

RESEARCH PAPER

Inhibition of transmitter release from rat sympathetic neurons via presynaptic M₁ muscarinic acetylcholine receptors

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Background and purpose: M₂, M₃ and/or M₄ muscarinic acetylcholine receptors have been reported to mediate presynaptic inhibition in sympathetic neurons. M₁ receptors mediate an inhibition of K_v7, Ca_v1 and Ca_v2.2 channels. These effects cause increases and decreases in transmitter release, respectively, but presynaptic M₁ receptors are generally considered facilitatory. Here, we searched for inhibitory presynaptic M₁ receptors.

Experimental approach: In primary cultures of rat superior cervical ganglion neurons, Ca²⁺ currents were recorded via the perforated patch-clamp technique, and the release of [³H]-noradrenaline was determined.

Key results: The muscarinic agonist oxotremorine M (OxoM) transiently enhanced ³H outflow and reduced electrically evoked release, once the stimulant effect had faded. The stimulant effect was enhanced by pertussis toxin (PTX) and was abolished by blocking M₁ receptors, by opening K_v7 channels and by preventing action potential propagation. The inhibitory effect was not altered by preventing action potentials or by opening K_v7 channels, but was reduced by PTX and ω-conotoxin GVIA. The inhibition remaining after PTX treatment was abolished by blockage of M₁ receptors or inhibition of phospholipase C. When [³H]-noradrenaline release was triggered independently of voltage-activated Ca²⁺ channels (VACCs), OxoM failed to cause any inhibition. The inhibition of Ca²⁺ currents by OxoM was also reduced by ω-conotoxin and PTX and was abolished by M₁ antagonism in PTX-treated neurons.

Conclusions and implications: These results demonstrate that M₁, in addition to M₂, M₃ and M₄, receptors mediate presynaptic inhibition in sympathetic neurons using phospholipase C to close VACCs.

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Abbreviations: GPCR, G-protein-coupled receptor; mAChRs, muscarinic acetylcholine receptors; MT-7, muscarinic toxin 7; OxoM, oxotremorine M; PTX, pertussis toxin; PIP₂, phosphatidylinositol-4,5-bisphosphate; SCG, superior cervical ganglia; TTX, tetrodotoxin; VACCs, voltage-activated Ca²⁺ channels

Introduction

The reduction of noradrenaline release due to the activation of muscarinic acetylcholine receptors (mAChRs) located at sympathetic axon terminals was described for the first time at least four decades ago (Lindmar *et al.*, 1968). Thus, this mechanism is, presumably, the best-documented example of presynaptic heteroinhibition in the sympathetic nervous system. Nevertheless, presynaptic modulation via muscarinic receptors remained controversial, as an enhancement of nora-

drenaline release via these receptors has also been observed (Fuder and Muscholl, 1995; Boehm and Kubista, 2002).

The family of mAChRs comprises at least five different subtypes (M₁ through M₅) which were originally characterized by the use of various antagonists that discriminate between them by up to 100-fold differences in affinities (Caulfield and Birdsall, 1998). More recently, toxins have been used instead which display more than 1000-fold differences in affinities (Onali *et al.*, 2005). Complementary information on the roles of single mAChRs has been obtained in mutant mice (Wess *et al.*, 2007). In the case of presynaptic mAChRs on sympathetic neurons, inhibitory effects have most commonly been reported to involve M₂, whereas facilitatory effects are rarely observed and rather assigned to M₁ receptors (Fuder and Muscholl, 1995; Boehm and Kubista, 2002). Results obtained with various sympathetically innervated tissues from mAChR

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knockout mice suggest that several combinations of presynaptic M₂, M₃ and M₄ receptors control noradrenaline release (Trendelenburg *et al.*, 2005). The different mAChRs are linked to various effector systems via one of the two following prototypic signalling cascades: M₁, M₃ and M₅ via pertussis toxin (PTX)-insensitive G_q-type G-proteins and phospholipase C β , M₂ and M₄ via PTX-sensitive G_i-type G-proteins and adenylyl cyclase (Caulfield and Birdsall, 1998; Wess *et al.*, 2007).

Inhibition of noradrenaline release via presynaptic G-protein-coupled receptors (GPCRs) is generally believed to rely on an inhibition of voltage-activated Ca²⁺ channels (VACCs) which in most, but not all, cases involves PTX-sensitive G-proteins (Kubista and Boehm, 2006). This mechanism has been investigated most thoroughly in sympathetic neurons derived from superior cervical ganglia (SCG) (Hille, 1994). With mAChRs, this effect involves several subtypes: the PTX-sensitive inhibition is mediated by M₂ and M₄, and the PTX-insensitive inhibition involves M₁ (Shapiro *et al.*, 1999; Liu and Rittenhouse, 2003b). This latter receptor not only controls the gating of VACCs, but also mediates an inhibition of K_v7 (KCNQ) channels which leads to a depolarization of SCG neurons and to increased action potential firing (Lechner *et al.*, 2003; Hernandez *et al.*, 2008). Moreover, the inhibition of K_v7 channels was shown to promote action potential-mediated noradrenaline release from sympathetic neurons (Kristufek *et al.*, 1999; Lechner *et al.*, 2003; Hernandez *et al.*, 2008) and was suggested to mediate the facilitation of dopamine release by presynaptic mAChRs in striatal nerve terminals (Martire *et al.*, 2007). Hence, in SCG neurons, M₁ receptors can activate signalling cascades that mediate either an inhibition or a facilitation of action potential-evoked noradrenaline release.

In previous experiments, activation of M₁ mAChRs has been demonstrated to trigger noradrenaline release from SCG neurons, but this effect was abolished by tetrodotoxin (TTX), that is, it required action potential propagation, and was thus not mediated by presynaptic receptors (Lechner *et al.*, 2003). The role of presynaptic M₁ mAChRs in SCG neurons, in contrast, remained elusive. Accordingly, this study was designed to clarify whether presynaptic M₁ receptors can control noradrenaline release from rat SCG neurons in either a facilitatory or an inhibitory manner and whether either of these effects involves a modulation of K_v7 channels or VACCs respectively. The results reveal that activation of presynaptic M₁ receptors reduces transmitter release due to an inhibition of VACCs via phospholipase C.

Methods

Primary cultures of rat superior cervical ganglion neurons

Primary cultures of dissociated SCG neurons from neonatal rats were prepared as described previously (Boehm, 1999). Newborn Sprague-Dawley rats were kept and killed 3 to 10 days after birth by decapitation in full accordance with all rules of the Austrian animal protection law (see http://ris1.bka.gv.at/App1/findbgbl.aspx?name=entwurf&format=pdf&docid=COO_2026_100_2_72288) and the Austrian animal experiment by-laws (see http://www.ris2.bka.gv.at/Dokumente/BgblPdf/2000_198_2/2000_198_2.pdf). Ganglia

were removed immediately after decapitation of the animals, cut into three to four pieces, and incubated in collagenase (1.5 mg·mL⁻¹) and dispase (3.0 mg·mL⁻¹) for 20 min at 36°C. Subsequently, they were further incubated in trypsin (0.25% trypsin) for 15 min at 36°C, dissociated by trituration and resuspended in Dulbeccos modified Eagle's medium containing 2.2 g·L⁻¹ glucose, 10 mg·L⁻¹ insulin, 25 000 iu·L⁻¹ penicillin and 25 mg·L⁻¹ streptomycin, 50 µg·L⁻¹ nerve growth factor, and 5% foetal calf serum. Finally, all cells were seeded onto 5 mm plastic discs for radiotracer release experiments and onto 35 mm culture dishes for electrophysiological experiments. All tissue culture plastic was coated with rat tail collagen. The cultures were stored for 4 to 8 days in a humidified 5% CO₂ atmosphere at 36°C. On days 1 and 4 after dissociation, the medium was exchanged entirely.

Determination of [³H]-noradrenaline release

[³H]-noradrenaline uptake and superfusion were performed as described previously (Boehm, 1999). The plastic discs with dissociated neurons were incubated in 0.05 µmol·L⁻¹ [³H]-noradrenaline (specific activity 37 Ci·mmol⁻¹) in culture medium supplemented with 1 mmol·L⁻¹ ascorbic acid at 36°C for 1 h. Thereafter, the culture discs were transferred to small chambers and superfused with a buffer containing (mmol·L⁻¹): NaCl 120, KCl 6.0, CaCl₂ 2.0, MgCl₂ 2.0, glucose 20, HEPES 10, fumaric acid 0.5, Na-pyruvate 5.0 and ascorbic acid 0.57, adjusted to pH 7.4 with NaOH. Superfusion was performed at 25°C at a rate of about 1.0 mL·min⁻¹. Collection of 4 min fractions of superfusate was started after a 60 min washout period during which excess radioactivity had been removed.

Depolarization-dependent tritium overflow was triggered either by electrical fields or by 30 mmol·L⁻¹ KCl (NaCl was reduced accordingly to maintain isotonicity), both being applied for periods of 60 s. Stimulation by monophasic rectangular electrical pulses (0.5 ms, 60 mA, 50 V·cm⁻¹) at 1.0 Hz was performed after 73 (S1) and 113 (S3) min of superfusion (see e.g. Figure 1A), stimulation by K⁺ after 73 (S1) and 93 (S3) min of superfusion (see e.g. Figure 4A). Tritium overflow independent of VACCs was elicited by 60 s applications of 100 µmol·L⁻¹ adenosine-5'-triphosphate (ATP) in the presence of TTX to selectively activate Ca²⁺-permeable presynaptic P2X receptors (Boehm, 1999), again after 73 (S1) and 93 (S3) min of superfusion. Muscarinic agonists [oxotremorine M (OxoM) and bethanecol] were included in the buffer from minute 88 (see e.g. Figure 4A) onward. As OxoM is known to stimulate noradrenaline release from SCG neurons (Lechner *et al.*, 2003), the accompanying transient rise in tritium outflow was defined as second stimulation period (S2). Modifying agents, such as TTX or retigabine, were included in the buffer after 50 min of superfusion (i.e. 10 min prior to the start of sample collection). The radioactivity remaining in the cells after the completion of experiments was extracted by immersion of the discs in 2% perchloric acid followed by sonication. Radioactivity in extracts and collected fractions was determined by liquid scintillation counting (Packard Tri-Carb 2800 TR). Radioactivity released in response to electrical field stimulation from rat sympathetic neurons after labelling with tritiated noradrenaline under conditions similar to those

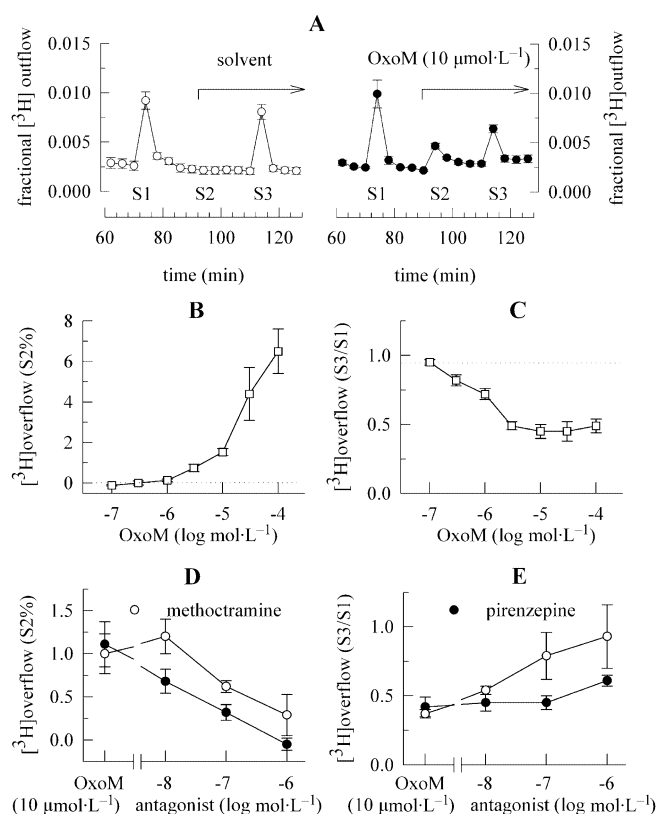


Figure 1 Enhancement of spontaneous and inhibition of electrically evoked noradrenaline release by OxoM via different sets of receptors. Primary cultures of rat SCG neurons were labelled with [³H]-noradrenaline for 1 h and then superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. Electrical field stimulation was applied at 73 (S1) and 113 (S3) min of superfusion. OxoM, either alone or together with pirenzepine or methoctramine, was present as indicated by the arrow on the right hand side in (A) and caused transient increases in the rate of spontaneous ³H outflow (S2); as a control for statistical comparison, solvent was applied instead of OxoM. (A) The time course of fractional ³H outflow·min⁻¹ and its alteration by 10 μmol·L⁻¹ OxoM on the right hand side (*n* = 3). For comparison, the effect of solvent is depicted on the left hand side (*n* = 3). (B) The concentration-dependence of OxoM-induced ³H overflow, as exemplified by S2% values. The indicated concentrations of OxoM were applied as shown in (A) (*n* = 5 to 6). (C) The concentration-dependence of the inhibition of electrically evoked tritium overflow by OxoM, as exemplified by S3/S1 ratios. The indicated concentrations of OxoM were applied as shown in (A) (*n* = 5 to 6). (D) The concentration-dependent attenuation of the ³H overflow induced by 10 μmol·L⁻¹ OxoM by pirenzepine or methoctramine, as exemplified by S2% values. OxoM (10 μmol·L⁻¹) was applied as shown in (A) either alone or together with the indicated concentrations of pirenzepine or methoctramine (*n* = 6 to 8). (E) The concentration-dependent attenuation of the inhibition of electrically evoked tritium overflow by 10 μmol·L⁻¹ OxoM in the presence of pirenzepine or methoctramine, as exemplified by S3/S1 ratios. OxoM (10 μmol·L⁻¹) was applied as shown in (A) either alone or together with the indicated concentrations of pirenzepine or methoctramine (*n* = 6 to 8). OxoM, oxotremorine M; SCG, superior cervical ganglia.

of the present study had previously been shown to consist predominantly of the authentic transmitter and to contain only small amounts (≤15%) of metabolites (Schwartz and Malik, 1993). Hence, the outflow of tritium measured in this study was assumed to reflect the release of noradrenaline and not that of metabolites.

The spontaneous (unstimulated) rate of ³H efflux was obtained by expressing the radioactivity retrieved during a collection period as a fraction of the total radioactivity in the cultures at the beginning of this period (fractional ³H outflow). Stimulation-evoked tritium overflow was calculated as the difference between the total tritium outflow during and after stimulation and the estimated basal outflow, which was assumed to follow a linear time course throughout experiments. Therefore, basal outflow during periods of stimulation was assumed to equate to the arithmetic mean of the samples preceding and those following stimulation, respectively. Differences between total and estimated basal outflow during periods of stimulation were expressed as percentages of total radioactivity in the cultures at the onset of stimulation (% of total radioactivity; S%). The amount of radioactivity in the cultures at the beginning of each collection period is calculated by summing up the radioactivity remaining in the cells at the end of experiments and that retrieved during the respective and all subsequent collection periods.

As the amount of depolarization- or drug-induced tritium overflow may vary considerably between different cultures (Scholze *et al.*, 2002), the effects of mAChR ligands on depolarization-dependent release were evaluated by determining changes in the ratio of tritium overflow evoked during the two periods of electrical or K⁺ stimulation (S3/S1). When cultures had been subjected to a certain treatment, for example PTX or muscarinic toxin 7 (MT-7), control experiments were also performed in sister cultures that had not been exposed to that treatment (i.e. remained 'untreated').

Electrophysiology

Ca²⁺ currents of sympathetic neurons were determined using the perforated patch clamp technique as described previously (Lechner *et al.*, 2005). Currents were recorded at room temperature (20–24°C) from single SCG neurons *in vitro* using an Axopatch 200B amplifier and the pCLAMP 8.0 hard- and software. Signals were low-pass filtered at 5 kHz, digitized at 10 to 50 kHz and stored on an IBM compatible computer. Traces were analysed off-line by the Clampfit 8.1 program (Axon). Patch electrodes were pulled (Flaming-Brown puller) from borosilicate glass capillaries, front-filled with a solution consisting of (mmol·L⁻¹): CsCl 130, tetraethylammonium chloride 20, CaCl₂ 0.24, glucose 10, HEPES 10, EGTA 5, adjusted to pH 7.3 with KOH, and then back-filled with the same solution containing 200 μg·mL⁻¹ amphotericin B (in 0.8% dimethyl sulfoxide (DMSO)) which yielded tip resistances of 2 to 3 MΩ. This combination of solutions results in small liquid junction potentials of about +2 mV, which, however, were ignored. Drugs were applied via a DAD-12 drug application device, which permits a complete exchange of solutions surrounding the cells being investigated within less than 100 ms (Boehm, 1999).

Ca²⁺ currents were elicited every 15 s by 30 ms depolarizations from a holding potential of -80 mV to +10 mV. Leakage currents were corrected for by an on-line leak subtraction protocol, which applies four hyperpolarizing pulses prior to the depolarization to +10 mV in order to determine the extent of leakage. The extent of current inhibition by muscarinic agonists or isradipine was quantified according to the

equation: % inhibition = $100 - (200 * B / (A + C))$, where B is peak current amplitudes determined 120 s after the start of drug application and A and C are the amplitudes of control currents measured directly before and 120 s after the end of the application of agonists. As ω -conotoxin GVIA causes an irreversible inhibition of voltage-gated Ca²⁺ currents in sympathetic neurons (Boehm and Huck, 1996), 1 $\mu\text{mol}\cdot\text{L}^{-1}$ of this toxin was applied until the reduction of current amplitudes reached a steady state. The amplitudes after this treatment were compared with those determined prior to the application of the toxin, and the effect was again expressed as % inhibition.

Statistics and nomenclature

All values are arithmetic means \pm s.e.mean. Values of *n* reflect single cells in electrophysiological experiments and numbers of cultures in radiotracer release experiments. Statistical significance of differences between two groups was determined by the Mann–Whitney test. Statistical significance of differences between multiple groups was performed by one-way analyses of variances followed by Bonferroni's multiple comparison corrections; *P* values <0.05 were considered as indicating statistical significance. The nomenclature for ion channels and receptors follows the guide to receptors and channels (Alexander *et al.*, 2008).

Materials

(-)-[Ring-2,5,6-³H]-noradrenaline was obtained from PerkinElmer (Vienna, Austria); collagenase, amphotericin B, OxoM, pirenzepine, methoctramine, bethanechol, UK 14304 (5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinolinamine), U73122 (1-[6-[[[(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione), U73343 (1-[6-[[[(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrollidinedione), isradipine and PTX from Sigma (Vienna, Austria); TTX from Latoxan (Rosans, France); MT-7 from the Peptide Institute Inc. (Osaka, Japan); ω -conotoxin GVIA from Alomone Labs Ltd. (Jerusalem, Israel), bulk chemicals were from Merck (Vienna, Austria). Retigabine was kindly donated by Dr B. Pechstein (Viatris, Frankfurt/Main, Germany). Water-insoluble drugs were first dissolved in DMSO and then diluted in buffer to yield final DMSO concentrations of up to 0.1% which were also included in control solutions. At these concentrations, DMSO did not affect any of the parameters investigated (Lechner *et al.*, 2003).

Dispase was obtained from Boehringer Mannheim (Vienna, Austria); trypsin, Worthington (Lakewood, NJ, USA); Dulbecco modified Eagle's medium, streptomycin and foetal calf serum, Invitrogen (Lofer, Austria); nerve growth factor, R&D Systems Inc. (Minneapolis, MN, USA); rat tail collagen, Biomedical Technologies Inc. (Stoughton, MA, USA). The pCLAMP 8.0 hard- and software was from Molecular Devices (Sunnyvale, CA, USA); the Flaming-Brown puller, Sutter Instruments (Novato, CA, USA); borosilicate glass capillaries, Science Products (Frankfurt/Main, Germany); DAD-12 drug application device, Adams & List (Westbury, NY, USA).

Results

Multiple effects of mAChR ligands on noradrenaline release from rat SCG neurons

Activation of M₁ mAChRs in primary cultures of rat SCG neurons is known to trigger noradrenaline release (Lechner *et al.*, 2003). This effect contrasts with the previously reported inhibition of sympathetic transmitter release via presynaptic mAChRs (Boehm and Kubista, 2002). When rat SCG neurons were exposed to 10 $\mu\text{mol}\cdot\text{L}^{-1}$ OxoM, there first was an increase in the rate of spontaneous tritium outflow, which despite the continuing presence of this muscarinic agonist returned to basal levels. No such effect was observed if solvent was applied instead of OxoM. The subsequent application of electrical pulses was able to trigger tritium overflow again, but the amount of stimulation-dependent overflow was smaller than that in the presence of solvent (Figure 1A). Accordingly, the S3/S1 ratio of electrically evoked ³H overflow was significantly smaller in the presence than in the absence of 10 $\mu\text{mol}\cdot\text{L}^{-1}$ OxoM (Figure 1C).

Both the stimulation of tritium overflow and the inhibition of electrically evoked overflow by OxoM were concentration-dependent, but the concentrations required to achieve a maximum inhibition were obviously lower than those required to result in a maximal stimulation (Figure 1B,C). In fact, even at 100 $\mu\text{mol}\cdot\text{L}^{-1}$, the stimulant effect of OxoM did not show an unequivocal sign of saturation (Figure 1B) as observed previously (Lechner *et al.*, 2003). From this, one may conclude that different receptors and/or different signalling mechanisms are involved in the opposing effects of OxoM on noradrenaline release.

To further substantiate this conclusion, the effects of two muscarinic antagonists with widely differing rank orders of receptor subtype affinities were used: the rank order of subtype affinities is M₂ > M₄ > M₁ > M₃ = M₅ for methoctramine and M₁ > M₄ > M₅ > M₃ > M₂ for pirenzepine (Caulfield and Birdsall, 1998; Eglen *et al.*, 2001). Pirenzepine was approximately 10-fold more potent than methoctramine at attenuating the stimulation of tritium overflow caused by 10 $\mu\text{mol}\cdot\text{L}^{-1}$ OxoM (Figure 1D), whereas methoctramine was more than 10-fold more potent than pirenzepine at attenuating the inhibition of electrically evoked tritium overflow caused by 10 $\mu\text{mol}\cdot\text{L}^{-1}$ OxoM (Figure 1E). Thus, the opposing effects of OxoM involve different receptor populations.

Evidence for M₁ receptors being involved in the inhibition of electrically evoked noradrenaline release by OxoM

In order to further characterize the receptors mediating the inhibition of electrically evoked noradrenaline release, we used the muscarinic agonist bethanechol which has been shown to selectively activate M₂ mAChRs in rat SCG neurons and to achieve maximal effects at a concentration of 100 $\mu\text{mol}\cdot\text{L}^{-1}$ (Liu and Rittenhouse, 2003b). Bethanechol (100 $\mu\text{mol}\cdot\text{L}^{-1}$) failed to significantly alter spontaneous (unstimulated) tritium outflow (Figure 2A,B), but did reduce electrically evoked overflow (Figure 2A,C). This latter effect appeared to be concentration-dependent between 10 and 100 $\mu\text{mol}\cdot\text{L}^{-1}$, and the maximum amounted to $24.5 \pm 3.7\%$ inhibition (*n* = 9; Figure 2C). For comparison, the maximum

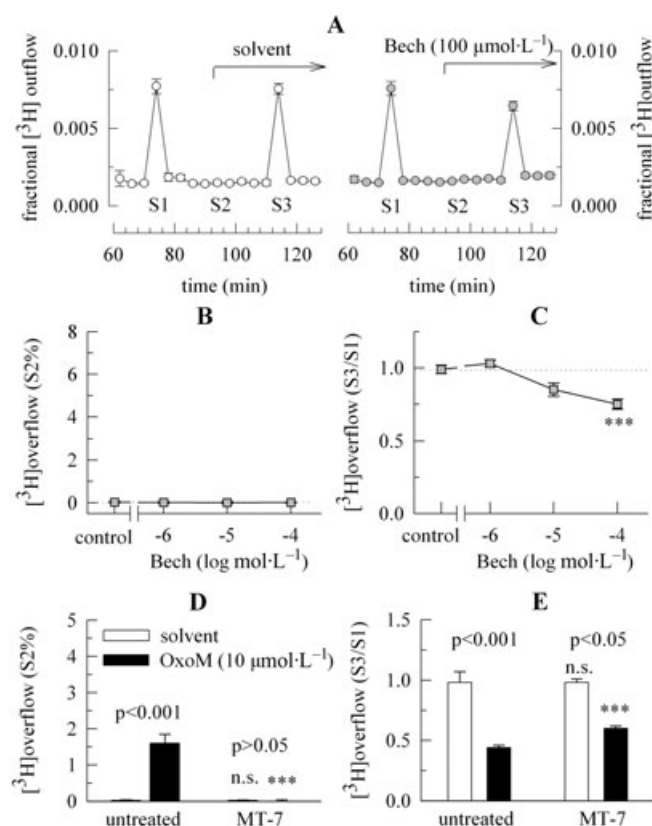


Figure 2 Characterization of the mAChRs mediating the inhibition of electrically evoked noradrenaline release. Primary cultures of rat SCG neurons were labelled with [³H]-noradrenaline for 1 h in the absence or presence of 100 nmol·L⁻¹ MT-7 and were then superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. Electrical field stimulation was applied at 73 (S1) and 113 (S3) min of superfusion. Either OxoM or bethanechol (Bech) was present as indicated by the arrow on the right hand side in (A); OxoM caused transient increases in the rate of spontaneous ³H overflow (S2) as shown in Figure 1A; as a control for statistical comparison, solvent was applied instead of OxoM or bethanechol. (A) The time course of fractional ³H outflow·min⁻¹ and its alteration by 100 μmol·L⁻¹ bethanechol on the right hand side (*n* = 3). For comparison, the effect of solvent is depicted on the left hand side (*n* = 3). (B) Indicates a lack of bethanechol-induced ³H overflow, as exemplified by S2% values. The indicated concentrations of bethanechol were applied as shown in (A) (*n* = 6 to 9). (C) The concentration-dependence of the inhibition of electrically evoked tritium overflow by bethanechol, as exemplified by S3/S1 ratios. The indicated concentrations of bethanechol were applied as shown in (A) (*n* = 6 to 9); *** indicates a significant difference versus the result obtained in the presence of solvent at *P* < 0.001. (D) ³H overflow induced by 10 μmol·L⁻¹ OxoM (as exemplified by S2% values) in either untreated or MT-7-treated cultures; its effect is compared with that of solvent (*n* = 9). (E) The inhibition of electrically evoked tritium overflow by 10 μmol·L⁻¹ OxoM in either untreated cultures or in cultures treated with 100 nmol·L⁻¹ MT-7 (as exemplified by S3/S1 ratios); the effect of OxoM is compared with that of solvent (*n* = 9). In (D) and (E) significances of differences between pairs of columns are indicated above the corresponding pairs. *** Indicates a significant difference versus the corresponding results obtained in untreated cultures at *P* < 0.001; n.s. indicates the lack of such a significance. mAChRs, muscarinic acetylcholine receptors; MT-7, muscarinic toxin 7; OxoM, oxotremorine M; SCG, superior cervical ganglia.

inhibition achieved with OxoM amounted to $52.4 \pm 7.1\%$ (*n* = 6; Figure 1C). This may be taken as an indication that M₂ mAChRs contribute to, but are not sufficient to entirely mediate, the inhibitory effects of OxoM.

To specifically search for a role of M₁ receptors in the inhibition of electrically evoked release, cultures were incubated in MT-7 (100 nmol·L⁻¹ for 60 min) which selectively and irreversibly blocks M₁ mAChRs (Olianas *et al.*, 2000). In cultures treated with MT-7, OxoM (10 μmol·L⁻¹) failed to cause significant changes in spontaneous tritium outflow when compared with the effects of solvent (Figure 2D), thus indicating that M₁ receptors were sufficiently blocked. In parallel, the inhibition of electrically evoked ³H overflow was significantly attenuated from $55.4 \pm 2.5\%$ to $39.0 \pm 2.3\%$ (*n* = 9; Figure 2E). This indicates a role for M₁ receptors in this latter effect.

Role of PTX-sensitive G-proteins in the inhibition of electrically evoked noradrenaline release by OxoM

Inhibition of noradrenaline release via presynaptic receptors involves in most, but not all, cases PTX-sensitive G-proteins (Kubista and Boehm, 2006). Therefore, cultures were treated with PTX (100 ng·mL⁻¹ for 24 h). This treatment greatly enhanced the stimulation of tritium outflow by OxoM (Figure 3A,B), but left the amount of tritium overflow evoked by electrical field stimulation unaltered (S1% values: untreated: 2.47 ± 0.23 , *n* = 17; PTX-treated: 2.63 ± 0.15 , *n* = 17; *P* > 0.1). In parallel, the inhibitory effect of OxoM was reduced from $47.8 \pm 3.9\%$ inhibition (*n* = 9) to $16.7 \pm 2.7\%$ inhibition (*n* = 9; Figure 3C), but was not abolished. This indicates that another, pertussis-toxin-insensitive, signalling cascade is also involved in the inhibition of electrically evoked noradrenaline release via mAChRs.

Role of K_v7 channels in the inhibition of electrically evoked noradrenaline release by OxoM

M₁ mAChRs are generally linked to G_q-type G-proteins and thereby couple to phospholipase C (Wess *et al.*, 2007). In SCG neurons, activation of phospholipase C leads to an inhibition of K_v7 channels via the depletion of membrane phosphatidylinositol-4,5-bisphosphate (PIP₂) (Suh and Hille, 2002). The stimulation of noradrenaline release via M₁ receptors is mediated by this K_v7 channel inhibition and is thus abolished by the K_v7 channel opener retigabine (Lechner *et al.*, 2003). In line with our previous results, 10 μmol·L⁻¹ retigabine entirely prevented the enhancement of the rate of spontaneous tritium outflow by OxoM (Figure 3D). However, neither electrically evoked ³H overflow itself, nor its inhibition by OxoM, was significantly affected by retigabine (Figure 3E). These results argue against a role of K_v7 channels in the inhibitory effect of OxoM.

Evidence for the presynaptic localization of inhibitory M₁ receptors

The stimulation of noradrenaline release via M₁ receptors is abolished when action potential propagation is blocked, thus indicating that these receptors are not directly localized at the presynaptic nerve terminals where exocytosis takes place (Lechner *et al.*, 2003). To determine whether the muscarinic receptors that mediate the inhibition of electrically evoked noradrenaline release are located at presynaptic sites, experiments were also performed in the presence of 0.1 μmol·L⁻¹

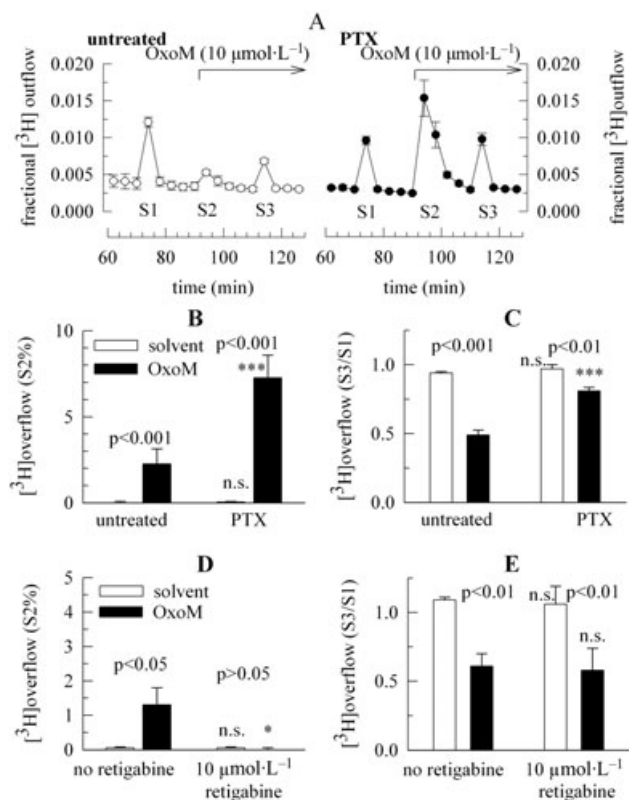


Figure 3 Characterization of signalling mechanisms involved in the inhibition of electrically evoked noradrenaline release via mAChRs. Primary cultures of rat SCG neurons were either treated with pertussis toxin (PTX; 100 ng·mL⁻¹ for 24 h) or remained untreated. Cultures were then labelled with [³H]-noradrenaline for 1 h and were superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. Electrical field stimulation was applied at 73 (S1) and 113 (S3) min of superfusion. OxoM 10 μmol·L⁻¹ was present as indicated by the arrows; OxoM caused transient increases in the rate of spontaneous ³H outflow (S2) as seen in (A); as a control for statistical comparison, solvent was applied instead of OxoM (see Figure 1A). (A) The time course of fractional ³H outflow·min⁻¹ and its alteration by 10 μmol·L⁻¹ OxoM in untreated (left) or PTX-treated cultures (right); n = 3. (B) ³H overflow induced by 10 μmol·L⁻¹ OxoM (as exemplified by S2% values) in either untreated or PTX-treated cultures; its effect is compared with that of solvent (n = 8 to 9). (C) The inhibition of electrically evoked tritium overflow (as exemplified by S3/S1 ratios) by OxoM; its effect is compared with that of solvent (n = 8 to 9). (D) ³H overflow induced by 10 μmol·L⁻¹ OxoM (as exemplified by S2% values) in either the absence or the presence of 10 μmol·L⁻¹ retigabine; the effect of OxoM is compared with that of solvent (n = 9). (E) The inhibition of electrically evoked tritium overflow by 10 μmol·L⁻¹ OxoM (as exemplified by S3/S1 ratios) in either the absence or the presence of 10 μmol·L⁻¹ retigabine; the effect of OxoM is compared with that of solvent (n = 9). In (B) to (E), significances of differences between pairs of columns are indicated above the corresponding pairs. *** and * Indicate significant differences versus the corresponding results obtained in untreated cultures in the absence of retigabine at P < 0.001 and P < 0.05 respectively; n.s. indicates the lack of such a significance. mAChRs, muscarinic acetylcholine receptors; OxoM, oxotremorine M; PTX, pertussis toxin; SCG, superior cervical ganglia.

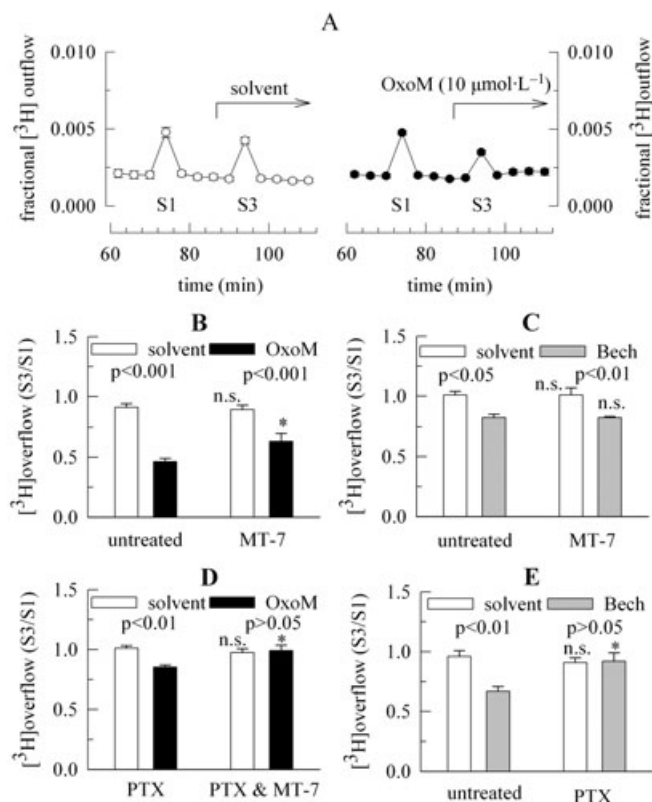


Figure 4 Characterization of the inhibition of K⁺-evoked noradrenaline release via mAChRs in the presence of TTX. Primary cultures of rat SCG neurons were either treated with pertussis toxin (PTX; 100 ng·mL⁻¹ for 24 h) or remained untreated. Cultures were then labelled with [³H]-noradrenaline for 1 h in the absence or presence of 100 nmol·L⁻¹ MT-7 and were superfused. From 50 min of superfusion onward, 0.1 μmol·L⁻¹ TTX was present. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. KCl 30 mmol·L⁻¹ (NaCl was reduced accordingly) was present at 73 (S1) and 93 (S3) min of superfusion. OxoM 10 μmol·L⁻¹ or 100 μmol·L⁻¹ bethanechol (Beth) was present as indicated by the arrow; as a control for statistical comparison, solvent was applied instead of OxoM or bethanechol. (A) The time course of fractional ³H outflow·min⁻¹ and its alteration by 10 μmol·L⁻¹ OxoM on the right hand side (n = 3). For comparison, the effect of solvent is depicted on the left hand side (n = 3). (B) The inhibition of K⁺-evoked tritium overflow (as exemplified by S3/S1 ratios) by 10 μmol·L⁻¹ OxoM in either untreated or MT-7-treated cultures; its effect is compared with that of solvent (n = 9). (C) The inhibition of K⁺-evoked tritium overflow (as exemplified by S3/S1 ratios) by 100 μmol·L⁻¹ bethanechol in either untreated or MT-7-treated cultures; its effect is compared with that of solvent (n = 6). (D) The inhibition of K⁺-evoked tritium overflow (as exemplified by S3/S1 ratios) by 10 μmol·L⁻¹ OxoM in cultures treated either with PTX or with PTX plus MT-7; the effect of OxoM is compared with that of solvent (n = 8 to 9). (E) The inhibition of K⁺-evoked tritium overflow (as exemplified by S3/S1 ratios) by 100 μmol·L⁻¹ bethanechol in either untreated or PTX-treated cultures; the effect of bethanechol is compared with that of solvent (n = 6). In (B) to (E), significances of differences between pairs of bars are indicated above the corresponding pairs. * Indicates a significant difference versus the corresponding results obtained in either untreated (B, C and E) or PTX-treated (D) cultures at P < 0.05; n.s. indicates the lack of such a significance. mAChRs, muscarinic acetylcholine receptors; MT-7, muscarinic toxin 7; OxoM, oxotremorine M; PTX, pertussis toxin; SCG, superior cervical ganglia; TTX, tetrodotoxin.

TTX. As expected, OxoM then failed to alter the rate of spontaneous ³H outflow (Figure 4A). As electrical field stimulation also fails to trigger noradrenaline release from SCG neurons when Na⁺ channels are blocked by TTX (Lechner *et al.*, 2003), exposure to 30 mmol·L⁻¹ K⁺ was used instead of electrical

stimulation to elicit depolarization-dependent tritium overflow. This type of overflow was also reduced by 10 $\mu\text{mol}\cdot\text{L}^{-1}$ OxoM, and the inhibition amounted to $48.9 \pm 3.1\%$ ($n = 9$; Figure 4B). When cultures had been treated with MT-7 (100 $\text{nmol}\cdot\text{L}^{-1}$ for 60 min), this inhibition by OxoM was significantly reduced to $29.0 \pm 7.4\%$ ($n = 8$; Figure 4B). When 100 $\mu\text{mol}\cdot\text{L}^{-1}$ bethanechol was used instead of OxoM to reduce K⁺-evoked tritium overflow in the presence of TTX, the inhibition amounted to $18.5 \pm 3.3\%$ ($n = 6$; Figure 4C). Treatment of the cultures with MT-7 as above did not alter the effect of bethanechol, which then caused $19.1 \pm 1.2\%$ inhibition ($n = 6$; Figure 4C). Conversely, in cultures treated with PTX, the inhibition by 10 $\mu\text{mol}\cdot\text{L}^{-1}$ OxoM amounted to $15.4 \pm 1.7\%$ ($n = 9$; Figure 4D). When cultures were treated with both MT-7 and PTX the inhibition of K⁺-evoked tritium overflow by OxoM was abolished (Figure 4D). Likewise, in PTX-treated cultures, 100 $\mu\text{mol}\cdot\text{L}^{-1}$ bethanechol failed to alter K⁺-evoked overflow, whereas it did cause a clear-cut inhibition in untreated sister cultures (Figure 4E). Once again, the PTX treatment did not alter the amount (S1%) of K⁺-evoked overflow (not shown). These results verify that the inhibitory effects of mAChR agonists on noradrenaline release are the same whether action potential propagation is blocked or not. Thus, this inhibition is mediated via a presynaptic site of action.

Presynaptic inhibition of noradrenaline release by OxoM is mediated by an inhibition of VACCs

OxoM inhibits VACCs in rat SCG neurons via multiple signalling cascades (Shapiro *et al.*, 1999), and the inhibition of VACCs is a predominant mechanism in the regulation of noradrenaline release via presynaptic GPCRs (Kubista and Boehm, 2006). To determine whether the inhibition of VACCs via muscarinic receptors displays the same pharmacological characteristics as the presynaptic inhibition of noradrenaline release, Ca²⁺ currents were measured in perforated patch-clamp recordings in order to maintain signalling cascades, including soluble components (Lechner *et al.*, 2005). Under these experimental conditions, the Ca_v1 (L-type) channel blocker isradipine (3 $\mu\text{mol}\cdot\text{L}^{-1}$) reversibly reduced Ca²⁺ current amplitudes by $9.9 \pm 2.6\%$ ($n = 9$), whereas the Ca_v2.2 (N-type) channel blocker ω -conotoxin GVIA (1 $\mu\text{mol}\cdot\text{L}^{-1}$) caused an irreversible reduction of current amplitudes by $75.4 \pm 3.1\%$ ($n = 8$); 10 $\mu\text{mol}\cdot\text{L}^{-1}$ OxoM reduced overall Ca²⁺ current amplitudes by $59.0 \pm 5.7\%$ ($n = 10$; Figure 5A). In the presence of 3 $\mu\text{mol}\cdot\text{L}^{-1}$ isradipine, the inhibition by OxoM remained unchanged, but it was significantly attenuated when the cells had been exposed to ω -conotoxin GVIA (Figure 5B). Thus, OxoM affects primarily, but not exclusively, Ca_v2.2 (N-type) Ca²⁺ channels. To determine whether the same holds true for the presynaptic inhibition, tritium overflow was triggered by 30 $\text{mmol}\cdot\text{L}^{-1}$ KCl in the presence of TTX in cultures that had either been treated with 1 $\mu\text{mol}\cdot\text{L}^{-1}$ ω -conotoxin GVIA for 24 h or had remained untreated. Such a treatment minimizes the contribution of Ca_v2.2 channels to depolarization-induced noradrenaline release from sympathetic neurons (Boehm and Huck, 1996). This treatment with ω -conotoxin GVIA reduced the amount of K⁺-evoked tritium overflow (S1%) by $69.7 \pm 2.5\%$ ($n = 17$; $P < 0.001$). Furthermore, in

ω -conotoxin-treated neurons the inhibition of tritium overflow by OxoM was significantly reduced when compared with that in untreated neurons (Figure 5C).

Bethanechol (100 $\mu\text{mol}\cdot\text{L}^{-1}$) reduced Ca²⁺ current amplitudes significantly less than 10 $\mu\text{mol}\cdot\text{L}^{-1}$ OxoM (Figure 5D). When cultures had been treated with PTX (100 $\text{ng}\cdot\text{mL}^{-1}$ for 24 h), the inhibition of Ca²⁺ currents by OxoM was significantly reduced, and the remaining inhibition was abolished when neurons had also been exposed to 100 $\text{nmol}\cdot\text{L}^{-1}$ MT-7 (Figure 5D). This confirms that the PTX-insensitive component of Ca²⁺ current inhibition is mediated by M₁ mAChRs.

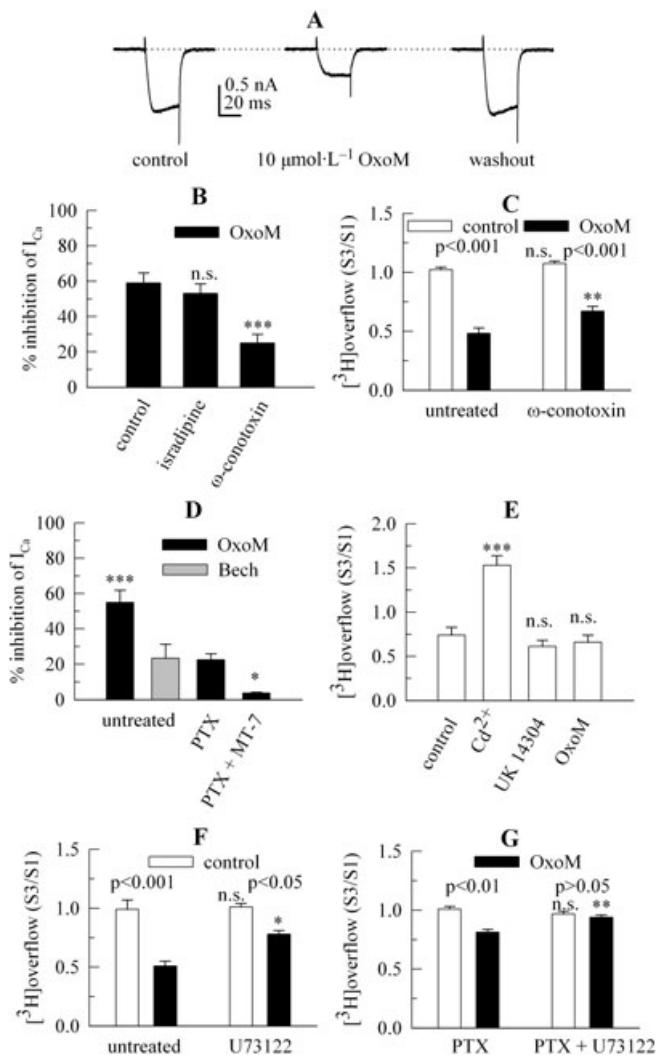
To prove that the inhibition of VACCs is a prerequisite for the presynaptic inhibition of transmitter release, tritium overflow was induced by a stimulation paradigm that bypasses VACCs, namely the activation of presynaptic P2X receptors (Boehm, 1999). TTX was present throughout these experiments to prevent the stimulant effects of OxoM and the contribution of non-presynaptic P2X receptors (Boehm, 1999). ATP 100 $\mu\text{mol}\cdot\text{L}^{-1}$ was applied as a secretagogue stimulus. With this stimulation paradigm, the blockade of VACCs by 100 $\mu\text{mol}\cdot\text{L}^{-1}$ Cd²⁺ did not abolish stimulation-evoked tritium overflow (as it does with depolarizing stimuli; Lechner *et al.*, 2003), but rather Cd²⁺ exerted an enhancing action on transmitter release (Figure 5E). A similar effect has been observed in PC12 cells and was shown to be caused by a direct allosteric potentiation of P2X receptors by Cd²⁺ ions (Ikeda *et al.*, 1996). Moreover, the α_2 -adrenoceptor agonist UK 14304 (3 $\mu\text{mol}\cdot\text{L}^{-1}$), which inhibits noradrenaline release exclusively through a blockade of VACCs (Boehm and Huck, 1995), failed to cause a significant reduction in ATP-induced tritium overflow (Figure 5E). OxoM (10 $\mu\text{mol}\cdot\text{L}^{-1}$) also failed to inhibit this type of overflow (Figure 5E), thus indicating that its inhibitory action requires the contribution of VACCs and is not caused by actions on vesicle exocytosis downstream of Ca²⁺ influx.

Presynaptic inhibition of noradrenaline release by OxoM is mediated by phospholipase C

The inhibition of Ca²⁺ currents in rat SCG neurons via M₁ mAChRs is mediated by phospholipase C and thus attenuated by U73122 (Liu and Rittenhouse, 2003a). This drug causes an irreversible inhibition of phospholipase C-dependent signalling cascades in SCG neurons (Bofill-Cardona *et al.*, 2000). When cultures had been treated with 3 $\mu\text{mol}\cdot\text{L}^{-1}$ U73122, the inhibition of K⁺-evoked tritium overflow by OxoM was reduced from $48.6 \pm 2.3\%$ ($n = 6$) to $24.4 \pm 1.6\%$ (Figure 5F). The inactive analogue, U73343, however, left the inhibition unaltered (not shown, but see Lechner *et al.*, 2005). Furthermore, the 19.5 $\pm 2.7\%$ inhibition remaining in PTX-treated neurons was abolished by U73122 (Figure 5G). Thus, the PTX-insensitive presynaptic inhibition induced by OxoM involves phospholipase C.

Discussion

Presynaptic inhibition via GPCRs is generally believed to be mediated by activated G-protein $\beta\gamma$ subunits that directly interact with VACCs and thereby reduce the Ca²⁺ influx



required for vesicle exocytosis (Stevens, 2004). Although this also holds true for sympathetic neurons in a large number of cases (Boehm and Kubista, 2002), another signalling pathway of inhibitory presynaptic GPCRs has been identified recently: one that involves B₂ bradykinin receptors, phospholipase C, depletion of membrane PIP₂ and a resulting closure of VACCs (Lechner *et al.*, 2005). This very signalling cascade is also linked to M₁ mAChRs (Gamper *et al.*, 2004; Liu *et al.*, 2004), but evidence for inhibitory presynaptic M₁ receptors has been lacking. The results shown above demonstrate that this receptor subtype contributes a small, but nevertheless significant, part to the presynaptic inhibition of noradrenaline release from rat SCG neurons elicited by mAChR agonists.

In primary cultures of rat SCG neurons, activation of M₁ mAChRs is sufficient to trigger action potential-dependent noradrenaline release in the absence of any other secretagogue stimulus (Lechner *et al.*, 2003). When OxoM was applied in the experiments shown above, a transient increase in the rate of noradrenaline release was also observed. In addition, OxoM caused a reduction of noradrenaline release evoked by electrical field stimulation once the secretagogue action of the muscarinic agonist had faded. This inhibitory effect was mediated by a different set of mAChRs than the

secretagogue action as evidenced by the following results: (i) pirenzepine was more potent than methoctramine in antagonizing the stimulant action of OxoM, and the reverse was true for the inhibitory effect; (ii) the mAChR agonist bethanechol mimicked the inhibitory, but not the stimulant, effect of OxoM; and (iii) the selective and irreversible M₁ receptor antagonist MT-7 (Olianas *et al.*, 2000) abolished the stimulant effect, but only attenuated the inhibitory action of OxoM. As bethanechol is selective for M₂ mAChRs in rat SCG neurons (Liu and Rittenhouse, 2003b), this receptor subtype can only be involved in the inhibition of noradrenaline release. The

Figure 5 Role of VACCs in the inhibition of noradrenaline release via presynaptic mAChRs. Primary cultures of rat SCG neurons were treated with pertussis toxin (PTX; 100 ng·mL⁻¹ for 24 h), with ω-conotoxin GVIA (1 μmol·L⁻¹ for 24 h), or remained untreated. Using the perforated patch clamp technique, Ca²⁺ currents were evoked by 30 ms depolarizations from -80 to +10 mV. Alternatively, cultures were labelled with [³H]-noradrenaline for 1 h in the absence or presence of 3 μmol·L⁻¹ U73122 and were superfused. From 50 min of superfusion onward, 0.1 μmol·L⁻¹ TTX was present. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. KCl 30 mmol·L⁻¹ (NaCl was reduced accordingly) or 100 μmol·L⁻¹ ATP were present at 73 (S1) and 93 (S3) min of superfusion. CdCl₂ 100 μmol·L⁻¹, 10 μmol·L⁻¹ UK 14304 or 10 μmol·L⁻¹ OxoM was present from 88 min onward (see Figure 4A); as a control for statistical comparison, solvent was applied instead of these agents. (A) Original current traces of one neuron before during and after the presence of 10 μmol·L⁻¹ OxoM. The dashed line indicates the zero current level. (B) The inhibition of Ca²⁺ currents by 10 μmol·L⁻¹ OxoM under control conditions, in the continuous presence of 3 μmol·L⁻¹ isradipine, and in cells that had been exposed to 1 μmol·L⁻¹ ω-conotoxin GVIA in order to irreversibly block Ca_v2.2 Ca²⁺ channels (*n* = 7 to 9). *** Indicates a significant difference versus the inhibition in the absence of Ca²⁺ channels blockers at *P* < 0.001; n.s. indicates the lack of such a significant difference. (C) Quantifies the effects of a treatment with 1 μmol·L⁻¹ ω-conotoxin GVIA for 24 h on the inhibition of K⁺-evoked tritium overflow (S3/S1) by 10 μmol·L⁻¹ OxoM; *n* = 8 to 9. Significances of differences between pairs of columns are indicated above the corresponding results. ** Indicates a significant difference versus the corresponding result obtained in untreated cultures at *P* < 0.01; n.s. indicates the lack of such a significance; *n* = 6 to 9. (D) The effects of 10 μmol·L⁻¹ OxoM or 100 μmol·L⁻¹ bethanechol (Beth) on Ca²⁺ current amplitudes in untreated, pertussis toxin (PTX)-treated, and PTX- plus MT-7-treated (PTX + MT-7) neurons; *n* = 6 to 8. *** and * Indicate significant differences versus the inhibition by OxoM observed in PTX-treated neurons at *P* < 0.001 and *P* < 0.05 respectively. (E) Quantifies the effects of 100 μmol·L⁻¹ CdCl₂, 10 μmol·L⁻¹ UK 14304 or 10 μmol·L⁻¹ OxoM on ATP-evoked tritium overflow (S3/S1); *n* = 8 to 9. *** Indicates a significant difference versus the results obtained with solvent (control) at *P* < 0.001; n.s. indicates the lack of such a significance. (F) Quantifies the effects of a treatment with 3 μmol·L⁻¹ U73122 on the inhibition of K⁺-evoked tritium overflow (S3/S1) by 10 μmol·L⁻¹ OxoM; *n* = 5 to 6. Significances of differences between pairs of columns are indicated above the corresponding pairs. * Indicates a significant difference versus the corresponding result obtained in cultures that had not been exposed to U73122 at *P* < 0.05; n.s. indicates the lack of such a significance. (G) Quantifies the effects of a treatment with 3 μmol·L⁻¹ U73122 on the inhibition of K⁺-evoked tritium overflow (S3/S1) by 10 μmol·L⁻¹ OxoM in neurons treated with PTX; *n* = 6 to 9. Significances of differences between pairs of columns are indicated above the corresponding pairs. ** Indicates a significant difference versus the corresponding results obtained in cultures that had not been exposed to U73122 at *P* < 0.01; n.s. indicates the lack of such a significance. ATP, adenosine-5'-triphosphate; mAChRs, muscarinic acetylcholine receptors; OxoM, oxotremorine M; PTX, pertussis toxin; SCG, superior cervical ganglia; VACCs, voltage-activated Ca²⁺ channels.

abolition of the secretagogue action of OxoM by MT-7 confirms that this latter effect is mediated by M₁ receptors only. The fact that this toxin attenuated the reduction of noradrenaline release by OxoM was the first indication that M₁ receptors may be involved in presynaptic inhibition.

One might argue that the MT-7-dependent attenuation of the inhibition of noradrenaline release by OxoM might be correlated with the loss of the secretagogue action of this agonist. However, the following results argue against such a conclusion. (i) Retigabine also abolished the secretagogue action of OxoM, but did not alter its inhibitory action. (ii) MT-7 attenuated the inhibition of depolarization-dependent release by OxoM even when the muscarinic agonist did not enhance the rate of otherwise unstimulated noradrenaline release, as was the case in the presence of TTX. Furthermore, the observation of an inhibition of depolarization-evoked transmitter release with action potential propagation being blocked by TTX demonstrates that all the mAChRs involved, including M₁, must be located close to the sites of vesicle exocytosis, that is must be presynaptic receptors.

In rat SCG neurons, activation of mAChRs modulates the functions of K_v7 K⁺ channels and of Ca_v2.2 (N-type) as well as Ca_v1 (L-type) Ca²⁺ channels (Hille, 1994). The resulting changes in the accompanying conductances may impinge on the excitability of the neurons and on the amount of transmitter being released (Boehm and Kubista, 2002; Hernandez *et al.*, 2008). To evaluate the role of these channels in the effects of OxoM on noradrenaline release, we first employed the K_v7 channel opener retigabine (Tatulian *et al.*, 2001) which abolishes the secretagogue action of OxoM (see above and Lechner *et al.*, 2003). However, 10 μmol·L⁻¹ retigabine did not alter the inhibitory action of OxoM on electrically evoked release. Thus, the presynaptic inhibition via mAChRs is independent of K_v7 channels.

Activation of primarily M₂, but also M₄, receptors mediates an inhibition of Ca_v2.2 Ca²⁺ channels in rat SCG neurons through a direct interaction of βγ subunits of inhibitory G-proteins with the channel proteins (Bernheim *et al.*, 1992; Liu and Rittenhouse, 2003b). This effect is abolished by PTX. Activation of M₁ receptors, in contrast, inhibits VACCs in a PTX-insensitive manner (Hille, 1994). In accordance with these data, the 45% to 50% inhibition of depolarization-evoked noradrenaline release by OxoM was reduced by PTX treatment to 15% to 20% inhibition. Moreover, the 15% to 20% inhibition by the M₂ selective agonist bethanechol was even abolished by PTX. And the 15% to 20% presynaptic inhibition by OxoM remaining in PTX-treated cultures was abolished by MT-7. These data concerning the inhibition of noradrenaline release were paralleled by electrophysiological results. OxoM caused a 55% to 60% reduction and bethanechol a 23% reduction of Ca²⁺ current amplitudes in untreated neurons. In PTX-treated neurons, however, the inhibition by OxoM was reduced to 23%, and this remainder was abolished by MT-7. The congruence of results obtained with the presynaptic inhibition of noradrenaline release, on the one hand, and with the inhibition of Ca²⁺ currents, on the other hand, suggests a causal association between these two phenomena. This causal association was challenged in experiments where tritium overflow was induced by a method that excludes VACCs from excitation-secretion coupling: the acti-

vation of presynaptic P2X receptors which mediate the transmembrane Ca²⁺ entry required for exocytosis themselves (Boehm, 1999). Under these conditions, OxoM failed to reduce noradrenaline release, as did the α₂-adrenoceptor agonist UK 14304. Activation of presynaptic α₂-adrenoceptors has been well documented to reduce noradrenaline release through an inhibition of VACCs (Kubista and Boehm, 2006). Accordingly, the present results demonstrate that the same holds true for the presynaptic inhibition via mAChRs.

Activation of M₁ mAChRs controls the gating of Ca_v1 and Ca_v2.2 Ca²⁺ channels in rat SCG neurons via signalling cascades that include Gαq G-protein subunits and the activation of phospholipase C with ensuing depletion of membrane PIP₂ and generation of arachidonic acid (Haley *et al.*, 2000; Liu and Rittenhouse, 2003a; Gamper *et al.*, 2004; Liu *et al.*, 2006). With respect to the presynaptic inhibition via M₁ receptors, the same signalling mechanism was found to operate as the inhibitory action of OxoM was attenuated by a phospholipase C inhibitor (and even abolished when neurons had also been treated with PTX). Both Ca²⁺ current amplitudes and noradrenaline release were reduced by ω-conotoxin GVIA by 70% to 75%. The remaining 25% to 30% of Ca²⁺ currents and evoked noradrenaline release were still reduced by OxoM, but the percentage of inhibition (about 30%) was significantly smaller than that in neurons with unblocked Ca_v2.2 channels. Moreover, one must not forget that this 30% inhibition of Ca²⁺ currents and noradrenaline release in ω-conotoxin-treated neurons equals no more than 10% of overall Ca²⁺ current amplitudes and evoked noradrenaline release. Thus, the inhibition of Ca_v2.2 Ca²⁺ channels is the most decisive, but not the only mechanism underlying the inhibition of noradrenaline release via presynaptic mAChRs.

The regulation of VACCs and K_v7 channels in SCG neurons via phospholipase C is initiated not only by M₁ mAChRs, but also by bradykinin B₂ receptors (Gamper *et al.*, 2004; Lechner *et al.*, 2005). As for M₁ receptors here, B₂ receptor activation has been found to trigger action potentials and thus noradrenaline release from SCG neurons (Boehm and Huck, 1997), on the one hand, and to simultaneously mediate presynaptic inhibition at the nerve terminals (Edelbauer *et al.*, 2005), on the other hand. Furthermore, these opposing effects of bradykinin were mediated by the inhibition of K_v7 channels and VACCs respectively (Edelbauer *et al.*, 2005). This leads to the question why one or the other effect triggered by these receptors predominates in presynaptic and non-presynaptic regions of SCG neurons respectively. One likely explanation is the diverging densities of the affected channels in different parts of a neuron. Ca_v2.2 Ca²⁺ channels are concentrated at the active zones of presynaptic nerve terminals (Khanna *et al.*, 2007), whereas K_v7 channels are rather located close to neuronal somata (Hu *et al.*, 2007; Rasmussen *et al.*, 2007). In addition, certain components of the signalling cascades involved, such as phospholipase A₂ (Liu *et al.*, 2006) and diacylglycerol lipase (Liu *et al.*, 2008) which mediate the inhibition of VACCs, but not that of K_v7 channels, may be differentially distributed throughout the SCG neurons. Moreover, the pattern of stimulating or inhibitory effects of G_q-coupled receptors on transmitter release may also vary between different types of neurons. Presynaptic M₃ mAChRs, for instance, have been shown to enhance dopamine release

from striatal neurons through an inhibition of K_v7 channels (Martire *et al.*, 2007), and evidence for stimulating presynaptic M₁ receptors has also been obtained in sympathetically innervated tissues (Boehm and Kubista, 2002). Here, we have demonstrated that presynaptic M₁ mAChRs can mediate an inhibition of transmitter release. It will thus be of interesting to determine how signalling cascades are organized to mediate either inhibitory or stimulating actions of one type of presynaptic GPCR.

In conclusion, the present data reveal that M₁ receptors contribute to the acetylcholine-dependent presynaptic inhibition in the sympathetic nervous system via a signal cascade that includes phospholipase C and VACCs.

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Conflict of interest

None.

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