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RESEARCH PAPER

An M_2-like muscarinic receptor enhances a delayed rectifier K^+ current in rat sympathetic neurones

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Background and purpose: Resting superior cervical ganglion (SCG) neurones are phasic cells that switch to a tonic mode of firing upon muscarinic receptor stimulation. This effect is partially due to the muscarinic inhibition of the M-current. Because delayed rectifier K^+ channels are essential to sustain tonic firing in central neurones, we asked whether the delayed rectifier current I_{KV} in SCG neurones was modulated by the muscarinic receptors expressed in these cells.
Experimental approach: Whole-cell patch-clamp records of M-current and I_{KV} were done in cultured or acutely dissociated rat SCG neurones. To characterize the receptor that regulates I_{KV}, cells were bathed with muscarinic agonists and antagonists, relatively specific for receptor subtypes.
Key results: The muscarinic agonist oxotremorine-M (Oxo-M) enhanced I_{KV} by $\sim$46% relative to its basal value. This effect remained unaltered when M-current was suppressed by linopirdine or Ba^{2+}. Enhancement of I_{KV} was insensitive to the M_1-antagonist pirenzepine, whereas it was inhibited ($\sim$60%) by the $M_{2/4}$-antagonist himbacine. Further, the relatively specific M_2-agonist bethanechol was as potent as Oxo-M in enhancing I_{KV}. The modulation of I_{KV} was insensitive to pertussis toxin (PTX), but was severely attenuated when internal ATP was replaced by its non-hydrolysable analogue AMP-PNP.
Conclusions and Implications: These results suggest that an M_2-like muscarinic receptor couples to a PTX-insensitive G-protein and to an ATP-dependent pathway to enhance I_{KV}. Modulation of I_{KV} must be taken into consideration in order to understand more precisely how muscarinic receptors acting on different ion channels regulate sympathetic excitability.
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Abbreviations: AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate; I_{CaN}, N-type Ca^{2+} current; I_{KV}, delayed rectifier K^+ current; mAChRs, muscarinic acetylcholine receptors; NA, noradrenaline; Oxo-M, oxotremorine-M; PTX, pertussis toxin; SCG, superior cervical ganglion

Introduction

In the nervous system, the slow synaptic actions of acetylcholine (ACh) are mediated by muscarinic ACh receptors (mAChRs) and their coupled G proteins. Stimulation of postsynaptic mAChRs very often elicits a slow membrane depolarization that increases firing frequency or switches firing pattern from a burst to a tonic mode (McCormick, 1993; Marrion, 1997). Most of the knowledge in relation to the signalling pathways activated by mAChRs that regulate cell excitability come from studies carried out in rat superior cervical ganglion (SCG) neurones. According to their similarity in amino-acid sequence and pharmacological profile, mAChRs are grouped into the M_1-like (M_1, M_3 and M_5) and M_2-like (M_2 and M_4) subtypes. Of these, SCG neurones mostly express the M_1, M_2 and M_4 receptors (Brown *et al.*, 1995).

SCG neurones are considered phasic nerve cells because during a maintained depolarizing current stimulus, they respond with few action potentials that show adaptation, that is, their firing rate slows down (Wang and McKinnon, 1995). This electrical behaviour is partially determined by the Kv7.2 and Kv7.3 (KCNQ) K^+ channel subunits that generate the voltage-activated and non-inactivating M-current (Wang *et al.*, 1998). As M-current dominates the membrane conductance around the cell firing threshold, its slow activation strongly damps the depolarizing effects by inward Na^+ or Ca^{2+} currents, thereby contributing to spike frequency adaptation (Wang *et al.*, 1998; Yue and Yaari, 2004). Conversely, inhibition of M-current by muscarinic agonists produces a slow depolarization that promotes high-frequency tonic firing (Brown and Constanti, 1980; Adams *et al.*, 1982; McCormick, 1993; Marrion, 1997). In SCG cells, the inhibition of M-current is disrupted by the relatively

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selective M_1-antagonist pirenzepine and its modulation disappears upon deletion of the m_1 gene (Hamilton *et al.*, 1997). This pharmacological and genetic evidence indicates that the M_1 subtype underlies the muscarinic modulation of the M-current (Marrion *et al.*, 1989; Bernheim *et al.*, 1992; Hamilton *et al.*, 1997). The signalling pathway downstream from the M_1 receptor has been elucidated and involves a $G\alpha_q$-activated phospholipase that depletes membrane PIP_2 (phosphatidylinositol-4,5-bisphosphate) as the ultimate signal for M-current inhibition (Haley *et al.*, 1998; Suh and Hille, 2002; Zhang *et al.*, 2003; Winks *et al.*, 2005). A second ionic current modulated by mAChRs is the N-type Ca^{2+} current (I_{CaN}). Its muscarinic inhibition develops with an initial fast ($\tau \sim 1$ s) and voltage-dependent component, followed by a slow ($\tau \sim 9$ s) and voltage-independent component (Zhou *et al.*, 1997). The slower component is blocked by pirenzepine, whereas the voltage-dependent inhibition is disrupted by the relatively selective $M_{2/4}$-antagonist himbacine (Bernheim *et al.*, 1992). Genetic deletion strategies indicate that the M_1 subtype mediates the slow component, whereas the M_2 subtype is responsible for the voltage-dependent inhibition of I_{CaN} (Shapiro *et al.*, 1999).

Despite the well-documented muscarinic inhibition of both M-current and I_{CaN}, it is not known whether other SCG-endogenous K^+ currents are modulated by mAChRs. One potential target for muscarinic action might be the delayed rectifier K^+ current (I_{KV}), because: (a) delayed rectifier K^+ currents are essential to sustain tonic firing of action potentials (McCrossan *et al.*, 2003; Melnick *et al.*, 2004), as muscarinic stimulation does in SCG neurones; (b) in SCG neurones, I_{KV} significantly contributes to both the late spike repolarization and the early fast spike afterhyperpolarization (Marsh and Brown, 1991), and its transient genetic elimination prolongs action potentials, thereby reducing the probability of tonic firing (Malin and Nerbonne, 2002); (c) it has been reported that muscarinic agonists modulate delayed rectifier K^+ currents from central neurones (Zhang *et al.*, 1992; ffrench-Mullen *et al.*, 1994). In this paper, we report that the muscarinic agonist oxotremorine-M (Oxo-M) enhanced the amplitude of I_{KV}, with a pharmacological profile that suggested the involvement of an M_2-like mAChR. Furthermore, some properties of this novel signalling pathway are characterized.

Methods

Cell culture

Experiments were mostly carried out on cultured SCG neurones taken from juvenile (2- to 4-week old) Wistar rats, although some current recordings were carried out in older animals (10 weeks old, 210 g weight). Rats were quickly anesthetized with chloroform and decapitated. Superior cervical ganglia were quickly removed, cleaned from non-neuronal tissue and placed for 18 min in modified Hank's solution containing papain (20 U ml^{-1} at 37°C). Thereafter, papain was replaced by a mixture of collagenase I (1.6 mg ml^{-1}) and dispase II (5 mg ml^{-1}) and further incubated for 14 min, with gentle pipette trituration every 7 min to increase cell dissociation. The acutely dissociated cells were suspended twice in Dulbecco's modified Eagle's medium (DMEM), centrifuged and plated on 4×4 mm glass chips (coated with poly-L-lysine). Cells were incubated for 10 h at 37°C (5% CO_2) with DMEM supplemented with 10% of heat-inactivated foetal bovine serum (FBS), before current recording. For some experiments, the acutely dissociated neurones were directly placed in the recording chamber for immediate patch-clamping.

Electrophysiology

Whole-cell patch-clamp recordings of I_{KV} or M-current were made with a List EPC-7 amplifier and seals were obtained with patch pipettes having 1–2 MΩ resistance. The resulting steady series resistance was 3–4 MΩ, 2 min after seal breakthrough. The cell membrane capacitance was measured by integrating the capacitive current generated during a 10 mV depolarizing step, from a holding of −80 mV. The experiments were carried out in SCG neurones with a mean membrane cell capacitance of 73 ± 3.4 pF (range: 47–88 pF). Voltage command pulses and current records were generated and acquired (sampling rate, 5 kHz) using a 12-bit interface (Indec Systems Inc., Sunnyvale, CA, USA). Membrane currents were low-pass filtered at 1 kHz and analysed using BASIC-FASTLAB (Indec Systems) and Sigma Plot (SPSS Inc.). The M-current was fully activated by setting the holding potential (V_h) at −25 mV and further deactivated by 500 ms pulses from −25 to −60 mV, every 4 s. The amplitude of M-current and its percentage of inhibition were measured as described (Cruzblanca *et al.*, 1998). For I_{KV} isolation, neurones were held at −50 mV to fully inactivate the I_A K^+ current (Wang and McKinnon, 1995) and Cd^{2+} (200 μM) was added to the external solution to block the voltage-activated Ca^{2+} currents and the Ca^{2+}-activated K^+ currents. Thereafter, I_{KV} was elicited by 100 ms command pulses from −50 to 40 mV, in steps of 10–20 mV. I_{KV} was quantified from leakage subtracted records, as the difference between the average of a 10 ms segment of basal current and the averaged outward current recorded during the last 5 ms of the command step. Thus, current density (pA pF^{-1}) was calculated by dividing the amplitude of I_{KV} (pA) and the cell membrane capacitance (pF). Modulation of I_{KV} was measured as the change in its current density at 0 mV. Membrane voltages were corrected on line for a −2 mV junction potential (standard internal solution).

Solutions

The composition of the modified Hank's solution was (in mM): 137 NaCl, 5.4 KCl, 0.44 KH_2PO_4, 0.34 NaH_2PO_4, 5 4-(2-hydroxyethyl)-1-piperazineethyl-sulphonic acid (HEPES), 5 glucose, pH = 7.4. SCG cells were transferred to a recording chamber (400 μl) and bathed (2.8 ml min^{-1}) with the appropriate external solution. For M-current recording, the external solution was (in mM): 160 NaCl, 2.5 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 8 glucose and 0.5 μM tetrodotoxin (TTX). The pH was adjusted to 7.4 with NaOH. For I_{KV} recording, Cd^{2+} (200 μM) was added to the external saline. Solution changes were accomplished in <10 s and the experiments were carried out at 25°C.

The composition of the standard pipette internal solution was (in mM): 175 KCl, 5 $MgCl_2$, 5 HEPES, 0.1 1,2-bis(2-

aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), 3 K_2-ATP (adenosine 5′-triphosphate), 0.1 Na-GTP, 0.08 leupeptin (pH = 7.4). For experiments requiring cell dialysis with a test molecule (adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP)) from the patch pipette, we waited for 5 min dialysis before beginning the K^+ current recordings. To avoid systematic bias, control and test measurements were alternated within each set of experiments.

Statistical analysis

Average results are given as the mean ± s.e.m. and sample means were compared using Student's *t*-test. Differences between means were considered significant at $P < 0.05$.

Materials

Reagents were obtained as follows: pertussis toxin (PTX) (List Biological Labs., Campbell, CA, USA); collagenase I, poly-L-lysine, HEPES, Na-GTP, pirenzepine, himbacine, linopirdine, Oxo-M, bethanechol (Sigma, St Louis, MO, USA); BAPTA (Molecular Probes, Eugene, OR, USA); papain, dispase II, leupeptin and K_2-ATP (Roche Diagnostics GmbH, Mannheim, Germany); DMEM and heat-inactivated FBS (GIBCO/Invitrogen Co, Carlsbad, CA, USA); TTX and AMP-PNP (Calbiochem, La Jolla, CA, USA). Stock solutions of AMP-PNP and Na-GTP were prepared in water and stored at −20°C.

Results

The muscarinic agonist Oxo-M enhances the amplitude of I_{KV}

In our preparations, by setting the V_h at −50 mV and bathing the cells with a TTX- and Cd^{2+}-containing external solution, it was possible to block the Na^+ and Ca^{2+} inward currents and to eliminate the contribution of the A-type and Ca^{2+}-activated K^+ currents, revealing a delayed-like outward current named here as I_{KV} (see inset in Figure 1a). I_{KV} was activated at membrane voltages more positive than −40 mV (Figure 1c) and was significantly more sensitive to external 4-AP than tetraethylammonium (TEA) (500 μM TEA or 4-AP suppressed I_{KV} tail current by 29 ± 2% and 56 ± 4%, respectively; $n = 6$). To assess whether I_{KV} is under regulation by mAChRs, the cultured SCG neurones were bathed with 10 μM of the muscarinic agonist Oxo-M. As shown in Figure 1, Oxo-M produced an increase of I_{KV} that was more noticeable at membrane potentials more positive than −20 mV (Figure 1a and b). For instance at 0 mV, Oxo-M increased the amplitude of I_{KV} from 4.1 to 5.5 nA (Figure 1c). For comparison purposes among cells, the enhancement of I_{KV} was normalized to the cell membrane capacitance. Thus, in eight cells Oxo-M increased I_{KV} current density by 16.1 ± 1.4 pA pF^{-1} from a non-stimulated basal level, at 0 mV, of 35 ± 3.6 pA pF^{-1}. In most of the cells tested (60%), there were neither changes on the holding current elicited at −50 mV nor on the leakage current generated by a 10 mV hyperpolarizing command pulse, from a V_h of −60 mV (control, 33 ± 4 pA; Oxo-M, 31 ± 4 pA). In the rest of the cells

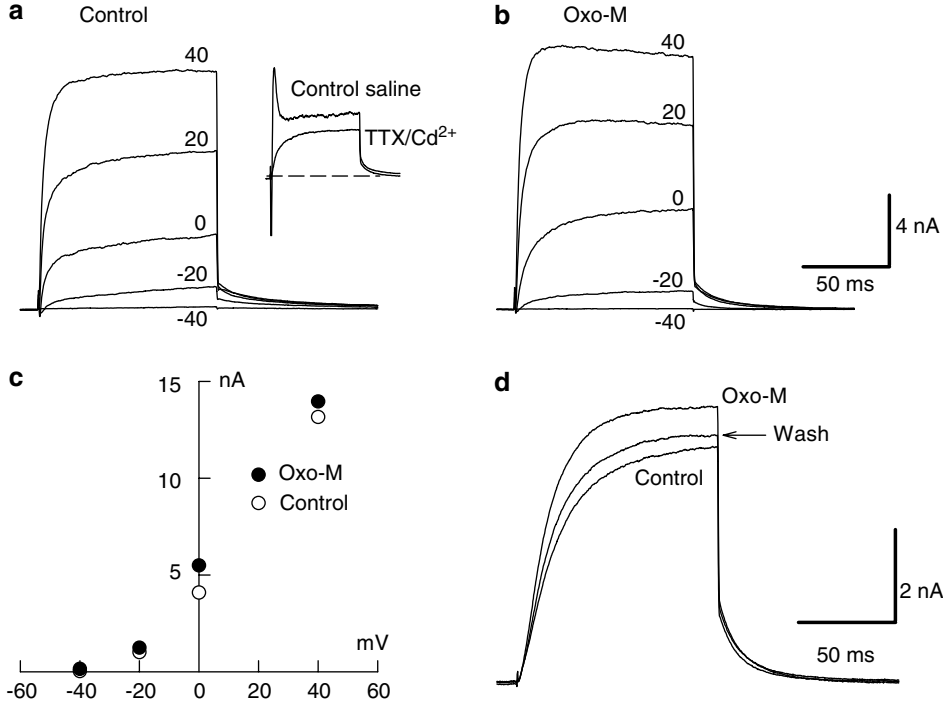

Figure 1 The muscarinic agonist Oxo-M enhances the amplitude of I_{KV}. I_{KV} records elicited from −40 to +40 mV ($V_h = -50$ mV), taken before (**a**) and during (**b**) Oxo-M (10 μM) exposure. The inset in (**a**) shows the superposition of the membrane currents recorded at 0 mV when a cultured SCG neurone was bathed with the normal external solution (Control saline) and during addition of TTX (500 nM) plus Cd^{2+} (200 μM); the dashed line indicates the zero current level. (**c**) *I–V* relationship taken before (open circles) and during (filled circles) Oxo-M bath application. (**d**) Superimposition of I_{KV} traces generated in an acutely dissociated SCG neurone from a juvenile rat, recorded before (Control) during Oxo-M exposure (Oxo-M) and after 2 min of the agonist removal (Wash).

(40%), the enhancement of I_{KV} occurred in parallel with a small inward shift (67 ± 11 pA) of the holding current and with a reproducible, but not statistically significant, increase of the leakage current from 40 ± 4.4 to 48 ± 4.6 pA.

It may be argued that the muscarinic enhancement of I_{KV} reported here could be induced by the culturing process or that modulation of I_{KV} could be mediated by a developmental process that disappears during the adult age. To assess for these possibilities, the effect of Oxo-M was tested on acutely dissociated SCG cells from juvenile rats (2–4 weeks old) and on cultured cells from adult (10 weeks old) animals. Stimulation of mAChRs resulted in a slowly reversible enhancement of I_{KV}, both in acutely dissociated juvenile SCG cells (Figure 1d) and cultured SCG neurones from adult rats as well. The mean increase in I_{KV} current density was 11.7 ± 1.5 pA pF^{-1} ($n = 5$) for juvenile recently dissociated SCG cells and 17.4 ± 2 pA pF^{-1} ($n = 6$) for cultured neurones from 10-week old rats.

The enhancement of I_{KV} *reflects a novel muscarinic signalling pathway operating in SCG neurones*

The modulation of I_{KV} was further characterized by clarifying whether its enhancement reflected the activity of a novel muscarinic pathway or whether it was a space-clamp artefact generated by the parallel strong muscarinic inhibition of the M-current (Adams *et al*., 1982; Marrion *et al*., 1989). To address this question, some experiments were carried out in the presence of linopirdine, a relatively selective M-channel blocker (Wang *et al*., 1998). As expected, linopirdine (10 μM) practically abolished M-current (Figure 2a, upper records). Nevertheless, in the presence of linopirdine, Oxo-M still enhanced I_{KV} (Figure 2a, bottom records). In summary, the increments of I_{KV} current density in the absence or presence of linopirdine were 16.1 ± 1.4 ($n = 8$) and 12.5 ± 1.6 pA pF^{-1} ($n = 5$), respectively, a difference not statistically significant. Similar results were found when Ba^{2+} was used to suppress M-current (Adams *et al*., 1982). Application of Ba^{2+} (4 mM) reduced the amplitude of I_{KV} by only $12.8 \pm 1.5\%$ ($n = 9$) (cf. Adams *et al*., 1982), however, the residual I_{KV} was still enhanced by Oxo-M (Figure 1b). Thus, Oxo-M enhanced I_{KV} by 16.9 ± 1.7 pA pF^{-1} ($n = 6$) in control cells, whereas in the presence of Ba^{2+}, the enhancement of current density was 12.1 ± 1.6 pA pF^{-1} ($n = 9$).

Furthermore, the time course of the enhancement of I_{KV} was relatively slower than that of the inhibition of M-current. For instance, a 20 s bath application of Oxo-M was enough to attain the maximum effect on M-current (Figure 2c), whereas the maximum enhancement of I_{KV} required at least 60 s (Figure 2d). The time course of the I_{KV} enhancement was described by a single exponential having a mean time constant of 20.4 ± 3.9 s ($n = 6$), whereas the corresponding time constant for M-current inhibition was only 3.6 ± 0.5 s ($n = 6$). Moreover, it was observed that recovery of I_{KV} modulation was slow with a mean time constant of 76.2 ± 5.4 s ($n = 6$).

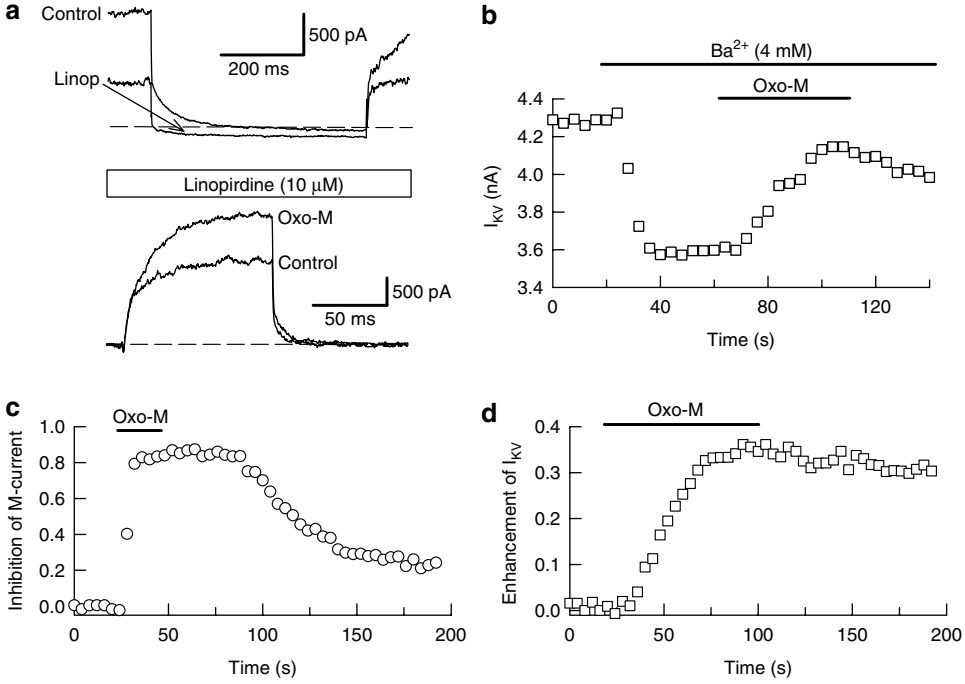

Figure 2 Stimulation of mAChRs produces independent modulation of M-current and I_{KV}. (**a**) Top panel, M-current deactivation records elicited before (Control) and during bath application of 10 μM of the M-channel blocker linopirdine (Linop). (**a**) Bottom panel, records of I_{KV} elicited before (Control) and during Oxo-M (10 μM) superfusion (Oxo-M); linopirdine was present throughout the experiment as indicated by the wide bar. The dashed line on current records indicates the zero current level. (**b**) The symbols represent the amplitude of I_{KV} measured every 4 s, before and when Ba^{2+} and Oxo-M were applied as indicated by the horizontal bars. Note that Ba^{2+} reduced I_{KV}; nevertheless, the residual outward current was enhanced by Oxo-M. Lower plots show the time course of the muscarinic-induced modulation of M-current (**c**) and I_{KV} (**d**). Inhibition of M-current was calculated as $1-$[(M-current (Oxo-M)/M-current (control)], whereas the enhancement of I_{KV} was measured as [I_{KV} (Oxo-M)$-I_{KV}$ (Control)]/I_{KV} (Control). Current records were taken every 4 s and Oxo-M (10 μM) was applied during periods as indicated by the horizontal bars. For comparison, note the slower enhancement of I_{KV} and its partial recovery 96 s after Oxo-M was removed.

The M_1-antagonist pirenzepine does not disrupt the modulation of I_{KV}

In SCG neurones, the M_1 receptor underlies the suppression of M-current (Hamilton *et al*., 1997) and the relatively selective M_1-antagonist pirenzepine ($pK_B = 8.2$; Ellis, 2002) blocks the effects of muscarinic agonists on M-current (Marrion *et al*., 1989; Bernheim *et al*., 1992). In order to assess whether the M_1 receptor underlies the modulation of I_{KV}, cells were treated with 100 nM pirenzepine 2 min before and throughout the Oxo-M challenge. Initially, modulation of M-current was used as a positive control to verify the potency of pirenzepine in our experimental conditions. M-current suppression by Oxo-M at 200 nM (a concentration close to its reported IC_{50}, 300 nM; Bernheim *et al*., 1992) was $43 \pm 2\%$ ($n = 11$) but, in the presence of pirenzepine, this effect of Oxo-M was significantly reduced ($n = 10$; Figure 3a and b). Conversely, pirenzepine did not prevent the enhancement of I_{KV} elicited by Oxo-M (Figure 3c and d).

The $M_{2/4}$-receptor antagonist himbacine disrupts the modulation of I_{KV}

The $M_{2/4}$-antagonist himbacine ($pK_B = 8.1$–8.2; Ellis, 2002) has been successfully used to distinguish between M_1- and $M_{2/4}$-mediated signalling pathways. Therefore, to test whether modulation of I_{KV} is mediated by an M_2-like receptor, cells were treated with himbacine (100 nM) 2 min before and during Oxo-M bath application. For these experiments, the M_1-mediated modulation of M-current was checked again because himbacine antagonizes M_1-receptors with a lower potency ($pK_B = 7.1$) than it does for $M_{2/4}$ mAChRs (Ellis, 2002) and because 100 nM himbacine is expected to produce some effect on M-current modulation (Bernheim *et al*., 1992). Indeed, in control neurones the suppression of M-current by 200 nM Oxo-M was $44.9 \pm 2.6\%$ ($n = 6$), whereas in himbacine-treated cells the suppression was slightly lower ($35.9 \pm 1\%$, $n = 6$). On the other hand and in contrast to pirenzepine, himbacine significantly attenuated the muscarinic enhancement of I_{KV} (Figure 4a, b).

So far, the muscarinic modulation of I_{KV} has been tested with the unspecific agonist Oxo-M. To confirm that an M_2-like mAChR subtype underlies the enhancement of I_{KV}, the effect of the agonist bethanechol was tested because this agonist selectively activated the M_2-mediated inhibition of I_{CaN} in neonatal SCG neurones (Liu and Rittenhouse, 2003). In our preparations of SCG neurones, bethanechol (10 μM) produced a small inhibition of M-current, as compared with the large effect generated by Oxo-M (Figure 4c and d). There was no significant difference in the potency between these muscarinic agonists (both at 10 μM) when they were tested on the modulation of I_{KV} (Figure 4e).

PTX does not block the muscarinic modulation of I_{KV}

In many cell types, the M_2 and M_4 mAChRs couple to and signal through PTX-sensitive G proteins (Ellis, 2002). To assess whether modulation of I_{KV} is sensitive to PTX, cells were incubated overnight with 500 ng ml^{-1} of that toxin. Surprisingly, PTX did not prevent the enhancement of I_{KV} elicited by 10 μM Oxo-M (Figure 5a). Summarizing, in non-

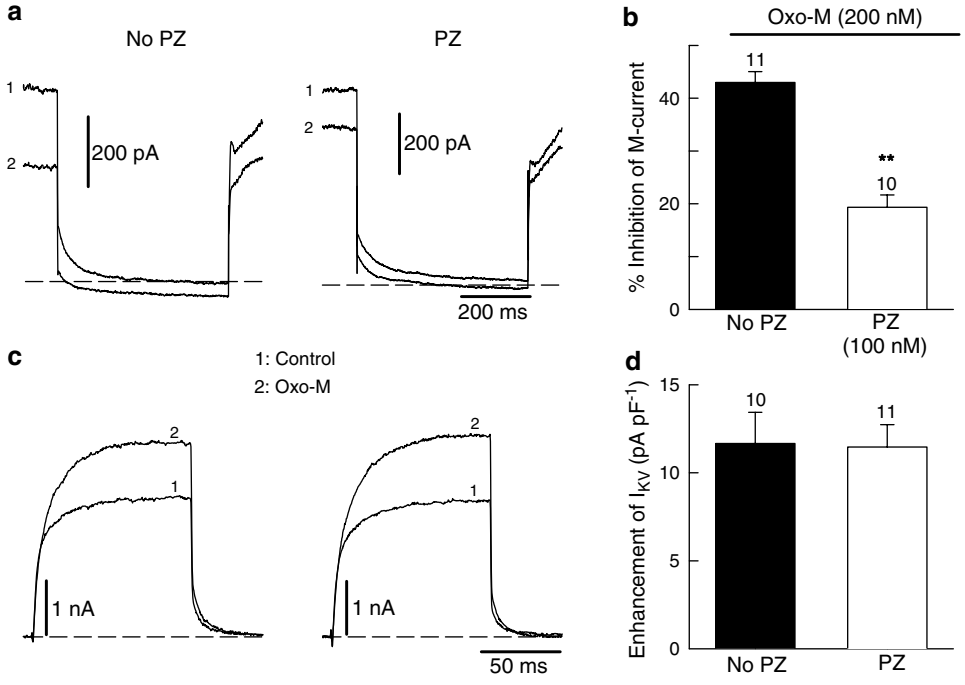

Figure 3 The M_1-antagonist pirenzepine disrupts the modulation of M-current, while leaves intact the muscarinic enhancement of I_{KV}. Representative traces of M-current (**a**) and I_{KV} (**c**) from non-treated neurones (No PZ) and those incubated with 100 nM pirenzepine (PZ). For each panel, recordings were taken before (1) and during (2) bath application of 200 nM Oxo-M and the dashed line indicates zero current level. Bar plots show the mean ($\pm$s.e.m.) Oxo-M-induced inhibition of M-current (**b**) and mean enhancement of I_{KV} current density (**d**) in control cells (solid bars) and in neurones treated with PZ (open bars). In PZ-treated cells only the muscarinic inhibition of M-current was significantly (**$P < 0.01$) attenuated from 43 ± 2 to $19.3 \pm 2.3\%$. Oxo-M increased I_{KV} current density by 11.6 ± 1.7 and 11.4 ± 1.2 pA pF^{-1} in No PZ- and PZ-treated cells, respectively. The numbers above bars indicate the numbers of cells tested for each condition.

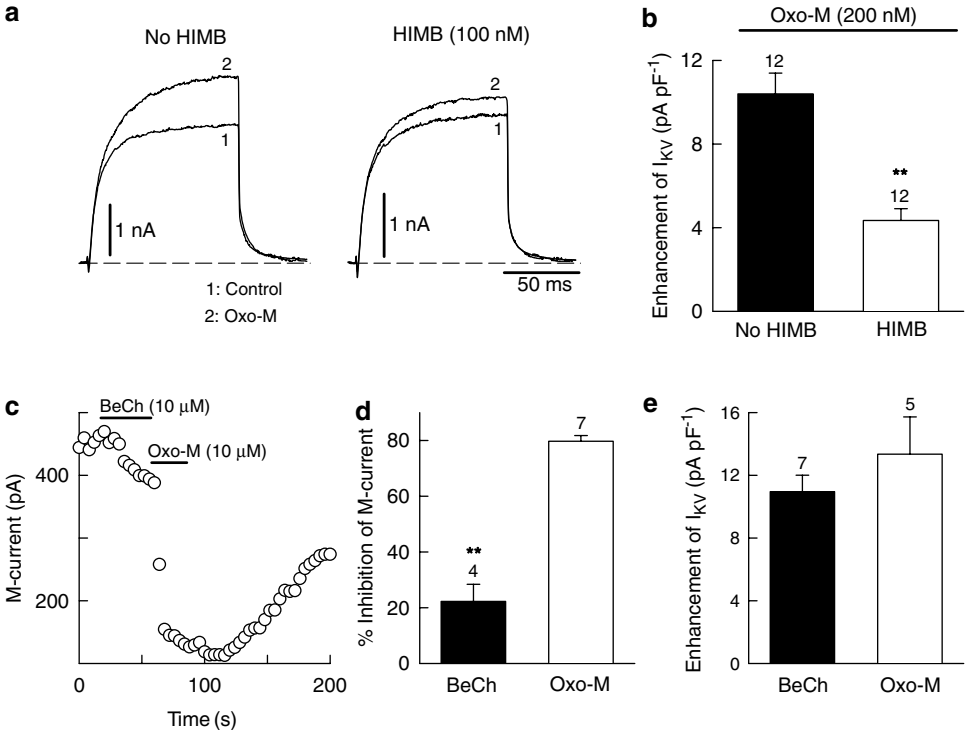

Figure 4 The muscarinic modulation of I_{KV} has a pharmacological $M_{2/4}$-profile. (**a**) Effect of Oxo-M on I_{KV} in a non-treated cell (No HIMB) and in a neurone incubated with the $M_{2/4}$-antagonist himbacine (HIMB). For each panel, I_{KV} was elicited before (1) and during (2) bath application of 200 nM Oxo-M. (**b**) Mean ($\pm$s.e.m.) increase of I_{KV} current density in control cells (solid bar) and in neurones treated with HIMB (open bar); HIMB significantly (**$P<0.01$) reduced the muscarinic enhancement of I_{KV} from 10.4 ± 1 to 4.3 ± 0.5 pA pF^{-1} (**c**) Effect on M-current of the sequential application of the muscarinic agonists bethanechol (BeCh) and Oxo-M; note that BeCh was applied for a longer period than Oxo-M, as indicated by the horizontal bars. (**d**) The mean suppression of M-current by BeCh (solid bar) and Oxo-M (open bar) were 22.2 ± 6.1 and 79.6 ± 2%, respectively; a difference statistically significant (**$P<0.01$). (e) BeCh (solid bar) and Oxo-M (open bar) applied at the same concentration (10 μM) enhanced I_{KV} current density by 10.9 ± 1 and 13.3 ± 2.3 pA pF^{-1}, respectively. For all bar plots, the numbers on columns indicate the numbers of cells tested.

treated neurones the muscarinic agonist increased current density by 17.9 ± 2.5 pA pF^{-1} ($n=8$), whereas in PTX-treated cells the increment was 16.7 ± 1.7 pA pF^{-1} ($n=10$), a difference not statistically significant ($P<0.05$%). To validate these results, we tested the well-known PTX sensitivity of the α_2-adrenoceptor-mediated inhibition of I_{CaN}, which is voltage-dependent and mediated by $G_{o/i}$ (Delmas *et al.*, 1999). The voltage-dependent inhibition of I_{CaN} was revealed with two 10 ms depolarizing test pulses from a V_h of -80 to $+10$ mV, interposed by a 25-ms depolarization to $+125$ mV. In the absence of agonist, each $+10$ mV pulses generated identical Ca^{2+} currents as shown in Figure 5b (C_1, C_2). Application of noradrenaline (NA) reduced I_{CaN}, although this effect was partially reversed by the $+125$ mV depolarization, facilitating I_{CaN} during the second test pulse (left panel in Figure 5b). As expected, PTX attenuated both the inhibitory effect of NA on I_{CaN} and current facilitation that results from the transient removal of its voltage-dependent inhibition (right panel in Figure 5b).

ATP is required for muscarinic modulation of I_{KV}

It is now well accepted that the activity of ion channels can be modulated by the activation of protein kinases linked to second-messenger systems (Hilgemann, 1997). Given that ATP is commonly used by protein kinases, we tested whether modulation of I_{KV} required the presence of ATP. Thus, cells were dialysed for $\sim$8 min with a standard internal solution containing the non-hydrolysable ATP analogue, AMP-PNP, instead of ATP. Figure 6a shows the Oxo-M response in a cell dialysed with the standard ATP-containing pipette solution. The long-term dialysis procedure, by itself, did not prevent the muscarinic-induced enhancement of I_{KV}. In contrast, in a cell dialysed with AMP-PNP, the muscarinic enhancement of I_{KV} was absent (Figure 6b). Similar results were found in two more paired experiments.

Discussion and conclusions

Our experiments have shown that stimulation of mAChRs induced a robust enhancement of I_{KV} in cultured or acutely dissociated SCG neurones from juvenile rats (Figure 1). This effect did not result from the improvement of the space-clamp conditions, secondary to the strong muscarinic inhibition of M-current, because: (a) the enhancement of I_{KV} by Oxo-M remained practically unaltered when M-channels were blocked either by linopirdine or Ba^{2+} (Figure 2); (b) the kinetic for muscarinic inhibition of M-current was, at least, five times faster than the time course of I_{KV} modulation (Figure 2); (c) the enhancement of I_{KV} was noticeable at membrane voltages more positive than -20 mV

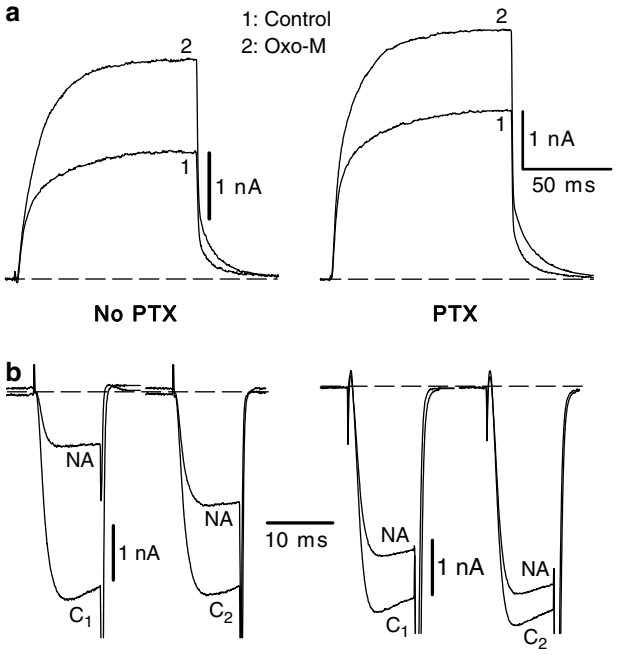

Figure 5 PTX does not prevent the muscarinic modulation of I_{KV}. (**a**) Representative delayed rectifier K^+ and (**b**) Ca^{2+} current records from non-treated neurones (No PTX) and those cells incubated overnight with 500 ng ml^{-1} of PTX. Note that PTX did not block the muscarinic enhancement of I_{KV} (traces were taken before (1) and during (2) bath application of 10 μM Oxo-M). (**b**) Typical I_{CaN} traces elicited by a two-pulse protocol (see text) in a control cell (left panel) and in a PTX-incubated neurone (right panel); I_{CaN} records were elicited before (C_1 and C_2) and during adrenergic stimulation (NA). As expected, PTX reduced the voltage-dependent inhibition of I_{CaN}. The dashed lines are the zero current level.

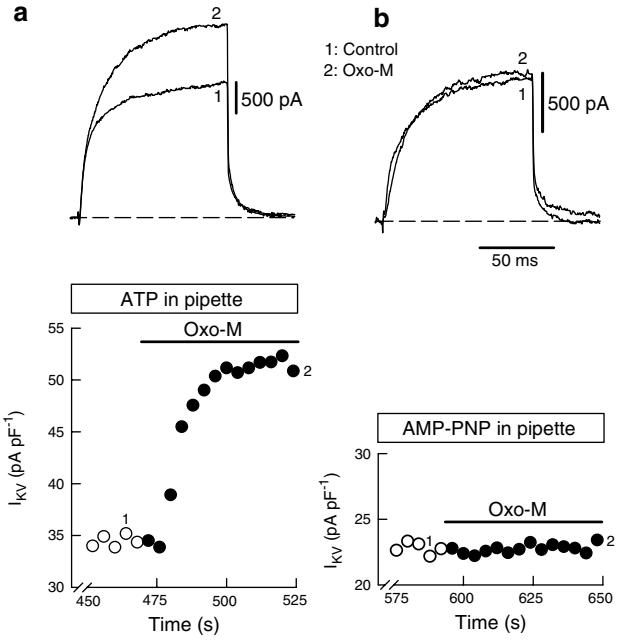

Figure 6 Muscarinic modulation of I_{KV} is mediated by an ATP-dependent process. Representative effect of Oxo-M on I_{KV} in a neurone dialysed with the ATP-containing pipette standard internal solution (**a**) and in a cell where ATP was replaced with its non-hydrolysable parent compound, AMP-PNP (**b**). The ATP-analogue readily blocked the muscarinic enhancement of I_{KV}, as are shown in the current records and plots describing the value of current density (pA pF^{-1}) measured every 4 s, before (open circles) and during Oxo-M challenge (filled circles and horizontal bars). Similar results were found in two more paired experiments.

(Figure 1), a voltage range where M-channels reached their maximal activation (Wang *et al*., 1998). Therefore, it is suggested that the enhancement of I_{KV} reflects a novel muscarinic pathway operating in rat sympathetic SCG neurones.

Given that these nerve cells mostly express the M_1, M_2 and M_4 receptor subtypes (Brown *et al*., 1995), the M_1-antagonist pirenzepine and the $M_{2/4}$-antagonist himbacine were used to pharmacologically characterize the mAChR that underlies the modulation of I_{KV}. The use of these muscarinic antagonists has been reported to be a valuable pharmacological tool to identify the subtypes of mAChR that modulate different SCG endogenous ion currents including the M-current, I_{CaN} and the Ca^{2+}-activated chloride current (Marrion *et al*., 1989; Bernheim *et al*., 1992; Marsh *et al*., 1995). Here, it was found that the enhancement of I_{KV} was insensitive to pirenzepine, whereas this M_1-antagonist reduced the inhibition of M-current by $\sim$50% (Figure 3). In contrast, the enhancement of I_{KV} was significantly reduced by himbacine (Figure 4). In agreement with these results, the relatively selective $M_{2/4}$-agonist bethanechol (Constanti, 2003; Liu and Rittenhouse, 2003) showed an ability to enhance I_{KV}, comparable to that of Oxo-M (Figure 4). Taken together, these results suggest that an M_2-like mAChR is responsible for the muscarinic modulation of I_{KV}. However, we cannot distinguish between the M_2 and M_4 because himbacine antagonizes both subtypes with similar potency (pK_B for $M_2 = 8.1$ and pK_B for $M_4 = 8.2$) (Ellis, 2002). Further genetic studies are required to identify the exact M_2-like receptor subtype that mediates the modulation of I_{KV}.

It is generally accepted that M_2-like receptors prefer to couple to G proteins of the PTX-sensitive G_i family (Ellis, 2002). Indeed, in SCG neurones M_2/M_4 subtypes couple to G_{oA}, the most abundant isoform of $G\alpha_o$ expressed in these cells, to induce the $G\beta\gamma$-mediated and voltage-dependent inhibition of I_{CaN} (Beech *et al*., 1992; Delmas *et al*., 1998). Surprisingly, PTX did not affect the muscarinic enhancement of I_{KV} (Figure 5) suggesting that in SCG cells the M_2-like receptor couples to a PTX-insensitive G protein as well. Such potential divergence at the level of G proteins has been reported for M_2 receptors co-expressed with $Ca_V2.3$ channels in human embryonic kidney cells. In this heterologous expression system, the agonist carbachol produces a rapid and PTX-sensitive inhibition, followed by a slow and PTX-insensitive stimulation of the Ca^{2+} current (Meza *et al*., 1999). Moreover, G-protein-activated, inwardly rectifying, K^+ (GIRK) currents also show a biphasic PTX-sensitive and -insensitive modulation by M_2 receptors, when receptors and GIRK channel subunits are co-expressed in Chinese hamster ovary K1 cells (Bünemann *et al*., 2000). For I_{KV} modulation, the most plausible candidate might be G_z because: (a) G_z is the unique PTX-insensitive member that belongs to the G_i

family; (b) SCG neurones express G_z and its overexpression in PTX-treated cells rescued the voltage-dependent inhibition of I_{CaN} by α_2-adrenergic or somatostatin receptors, thereby indicating that G_z is able to link G_i-coupled receptors to ion channels (Jeong and Ikeda, 1998). Whatever the nature of the PTX-insensitive G protein might be, it stimulated an ATP-dependent, slow signalling pathway to modulate I_{KV}.

Muscarinic modulation of delayed rectifier K^+ currents has been reported to occur in some central neurones as well. However, the effect depends on the cell type because in CA1 hippocampal neurones, like in SCG cells, stimulation of mAChRs potentates a voltage-activated sustained K^+ current (Zhang *et al*., 1992), whereas in hypothalamic neurones a I_{KV} is inhibited (ffrench-Mullen *et al*., 1994). It is possible that the muscarinic enhancement of I_{KV} seen both in CA1 and SCG neurones reflects the modulation of either Kv2.1 or Kv2.2 subunits, because they mostly contribute to the delayed rectifier currents generated in these neurone types (Murakoshi and Trimmer, 1999; Malin and Nerbonne, 2002). In agreement with this suggestion, I_{KV} had a pharmacological profile consistent with the involvement of Kv2.1 subunits. For instance, (a) like rat cloned Kv2.1 channels (Chandy and Gutman, 1995), I_{KV} had a greater sensitivity to 4-AP than to TEA; (b) it was reported that 500 μM of 4-AP blocked homomeric Kv2.1 currents by 50% (Chandy and Gutman, 1995) and this concentration of 4-AP applied to the SCG cells reduced I_{KV} by $\sim$55%; (c) 500 μM 4-AP practically abolished the enhancement of I_{KV} by Oxo-M or angiotensin II (unpublished observations).

An effect of muscarine on action potential shape was not observed in microelectrode recordings from adult rat or bullfrog sympathetic neurones (Brown and Constanti, 1980; Adams *et al*., 1982). The data presented in this study would have predicted an effect. This apparent discrepancy is not due to the different ages among the animals used or to the cell culture process *per se*, because: (a) it was found that Oxo-M enhanced I_{KV}, both in cultured SCG neurones from juvenile (2–4 weeks old) or adult (10 weeks old) rats; (b) in SCG cells from juvenile rats, the enhancement of I_{KV} was similar both in cultured and fresh dissociated cells. There is evidence from SCG cells that a delayed rectifier K^+ current ($I_{K(DR)}$) with a pharmacological profile similar to that of I_{KV}, significantly contributes to the early fast spike afterhyperpolarization (Marsh and Brown, 1991). Thus, it might be expected that the effect of Oxo-M on I_{KV} would cause an increase of the fast spike afterhyperpolarization. This had been reported previously (see Figure 8 in Brown and Constanti, 1980). It is possible that the final effect of muscarinic agonists on single action potential shape would be difficult to discern, because of specific roles that each voltage- and Ca^{2+}-activated K^+ currents have in shaping the SCG action potential. For instance, when I_{KV} was blocked in bullfrog sympathetic neurones its contribution to the action potential was only revealed (prolonged action potential duration and reduced fast afterhyperpolarization) when the Ca^{2+}-activated K^+ currents I_C and I_{AHP} were also blocked (Goh *et al*., 1989). In SCG cells, I_C and I_{AHP} are mediated by BK- and SK-type K^+ channels that are activated by Ca^{2+} entry through L-type and N-type Ca^{2+} channels, respectively (Davies *et al*., 1996). Given that BK- and SK channels contribute to spike repolarization and afterhyperpolarization, respectively, their indirect suppression secondary to the well-known muscarinic inhibition of the L- and N-type Ca^{2+} currents (Bernheim *et al*., 1992; Shapiro *et al*., 1999), could mask the effect of the muscarinic enhancement of I_{KV} on spike repolarization (faster) and afterhyperpolarization (larger amplitude), thereby resulting in no noticeable changes in single action potential morphology. So, the impact of each of these K^+ currents on the action potential repolarization and fast afterhyperpolarization may depend on the corresponding K^+ channel-type density (Malin and Nerbonne, 2002) and the strength and duration of their muscarinic modulation.

It is concluded that in SCG neurones from juvenile and adult rats, an M_2-like muscarinic receptor couples to a PTX-insensitive G protein and to an ATP-dependent pathway to modulate the delayed rectifier current I_{KV}. The muscarinic enhancement of I_{KV} must be taken into consideration in order to understand better how mAChRs acting on different ion channels regulate sympathetic excitability.

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Conflict of interest

The author states no conflict of interest.

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