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$G\alpha_q$-Mediated Regulation of TASK3 Two-Pore Domain Potassium Channels: The Role of Protein Kinase C

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ABSTRACT

The TASK subfamily of two pore domain potassium channels (K2P) gives rise to leak potassium currents, which contribute to the resting membrane potential of many neurons and regulate their excitability. K2P channels are highly regulated by phosphorylation and by G protein-mediated pathways. In this study, we show that protein kinase C (PKC) inhibits recombinant TASK3 channels. Inhibition by PKC is blocked by the PKC inhibitors bisindolylmaleimide 1 hydrochloride (BIM) and 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole (Gö6976). Gene-silencing experiments with a validated small interfering RNA sequence against PKCα ablates the effect of PKC. PKC acts directly on hTASK3 channels to phosphorylate an identified amino acid in the C terminus region (Thr341), thereby reducing channel current. PKC also inhibits mTASK3 channels despite their having a quite different C-terminal structure to hTASK3 channels. Activation of M_3 muscarinic receptors inhibits both hTASK3 channels expressed in tsA-201 cells and standing outward potassium current (IK_{SO}) in mouse cerebellar granule neurons through the activation of the G protein $G\alpha_q$, because both effects are abolished by the selective $G\alpha_q$ antagonist YM-254890 (*J Biol Chem* **279:**47438–47445, 2004). This inhibition is not directly transduced through activation of PKC because inhibition persists in mutated PKC-insensitive hTASK3 channels. Instead, inhibition seems to occur through a direct action of $G\alpha_q$ on the channel. Nevertheless, preactivation of PKC blocks muscarinic inhibition of both TASK3 channels and IK_{SO}. Our results suggest that activation of PKC (via phospholipase C) has a role in opposing inhibition after M_3 receptor activation rather than transducing it and may act as a negative regulator of G protein modulation to prevent prolonged current inhibition.

Two pore domain potassium channels (K2P) underlie leak potassium currents and are expressed throughout the central nervous system (Talley et al., 2001; Aller et al., 2005). Currents through these channels contribute to the resting membrane potential of neurons and regulate their excitability. There are 15 members of the K2P channel family in humans, which can be divided into 6 subfamilies based on their structural and functional properties (Goldstein et al., 2005; Kim, 2005). K2P channels are regulated by many different pharmacological agents and physiological mediators and by a number of G protein-coupled receptor-activated pathways and protein kinases (Lesage, 2003; Kim, 2005). Phosphorylation of K2P channels is a primary mechanism underpinning their regulation. For example, the K2P channel, TREK1 ($K_{2P}2.1$), can switch from being a voltage-independent to a voltage-dependent conductance depending on the phosphorylation state of the channel (Bockenhauer et al., 2001).

The TASK subfamily of K2P channels [TASK1 ($K_{2P}3.1$), TASK3 ($K_{2P}9.1$), and the nonfunctional TASK5 ($K_{2P}15.1$)] underlie leak currents in a variety of neuronal populations, including cerebellar granule neurons (CGNs) (Millar et al., 2000; Talley et al., 2000; Kang et al., 2004; Aller et al., 2005), and their activity is strongly inhibited after activation of $G\alpha_q$-coupled receptors. There are currently three competing hypotheses as to how the inhibition of TASK channels may occur (Mathie, 2007). First, it has been suggested that inhibition is mediated by one or more of the hydrolysis products

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ABBREVIATIONS: K2P, two pore domain potassium channels; BIM, bisindolylmaleimide 1 hydrochloride; CGN, cerebellar granule neuron; IK_{SO}, standing outward potassium current; DAG, diacylglycerol; RT-PCR, reverse transcription-polymerase chain reaction; WT, wild type; PMA, phorbol 12-myristate 13-acetate; siRNA, small interfering RNA; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PKC, protein kinase C; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole.

of phosphatidylinositol 4,5-bisphosphate (PIP_2) generated after activation of phospholipase C (PLC) (Besana et al., 2004). A second hypothesis proposes that inhibition occurs directly as a result of depletion of PIP_2, which normally acts to maintain channel activity (Czirjak et al., 2001; Chemin et al., 2003; Lopes et al., 2005). Most recently, a third proposal suggests that activated $G\alpha_q$ acts directly to inhibit TASK3 channels (Chen et al., 2006). There is no reason why these three potential pathways should be mutually exclusive (Mathie, 2007).

Whereas TASK1 channels seem to be modestly inhibited after activation of protein kinase C (PKC) (e.g., Lopes et al., 2000), and this underlies the platelet-activating factor receptor-mediated inhibition of TASK1 current in cardiac cells (Besana et al., 2004), the evidence regarding PKC-mediated inhibition of TASK3 is more equivocal. Initial studies of both rat and human TASK3 suggested that treatment with the phorbol ester, phorbol 12-myristate 13-acetate (PMA), had no effect on current through TASK3 channels (Kim et al., 2000; Meadows and Randall, 2001). However, a similar series of experiments by Vega-Saenz de Miera et al. (2001) showed substantial reduction in current through hTASK3 channels after activation of PKC.

In this study, we ask whether activation of PKC does indeed regulate TASK3 channels and try to explain the discrepancy between the observations above. Furthermore, we ask whether PKC activation is important in the transduction pathway stimulated by M_3 muscarinic receptor-mediated activation of $G\alpha_q$, which leads to inhibition of both TASK3 channels and the native correlate of TASK3 channel current, standing outward potassium current (IK_{SO}), in mouse CGNs.

Materials and Methods

tsA-201 Cell Culture Preparation. Modified human embryonic kidney 293 cells (tsA-201) were maintained in 5% CO_2 in a humidified incubator at 37°C in growth media [89% Dulbecco's modified Eagle's medium, 10% heat-inactivated fetal bovine serum, 1% penicillin (10,000 U/ml), and streptomycin (10 mg/ml)]. When the cells were 80% confluent, they were split and plated for transfection onto glass coverslips coated with poly(D-lysine) (1 mg/ml) to ensure good cell adhesion. The cells were transiently transfected using the calcium phosphate method. cDNA expression vector (0.3–1 μg) encoding a mouse or human TASK3 subunit was added to each 15-mm well, and 0.3 to 1 μg of a plasmid encoding the cDNA of green fluorescent protein was included to identify cells expressing K2P channels. After an 18- to 24-h incubation period at 3% CO_2, the cells were rinsed with saline, and fresh growth medium was added to the wells. The cells were incubated at 37°C with 5% CO_2 for 24 to 60 h before electrophysiological measurements were made.

Mutations and Truncations. To generate mutations and truncations, point mutations were introduced by site-directed mutagenesis into the TASK3 channel clones using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). A pair of short (25–35 bases) complementary oligonucleotide primers, incorporating the intended mutation, were synthesized (MWG-Biotech, Ebersberg, Germany). Mutant DNA constructs were sequenced (MWG-Biotech) to confirm the introduction of the correct mutated bases.

siRNA Experiments. We used predesigned validated siRNAs targeting human PKCα (Hs_PRKCA_6_HP, SI00605927) and human PKCε (Hs_PRKCE_6_HP, SI02622088; Qiagen, West Sussex, UK), which provide >70% target gene knockdown when functionally tested for knockdown efficiency by quantitative RT-PCR. Sequence information for each of the individual siRNAs is unavailable; however, the sequences of the siRNAs (SI00605927, SI02622088) against human PKCα (NM RT-PCR 002737) and PKCε (NM RT-PCR 005400) are shown to target a region between 400 to 800 and 1500 to 1750 base pairs relative to the first nucleotide of the start codon, respectively. tsA-201 cells were transiently cotransfected with either PKCα siRNA or PKCε siRNA (1 μg), together with the reporter plasmid, green fluorescent protein, and cDNA encoding human TASK3 DNA as described above.

Tissue Culture of CGNs. CCNs were isolated using methods described previously (Cambray-Deakin, 1995) from the cerebella of 7- to 8-day-old mice of either gender that had been killed by decapitation. After dispersion, cells were plated at a density of 2×10^6 cells/ml onto 13-mm glass coverslips coated with poly(D-lysine) and allowed to adhere. Cells were grown in minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 30 mM glucose, 5 ml of insulin-transferrin-sodium selenite supplement, 0.5% penicillin (10,000 U/ml), streptomycin (10 mg/ml), and 25 mM K^+ (shown to enhance the viability of CGNs in culture). Cultures were maintained in 5% CO_2 at 37°C, and medium was replaced after a minimum of 7 days.

Electrophysiological Recordings from tsA-201 Cells and CGNs. Whole-cell voltage-clamp recordings were made from tsA-201 cells transiently transfected with hTASK3 or mTASK3 wild-type or mutated channels or from cultures of CGNs aged from 9 to 10 days. The composition of the control extracellular solution was 145 mM NaCl, 2.5 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES, titrated to pH 7.4 with NaOH. Glass microelectrodes were pulled from thick-walled borosilicate glass capillaries. Fire-polished pipettes were back-filled with 0.2 μm of filtered intracellular solution (composition: 150 mM KCl, 3 mM $MgCl_2$, 5 mM EGTA, and 10 mM HEPES, titrated to pH 7.4 with KOH). For CGNs, amphotericin B-permeabilized patches were used to minimize the disruption of intracellular composition and rundown of IK_{SO} (Watkins and Mathie, 1996). For these cells, the pipette solution contained 120 mM KCH_3SO_4, 4 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 10 mM EGTA, 3 mM ATP, 0.3 mM GTP, and 240 μg/ml amphotericin, titrated to pH 7.4 with KOH. Cells were voltage-clamped using an Axopatch 1D amplifier (Molecular Devices, Sunnyvale, CA) and low pass-filtered at 5 kHz before sampling (2–10 kHz) and online capture. Data acquisition was carried out using pClamp software (Axon Instruments). tsA-201 cells were usually held at −80 mV and then subjected to a step to −40 mV for 500 ms, followed by a 500- or 1000-ms voltage ramp from −110 to +20 mV (or similar voltage ranges) once every 5 or 6 s (see also legend to Fig. 4). CGNs were typically held at −20 mV and stepped to −60 mV for 250 ms before returning to −20 mV. This protocol was repeated every 6 s. IK_{SO} was measured as a 200-ms average of the current at −20 mV after the CGN had been at this potential for over 5 s. All electrophysiological measurements were carried out at room temperature (21–23°C).

Data Analysis. Data were analyzed using Clampfit software (Axon Instruments), Excel (Microsoft Corporation, Redmond, WA) and Origin (OriginLab Corp, Northampton, MA). Statistical comparisons were carried out using Student's t test, and p values <0.05 were regarded as significant. Results are given as mean ± S.E.M., with n as the number of experiments. When current amplitudes were used for comparisons, control currents were always recorded on the same experimental days as the experimental manipulations of interest.

Drugs, Chemicals, and cDNA. PMA, 4-α-PMA, bisindolylmaleimide 1 hydrochloride (BIM), amphotericin B, ionomycin, and muscarine chloride were all obtained from Sigma (Poole, Dorset, UK). Gö6976 was from Calbiochem (Merck Biosciences, Nottingham, UK). YM-254890 was from Dr. J. Takasaki (Astellas Pharma Inc., Tokyo, Japan). All compounds were made up in either dimethyl sulfoxide or water and diluted in external or internal solution before experimentation. For the PMA plus ionomycin experiments, cells were incubated for at least 20 min before recording with care taken to protect degradation of PMA from external light sources. The human TASK3

K2P channel clone in the pcDNA 3.1 vector was from Helen Meadows (GlaxoSmithKline, Uxbridge, Middlesex, UK). M_2 and M_3 muscarinic acetylcholine receptors and all constitutively active Gα constructs were from the Guthrie cDNA Resource Center (Rollo, MO). The PLC-disrupted mutant Gα_q*RT (R256A, T257A) was generated as described above based on the earlier work of Chen et al. (2006).

Results

A number of groups have shown that TASK currents can be inhibited after activation of G protein-coupled receptors, such as muscarinic acetylcholine receptors, which couple primarily to the G protein family Gα_q (see Introduction); however, no direct evidence exists that this pathway is the sole pathway underlying TASK3 current inhibition after M_3 receptor activation. To address this issue, we have used a recently described selective inhibitor of Gα_q, YM-254890 (structure in Takasaki et al., 2004) to determine the G protein used by M_3 muscarinic receptors to inhibit currents through hTASK3 channels. Figure 1*a* shows the powerful inhibition of hTASK3 channels after activation of cotransfected M_3 muscarinic receptors. Muscarine (1 μM) inhibited hTASK3 current by 88 ± 2% (n = 21). In contrast, cotransfected M_2 muscarinic receptors had little effect on current amplitude when activated by 1 μM muscarine (Fig. 1*b*; 8 ± 4%, n = 9). It can be seen that pretreatment of cells with YM-254890 completely abolished the inhibition of hTASK3 by M_3 muscarinic receptor activation with an IC_{50} value of 27 ± 8 nM (Fig. 1, *c* and *d*). Even at the highest concentration used (1 μM), YM-254890 had no effect on hTASK3 current amplitude itself.

Because Gα_q stimulates PLC, which will, among other actions, activate PKC, it is of some interest to determine whether PKC activation can transduce all or part of the M_3 mediated inhibition of hTASK3 channels, as has been suggested for other K2P channels (Mathie, 2007). There is evidence in the literature both in favor of (Vega-Saenz de Miera et al., 2001) and against (Kim et al., 2000; Meadows and Randall, 2001) regulation of TASK3 currents by PKC. To revisit this issue, we considered the regulation of hTASK3 channels expressed in tsA-201 cells by PMA (100 nM) treatment, either alone or in the additional presence of the calcium ionophore, ionomycin (1 μM), the latter a combination often used experimentally to activate classic or calcium-dependent PKCs (Foey and Brennan, 2004). Figure 2*a* illustrates the effect of bath application of PMA and ionomycin on normalized currents through hTASK3 channels. PMA and ionomycin produced a 63 ± 4% (n = 4) inhibition of current, which reached steady state after approximately 12 min. Figure 2*b* shows mean current amplitudes for hTASK3 channels measured at −80 and −40 mV (see *Materials and Methods*) in control conditions and after various treatments each applied for at least 20 min before recording. It can be seen that PMA had no effect on hTASK3 mean current amplitude when applied alone ($p > 0.05$) but reduced mean current amplitude ($p < 0.05$) when applied together with ionomycin. This effect is not caused by ionomycin, per se, because treatment with the inactive phorbol ester 4-α-PMA and ionomycin or ionomycin alone had no effect on current amplitude compared with control ($p > 0.05$). The inset shows mean currents

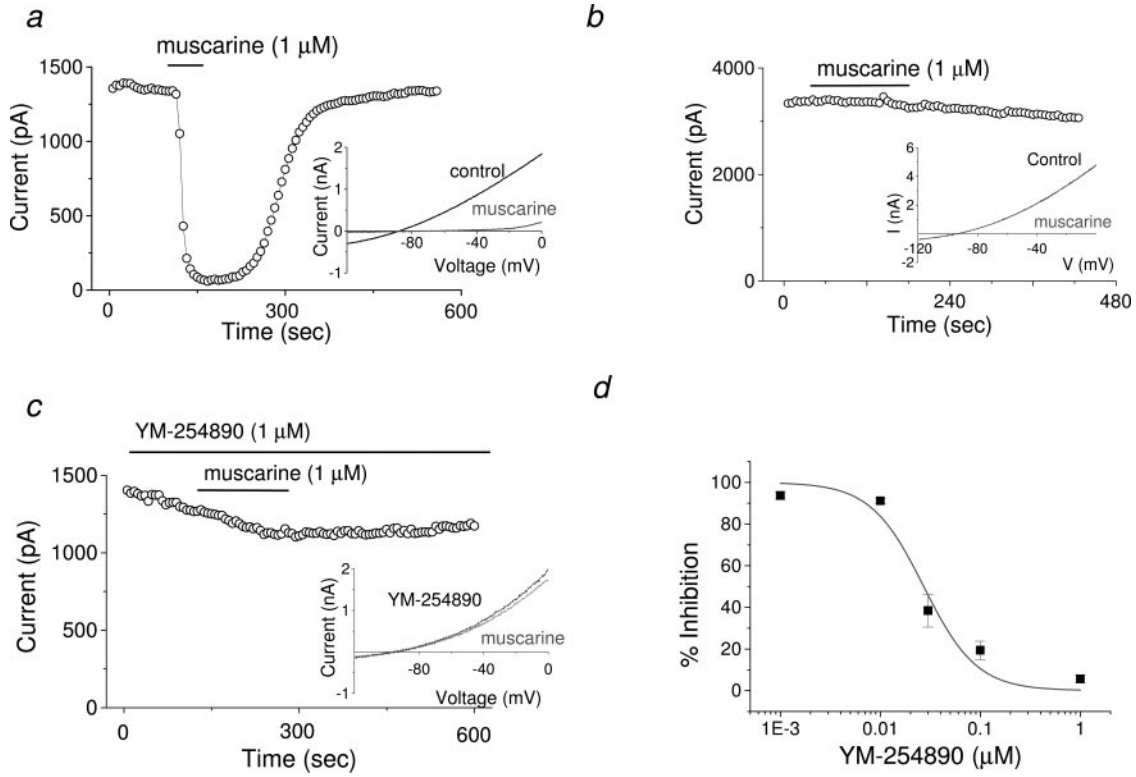

Fig. 1. M_3 receptor-mediated inhibition of hTASK3 channels is blocked by YM-254890. *a* and *b*, inhibition of hTASK3 channels by muscarine in cells transfected with M_3 (*a*) or M_2 (*b*) muscarinic receptors. *c*, inhibition of hTASK3 current through activation of M_3 muscarinic receptors is blocked by YM-254890 (1 μM). Insets, representative current traces evoked by ramp changes in voltage from −120 to 0 mV in the presence and absence of muscarine. *D*, concentration-response curve for YM-254890 inhibition of responses to muscarine (1 μM). YM-254890 causes complete inhibition of the muscarine response with an IC_{50} value of 27 ± 8 nM.

through hTASK3 channels in control conditions and after treatment with PMA alone or PMA and ionomycin.

The effect of PMA and ionomycin could be completely blocked by the nonselective PKC inhibitor BIM (1 μM) (Fig. 2*b*). It was also completely blocked by the PKC inhibitor Gö6976 (Fig. 2b), which, at the concentration used here (100 nM), is a selective inhibitor for classic PKCs such as PKCα, PKCβI and βII, and PKCγ. tsA-201 cells express high levels of PKCα, so we complemented our pharmacological evidence through the use of an RNAi approach to selectively silence PKCα activity in tsA-201 cells. tsA-201 cells were transfected with a validated siRNA sequence targeted against hPKCα (see *Materials and Methods*) at the same time as normal transfection with TASK3. As a control, additional cells were transfected with a validated siRNA sequence targeted against hPKCε. PKCε is also expressed in tsA-201 cells, but it is not a calcium-dependent PKC and is unaffected by Gö6976 at the concentrations used in this study. Our results are shown in Fig. 2 (*c* and *d*). It can be seen that the PKCα siRNA caused a slight but significant reduction ($p < 0.05$) in the mean current through hTASK3 channels compared with control cells. This current was unaffected, however, after treatment with PMA and ionomycin ($p > 0.05$). In contrast, PMA and ionomycin treatment still inhibited hTASK3 current after transfection with siRNA against PKCε ($p < 0.05$). Thus, our pharmacological experiments suggest that hTASK3 channels can be strongly inhibited after activation of a classic PKC (which on the basis of our siRNA experiments may be PKCα) and that the activation requires an elevation of intracellular calcium.

Activation of PKC may reduce current through hTASK3 channels, either through a direct phosphorylation of the channel protein itself or through an intermediary pathway. The large C terminus of K2P channels generally, and TASK3 in particular, contains several potential sites for regulation. Truncation of hTASK3 channels to remove the C terminus (removal of the last 124 amino acids) results in functional channels. These truncated channels, however, are no longer inhibited after activation of PKC (from 1892 ± 222 pA, n = 20, to 2254 ± 190 pA, n = 11; $p > 0.05$). This would suggest that PKC (either directly or through an intermediary pathway) acts on the C terminus of hTASK3 to inhibit channel current.

The C terminus of hTASK3 contains three potential consensus sequence sites for PKC phosphorylation (Fig. 3*a*). We have made single point mutations to each of these sites in turn (S319A, S331A, and T341A) to determine whether one or more of these sites is acted on after activation of PKCα. From Fig. 3*b*, it can be seen that hTASK3$_{(S319A)}$ and hTASK3$_{(S331A)}$ were still inhibited as normal by PMA and ionomycin. However, hTASK3$_{(T341A)}$ channels gave currents with a smaller amplitude than wild type, and these currents were no longer affected by activation of PKCα (from $p >$ 0.05). These data suggest that PKCα acts directly on hTASK3 channels to phosphorylate the channel at position Thr341. Mutation of all three amino acids together [S319A_S331A_T341A; TASK3$_{(PKC-)}$] gave similar results to the single T341A mutation.

If we wish to relate the recombinant channel experiments to the situation for native neuronal currents such as IK_{SO} in

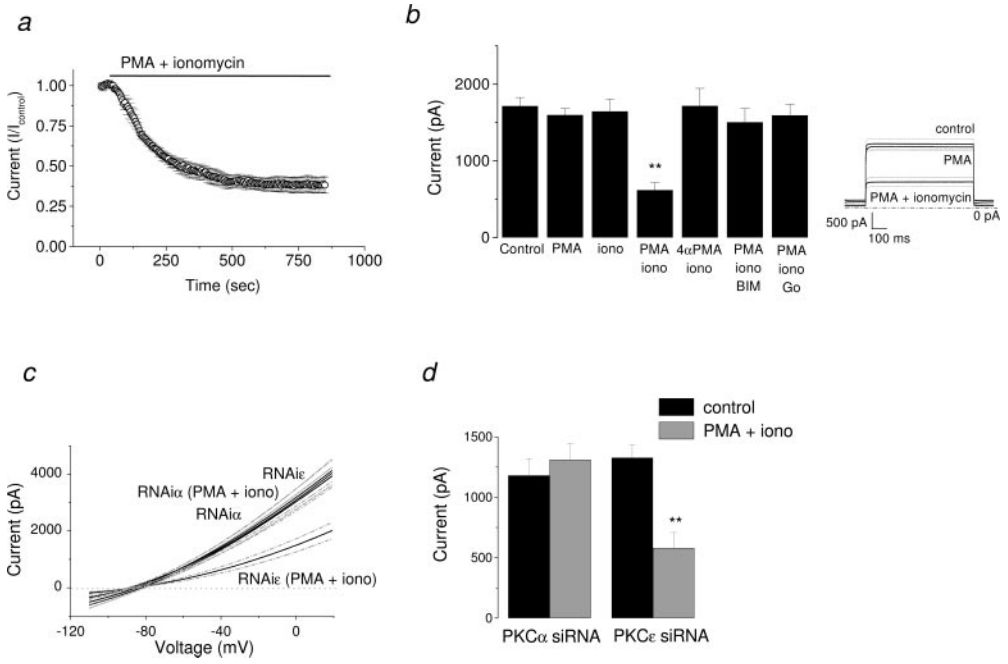

Fig. 2. Inhibition of hTASK3 channels by activation of PKC. *a*, averaged currents through hTASK3 channels showing the effect of perfusion of PMA (100 nM) plus ionomycin (1 μM). Currents were normalized to the mean control amplitude of the first 10 data points for each cell (n = 4). *b*, histogram of mean current through hTASK3 channels (measured as the difference in current between that at −40 and that at −80 mV) in control conditions (n = 28), the presence of PMA (100 nM, n = 15), ionomycin (1 μM, n = 8), PMA plus ionomycin (1 μM, n = 16), 4-α-PMA (100 nM) plus ionomycin (n = 5), PMA plus ionomycin plus BIM (1 μM, n = 7), and PMA plus ionomycin plus Gö6976 (100 nM, Go, n = 8). Inset, averaged currents (±S.E.) through hTASK3 channels in control conditions or after incubation with either PMA alone (100 nM) or PMA plus ionomycin (1 μM) for at least 20 min. *c*, averaged currents (±S.E.) through hTASK3 channels evoked between −110 and +20 mV after cotransfection with siRNA against PKCα (RNAiα) or PKCε (RNAiε) in control conditions or after treatment with PMA and ionomycin. *d*, histogram of mean current through hTASK3 channels (measured as the difference in current between that at −40 and that at −80 mV) after cotransfection with siRNA against PKCα or PKCε in control conditions (n = 9 and 6, respectively) or after treatment with PMA and ionomycin (n = 10 and 8, respectively).

mouse CGNs, we need to consider regulation of mouse TASK3 channels. Our original pharmacological characterization suggested little difference between mTASK3 and hTASK3 functional properties (Aller et al., 2005); however, the C terminus of mTASK3 is substantially different from hTASK3 with only 44% amino acid similarity (using Blosum 62 similarity matrix) or 28% identity, compared with 98% similarity and 94% identity for the remainder of the TASK3 channel protein (Fig. 3*a*). Furthermore mTASK3 contains four (rather than three) putative PKC phosphorylation sites, which for the most part do not align with the corresponding hTASK3 sites. As such, it is not clear whether mTASK3 will be phosphorylated after activation of PKC and, even if so, which site(s) are targeted by PKC.

Our data for mTASK3 are summarized in Fig. 3*c*. mTASK3 was indeed inhibited after activation of PKC ($p < 0.05$) and, as for hTASK3, this effect was ablated after C-terminal truncation (removal of last 134 amino acids; data not shown). Mutation of each of the four PKC sites individually gives results that are not so clearcut as for hTASK3. Whereas no one mutation completely ablated the effect of PKC, Thr348 is most likely the amino acid phosphorylated by PKC to give the analogous effect to that seen for hTASK3 channels, because the effect of PMA and ionomycin was significantly reduced ($p < 0.05$) for this mutation. We cannot, however, rule out a contribution of other sites such as the neighboring PKC site (Ser351), particularly because the double mutant $mTASK3_{(T348A\_S351A)}$ was completely unaffected by PMA and ionomycin (Fig. 3*c*).

To determine whether PKC directly transduces M_3-mediated inhibition of hTASK3 channels, muscarinic inhibition was tested on hTASK3 channels in which either the PKCα site (Fig. 3) had been mutated (T341A) to abolish direct PKC action on the channel or in which each of the three consensus sequence sites for PKC in the C terminus of hTASK3 was mutated [$TASK3_{(PKC-)}$]. In both sets of experiments, the degree of inhibition of hTASK3 by M_3 receptor activation was completely unaltered compared with wild-type (WT) hTASK3, with 0.1 μM muscarine producing 76 ± 9% ($n = 4$) and 80 ± 3% ($n = 6$) inhibition, respectively (Fig. 4). These data suggest that PKC activation does not directly transduce $G\alpha_q$-mediated inhibition of TASK3 channels.

Although not the primary transducer of inhibition, PKC does have a role in M_3 receptor-mediated inhibition of hTASK3 channels. It can be seen (Fig. 4, *a*, *b*, and *e*) that pretreatment with PMA and ionomycin virtually abolished inhibition of hTASK3 currents after activation by 0.1 μM muscarine [from 71 ± 7% ($n = 5$) in control to 8 ± 3% ($n = 6$) in the presence of PMA and ionomycin]. PMA alone, although not as effective as PMA plus ionomycin, was also able to significantly reduce ($p < 0.05$) the effectiveness of muscarine [31 ± 3% ($n = 5$) inhibition]. Current amplitude in these cells was reduced to 886 ± 126 pA ($n = 17$) by PMA and ionomycin pretreatment before application of muscarine. YM-254890 could not reverse the effect of PMA and ionomycin (1074 ± 136 pA, $n = 5$). PKCα siRNA blunted (but did not abolish) this effect of PMA and ionomycin so that muscarine (0.1 μM) was now able to inhibit TASK3 current (37 ± 7%, $n = 3$). Furthermore, muscarinic inhibition of TASK3 current was still observed when cells were pretreated with ionomycin and 4-α-PMA or when cells were pretreated with the nonselective PKC inhibitor BIM (in addition to PMA and ionomycin).

This action of PMA and ionomycin was fully retained in $hTASK3_{(PKC-)}$ channels [Fig. 4, *c*, *d*, and *e*; from 80 ± 3% ($n = 6$) in control to −1 ± 1% ($n = 4$) in the presence of PMA and ionomycin and to 21 ± 9% ($n = 5$) in the presence of PMA alone], which suggests that this effect is not mediated through an action of PKC on the TASK3 channel itself. This action of

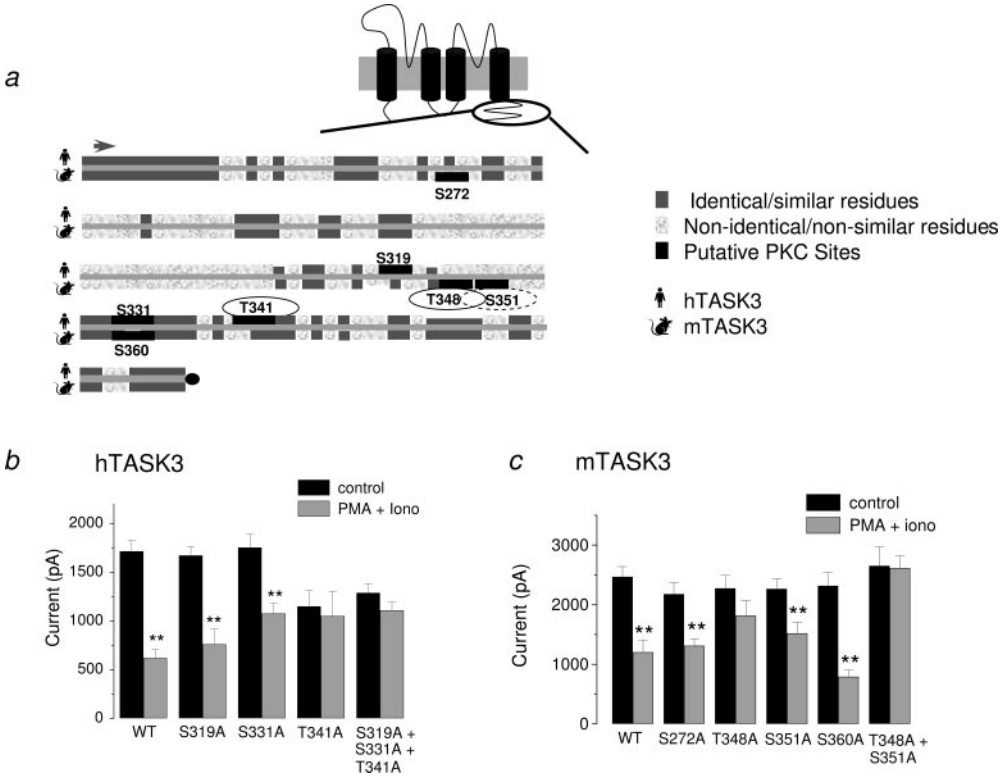

Fig. 3. PKC acts on identified amino acids in the C terminus of hTASK3 and mTASK3. *a*, sequence comparison and putative PKC phosphorylation sites for the C terminus of hTASK3 and mTASK3. hTASK3 and mTASK3 show only 44% similarity in their C-terminal regions (see *Results*) as shown. Putative PKC phosphorylation sites, three for hTASK3 and four for mTASK3, are shown. *b* and *c*, histogram of mean current through hTASK3 (*b*) or mTASK3 (*c*) channels (measured as the difference in current between that at −40 and that at −80 mV) for WT channels and for channels with a single point mutation, or a triple point mutation for hTASK3 and a double point mutation for mTASK3, for each of the putative PKC sites in turn in control conditions and after treatment with PMA and ionomycin.

PMA and ionomycin was also seen in the mouse PKC-resistant TASK3 channel mutant, mTASK3$_{(T348A_S351A)}$, with muscarine producing no detectable inhibition after pretreatment with PMA and ionomycin (2 ± 6%, $n = 3$).

TASK3 channels are believed to underlie at least part of the whole-cell IK_{SO} in rodent CGNs (Clarke et al., 2004; Kang et al., 2004; Aller et al., 2005), which is inhibited after activation of M_3 muscarinic acetylcholine receptors in rat CGNs (Boyd et al., 2000). We show that IK_{SO} in cultured mouse CGNs was also inhibited after muscarinic receptor activation (Fig. 5*a*), albeit to a lesser degree than in rat (Watkins and Mathie, 1996). As for hTASK3 channels, YM-254890 completely abolished inhibition of IK_{SO} by muscarinic receptor activation in mouse CGNs (Fig. 5*c*) without having any effect on the amplitude of IK_{SO} itself. These data show directly that M_3 muscarinic receptors activate the G protein $G\alpha_q$ to inhibit IK_{SO}. This muscarinic inhibition was significantly attenuated ($p < 0.05$) after pretreatment of mouse CGNs with PMA and ionomycin (Fig. 5, *b* and *c*) from 37 ± 5% ($n = 13$) in control to 11 ± 1% ($n = 4$) in the presence of PMA plus ionomycin. Likewise, in rat CGNs, PMA alone significantly reduced ($p < 0.05$) muscarinic inhibition of IK_{SO} from 74 ± 2% ($n = 27$) in control to 32 ± 5% ($n = 9$) in the presence of PMA. Muscarinic inhibition of IK_{SO} was unaffected by pretreatment of the cells with BIM, but recovery from muscarinic inhibition was only partial (see below). Although we observed a small reduction in the amplitude of IK_{SO} during pretreatment with PMA and ionomycin, we could not obtain enough data to see a significant inhibition of IK_{SO}, at least in part because CGNs were generally unhappy when incubated for prolonged periods in the presence of these agents. We are unclear why this effect is smaller than seen for the recombinant TASK3 channels, but one complication may be the fact that a number of different K2P channels underlie IK_{SO} in CGN cultures (Kang et al., 2004).

Because PKC does not seem to be acting at the level of the

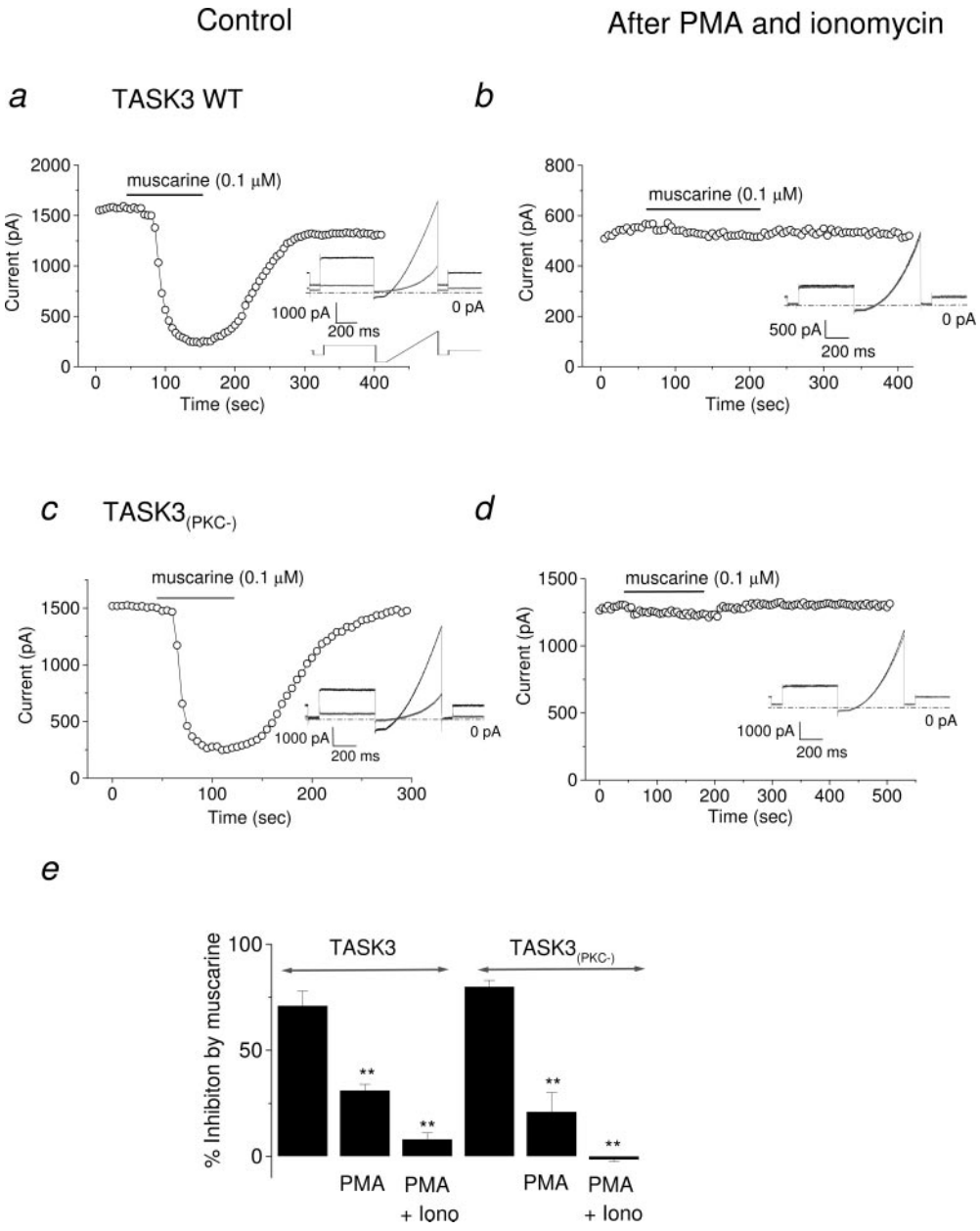

Fig. 4. Activation of PKC attenuates M_3 receptor-mediated inhibition of hTASK3 channels. *a* and *b*, inhibition of hTASK3 channels by muscarine (0.1 μM) in control conditions (*a*) and after preactivation of PKC with PMA (100 nM) and ionomycin (1 μM) (*b*). Insets, representative current traces before and after perfusion of muscarine. The voltage protocol shows a step to −80 from −60 mV for 100 ms followed by a 500-ms step to −40 mV. A 100-ms step to −110 mV is followed by a 500-ms ramp change in voltage to +20 mV. Finally, a 100-ms step to −80 mV precedes a step to the holding potential of −60 mV. This protocol was repeated once every 5 s. *c* and *d*, inhibition of triple-mutated TASK3$_{(PKC-)}$ channels by muscarine (0.1 μM) in control conditions (*c*) and after preactivation of PKC with PMA (100 nM) and ionomycin (1 μM) (*d*). Insets, representative current traces in the presence and absence of muscarine. *e*, histogram of mean percentage inhibition by 0.1 μM muscarine for hTASK3 channels and hTASK3$_{(PKC-)}$ channels in control conditions and after treatment with PMA (100 nM) and ionomycin (1 μM) or PMA alone.

TASK3 channel to occlude inhibition by muscarine, it must act at some other point in the regulatory pathway. We tested the importance of the receptor itself, by considering the regulation of channel current by the nonhydrolyzable form of GTP, GTPγS. This is illustrated in Fig. 6, *a* to *c*. When recordings were made with GTPγS in the recording pipette, it can be seen that hTASK3 current amplitude decreased significantly with time compared with control cells (Fig. 6*a*), with current reaching 64 ± 6% ($n = 7$) of its original amplitude after 10-min recording compared with 102 ± 9% ($n = 7$) for control cells ($p < 0.05$). Furthermore, muscarine treatment now caused an irreversible inhibition of hTASK3 current (Fig. 6b; Chemin et al., 2003). Pretreatment with PMA and ionomycin abolished the decrease of TASK3 currents in cells dialyzed with GTPγS, and muscarine no longer induced inhibition of TASK3 current (Fig. 6, *a* and *c*). Thus, PKC acts at a site downstream from the muscarinic receptor to inhibit this regulatory pathway.

The recent suggestion that activated $G\alpha_q$ may inhibit TASK3 channels directly (Chen et al., 2006) simplifies considerably the potential targets for PKC modulation. We addressed this issue by repeating the experiments of Chen et al. (2006) by considering the effects of constitutively activated $G\alpha_q$ ($G\alpha_q$*) on hTASK3 channel currents and extending these observations to $hTASK3_{(PKC-)}$ channel currents. $G\alpha_q$* significantly inhibited ($p < 0.05$) current through both WT and mutated hTASK3 channels, and this effect was retained with further mutated $G\alpha_q$* ($G\alpha_q$*RT), which does not activate PLC (Fig. 6, *d* and *e*). The activated form of the $G\alpha_q$-like G protein, $G\alpha_{11}$*, mimicked the effect of $G\alpha_q$* (Fig. 6*e*), but there was no difference from control currents for either $G\alpha_{i2}$* or $G\alpha_{12}$* (Fig. 6*d*).

PMA and ionomycin did not reverse the effect of either $G\alpha_q$* or $G\alpha_q$*RT on $hTASK\text{-}3_{(PKC-)}$ channels (Fig. 6*e*). This suggests that PKC acts to alter the activity of $G\alpha_q$ but is ineffective when $G\alpha_q$ is constitutively active. The differential effect of PKC and ionomycin on GTPγS-induced modulation compared with that mediated by $G\alpha_q$* may be due to the fact that PKC is already activated in the GTPγS experiments before cell dialysis and recording. The opposite is true for the $G\alpha_q$* experiments in which the constitutively active G protein can regulate channel activity long before PMA and ionomycin treatment. It is possible, for example, that $G\alpha_q$ and TASK3 form part of a signaling complex that can only assemble and function properly in the absence of PKC-mediated phosphorylation of a regulatory protein involved.

To determine whether the regulation by PKC has a potential negative-feedback role during normal muscarinic receptor activation, inhibition was tested in the presence of the nonselective PKC inhibitor BIM (1 μM). Whereas the degree of inhibition of hTASK3 by M_3 receptor activation was completely unaltered (Fig. 7, *a* and *b*), it can be seen that recovery from inhibition of hTASK3 by 1 μM muscarine was changed, with significantly less ($p < 0.05$) recovery from inhibition obtained in the presence of BIM (Fig. 7, *c* and *d*). Similar results were obtained with BIM for $hTASK3_{T341A}$-mutated channels. This suggests that activation of PKC (via PLC) has a role in opposing inhibition after M_3 receptor activation rather than transducing it and may act as a negative regulator of G protein modulation to prevent prolonged current inhibition.

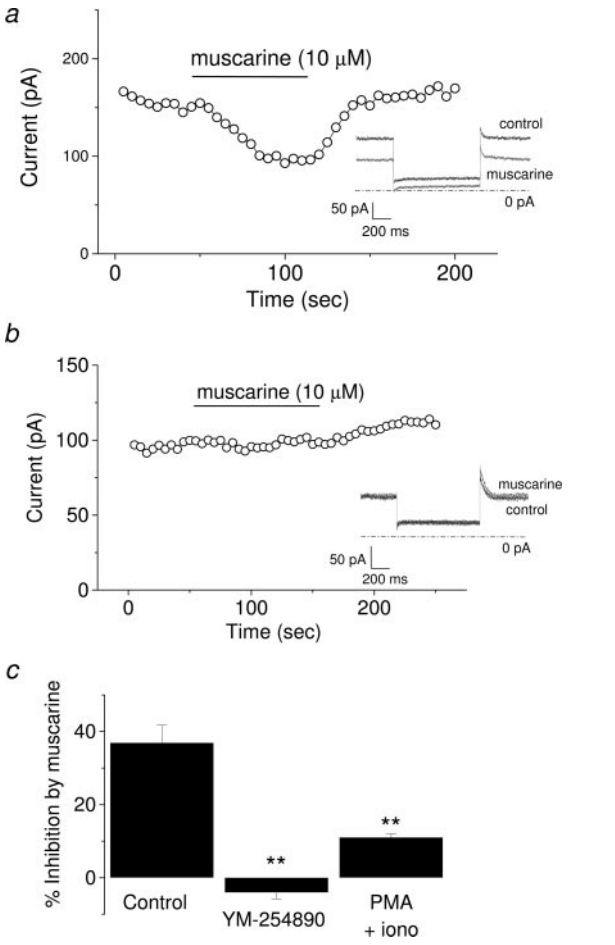

Fig. 5. Activation of PKC attenuates muscarinic receptor-mediated inhibition of IK_{SO} in mouse CGNs. *a* and *b*, inhibition of IK_{SO} by muscarine (10 μM) in control conditions (*a*) and after preactivation of PKC with PMA (100 nM) and ionomycin (1 μM). Insets, representative current traces evoked by steps from −20 to −60 mV and back in the presence and absence of muscarine. *c*, histogram of mean percentage inhibition of IK_{SO} by muscarine (10 μM) in control conditions, after YM-254890 (1 μM), or after pretreatment with PMA (100 nM) and ionomycin (1 μM).

Discussion

We have shown that activation of PKC inhibits TASK3 current when there is a concomitant increase in intracellular calcium, suggesting the involvement of a classic, calcium-dependent PKC such as PKCα. Kim et al. (2000) and Meadows and Randall (2001) preincubated cells in PMA (100 nM) and found no effect of the compound on the amplitude of TASK3 currents compared with control. Vega-Saenz de Miera et al. (2001), on the other hand, continually measured current through TASK3 channels and found a 50% reduction in current after 20-min treatment, similar to the results obtained in this study. One possible explanation for this difference is that in the latter experiments, by continually measuring current (and thus regularly depolarizing the oocytes), the resultant calcium influx was sufficient to act synergistically with PMA to optimally activate classic calcium-dependent PKCs. A similar calcium requirement has been found for phorbol ester-induced inhibition of potassium currents in rat sensory neurons, which requires calcium entry through voltage-gated calcium channels (Zhang et al., 2001).

Classic PKCs such as PKCα are activated by diacylglycerol

(DAG) (or phorbol esters) and calcium (Nishizuka, 1995). DAG binds to the C1 domain of PKCα and is required for full enzymatic activity, whereas calcium binds to the C2 domain, which (together with phosphatidylserine binding) promotes translocation of the PKCα to the plasma membrane (Evans et al., 2006). The physiological requirement for a coincident increase in intracellular calcium and generation of DAG to activate PKC and inhibit TASK3 channels may suggest that this pathway functions optimally during periods of high neuronal activity when intracellular calcium concentration increases.

Although the closely related K2P channel mTASK1 is regulated modestly after PKC activation (Lopes et al., 2000), this seems to occur through a different PKC isoenzyme, the calcium-independent PKCε (Besana et al., 2004). Our pharmacological and RNAi data suggest that inhibition of PKCε has little influence on the large inhibition of TASK3 current by PMA and ionomycin, suggesting that for TASK3, activation of this isoenzyme induces little, if any, inhibition.

Both human and mouse TASK3 are equally sensitive to activation of PKC. We were surprised to find that the C termini of hTASK3 and mTASK3 differ markedly (only 44% sequence similarity, see *Results*) and do not share either the same number or position of putative PKC consensus sites. Although both hTASK3 and mTASK3 are inhibited after PKCα activation, at least in terms of amino acid sequence, the sites of action on the respective C termini differ. It is possible that despite the large differences in sequence between the C terminus of TASK3 in the two species, the tertiary structure of the C terminus of hTASK3 and mTASK3 places the phosphorylation sites in a similar physical location relative to the rest of the channel protein.

A number of groups have shown that TASK currents can be inhibited after activation of G protein-coupled receptors, such as muscarinic acetylcholine receptors, which couple primarily to the G protein family Gα_q (Mathie, 2007). No direct evidence exists, however, that this pathway is the sole pathway stimulated after activation of M_3 receptors, and it is known that these receptors may activate transduction pathways mediated by alternative G proteins (for example, M_3 receptors can stimulate phospholipase D activity through the G protein, Gα_{12}) (Rümenapp et al., 2001). Our data with the selective inhibitor of Gα_q YM-254890 (Takasaki et al., 2004) and with constitutively active Gα_{12} suggest that both M_3 muscarinic receptor-mediated inhibition of recombinant TASK3 channels and of IK_{SO} in native mouse CGNs occurs solely through activation of Gα_q.

Although PKC activation can inhibit TASK3 channels, thereby reaching the same functional endpoint as Gα_q-mediated inhibition, it does not have a direct role in transducing M_3-mediated inhibition of hTASK3 channels because mutated hTASK3 channels, which are no longer sensitive to PKC activation, are still inhibited as normal. This is in contrast to Gα_q-mediated inhibition of the related K2P channels

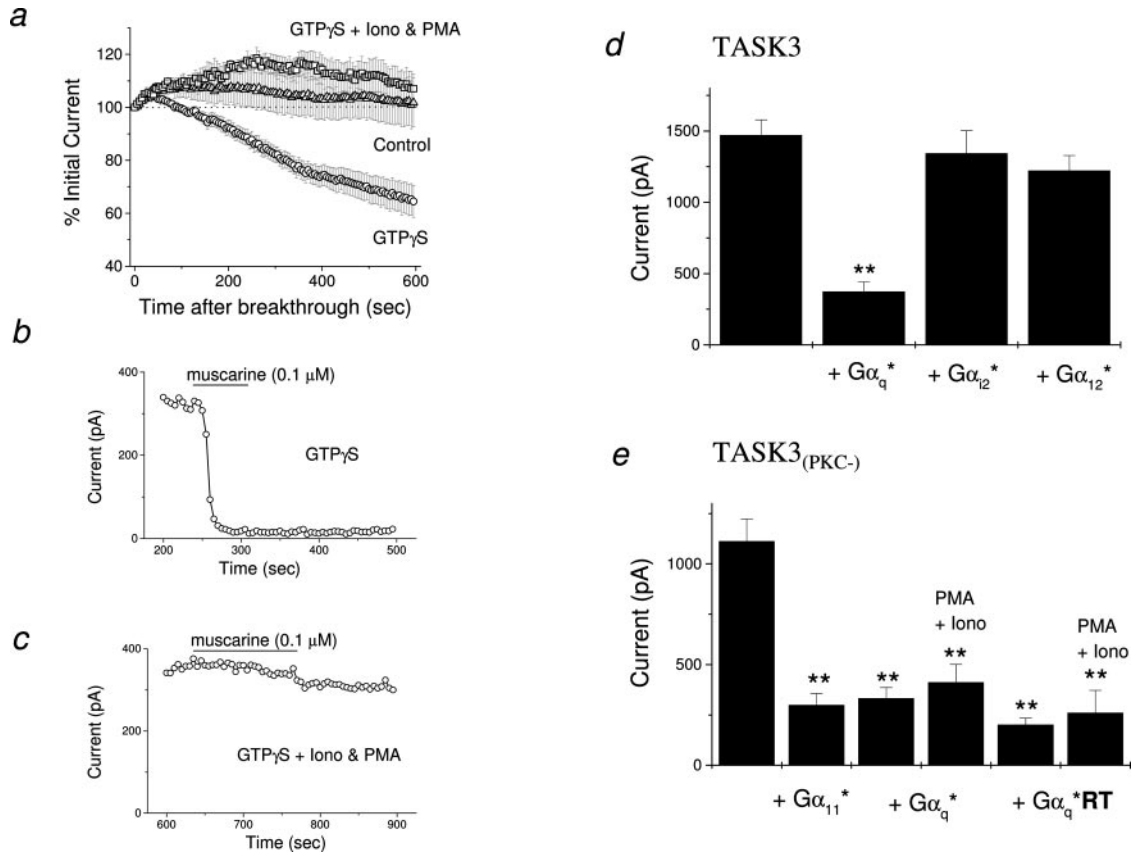

Fig. 6. TASK3 channel currents are inhibited by GTPγS and by constitutively active Gα_q. *a*, decrease in TASK3 current over 10 min when GTPγS is included in the recording pipette ($n = 7$) compared with control cells ($n = 7$). This effect is occluded by pretreatment of the cells with PMA (100 nM) and ionomycin (1 μM) ($n = 5$). *b*, in cells dialyzed with GTPγS, muscarine (0.1 μM) produces a strong and irreversible inhibition of TASK3 current. *c*, no effect of muscarine is seen in cells pretreated with PMA (100 nM) and ionomycin (1 μM). *d*, WT TASK3 channel currents are inhibited by Gα_q* but not Gα_{i2}* or Gα_{12}*. *e*, TASK3$_{(PKC-)}$ channel currents are inhibited by Gα_{11}*, Gα_q*, and Gα_q*RT, but the inhibitions of Gα_q* and Gα_q*RT are unaltered by PMA (100 nM) and ionomycin (1 μM).

TASK1, TREK1, and TREK2, which all require activation of PKC for channel inhibition (Besana et al., 2004; Murbartián et al., 2005; Kang et al., 2006).

The sequence of events that occur after activation of $G\alpha_q$, which lead to TASK3 channel inhibition, remains to be established (Talley and Bayliss, 2002; Chen et al., 2006; Mathie, 2007). There is evidence in favor of the hypothesis that the breakdown of the signaling molecule, PIP_2, is critical. Because PIP_2 has been shown to enhance the current through TASK3 channels (Chemin et al., 2003; Lopes et al., 2005) and other K2P channels (Chemin et al., 2005), it follows that breakdown of PIP_2 after $G\alpha_q$-mediated activation of PLC would be predicted to inhibit the current. Furthermore, recovery of K2P channel currents after receptor-induced inhibition is slowed or prevented in the presence of the inhibitor of PIP_2 resynthesis, wortmannin (Czirjak et al., 2001; Lopes et al., 2005). Such a process has been shown to underlie the regulation of many other ion channels (Suh and Hille, 2005).

Nevertheless, not all studies find evidence to support this hypothesis. A recent study by Chen et al. (2006) showed that TASK3 channel inhibition can proceed independently of either PLC activity or PIP_2 depletion. This has led to an alternative proposal, whereby activation of PLC is not required for TASK3 channel inhibition, and instead, activated $G\alpha_q$ acts directly (or via an alternative, as-yet-unknown second-messenger pathway) to inhibit TASK3 channels (Chen et al., 2006). Our data with $G\alpha_q$*RT on both WT TASK3 and $TASK3_{(PKC-)}$ channels support this hypothesis. In fact, there is no reason why more than one component of the signaling cascade may not act in parallel to transduce $G\alpha_q$-mediated inhibition of TASK3 current, as would seem to be the case for inhibition of TREK1 channels after activation of $G\alpha_q$ (Chemin et al., 2005; Murbartián et al., 2005).

Despite not being the primary transducer of $G\alpha_q$-mediated inhibition of TASK3 channels, our data do, however, show a clear regulatory role for PKC in this process, because preactivation of PKC with PMA, either alone or in the presence of ionomycin, protects both TASK3 channels and the native neuronal current IK_{SO} from inhibition by M_3 receptor activation.

A number of studies have shown that there is cross-talk between PKC and G protein inhibition of N-type voltage-gated calcium channels (Shapiro et al., 1996), whereby PKC activation occludes inhibition of the channel by certain G protein $\beta\gamma$ subunits. For some (Hamid et al., 1999) of the G protein $\beta\gamma$ subunits involved, this cross-talk has been suggested to be at the level of the calcium channel protein, in which one of the PKC consensus sequence sites acts as an "integration center" for this reciprocal regulation. This mechanism does not seem to underlie muscarinic regulation of TASK3 channels, because PKC activation still occluded $G\alpha_q$-mediated inhibition of TASK3 channels when all three consensus sequence site for PKC had been mutated. Because PMA and ionomycin pretreatment inhibits GTPγS-induced rundown of TASK3 current and because muscarinic regulation of M current is unaffected by PKC activation (Shapiro et al., 1996), this cross-talk also seems unlikely to be at the level of the muscarinic receptor itself.

Instead, our results point to the recruitment of a PKC-activated pathway that acts to oppose muscarinic modulation of TASK3 channels and reset channel activity after cessation

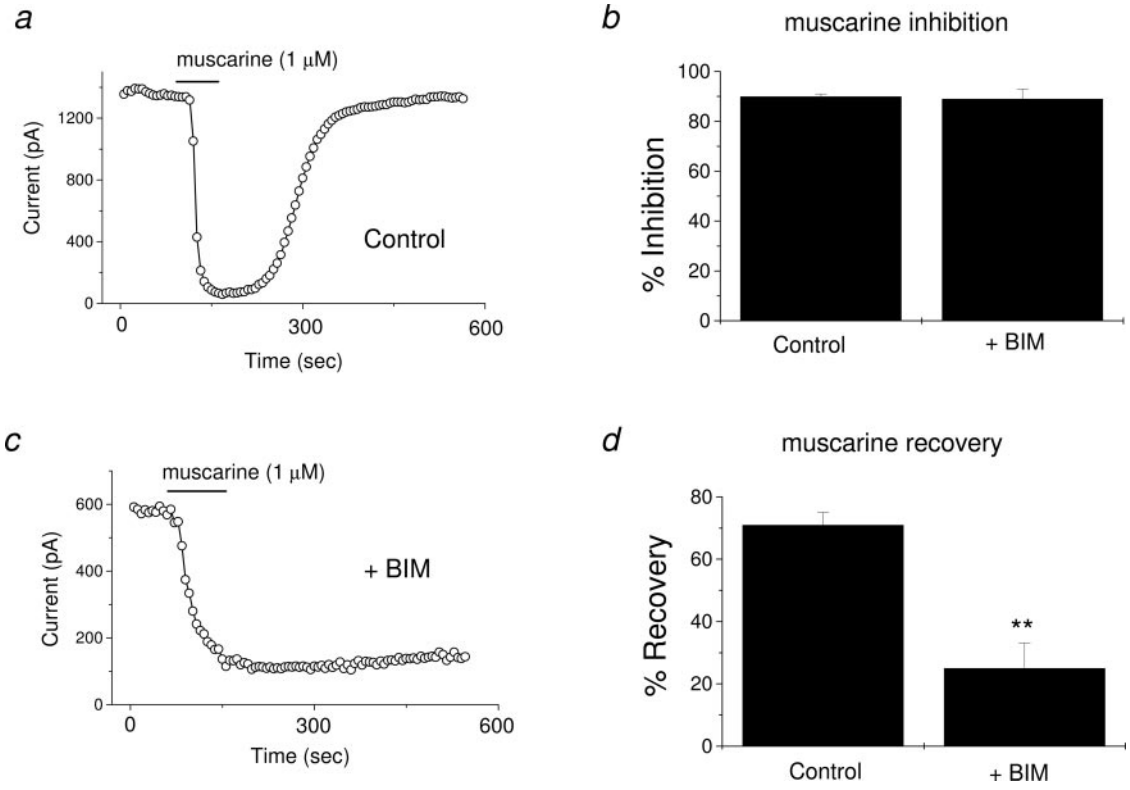

Fig. 7. Recovery from inhibition of hTASK3 channels by muscarine is attenuated by BIM. *a* and *c*, time courses of inhibition by muscarine (1 μM) for control (*a*) and BIM (1 μM)-treated (*c*) cells expressing hTASK3 channels. The magnitude of inhibition is unaltered by BIM (*b*), but the degree of recovery from inhibition is reduced (*d*).

of muscarinic receptor activation. This reversal of inhibition is attenuated in the presence of the PKC antagonist BIM. A similar attenuation by a PKC antagonist has been observed for thyrotropin-releasing hormone receptor-mediated inhibition of rat *ether-a-go-go*–related gene (rERG) (Gomez-Varela et al., 2003) and has been attributed to the block of an intracellular signaling cascade that normally mediates recovery of channel activity after thyrotropin-releasing hormone withdrawal. Likewise, inhibition of recovery by wortmannin (Czirjak et al., 2001; Lopes et al., 2005) need not solely be attributed to a decreased level of PIP_2 for maintaining TASK3 current, but may also be due to a decreased PLC-transduced activation of PKC to limit direct $G\alpha_q$-mediated inhibition of TASK3 channels. Although $G\alpha_q$ itself is unlikely to be directly phosphorylated by PKC (Aragay and Quick, 1999), a number of different RGS proteins of the B/R4 family act on $G\alpha_q$ to attenuate M_3 receptor-mediated signaling (Tovey and Willars, 2004), and the activity of these proteins is often regulated by phosphorylation (Willars, 2006).

Thus, although activation of PKCα can inhibit TASK3 channels, this is not the primary mechanism underlying $G\alpha_q$-mediated inhibition of these channels. Instead, $G\alpha_q$ seems to inhibit TASK3 channels directly (Chen et al., 2006). In addition, we propose that $G\alpha_q$ stimulates PKC after activation of PLC, and this acts as a negative feedback signal to limit the magnitude and duration of muscarinic receptor-mediated modulation of both TASK3 channels and the native leak potassium current in CGNs, IK_{SO}.

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References

Aller MI, Veale EL, Linden AM, Sandu C, Schwaninger M, Evans LJ, Korpi ER, Mathie A, Wisden W, and Brickley SG (2005) Modifying the subunit composition of TASK channels alters the modulation of a leak conductance in cerebellar granule neurons. *J Neurosci* **25:**11455–11467.

Aragay AM and Quick MW (1999) Functional regulation of $G\alpha_{16}$ by protein kinase C. *J Biol Chem* **274:**4807–4815.

Besana A, Barbuti A, Tateyama MA, Symes AJ, Robinson RB, and Feinmark SJ (2004) Activation of protein kinase C ε inhibits the two-pore domain K^+ channel, TASK-1, inducing repolarization abnormalities in cardiac ventricular myocytes. *J Biol Chem* **279:**33154–33160.

Bockenhauer D, Zilberberg N, and Goldstein SA (2001) KCNK2: reversible conversion of a hippocampal potassium leak into a voltage-dependent channel. *Nature Neurosci* **4:**486–491.

Boyd DF, Millar JA, Watkins CS, and Mathie A (2000) The role of Ca^{2+} stores in the muscarinic inhibition of the K^+ current IK_{SO} in neonatal rat cerebellar granule cells. *J Physiol* **529:**321–331.

Cambray-Deakin MA (1995) Cerebellar granule cells, in *Neural Cell Culture* (Cohen J and Wilkin GP eds) pp 3–13, Oxford University Press, New York.

Chemin J, Girard C, Duprat F, Lesage F, Romey G, and Lazdunski M (2003) Mechanisms underlying excitatory effects of group I metabotropic glutamate receptors via inhibition of 2P domain K^+ channels. *EMBO (Eur Mol Biol Organ) J* **22:**5403–5411.

Chemin J, Patel AJ, Duprat F, Lauritzen I, Lazdunski M, and Honore E (2005) A phospholipid sensor controls mechanogating of the K^+ channel TREK-1. *EMBO (Eur Mol Biol Organ) J* **24:**44–53.

Chen X, Talley EM, Patel N, Gomis A, McIntire WE, Dong B, Viana F, Garrison JC, and Bayliss DA (2006) Inhibition of a background potassium channel by Gq protein α-subunits. *Proc Natl Acad Sci USA* **103:**3422–3427.

Clarke CE, Veale EL, Green PJ, Meadows HJ, and Mathie A (2004) Selective block of the human 2-P domain potassium channel, TASK-3, and the native leak potassium current, IK_{SO}, by zinc. *J Physiol* **560:**51–62.

Czirjak G, Petheo GL, Spat A, and Enyedi P (2001) Inhibition of TASK-1 potassium channel by phospholipase C. *Am J Physiol* **281:**C700–C708.

Evans JH, Murray D, Leslie CC, and Falke JJ (2006) Specific translocation of protein kinase Calpha to the plasma membrane requires both Ca^{2+} and PIP2 recognition by its C2 domain. *Mol Biol Cell* **17:**56–66.

Foey AD and Brennan FM (2004) Conventional protein kinase C and atypical protein kinase Czeta differentially regulate macrophage production of tumour necrosis factor-alpha and interleukin-10. *Immunology* **112:**44–53.

Goldstein SAN, Bayliss DA, Kim D, Lesage F, Plant LD, and Rajan S (2005) International Union of Pharmacology. LV. Nomenclature and molecular relationships of two-P potassium channels. *Pharmacol Rev* **57:**527–540.

Gomez-Varela D, Giráldez T, de la Peña P, Dupuy SG, García-Manso D, Barros F (2003) Protein kinase C is necessary for recovery from the thyrotropin-releasing hormone-induced r-ERG current reduction in GH3 rat anterior pituitary cells. *J Physiol* **547:**913–929.

Hamid J, Nelson D, Spaetgens R, Dubel SJ, Snutch TP, and Zamponi GW (1999) Identification of an integration center for cross-talk between protein kinase C and G protein modulation of N-type calcium channels. *J Biol Chem* **274:**6195–6202.

Kang D, Han J, and Kim D (2006) Mechanism of inhibition of TREK-2 (K2P10.1) by the Gq-coupled M3 muscarinic receptor. *Am J Physiol* **291:**C649–C656.

Kang D, Han J, Talley EM, Bayliss DA, and Kim D (2004) Functional expression of TASK-1/TASK-3 heteromers in cerebellar granule cells. *J Physiol* **554:**64–77.

Kim D (2005) Physiology and pharmacology of two-pore domain potassium channels. *Curr Pharm Des* **11:**2717–2736.

Kim Y, Bang H, and Kim D (2000) TASK-3, a new member of the tandem pore K^+ channel family. *J Biol Chem* **275:**9340–9347.

Lesage F (2003) Pharmacology of neuronal background potassium channels. *Neuropharmacology* **44:**1–7.

Lopes CM, Gallagher PG, Buck ME, Butler MH, and Goldstein SA (2000) Proton block and voltage gating are potassium-dependent in the cardiac leak channel Kcnk3. *J Biol Chem* **275:**16969–16978.

Lopes CM, Rohacs T, Czirjak G, Balla T, Enyedi P, and Logothetis DE (2005) PIP2 hydrolysis underlies agonist-induced inhibition and regulates voltage gating of two-pore domain K^+ channels. *J Physiol* **564:**117–129.

Mathie A (2007) Neuronal two pore domain potassium channels and their regulation by G protein coupled receptors. *J Physiol* **578:**377–385.

Meadows HJ and Randall AD (2001) Functional characterisation of human TASK-3, an acid-sensitive two-pore domain potassium channel. *Neuropharmacology* **40:**551–559.

Millar JA, Barratt L, Southan AP, Page KM, Fyffe RE, Robertson B, and Mathie A (2000) A functional role for the two-pore domain potassium channel TASK-1 in cerebellar granule neurons. *Proc Natl Acad Sci USA* **97:**3614–3618.

Murbartián J, Lei Q, Sando JJ, and Bayliss DA (2005) Sequential phosphorylation mediates receptor- and kinase-induced inhibition of TREK-1 background potassium channels. *J Biol Chem* **280:**30175–30184.

Nishizuka Y (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* **9:**484–496.

Rümenapp U, Asmus M, Schablowski H, Woznicki M, Han L, Jakobs KH, Fahimi-Vahid M, Michalek C, Wieland T, and Schmidt M (2001) The M3 muscarinic acetylcholine receptor expressed in HEK-293 cells signals to phospholipase D via G_{12} but not G_q-type G proteins: regulators of G proteins as tools to dissect pertussis toxin-resistant G proteins in receptor-effector coupling. *J Biol Chem* **276:**2474–2479.

Shapiro MS, Zhou J, and Hille B (1996) Selective disruption by protein kinases of G-protein-mediated Ca^{2+} channel modulation. *J Neurophysiol* **76:**311–320.

Suh BC and Hille B (2005) Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. *Curr Opin Neurobiol* **15:**370–378.

Takasaki J, Saito T, Taniguchi M, Kawasaki T, Moritani Y, Hayashi K, and Kobori M (2004) A novel $G\alpha_{q/11}$-selective inhibitor. *J Biol Chem* **279:**47438–47445.

Talley EM and Bayliss DA (2002) Modulation of TASK-1 (Kcnk3) and TASK-3 (Kcnk9) potassium channels: volatile anesthetics and neurotransmitters share a molecular site of action. *J Biol Chem* **277:**17733–17742.

Talley EM, Lei Q, Sirois JE, and Bayliss DA (2000) TASK-1, a two-pore domain K^+ channel, is modulated by multiple neurotransmitters in motoneurons. *Neuron* **25:**399–410.

Talley EM, Solorzano G, Lei Q, Kim D, and Bayliss DA (2001) CNS distribution of members of the two-pore-domain (KCNK) potassium channel family. *J Neurosci* **21:**7491–7505.

Tovey SC and Willars GB (2004) Single-cell imaging of intracellular Ca^{2+} and phospholipase C activity reveals that RGS 2, 3, and 4 differentially regulate signaling via the $G\alpha_{q/11}$-linked muscarinic M3 receptor. *Mol Pharmacol* **66:**1453–1464.

Vega-Saenz de Miera E, Lau DH, Zhadina M, Pountney D, Coetzee WA, and Rudy B (2001) KT3.2 and KT3.3, two novel human two-pore K^+ channels closely related to TASK-1. *J Neurophysiol* **86:**130–142.

Watkins CS and Mathie A (1996) A non-inactivating K^+ current sensitive to muscarinic receptor activation in rat cultured cerebellar granule neurons. *J Physiol* **491:**401–412.

Willars GB (2006) Mammalian RGS proteins: multifunctional regulators of cellular signalling. *Semin Cell Dev Biol* **17:**363–376.

Zhang YH, Kenyon JL, and Nicol GD (2001) Phorbol ester-induced inhibition of potassium currents in rat sensory neurons requires voltage-dependent entry of calcium. *J Neurophysiol* **85:**362–373.

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