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Gα_q MEDIATED REGULATION OF TASK3 TWO PORE DOMAIN POTASSIUM CHANNELS: THE ROLE OF PROTEIN KINASE C

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PKC modulation of TASK3 channels.

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Abstract

The TASK subfamily of two pore domain potassium channels (K2P) gives rise to leak K 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 BIM and Go 6976. Gene silencing experiments with a validated siRNA 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 (T341), thereby reducing channel current. PKC also inhibits mTASK3 channels despite them having a quite different C terminus structure to hTASK3 channels. Activation of M_3 muscarinic receptors inhibits both hTASK3 channels expressed in tsA-201 cells and IK_{SO} in mouse cerebellar granule neurons through the activation of the G protein $G\alpha_q$ since both effects are abolished by the selective $G\alpha_q$ antagonist, YM-254890. This inhibition is not directly transduced through activation of PKC since 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, pre-activation 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 following M_3 receptor activation rather than transducing it and may act as a negative regulator of G protein modulation to prevent prolonged current inhibition.

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Two pore domain potassium channels (K2P) underlie leak K 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 fifteen members of the K2P channel family, in humans, which can be divided into six 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 (GPCR) 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 non functional TASK5, $K_{2P}15.1$) underlie leak currents in a variety of neuronal populations including cerebellar granule neurons (CGNs) (e.g. Millar et al., 2000; Talley et al., 2000; Kang et al., 2004; Aller et al., 2005) and their activity is strongly inhibited following activation of $G\alpha_q$-coupled receptors. There are currently three competing hypotheses as to how inhibition of TASK channels may occur (Mathie, 2007). Firstly, it has been suggested that inhibition is mediated by one or more of the hydrolysis product of phosphatidylinositol 4,5-bisphosphate (PIP_2) generated following activation of phospholipase C (PLC) (e.g. 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 (e.g. 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

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channels (e.g. Chen et al., 2006). There is no reason why these three potential pathways should be mutually exclusive (Mathie, 2007).

Whilst TASK1 channels seem to be modestly inhibited following activation of protein kinase C (PKC) (e.g. Lopes et al., 2000) and this underlies the PAF 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, 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 following 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, IK_{SO}, in mouse CGNs.

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Materials and Methods

tsA-201 cell culture preparation.

Modified HEK-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 units ml^{-1}) and streptomycin (10 mg ml^{-1}). When the cells were 80% confluent, they were split and plated for transfection onto glass coverslips coated with poly-D-lysine (1 mg ml^{-1}) to ensure good cell adhesion. The cells were transiently transfected using the calcium phosphate method. 0.3 - 1 µg of cDNA expression vector encoding a mouse or human TASK3 subunit was added to each 15 mm well, and 0.3 - 1 µg of a plasmid encoding the cDNA of green fluorescent protein was included to identify cells expressing K2P channels. Following an 18 - 24 hour 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 - 60 hours 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 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.

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siRNA experiments.

We used pre-designed 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 sequence of the siRNAs (SI00605927; SI02622088) against human PKCα (NM_002737) and PKCε (NM_005400) are shown to target a region between 400 – 800 and 1500 – 1750 bp relative to the first nucleotide of the start codon, respectively. tsA-201 cells were transiently co-transfected with either PKCα siRNA or PKCε siRNA (1μg), together with the reporter plasmid, green fluorescent protein (GFP) and cDNA encoding human TASK3 DNA as described above.

Tissue culture of CGNs.

CCNs were isolated using previously described methods (Cambray-Deakin, 1995) from the cerebella of 7- to 8- day old mice of either sex, which had been killed by decapitation. Following dispersion, cells were plated at a density of 2 x 10^6 cells ml^{-1} onto 13 mm glass coverslips coated with poly-D-lysine and allowed to adhere. Cells were grown in minimum essential medium (MEM) supplemented with 10% heat inactivated fetal bovine serum, 30 mM glucose, 5 ml ITS supplement, 0.5 % penicillin (10, 000 units ml^{-1}) and streptomycin (10 mg ml^{-1}) and 25 mM K^+ (shown to enhance 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.

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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 (in mM) 145 NaCl, 2.5 KCl, 3 $MgCl_2$, 1 $CaCl_2$, 10 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-filtered intracellular solution (composition in mM: 150 KCl, 3 $MgCl_2$, 5 EGTA, 10 HEPES (titrated to pH 7.4 with KOH). For CGNs, amphotericin B-permeablised patches were used to minimise disruption of intracellular composition and run down of IK_{SO} (Watkins and Mathie, 1996). For these cells, the pipette solution contained (mM): 120 KCH_3SO_4, 4 NaCl, 1 $MgCl_2$, $CaCl_2$, 10 HEPES, 10 EGTA, 3 ATP, 0.3 GTP and 240 μg ml^{-1} amphotericin (titrated to pH 7.4 with KOH). Cells were voltage-clamped using an Axopatch 1D amplifier (Axon instruments, USA) 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, USA). 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 seconds (see also legend to figure 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 seconds. 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 seconds. All electrophysiological measurements were carried out at room temperature (21 - 23˚C).

Data analysis.

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Data were analysed using Clampfit software (Axon Instruments, USA), Excel (Microsoft Corporation, USA) and Origin (Microcal, USA). Statistical comparisons were carried out using Student's *t* test and *p* values < 0.05 were regarded as significant. Results are given as mean ± standard error of the mean 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.

Phorbol 12-myristate 13-acetate (PMA); 4-α-phorbol 12-myristate-13-acetate (4-α-PMA); bisindolylmaleimide 1 hydrochloride (BIM); amphotericin B; ionomycin; muscarine chloride, were all obtained from Sigma, UK. Go 6976 was from Calbiochem, Merck Biosciences, UK. YM-254890, was from Dr J Takasaki, Astellas Pharma Inc, Japan. All compounds were made up in either DMSO or water and diluted in external or internal solution prior to experimentation. For the PMA, plus ionomycin experiments, cells were incubated for at least 20 mins 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, UK). M_2 & M_3 muscarinic acetylcholine receptors and all constitutively active Gα constructs were from the Guthrie cDNA resource center, USA. The PLC-disrupted mutant $G\alpha_q$*RT (R256A, T257A) was generated as described above, based on the earlier work of Chen et al., (2006).

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Results

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

Since $G\alpha_q$ 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 (see Mathie, 2007). There is evidence in the literature both in favour 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

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alone or in the additional presence of the calcium ionophore, ionomycin (1 μM), the latter a combination often used experimentally to activate classical or calcium-dependent PKCs (e.g. Foey and Brennan, 2004). Figure 2a illustrates the effect of bath application of PMA and ionomycin on normalised currents through hTASK3 channels. PMA and ionomycin produced a 63 ± 4 % (n = 4) inhibition of current which reached steady-state after around 12 mins. Figure 2b shows mean current amplitudes for hTASK3 channels measured at -80 and -40 mV (see methods) in control conditions and following 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 due to ionomycin, *per se*, since treatment with the inactive phorbol ester, 4αPMA and ionomycin or ionomycin alone had no effect on current amplitude when compared with control ($p > 0.05$). The inset shows mean currents through hTASK3 channels in control conditions and following treatment with PMA alone or PMA and ionomycin.

The effect of PMA and ionomycin could be completely blocked by the non selective PKC inhibitor, BIM (1 μM) (figure 2b). It was also completely blocked by the PKC inhibitor Go 6976 (figure 2b) which, at the concentration used here (100 nM) is a selective inhibitor for classical PKCs such as PKCα, PKCβI & 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 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

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is also expressed in tsA-201 cells but it is not a calcium dependent PKC and is unaffected by Go 6976 at the concentrations used in this study. Our results are shown in figure 2 (c, 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 to control cells. This current was unaffected, however, following treatment with PMA and ionomycin ($p > 0.05$). In contrast, PMA and ionomycin treatment still inhibited hTASK3 current following transfection with siRNA against PKCε ($p < 0.05$). Thus our pharmacological experiments suggest that hTASK3 channels can be strongly inhibited following activation of a classical 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 are, however, no longer inhibited following 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 (figure 3a). We have made single point mutations to each of these sites in turn (S319A, S331A, T341A) to determine whether one or more of these sites is acted on following activation of PKCα. From figure 3b, it can be seen that $hTASK3_{(S319A)}$ and

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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 T341. 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 mouse CGNs, we need to consider regulation of mouse TASK3 channels. Our original pharmacological characterisation suggested little difference between mTASK3 and hTASK3 functional properties (see 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 to 98% similarity and 94% identity for the remainder of the TASK3 channel protein (see figure 3a). 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 following activation of PKC and, even if so, which site(s) are targeted by PKC.

Our data for mTASK3 are summarised in figure 3c. mTASK3 was indeed inhibited following activation of PKC ($p < 0.05$) and, as for hTASK3, this effect was ablated following C terminus 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 clear-cut as for hTASK3. Whilst no one mutation completely ablated the effect of PKC, T348 is most likely the amino acid phosphorylated by PKC to give the analogous effect to that

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seen for hTASK3 channels, since 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 neighbouring PKC site (S351), particularly since the double mutant mTASK3$_{(T348A_S351A)}$ was completely unaffected by PMA and ionomycin (figure 3c).

To determine whether PKC directly transduces M_3 mediated inhibition of hTASK3 channels, muscarinic inhibition was tested on hTASK3 channels where either the PKCα site (see figure 2) has been mutated (T341A) to abolish direct PKC action on the channel or where each of the three consensus sequence sites for PKC in the C terminus of hTASK3 were mutated (TASK3$_{(PKC-)}$). In both sets of experiments, the degree of inhibition of hTASK3 by M_3 receptor activation was completely unaltered compared with WT hTASK3, with 0.1 μM muscarine producing 76 ± 9 % (n = 4) and 80 ± 3 % (n = 6) inhibition, respectively (see figure 4). These data suggest that, PKC activation does not directly transduce Gα_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 (figure 4a, b, e) that pre-treatment with PMA and ionomycin virtually abolished inhibition of hTASK3 currents following 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, whilst 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 pre-treatment 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,

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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 4αPMA or when cells were pretreated with the non-selective PKC inhibitor BIM (in addition to PMA and ionomycin).

This action of PMA and ionomycin was fully retained in $hTASK3_{(PKC-)}$ channels (figure 4 c, d, 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 PMA and ionomycin was also seen in the mouse PKC-resistant TASK3 channel mutant, $mTASK3_{(T348A_S351A)}$, with muscarine producing no detectable inhibition following pre-treatment with PMA and ionomycin (2 ± 6 %, n = 3).

TASK3 channels are thought to underlie at least part of the whole-cell standing outward potassium current (IK_{SO}) in rodent CGNs (Clarke et al., 2004; Kang et al., 2004; Aller et al., 2005) which is inhibited following 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 following muscarinic receptor activation (figure 5a) albeit to a lesser degree than in rat (see Watkins and Mathie, 1996). As for hTASK3 channels, YM-254890 completely abolished inhibition of IK_{SO} by muscarinic receptor activation in mouse CGNs (figure 5c), 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 ($p < 0.05$) following pre-treatment of mouse CGNs with PMA and ionomycin (figure 5b, c) from 37 ± 5 % (n = 13) in control to 11 ± 1 % (n = 4) in the presence of PMA plus ionomycin. Similarly in rat CGNs, PMA alone significantly reduced ($p < 0.05$) muscarinic inhibition of IK_{SO} from 74 ± 2 % (n = 27)

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in control to 32 ± 5 % (n = 9) in the presence of PMA. Muscarinic inhibition of IK_{SO} was unaffected by pre-treatment of the cells with BIM but recovery from muscarinic inhibition was only partial (see below). Whilst 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 (e.g. Kang et al., 2004).

Since PKC does not seem to be acting that the level of the 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 regulation of channel current by the non-hydrolysable form of GTP, GTPγS. This is illustrated in figure 6 (a-c). When recordings were made with GTPγS in the recording pipette, it can be seen that hTASK3 current amplitude declined, significantly, with time when compared to control cells (figure 6a), with current reaching 64 ± 6% (n = 7) of it's original amplitude after 10 min recording compared to 102 ± 9 % (n = 7) for control cells ($p < 0.05$). Furthermore, muscarine treatment now caused an irreversible inhibition of hTASK3 current (figure 6b, see also Chemin et al., 2003). Pretreatment with PMA and ionomycin abolished the decline of TASK3 currents in cells dialysed with GTPγS and, in addition, muscarine no longer induced inhibition of TASK3 current (figure 6a, c). Thus PKC acts at a site downstream from the muscarinic receptor to inhibit this regulatory pathway.

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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 (figure 6 d, e). The activated form of the $G\alpha_q$-like G protein, $G\alpha_{11}$* mimicked the effect of $G\alpha_q$* (figure 6e) but there was no difference from control currents for either $G\alpha_{i2}$* or $G\alpha_{12}$* (figure 6d).

PMA and ionomycin did not reverse the effect of either $G\alpha_q$* or $G\alpha_q$*RT on $hTASK\text{-}3_{(PKC-)}$ channels (figure 6e). 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 to 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 where 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 signalling complex that can only assemble and function properly in the absence of PKC mediated phosphorylation of a regulatory protein involved.

To determine whether regulation by PKC has a potential negative-feedback role during normal muscarinic receptor activation, inhibition was tested in the presence of the non selective PKC inhibitor BIM (1 μM). Whilst the degree of inhibition of hTASK3 by M_3

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receptor activation was completely unaltered (figure 7 a, 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 (figure 7 c, 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 following M_3 receptor activation rather than transducing it and may act as a negative regulator of G protein modulation to prevent prolonged current inhibition.

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Discussion

We have shown that activation of PKC inhibits TASK3 current when there is a concomitant rise in intracellular calcium, suggesting the involvement of a classical, calcium-dependent PKC such as PKCα. Previously, Kim et al., (2000) and Meadows and Randall, (2001) pre-incubated cells in PMA (100 nM) and found no effect of the compound on the amplitude of TASK3 currents when compared to 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 depolarising the oocytes) the resultant calcium influx was sufficient to act synergistically with PMA to optimally activate classical 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).

Classical 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 whilst calcium binds to the C2 domain which (together with phosphatidylserine binding) promotes translocation of the PKCα to the plasma membrane (see e.g. Evans et al., 2006). Physiologically, the requirement for a coincident rise in intracellular calcium and generation of DAG in order to activate PKC and inhibit TASK3 channels may suggest that this pathway functions optimally during periods of high neuronal activity when intracellular calcium concentration rises.

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Whilst the closely related K2P channel, mTASK1, is regulated modestly following 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. Surprisingly, 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. Whilst both hTASK3 and mTASK3 are inhibited following 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 h & 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 following activation of G protein coupled receptors, such as muscarinic ACh receptors, which couple, primarily, to the G protein family, $G\alpha_q$ (Mathie, 2007). No direct evidence exists, however, that this pathway is the sole pathway stimulated following 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 PLD activity through the G protein, $G\alpha_{12}$, Rumenapp et al., 2001). Our data with the selective

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inhibitor of $G\alpha_q$, YM-254890 (Takasaki et al., 2004) and with constitutively active $G\alpha_{12}$, 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 $G\alpha_q$.

Whilst PKC activation can inhibit TASK3 channels thereby reaching the same functional endpoint as $G\alpha_q$ mediated inhibition, it does not have a direct role in transducing M_3 mediated inhibition of hTASK3 channels since mutated hTASK3 channels which are no longer sensitive to PKC activation are still inhibited as normal. This is in contrast to $G\alpha_q$ mediated inhibition of the related K2P channels TASK1, TREK1 and TREK2 which all require activation of PKC for channel inhibition (Besana et al., 2004; Murbartian et al., 2005; Kang et al., 2006).

The sequence of events that occur subsequent to activation of $G\alpha_q$ which lead to TASK3 channel inhibition remain to be established (Talley et al., 2002; Chen et al., 2006; Mathie, 2007). There is evidence in favour of the hypothesis that the breakdown of the signalling molecule, PIP_2, is critical. Since 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 following $G\alpha_q$ mediated activation of PLC would be predicted to inhibit the current. Furthermore, recovery of K2P channel currents following receptor induced inhibition is slowed or prevented in the presence of the inhibitor of PIP_2 re-synthesis, wortmannin (Czirjak et al., 2001; Lopes et al., 2005). Such a process has been shown to underlie the regulation of many other ion channels (see Suh and Hille, 2005).

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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 lead 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 signalling cascade may not act in parallel to transduce $G\alpha_q$ mediated inhibition of TASK3 current, as would appear to be the case for inhibition of TREK1 channels following activation of $G\alpha_q$ (see Murbartian et al., 2005; Chemin 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, since pre-activation 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 (e.g. 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 Ca channel protein, where one of the PKC consensus sequence sites acts as an "integration centre" for this reciprocal regulation. This mechanism does not seem to underlie muscarinic regulation of TASK3 channels since

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PKC activation still occluded $G\alpha_q$ mediated inhibition of TASK3 channels when all three consensus sequence site for PKC had been mutated. Since PMA and ionomycin pre-treatment inhibits GTPγS induced rundown of TASK3 current and since 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 which acts to oppose muscarinic modulation of TASK3 channels and reset channel activity following cessation 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 TRH-receptor mediated inhibition of r-ERG (Gomez-Varela et al., 2003) and has been attributed to the block of an intracellular signalling cascade that normally mediates recovery of channel activity following TRH withdrawal. Similarly, 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 (see 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 signalling (Tovey and Willars, 2004) and the activity of these proteins is often regulated by phosphorylation (Willars, 2006).

Thus while 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). Additionally, we propose that $G\alpha_q$

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stimulates PKC following 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 K current in CGNs, IK_{SO}.

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Footnotes

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Figure Legends

Figure 1. M_3 receptor mediated inhibition of hTASK3 channels is blocked by YM-254890. a, 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 show 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} of 27 ± 8 nM.

Figure 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 normalised to the mean control amplitude of the first ten data points for each cell (n = 4). b) Histogram of mean current through hTASK3 channels (measured as the difference current between that at -40 mV 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 Go 6976 (100 nM, "Go" n = 8). Inset shows averaged currents (± standard error) through hTASK3 channels in control conditions or following incubation with either PMA alone (100 nM) or PMA plus ionomycin (1 μM) for at least 20 min. c) Averaged currents (± standard error) through hTASK3 channels evoked between -110 and +20 mV following co-transfection with siRNA against PKCα ("RNAiα") or PKCε ("RNAiε") in control conditions or following treatment with PMA and ionomycin. d) Histogram of mean current through hTASK3

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channels (measured as the difference current between that at -40 mV and that at -80 mV) following co-transfection with siRNA against PKCα or PKCε in control conditions (n = 9 & n = 6, respectively) or following treatment with PMA and ionomycin (n = 10 & n = 8, respectively).

Figure 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 terminus regions (see results) as shown. Putative PKC phosphorylation sites, three for hTASK3 and four for mTASK3 are shown. b,c) Histogram of mean current through hTASK3 (b) or mTASK3 (c) channels (measured as the difference current between that at -40 mV and that at -80 mV) for wild type (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 following treatment with PMA and ionomycin.

Figure 4. Activation of PKC attenuates M_3 receptor mediated inhibition of hTASK3 channels. a, b) Inhibition of hTASK3 channels by muscarine (0.1 μM) in control conditions (a) and following pre-activation of PKC with PMA (100 nM) and ionomycin (1 μM) (b). Insets show representative current traces before and after perfusion of muscarine. The voltage protocol shows a step to -80 mV 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 seconds. c,d) Inhibition of triple mutated $TASK3_{(PKC-)}$ channels by muscarine (0.1 μM) in control

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conditions (c) and following pre-activation of PKC with PMA (100 nM) and ionomycin (1 μM) (d). Insets show 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 following treatment with PMA (100 nM) and ionomycin (1 μM) or PMA alone.

Figure 5. Activation of PKC attenuates muscarinic receptor mediated inhibition of IK_{SO} in mouse CGNs. a, b) Inhibition of IK_{SO} by muscarine (10 μM) in control conditions (a) and following pre-activation of PKC with PMA (100 nM) and ionomycin (1 μM). Insets show 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, following YM-254890 (1 μM) or following pre-treatment with PMA (100 nM) and ionomycin (1 μM).

Figure 6. TASK3 channel currents are inhibited by GTPγS and by constitutively active $G\alpha_q$. a) Decrease in TASK3 current over 10 min when GTPγS is included in the recording pipette (n = 7) compared to control cells (n = 7). This effect is occluded by pre-treatment of the cells with PMA (100 nM) and ionomycin (1 μM) (n = 5). b) In cells dialysed 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 $G\alpha_q$* but not $G\alpha_{i2}$* or $G\alpha_{12}$*. e) $TASK3_{(PKC-)}$ channel currents are inhibited by $G\alpha_{11}$*, $G\alpha_q$* and $G\alpha_q$*RT but the inhibitions $G\alpha_q$* and $G\alpha_q$*RT are unaltered by PMA (100 nM) and ionomycin (1 μM).

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Figure 7. Recovery from inhibition of hTASK3 channels by muscarine is attenuated by BIM. a, 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).

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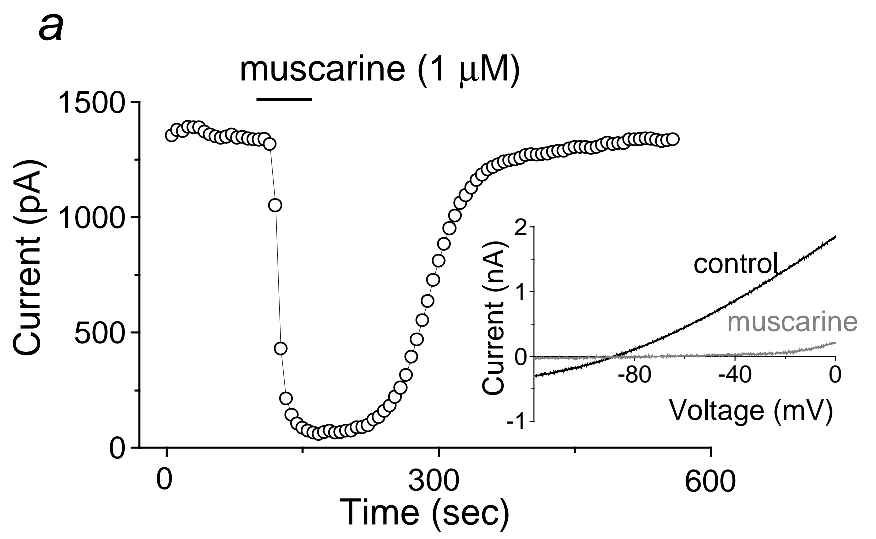
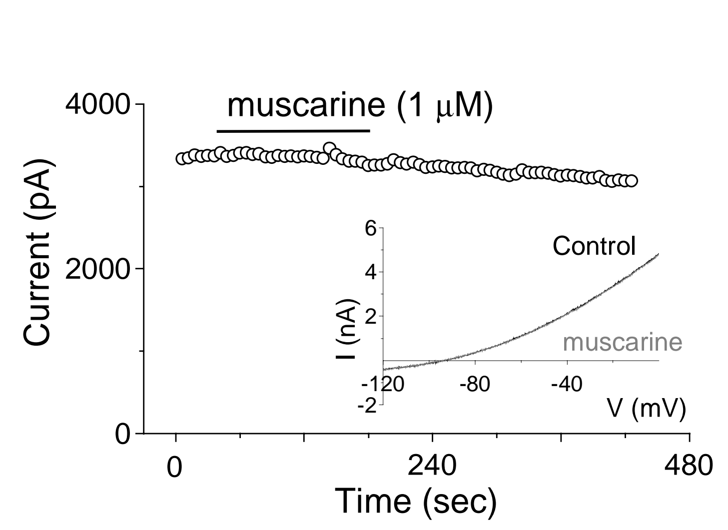
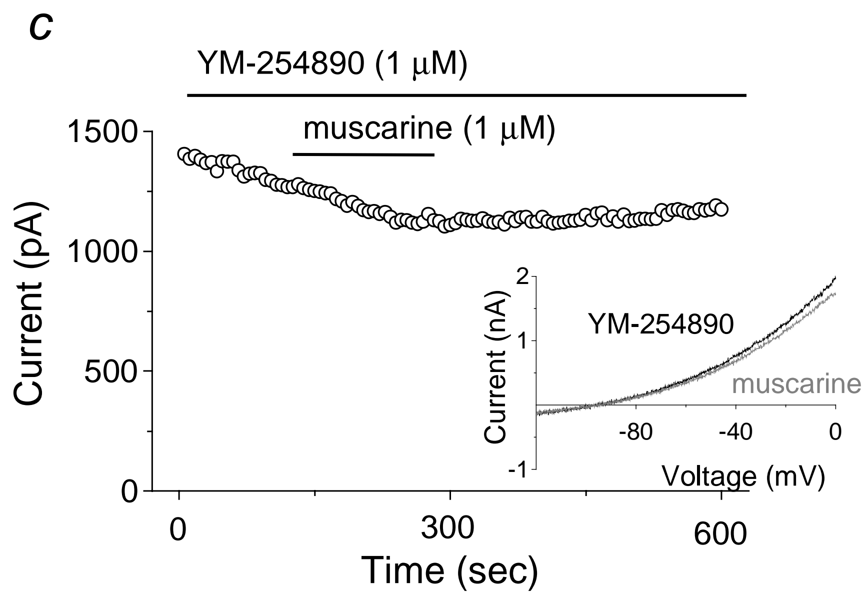
Figure 1







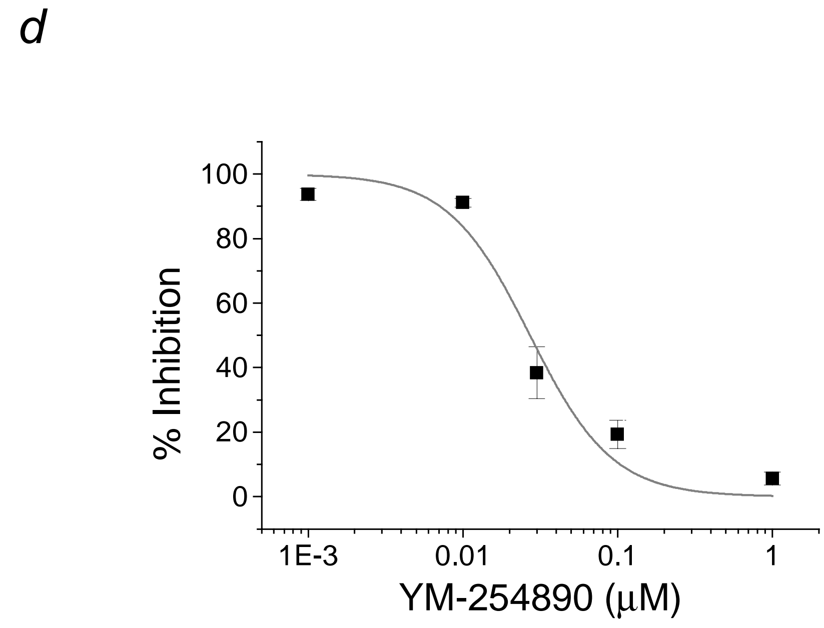


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Figure 2

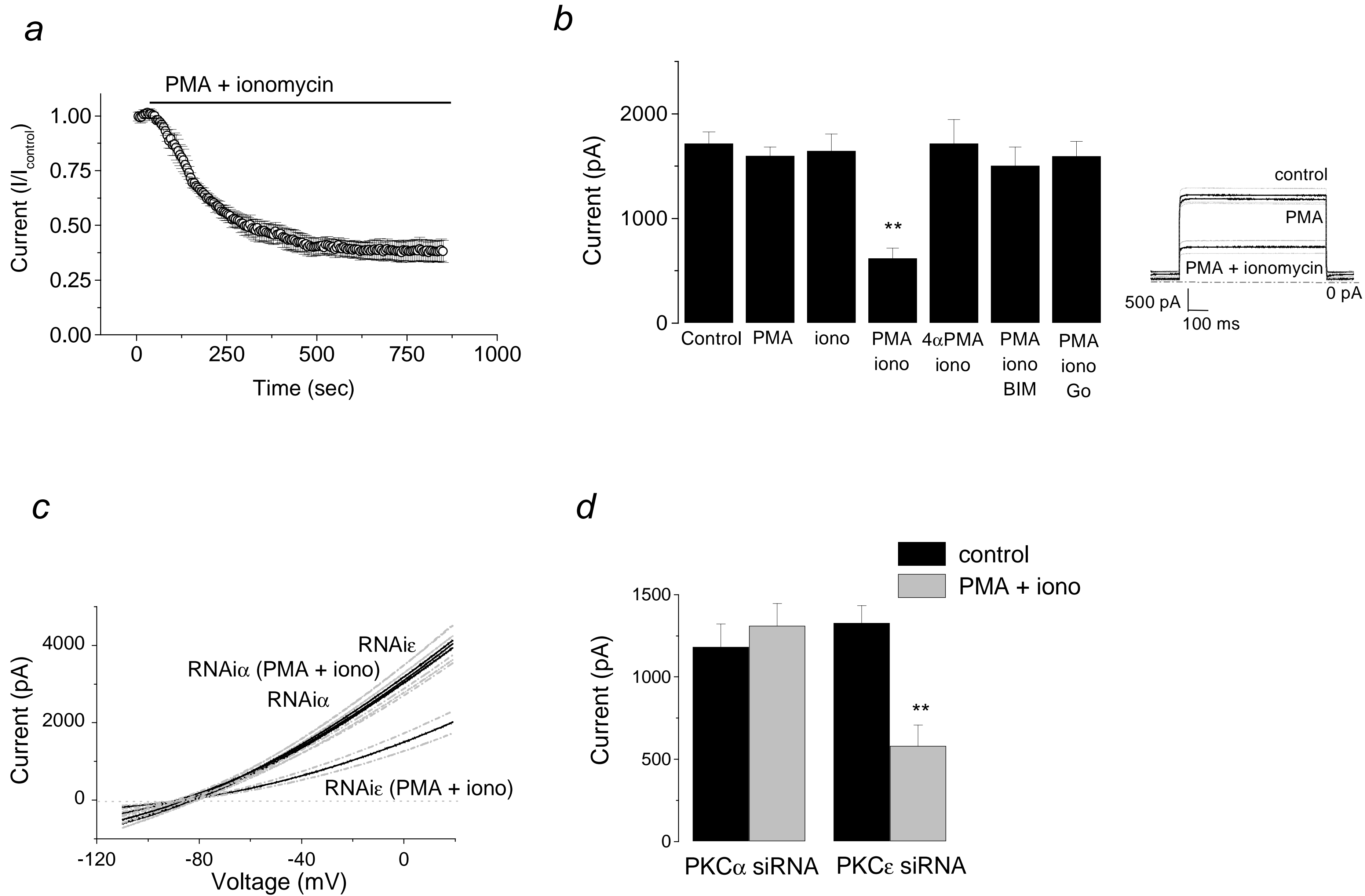

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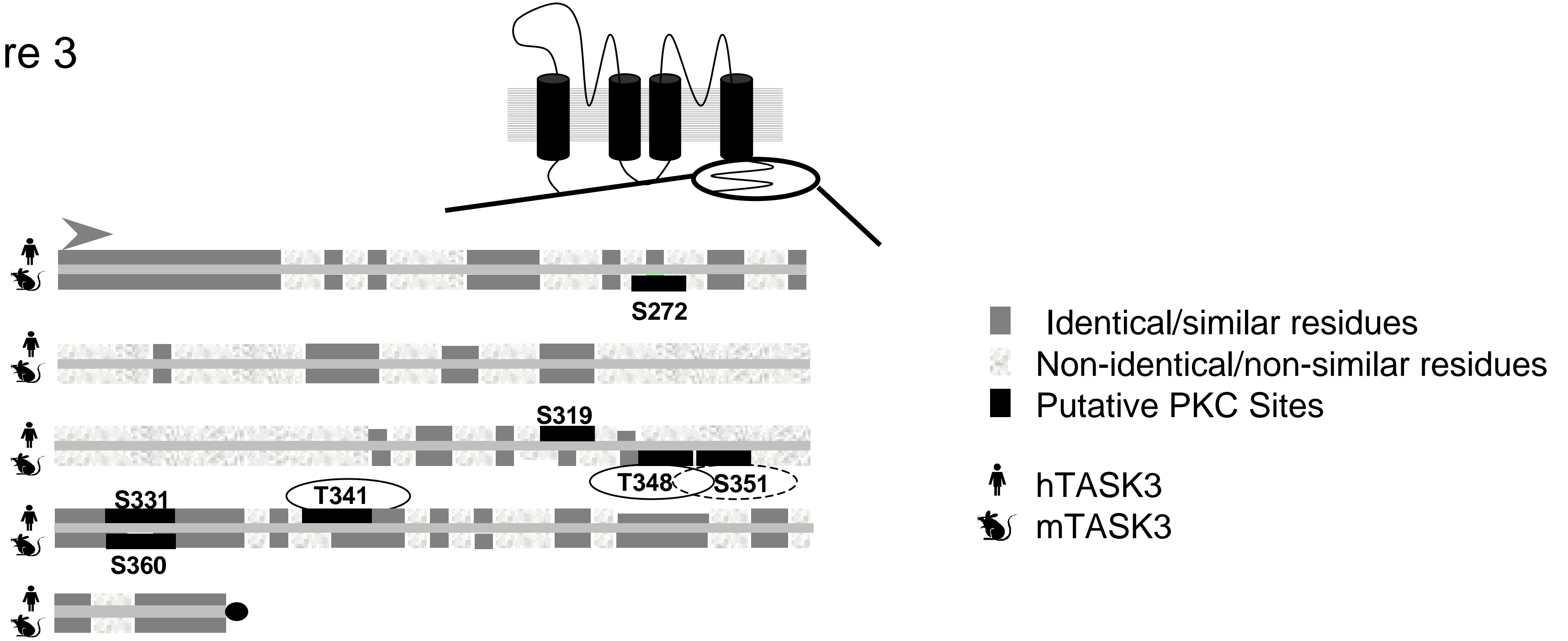
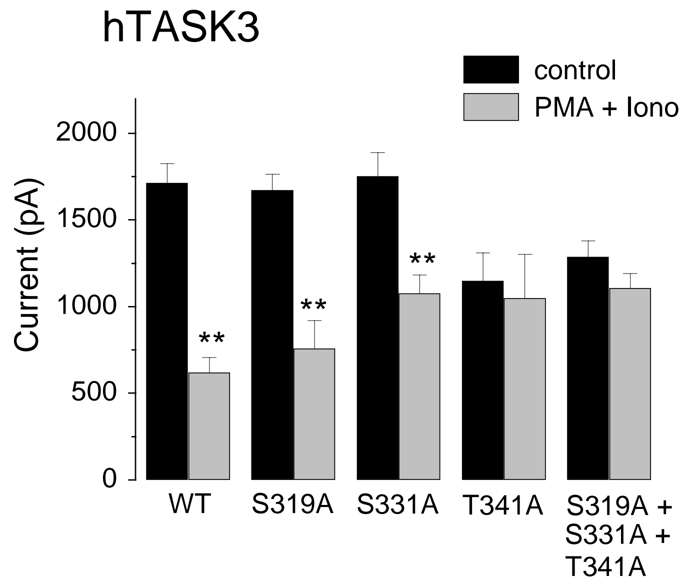
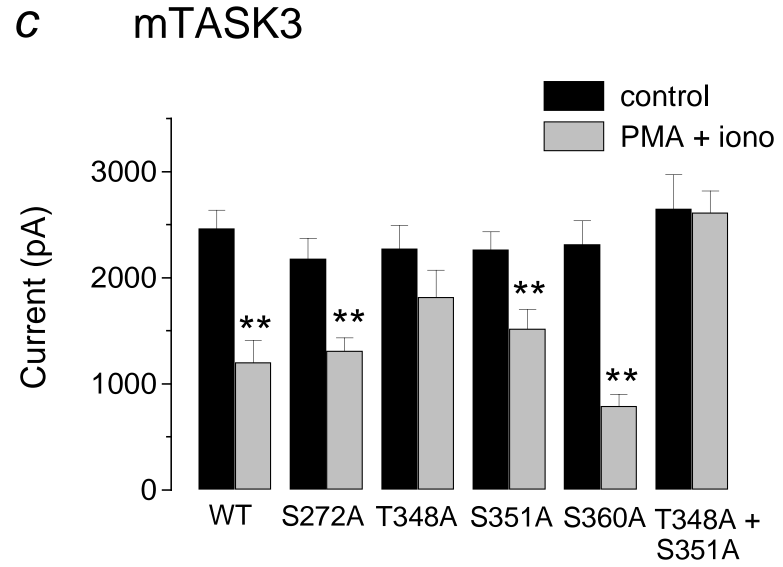
Figure 3







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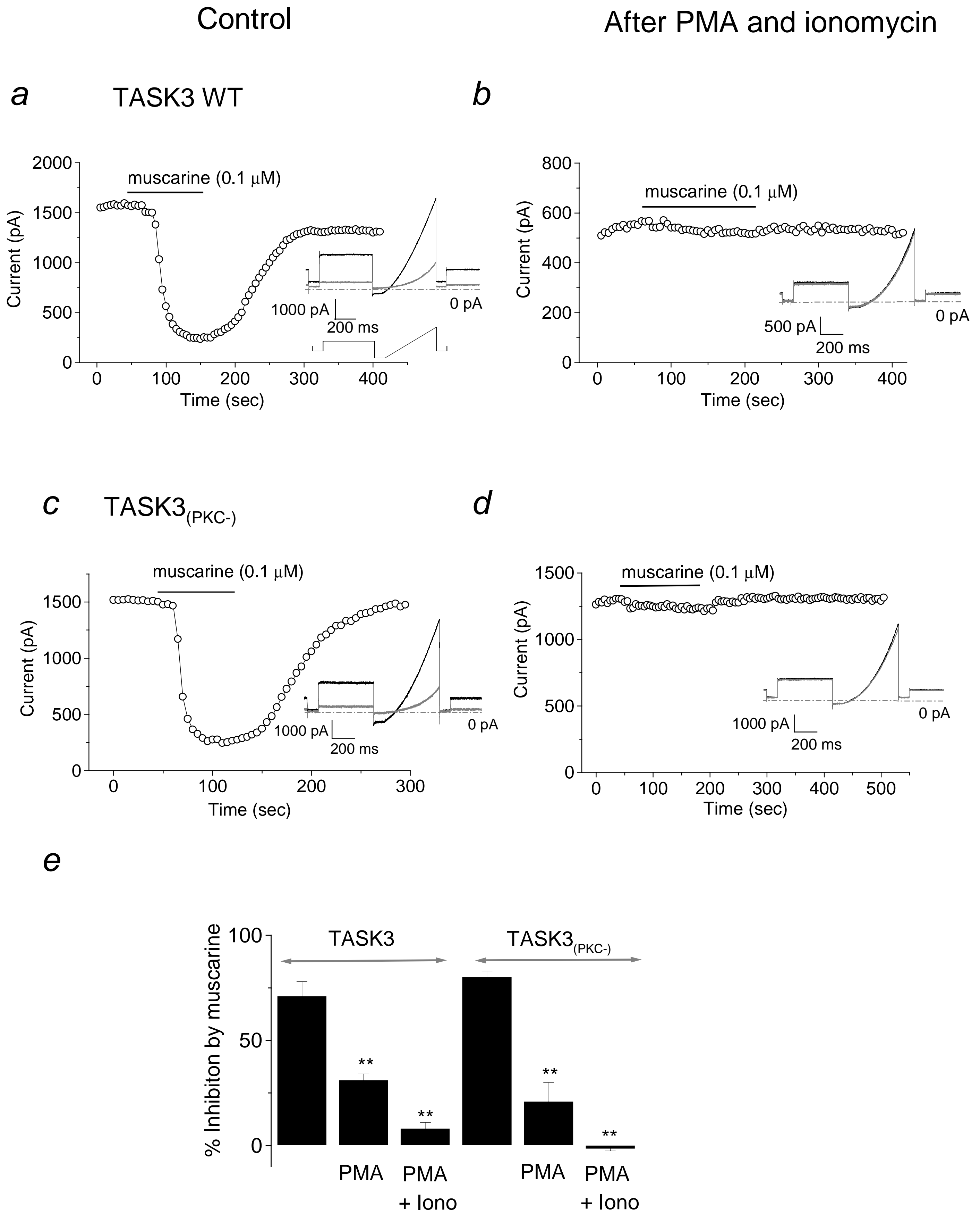

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Figure 5

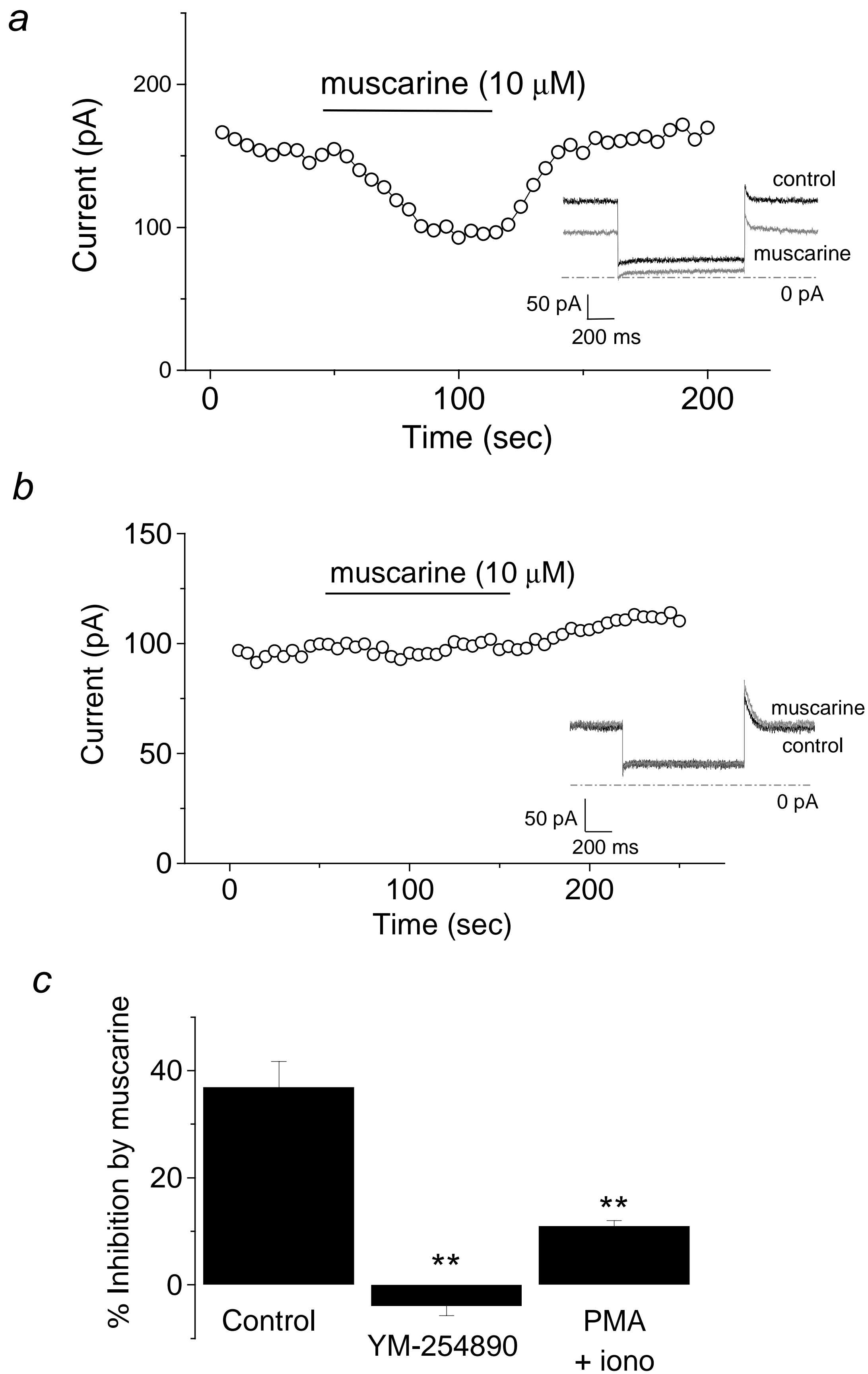

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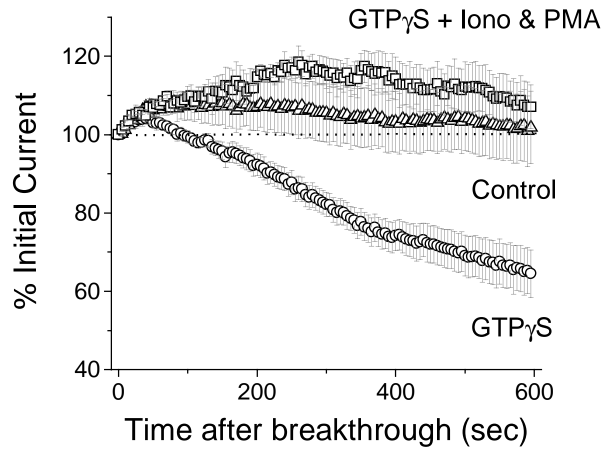
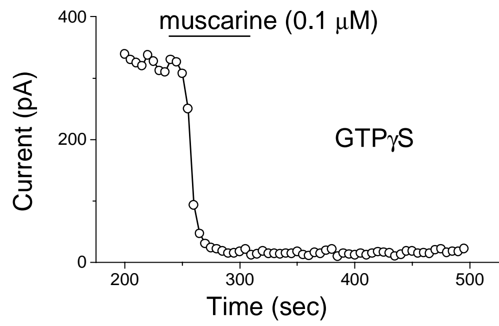
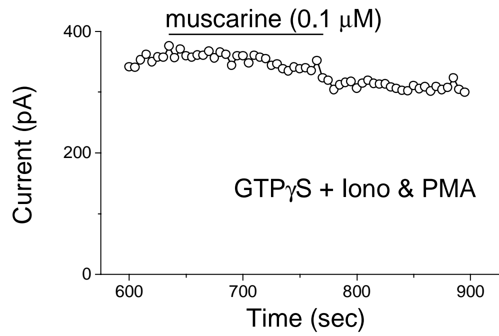
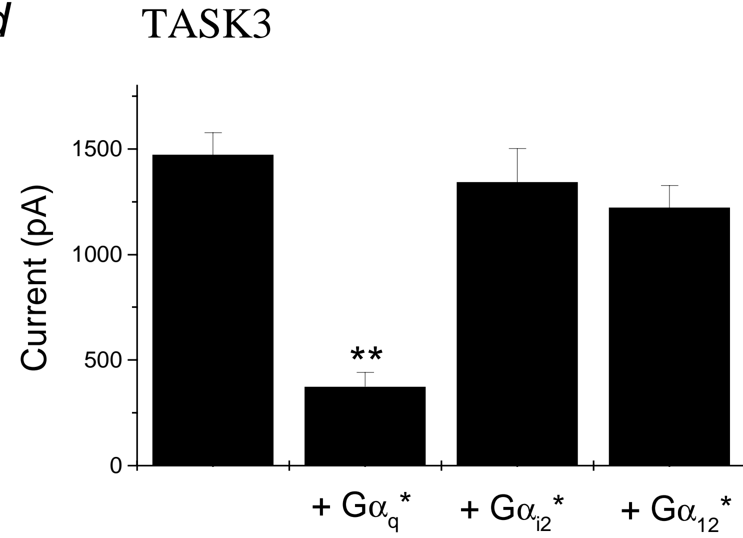
Figure 6









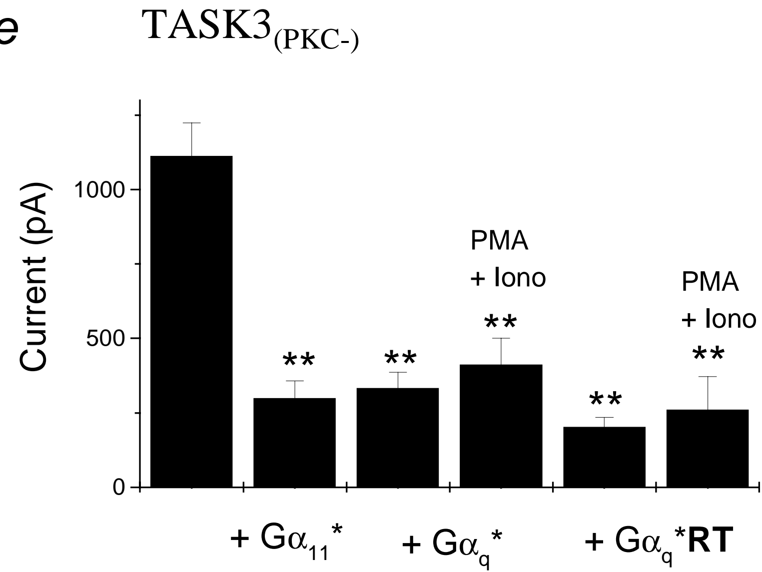


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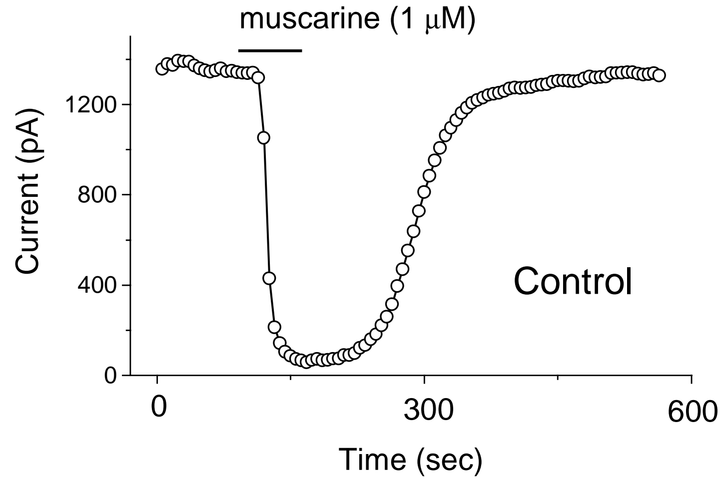
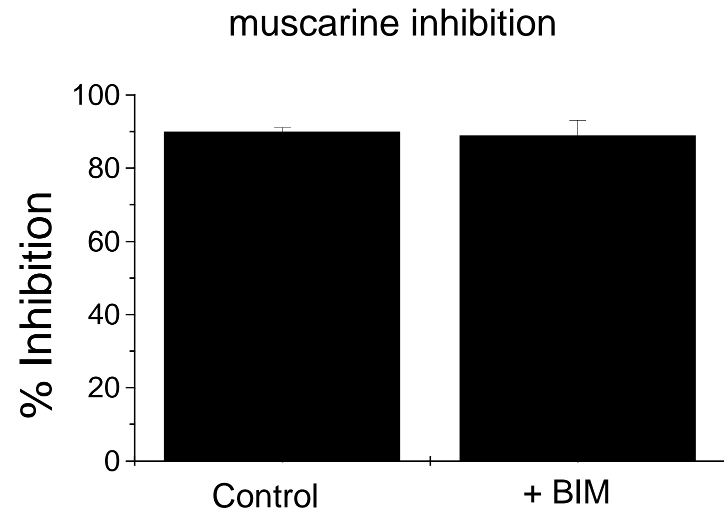
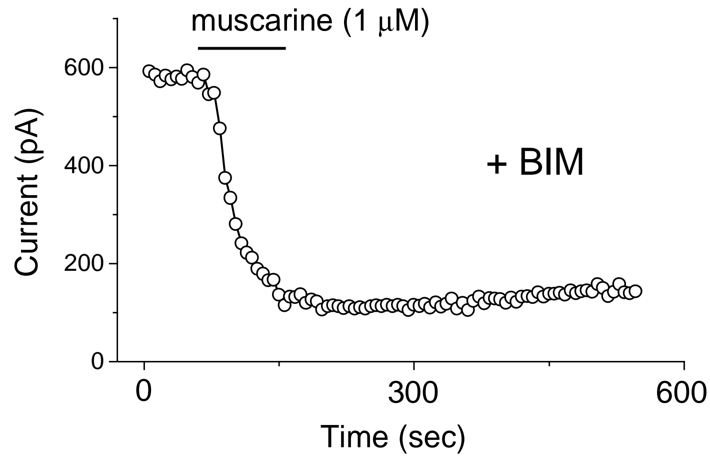
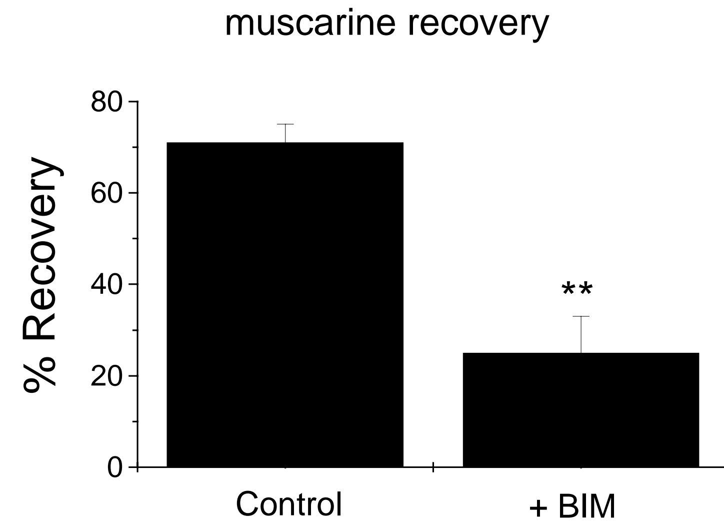
Figure 7









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