abbreviations

Bis I, bisindolylmaleimide I; CsA, cyclosporine A; DMSO, dimethyl sulfoxide; K_{2P}, two-pore-domain K^+ channel; NFAT, nuclear factor of activated T cells; OAG, 2-oleoyl-2-acetyl-sn-glycerol; PMA, phorbol 12-myristate-13-acetate; RMP, resting membrane potential; TM, transmembrane domain; TMX, thymeleatoxin; TRESK, TWIK-related spinal cord K^+ channel; TWIK, tandem of P domains in a weak inward rectifying K^+ channel

Introduction

Two-pore-domain K^+ channels (K_{2P}) mediate potassium background (or 'leak') currents. They exert control over cellular excitability by stabilizing the resting membrane potential (RMP) and by influencing duration and frequency of action potentials in excitable cells (Goldstein *et al.*, 2001; Thomas *et al.*, 2008; Thomas and Goldstein, 2009; Enyedi and Czirjak,

2010; Gierten *et al.*, 2010; 2012; Seyler *et al.*, 2011; Staudacher *et al.*, 2011a,b). K_{2P} α-subunits are identified by their unique structure of two pore-forming domains (P) and four transmembrane segments (TM). Two α-subunits assemble to form a functional potassium channel. The human (h) $K_{2P}18.1$ channel (TRESK, TWIK-related spinal cord K^+ channel) is expressed in spinal cord and brain and is regulated by protons, polyunsaturated fatty acids and anaesthetics (Sano *et al.*, 2003; Liu *et al.*, 2004). $I_{K2P18.1}$ contributes to the K^+ background current in dorsal root ganglion neurons and has been implicated in pain and anaesthesia (Liu *et al.*, 2004; Keshavaprasad *et al.*, 2005; Kang and Kim, 2006; Dobler *et al.*, 2007; Yang *et al.*, 2007; Chae *et al.*, 2010; Tulleuda *et al.*, 2011). In addition, $K_{2P}18.1$ is involved in paraesthesia associated with hydroxy-α-sanshool found in Sichuan peppers, in nociception and in calcineurin-inhibitor induced pain syndrome (Bautista *et al.*, 2008; Smith, 2009). Furthermore, a dominant-negative loss-of-function mutation in the *kcnk*18 gene (encoding for $K_{2P}18.1$) causes migraine with aura (Lafreniere *et al.*, 2010). Despite its potential significance as a pharmacological target in migraine, migraine-related disorders and other pain syndromes, information on the regulation of $K_{2P}18.1$ is limited. Activation of $K_{2P}18.1$ channels by a calcium-dependent pathway via calcineurin binding to a nuclear factor of activated T cells (NFAT)-like binding site in the intracellular loop of the channel protein has been described previously (Czirjak *et al.*, 2004; Czirjak and Enyedi, 2006; 2010). In addition, channel activation through PKA-mediated binding of 14-3-3-proteins has been demonstrated (Czirjak *et al.*, 2008). Regulation of $hK_{2P}18.1$ by PKC, a key modulator of ion channel activity in response to extracellular signalling, has not been studied to date. In the present study, we investigated the PKC-dependent activation of $hK_{2P}18.1$ currents in *Xenopus* oocytes. Our results suggest that activation of $K_{2P}18.1$ channels can silence neuronal firing by hyperpolarization of the RMP and this might represent a novel therapeutic target for migraine treatment.

Methods

Molecular biology

The $hK_{2P}18.1$ clone was kindly provided by Dr C. Spencer Yost (San Francisco, CA, USA). Drug target nomenclature conforms with British Journal of Pharmacology's *Guide to Receptors and Channels* (Alexander *et al.*, 2011). After vector linearization with PmeI, cDNA was transcribed using T7 DNA polymerase and the mMessage mMachine Kit (Ambion, Austin, TX, USA). RNA transcripts were quantified by spectrophotometry after separation by agarose gel electrophoresis. Putative consensus PKC phosphorylation sites were identified using Scansite, NetPhos, KinasePhos, and Human Protein Reference Database software, and targeted by alanine scanning. Site-directed mutagenesis was performed using the QuikChange kit (Stratagene Products Division, Agilent Technologies, La Jolla, CA, USA) and synthetic oligonucleotide primers (Sigma-Aldrich, Steinheim, Germany) (Table 1). Mutants were confirmed by DNA sequencing (GATC, Karlsruhe, Germany). Mutated cDNA plasmids were subcloned into pcDNA3.1-TOPO (Invitrogen, San Diego, CA, USA) using HindIII and SacII (Roche, Mannheim, Germany).

Electrophysiology

Two-electrode voltage clamp measurements were performed as described previously (Kiehn *et al.*, 1999). All animal care and experimental procedures were carried out in accordance with the National Institute of Health Guide for the Care and

Table 1

Forward (F) and reverse (R) oligonucleotide primers used for site-directed mutagenesis of putative PKC phosphorylation sites in $hK_{2P}18.1$

Mutation	Primer sequences
S162A	F: 5′-CAACCATCTTATCTACAGCTTATAATCGGTTCCG-3′
	R: 5′-CGGAACCGATTATAAGCTGTAGATAAGATGGTTG-3′
S178A	F: 5′-CTTTACCCGCCCCCTCCTCGCCAAGTGGTGCCCCAAATC-3′
	R: 5′-GATTTGGGGCACCACTTGGCGAGGAGGGGGCGGGTAAAG-3′
S184A	F: 5′-CTCCAAGTGGTGCCCCAAAGCTCTCTTCAAGAAAAAAC-3′
	R: 5′-GTTTTTTCTTGAAGAGAGCTTTGGGGCACCACTTGGAG-3′
S205A	F: 5′-GTCCCTCAGATCATCATCGCTGCTGAAGAGCTTCCAG-3′
	R: 5′-CTGGAAGCTCTTCAGCAGCGATGATGATCTGAGGGAC-3′
S224A	F: 5′-CTTCACGCCCAAGCTGCGCCATGGAGCTGTTTGAG-3′
	R: 5′-CTCAAACAGCTCCATGGCGCAGCTTGGGCGTGAAG-3′
T161A	F: 5′-CCTGGCAACCATCTTATCTGCATCTTATAATCGGTTCCG-3′
	R: 5′-CGGAACCGATTATAAGATGCAGATAAGATGGTTGCCAGG-3′
T216A	F: 5′-GCCCCAAACTTGGCGCATGTCCTTCACG-3′
	R: 5′-CGTGAAGGACATGCGCCAAGTTTGGGGC-3′
T239A	F: 5′-CGCTAGAGAAACAGAACGCACTGCAACTGCCCCCAC-3′
	R: 5′-GTGGGGGCAGTTGCAGTGCGTTCTGTTTCTCTAGCG-3′

Use of Laboratory Animals and with the German Law on the Protection of Animals. Surgical procedures on female *Xenopus* laevis frogs were performed as previously described (Gierten *et al.*, 2008). Briefly, stage V and VI defolliculated *Xenopus* oocytes were injected with 10–30 ng cRNA encoding study channels and measured 2–4 days after injection. Currents were recorded using an Oocyte Clamp amplifier (Warner Instruments, Hamden, CT, USA) and pCLAMP software (Axon Instruments, Foster City, CA, USA). Data were sampled at 2 kHz and filtered at 1 kHz. Leak currents were not subtracted. Current amplitudes were determined at the end of +20 mV pulses.

Solutions and drug administration

Voltage clamp electrodes filled with 3 mM KCl solution had tip resistances of 3–5 MΩ. Experiments were performed at room temperature (20–22°C) and under constant perfusion by a gravity-driven perfusion system. The standard extracellular solution contained 96 mM NaCl, 4 mM KCl, 1.1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES. The pH was titrated to 7.4 with NaOH. The following chemicals were dissolved in dimethyl sulfoxide (DMSO) to stock solutions of 10 mM and stored at –20°C: phorbol 12-myristate-13-acetate (PMA, Calbiochem, La Jolla, CA, USA), 4α-PMA, KT 5720, Ro-32-0432, bisindolylmaleimide I (Bis I), chelerythrine, 2-oleoyl-2-acetyl-sn-glycerol (OAG) and thymeleatoxin (TMX) (all from Sigma-Aldrich). Cyclosporine A (CsA; Sigma-Aldrich) was dissolved in DMSO (10 mM) and stored at 4°C. On the day of experiments, aliquots of respective stock solutions were diluted to the desired concentration with bath solution.

Data analysis and statistics

Concentration–response relationships for drug-induced current activation were fitted to a logistic dose–response function [$y = (A_1 - A_2)/(1 + (x/x_0)) + A_2$; x_0, EC_{50} value]. Data are expressed as mean ± SEM. We used Student's paired and unpaired *t*-tests to compare statistical significance of the results, as appropriate. $P < 0.05$ was considered significant. Data were analysed using Origin (OriginLab, Northampton, MA, USA) and Microsoft Excel software (Microsoft, Redmond, WA, USA).

Results

Human $K_{2P}18.1$ background currents are activated by PMA

Whole-cell $hK_{2P}18.1$ currents were recorded using a voltage protocol consisting of 20 mV steps from –140 to +60 mV (460 ms duration; Figure 1A). The holding potential was –80 mV. This protocol was applied in all experiments, unless indicated otherwise. First, we examined the effect of the phorbol ester PMA, a non-specific PKC activator that stimulates conventional PKC (cPKC) and novel PKC (nPKC) isoenzymes (Nishizuka, 1984; Ron and Kazanietz, 1999). Treatment of cells with PMA (100 nM; 30 min) induced 3.1 ± 0.2-fold $hK_{2P}18.1$ current activation (n = 7; Figure 1A and B). Current activation displayed a biphasic time course (Figure 1C). Currents reached a steady state after 30 min and decreased gradually under constant PMA application indicative of a metabolic process. The corresponding time control experiments (30 min) revealed moderate current run-up that was not statistically significant (+27.5 ± 13%; $n = 7$; $P = 0.45$). Activation of $hK_{2P}18.1$ channels after PKC stimulation led to hyperpolarization of *Xenopus* oocytes by 7.1 ± 2.8 mV ($n = 7$; $P = 0.02$), as expected for background K^+ channel stimulation (Figure 1D and E). The EC_{50} for PMA-dependent $hK_{2P}18.1$ activation was 17.9 ± 9.0 nM (Figure 1F). $K_{2P}18.1$ current–voltage relationships or activation thresholds were not altered by PMA stimulation (Figure 1G and H).

To assess the effects of PMA on $hK_{2P}18.1$ rectification, linear ramp voltage protocols were applied between –140 and +60 mV (500 ms) before and after application of 100 nM PMA for 30 min (Figure 2A). Outward rectification was observed before and after drug application (n = 5). To exclude any non-specific interaction of PMA molecules with $hK_{2P}18.1$ channels as a hypothetical mechanism underlying PMA-mediated $hK_{2P}18.1$ activation, control experiments were performed with the inactive analogue 4α-PMA using the ramp protocol. 4α-PMA did not produce significant current augmentation after an incubation time of 30 min compared with time controls ($I_{4\alpha\text{-PMA}}/I_{control}$ = 1.11 ± 0.13; n = 5; P = 0.39), ruling out any non-specific interaction of PMA with $hK_{2P}18.1$ proteins (Figure 2B).

Additional PKC activators were applied to further elucidate $hK_{2P}18.1$ activation. First, OAG was tested; OAG is a naturally occurring DAG that activates both conventional and nPKC isoenzymes. However, OAG application (10 μM; 30 min) did not significantly affect $hK_{2P}18.1$ currents (1.04 ± 0.06-fold change; n = 5; P = 0.15) (Figure 3A). OAG is a less potent PKC activator than PMA, which could explain its lack of efficacy under the given experimental conditions. Next, the isoenzyme-specific activator of cPKCs, TMX, was tested (Ryves *et al.*, 1991). Whole-cell $hK_{2P}18.1$ currents determined after TMX incubation (100 nM; 30 min) were not significantly modified (1.10 ± 0.06-fold change; n = 5; P = 0.28), ruling out any relevant contribution of cPKCs to $hK_{2P}18.1$ activation (Figure 3A).

$hK_{2P}18.1$ current activation is PKC-dependent

To investigate whether PMA-induced $hK_{2P}18.1$ activation is mediated by PKC or by cross-talk with PKA-dependent signal transduction, PMA was applied together with protein kinase inhibitors. In addition, control measurements were performed for each pharmacological inhibitor to evaluate effects of selective PKA/PKC inhibition in the absence of kinase stimulation. For co-administration of PMA and inhibitors, cells were first pre-incubated with the respective compound (1 μM staurosporine, 1–2 h; 3 μM Ro-32-0432, 3–4 h; 1 μM Bis I, 2–3 h; 2.5 μM KT 5720, 30 min; Figure 3B–F) before PMA effects were determined (30 min; 100 nM PMA + PKA/PKC inhibitor; Figure 3G–K). Chelerythrine (10 μM) was applied for 30 min in the absence (Figure 3E) or presence of 100 nM PMA (Figure 3K) without drug pre-incubation.

The non-specific protein kinase inhibitor staurosporine produced a $hK_{2P}18.1$ current increase (1.36 ± 0.22-fold; n = 6; P = 0.70) that was not significantly different from the run-up observed under control conditions (4 mM K^+ solution; 30 min) (Figure 3B). No difference (P = 0.74) was observed between current activation after co-application of PMA and staurosporine (3.30 ± 0.48-fold; n = 7) and PMA alone (3.11 ±

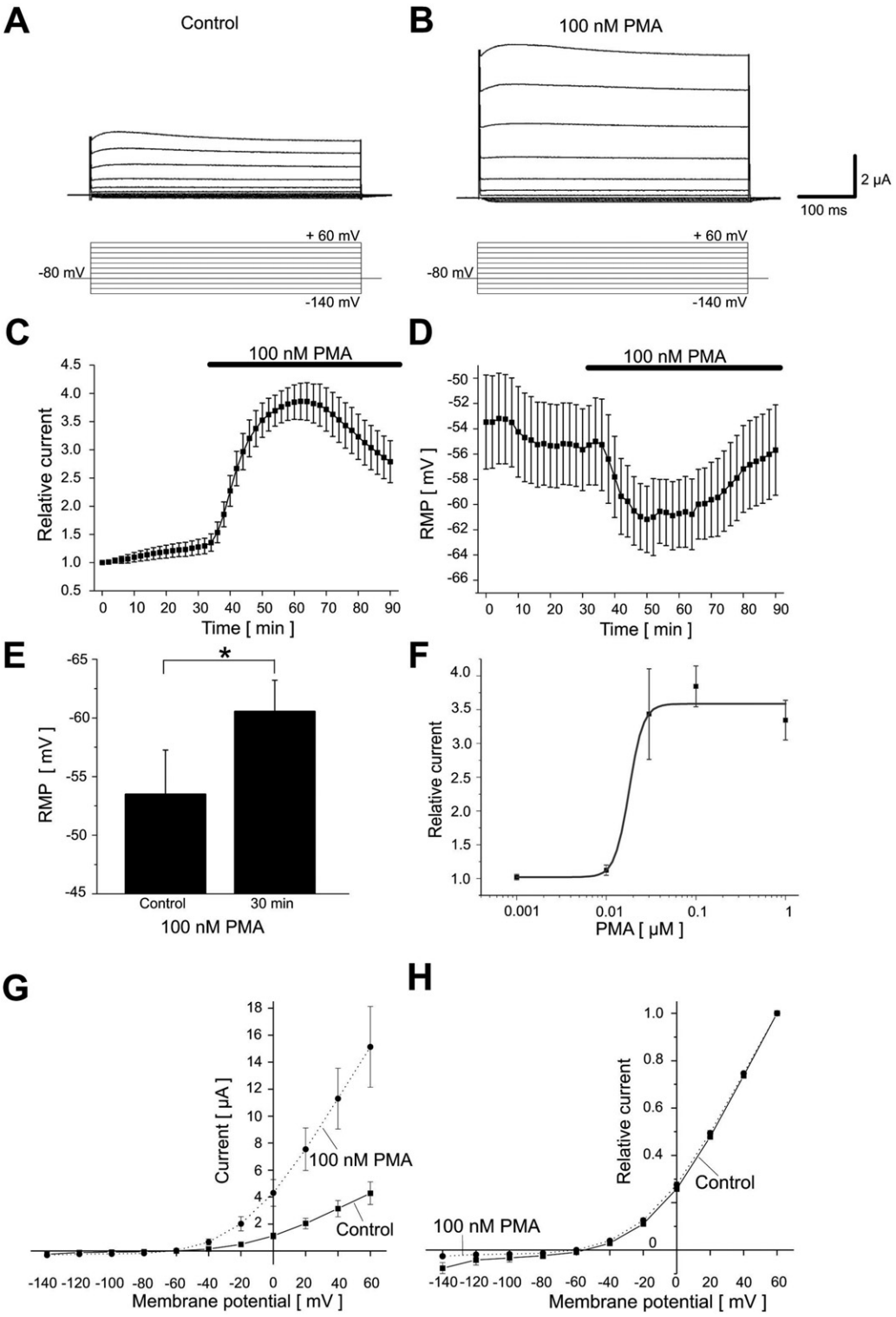

Figure 1

Activation of human $K_{2P}18.1$ channels by PMA in *Xenopus* oocytes. (A) $hK_{2P}18.1$ currents activated instantaneously and displayed virtually no inactivation under control conditions. (B) Stimulation of $hK_{2P}18.1$ background currents by PMA application (100 nM, 30 min). (C) biphasic time course of current response to PMA stimulation. PMA (100 nM) induced maximum stimulation after 30 min, followed by current decrease ($n = 7$). (D, E) The increase in $hK_{2P}18.1$ background currents correlates with significant RMP hyperpolarization ($n = 7$; $^*P < 0.05$). (F) Concentration–response relationship for $hK_{2P}18.1$ channel activation by PMA (EC_{50} = 7.9 nM; n = 4–7 cells). (G, H) Activation curves, that is, step current amplitudes as a function of test potentials, recorded under isochronal conditions (G, original current amplitudes; H, values normalized to maximum currents). Current–voltage relationships were not affected by PMA ($n = 7$).

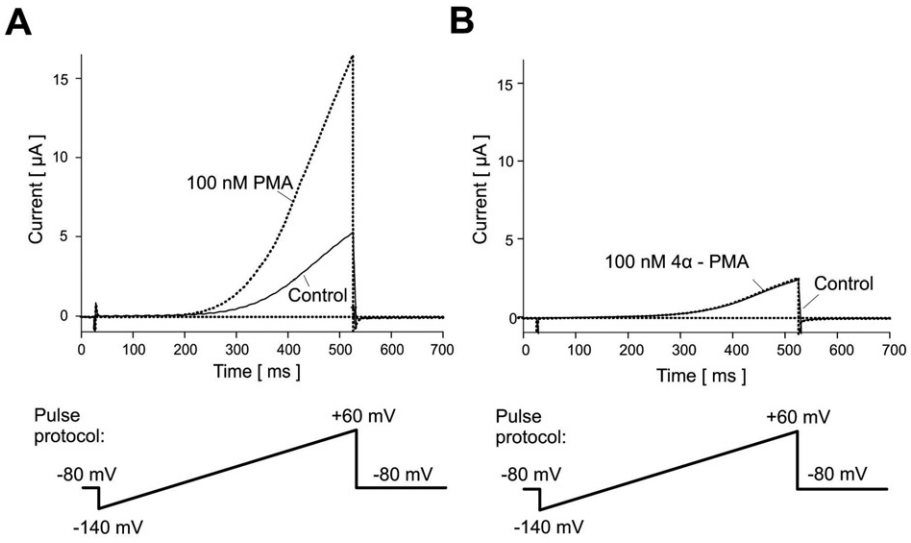

Figure 2

Open rectification of $hK_{2P}18.1$ currents elicited by voltage ramps from −140 to +60 mV. (A) Representative current traces in the absence and presence of 100 nM PMA are superimposed, revealing 2.52 ± 0.21-fold current activation ($n = 5$; $P = 0.06$) in comparison to control currents (bath solution; 30 min). (B) 4α-PMA, an inactive analogue of PMA, used as negative control, did not activate $hK_{2P}18.1$ currents ($n = 5$; $P = 0.39$).

0.22-fold; $n = 7$) (Figure 3G). Specific inhibition of PKC was achieved using three selective inhibitors, Ro-32-0432, Bis I and chelerythrine. These drugs were previously shown to inhibit PKC in *Xenopus* oocytes (Barros *et al.*, 1998; Skeberdis *et al.*, 2001; Karle *et al.*, 2002; Kathöfer *et al.*, 2003; Thomas *et al.*, 2004; Kiesecker *et al.*, 2006; Scherer *et al.*, 2007; Zhang *et al.*, 2007; Zitron *et al.*, 2008; Chen *et al.*, 2010). PMA-dependent $hK_{2P}18.1$ activation was reduced by Ro-32-0432 (–43.0%; $n = 5$; $P < 0.001$) or Bis I (–25.2%; $n = 6$; $P = 0.015$) (Figure 3H and I), respectively, confirming a significant contribution of PKC to the response. The apparent attenuation of PMA-induced $hK_{2P}18.1$ activation by chelerythrine did not reach statistical significance (–18.6%; $n = 5$; $P = 0.067$) (Figure 3J). In the absence of PMA, Ro-32-0432 caused only a small reduction in the $hK_{2P}18.1$ current (0.94 ± 0.02-fold inhibition; $n = 6$; $P = 0.04$), while Bis I (1.03 ± 0.03-fold change; $n = 5$; $P = 0.11$) and chelerythrine (0.97 ± 0.07-fold change; $n = 5$; $P = 0.08$) had no significant effects compared with time controls (Figure 3C–E), indicating negligible baseline PKC activity.

PKC-dependent $hK_{2P}18.1$ activation might involve cross-talk with PKA pathways. Inhibition of $hK_{2P}18.1$ by PKA (Czirjak and Enyedi, 2010) was confirmed by significant current activation (2.09 ± 0.28-fold; $n = 5$; $P = 0.04$) upon application of 2.5 µM KT 5720, a specific PKA inhibitor, for 30 min (Figure 3F). However, combined treatment with KT 5720 (2.5 µM) and PMA (100 nM) increased $hK_{2P}18.1$ by 3.56 ± 0.20-fold ($n = 5$), similar to the current increase observed after PMA treatment alone($P = 0.17$) (Figure 3K).

Significance of $hK_{2P}18.1$ subunit phosphorylation in PMA-induced current activation

To distinguish between a direct action of PKC on $hK_{2P}18.1$ channels and indirect effects via intermediate signalling cascades, putative PKC consensus sequences in $hK_{2P}18.1$ were identified and replaced by alanine residues (S162A, S178A, S184A, S205A, S224A, T161A, T216A, T239A) (Figure 4A). All cRNAs expressed in *Xenopus* oocytes yielded functional potassium channels with kinetics similar to wild-type (WT) channels (data not shown). Oocytes were injected with the same amount of cRNAs (14 ng) for mutated and WT channels 3 days before the electrophysiological measurements. Relative PMA-induced channel activation (100 nM; 30 min) compared with WT currents was not significantly attenuated in any of the mutants tested (Figure 4B). Thus, direct PKC-mediated phosphorylation of $hK_{2P}18.1$ proteins at single phosphorylation sites investigated here was not required for PMA-triggered current activation.

The PMA-induced $hK_{2P}18.1$ activation is independent of calcineurin

Mobilization of cellular calcium enables calcineurin to bind to an NFAT-like binding site in the intracellular loop of $K_{2P}18.1$, leading to channel activation (Czirjak *et al.*, 2004). PKC and calcineurin interact in response to myocardial stretch (Vincent *et al.*, 2006). Thus, we tested whether this interaction is relevant to PMA-dependent $hK_{2P}18.1$ activation. Inhibition of calcineurin, using CsA 1 µM, did not modulate $hK_{2P}18.1$ currents at baseline (Figure 4C). Cells were pre-incubated for 2–2.5 h in 1 µM CsA solution before the application of PMA, followed by treatment with CsA and 100 nM PMA for 30 min ($n = 5$). Current activation by PMA was not affected by CsA ($P = 0.34$) (Figure 4C), ruling out a significant contribution of calcineurin to PKC-dependent $hK_{2P}18.1$ activation.

Discussion

We demonstrated that PKC activates $hK_{2P}18.1$ (TRESK); this represents a previously unrecognized regulatory pathway of

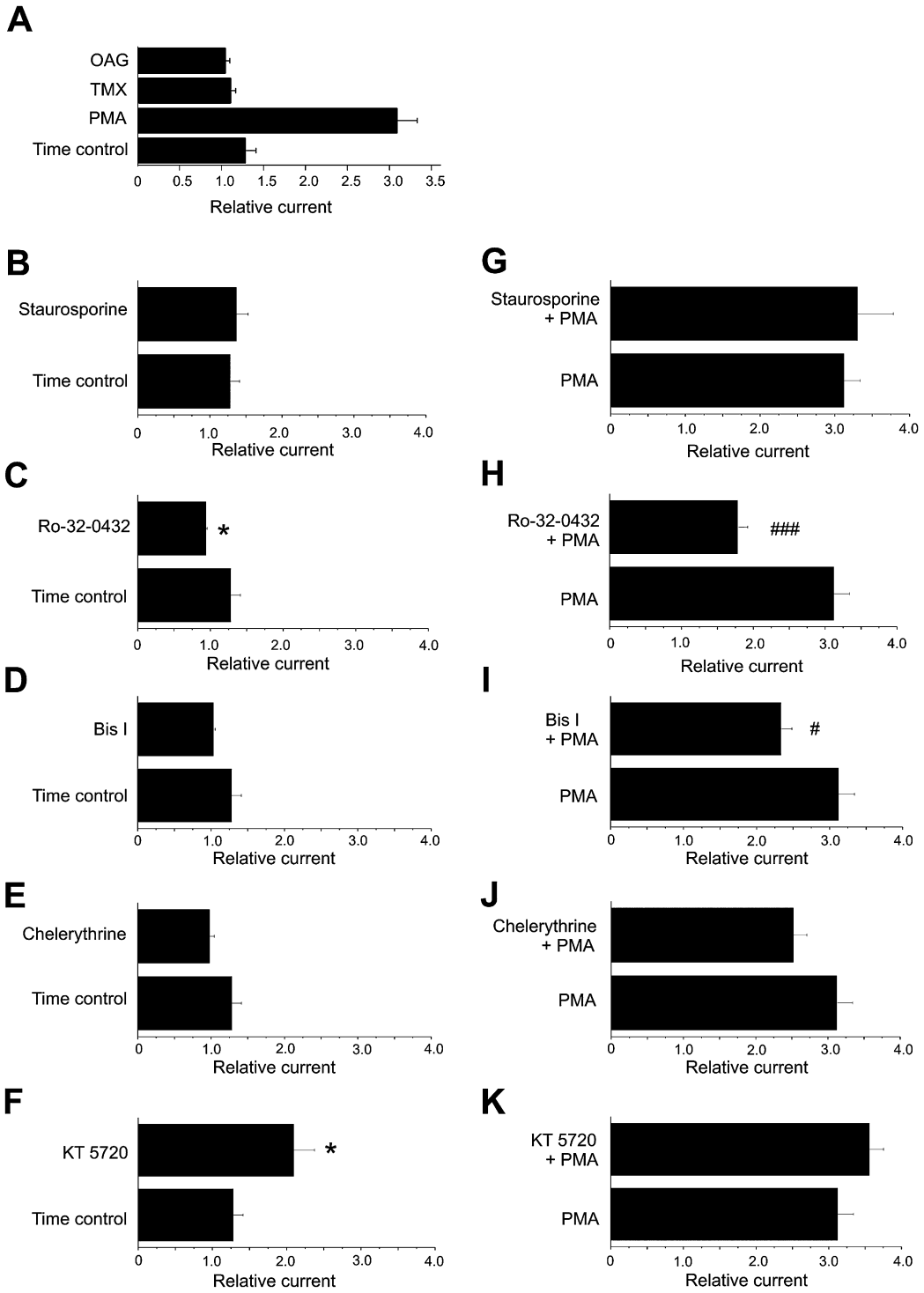

Figure 3

Protein kinase-dependent modulation of PMA-dependent $I_{K2P18.1}$ activation. (A) Efficacy of selected PKC agonists. Neither OAG (10 μM; 30 min) nor TMX (100 nM; 30 min), an activator of conventional PKC isoenzymes, caused significant $hK_{2P}18.1$ current activation ($n = 5$ each) compared with time control experiments ($n = 7$). PMA-induced current activation is provided as reference ($n = 7$). (B–K) Oocytes were pre-incubated with different PKC or PKA inhibitors before the application of 100 nM PMA (30 min). (B–E) Administration of staurosporine, Bis I and chelerythrine did not significantly modulate $I_{K2P18.1}$ compared with time controls (4 mM K^+), whereas Ro-32-0432 slightly reduced $I_{K2P18.1}$. (F) The PKA inhibitor KT 5720 (2.5 μM, 30 min) significantly reduced the increase in current induced by PMA (*$P < 0.05$ vs. time controls). (G) The broad spectrum PKC inhibitor staurosporine did not modulate PMA-dependent activation. (H–J) Selective PKC inhibition by Ro-32-0432, Bis I or chelerythrine reduced the effect of 100 nM PMA. (K) $hK_{2P}18.1$ current activation induced by dual incubation in KT 5720 and PMA was not different from that observed after PMA treatment. $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ versus 100 nM PMA.

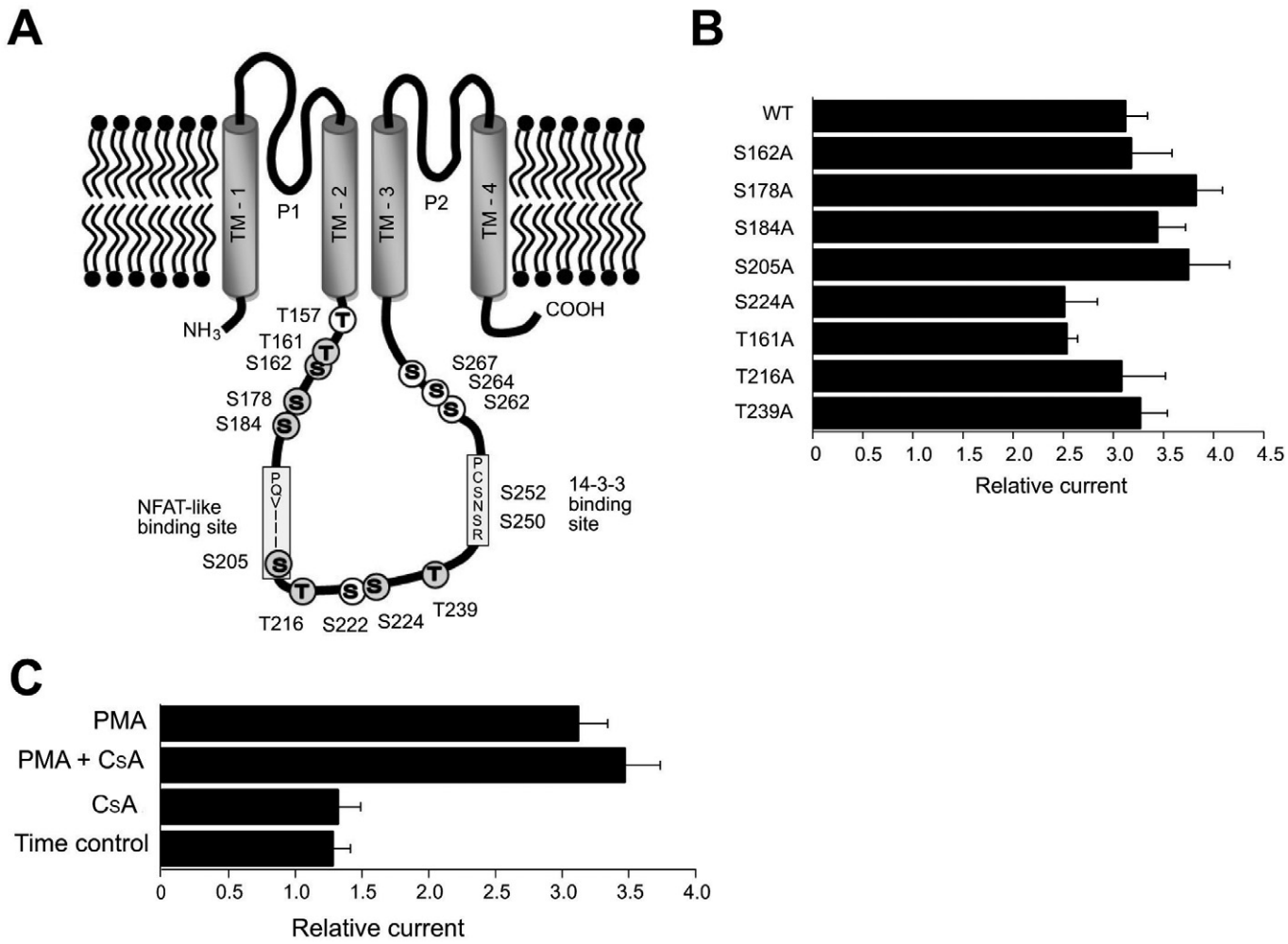

Figure 4

Mutation of putative PKC sites in $hK_{2P}18.1$ did not abolish activation by PMA. (A) Hypothetical membrane folding model showing a single $hK_{2P}18.1$ α-subunit consisting of four TM and two pore (P) domains. The large intracellular loop connects TM2 with TM3 and harbours NFAT-like and 14-3-3 protein-binding sites. Predicted PKC phosphorylation sites were replaced with alanine residues. (B) Relative current activation of $hK_{2P}18.1$ mutants after PMA application (100 nM, 30 min) compared with wild-type channels (n = 5–7 cells). (C) PMA-mediated $hK_{2P}18.1$ stimulation was independent of calcineurin activity. The calcineurin inhibitor CsA (1 μM) did not affect $hK_{2P}18.1$ currents after 30 min (n = 5) compared with time controls (n = 7). In addition, stimulation of CsA-pretreated cells with PMA (100 nM) had no effect on current activation (n = 5) compared with PMA administration in the absence of CsA (n = 7).

neuronal excitability. The present results also indicate that PKC-induced $hK_{2P}18.1$ current activation is independent of direct phosphorylation of the ion channel protein. Activation (as opposed to inhibition) of $K_{2P}18.1$ by Gq protein-coupled intracellular signalling is unique among K_{2P} channels (Enyedi and Czirjak, 2010). The underlying molecular pathways were investigated by analysing the effects of protein kinase activators and inhibitors on WT $hK_{2P}18.1$ channels and using PKC site-deficient channel proteins.

PKC-dependent signalling mechanisms regulate $I_{K2P18.1}$

PKC stimulation by PMA led to robust $hK_{2P}18.1$ channel activation in a dose-dependent manner (EC_{50} = 7.9 nM) (Figure 1). In contrast to activation of human $K_{2P}18.1$, the murine orthologue was reported to be insensitive to 100 nM PMA (Sano *et al.*, 2003). Mouse and human $K_{2P}18.1$ channels share relatively low homology (65%; Enyedi and Czirjak, 2010), providing a molecular basis for species-specific regulation of $K_{2P}18.1$.

PKC isoforms are grouped into three subtypes. While conventional cPKCs (isoforms α, βI, βII, γ) require calcium and/or DAG for activation, nPKCs (isoforms δ, ε, η, θ) can be activated by DAG but are insensitive to calcium, and atypical PKCs (aPKCs) (isoforms ζ and ι/λ) are unresponsive to calcium or DAG (Ron and Kazanietz, 1999; Thomas *et al.*, 2003). Phorbol esters activate cPKC and nPKC isoforms but do not affect aPKC. $K_{2P}18.1$ stimulation could not be mimicked by OAG or the specific cPKC activator TMX (activating cPKC isoforms) (Figure 3). However, pre-incubation of cells with PKC inhibitors (Ro-32-0432, Bis I, chelerythrine)

reduced PMA-dependent $hK_{2P}18.1$ channel activation, implicating the involvement of nPKC isoenzymes that are activated by PMA but insensitive to TMX (Figure 3).

Protein kinase regulation of K_{2P} channels through channel phosphorylation has been demonstrated in previous studies (Fink *et al.*, 1996; Patel *et al.*, 1998; Maingret *et al.*, 2000; Koh *et al.*, 2001; Gu *et al.*, 2002; Honoré *et al.*, 2002; Besana *et al.*, 2004; Murbartian *et al.*, 2005; Kang *et al.*, 2007; Veale *et al.*, 2007; Cain *et al.*, 2008; Czirjak *et al.*, 2008; Czirjak and Enyedi, 2010; Mant *et al.*, 2011; Seyler *et al.*, 2011). To address the involvement of $hK_{2P}18.1$ phosphorylation in the response to PMA, alanine scanning mutagenesis was performed on putative PKC sites located in the intracellular loop connecting TM2 and TM3. Mutation of PKC sites did not alter PMA-induced current stimulation, suggesting that PMA-dependent activation of $hK_{2P}18.1$ currents is not mediated by direct phosphorylation of channel subunits at putative consensus sites tested in this study (Figure 4). However, we cannot exclude the possibility that other less specific seronine/threonine residues or a combination of multiple putative phosphorylation sites are targeted by PKC. Maximum stimulation of $hK_{2P}18.1$ channels was observed after 30 min. A similar time course was reported with PMA-induced modulation of human ether-a-go-go-related gene channels via intermediate signalling (Thomas *et al.*, 2003), supporting the hypothesis that intermediate signal transduction mechanisms mediated $hK_{2P}18.1$ activation observed in the present study.

Signalling cross-talk between regulatory pathways

$K_{2P}18.1$ regulation by calcineurin and PKA has been reported previously (Czirjak *et al.*, 2004; Czirjak and Enyedi, 2010). In mice, $K_{2P}18.1$ channels are activated by calcineurin that binds to an NFAT-like binding site and consequently de-phosphorylates a cluster of serine residues (S274, S276, S279), thereby increasing channel activity. Both, the NFAT-like motif and the serine cluster (S262, S264, S267) are found in human $K_{2P}18.1$ as well (Figure 4). Calcineurin inhibition using CsA did not modulate PMA-induced $hK_{2P}18.1$ activation, ruling out the possibility that any relevant interactions between PKC- and calcineurin-dependent pathways are involved in this response (Figure 4). Furthermore, PKA-dependent inhibition of $K_{2P}18.1$ was confirmed in our study. Current increase upon application of the PKA inhibitor KT 5720 is consistent with reduced inhibitory baseline PKA activity (Figure 3). However, the magnitude of the $K_{2P}18.1$ current induced by PMA was similar in the absence or presence of KT 5720, potentially indicating that PMA increased $K_{2P}18.1$ current irrespective of PKA blockade. Independent modulatory pathways of PKA inhibition by KT 5720 (~2-fold current increase; Figure 3F) and of PKC activation by PMA (~3-fold current increase; Figure 3K) were expected to produce a cumulative current increase, but this was not observed. These findings could be explained by entirely independent pathways of PKC- and PKA-dependent stimulation of $K_{2P}18.1$ that are limited by maximum current enhancement. Alternatively, activation of PKC may prevent additional current increase by KT 5720 by preserving the baseline PKA inhibition, and *vice versa*. This mechanism of action could be exerted via changes in channel conformation.

The non-specific protein kinase inhibitor, staurosporine, did not modulate the $hK_{2P}18.1$ current response to PMA. Staurosporine blocks kinase activity by preventing the binding of ATP to ATP-dependent protein kinases. This drug also affects PKC, PKA and additional enzymes including PKG, calcium/calmodulin-dependent protein kinase II and myosin light chain kinase. Furthermore, staurosporine modulates pathways involved in cell growth and apoptosis. The apparent lack of effect observed with staurosporine in this work may be explained by multiple antagonistic actions of staurosporine on $hK_{2P}18.1$ channel function through regulation of PKA and PKC activity. These effects presumably offset each other under baseline conditions and allow for unaltered channel response to PMA (Figure 3).

Conclusion

We describe a novel regulatory pathway of $hK_{2P}18.1$ (TRESK) activation via PKC stimulation. We hypothesize that activation of $hK_{2P}18.1$ is mediated through intermediate signalling pathway components. Further studies are required to elucidate intracellular mechanisms of $K_{2P}18.1$ activation. Loss of $K_{2P}18.1$ function as a result of a frame shift mutation is associated with migraine in humans. Thus, PKC-dependent activation of $K_{2P}18.1$ background currents implies a novel therapeutic strategy. Investigation of the underlying pathway and screening for more selective agonists may identify treatment alternatives for migraine and other $K_{2P}18.1$-related disorders.

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Conflict of interest

None.

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