BJP British Journal of Pharmacology

DOI:10.1111/bph.13011
www.brjpharmacol.org

RESEARCH PAPER

Identification of muscarinic receptor subtypes involved in catecholamine secretion in adrenal medullary chromaffin cells by genetic deletion

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Received
20 May 2014
Revised
26 October 2014
Accepted
31 October 2014

BACKGROUND AND PURPOSE

Activation of muscarinic receptors results in catecholamine secretion in adrenal chromaffin cells in many mammals, and muscarinic receptors partly mediate synaptic transmission from the splanchnic nerve, at least in guinea pigs. To elucidate the physiological functions of muscarinic receptors in chromaffin cells, it is necessary to identify the muscarinic receptor subtypes involved in excitation.

EXPERIMENTAL APPROACH

To identify muscarinic receptors, pharmacological tools and strains of mice where one or several muscarinic receptor subtypes were genetically deleted were used. Cellular responses to muscarinic stimulation in isolated chromaffin cells were studied with the patch clamp technique and amperometry.

KEY RESULTS

Muscarinic M_1, M_4 and M_5 receptors were immunologically detected in mouse chromaffin cells, and these receptors disappeared after the appropriate gene deletion. Mouse cells secreted catecholamines in response to muscarinic agonists, angiotensin II and a decrease in external pH. Genetic deletion of M_1, but not M_3, M_4 or M_5, receptors in mice abolished secretion in response to muscarine, but not to other stimuli. The muscarine-induced secretion was suppressed by MT7, a snake peptide toxin specific for M_1 receptors. Similarly, muscarine failed to induce an inward current in the presence of MT7 in mouse and rat chromaffin cells. The binding affinity of VU0255035 for the inhibition of muscarine-induced currents agreed with that for the M_1 receptor.

CONCLUSIONS AND IMPLICATIONS

Based upon the effects of genetic deletion of muscarinic receptors and MT7, it is concluded that the M_1 receptor alone is responsible for muscarine-induced catecholamine secretion.

Abbreviations

HA, haemagglutinin; IR, immunoreactivity; KO, knockout; nAChR, nicotinic ACh receptor

Tables of Links

TARGETS
GPCRs[a]
Muscarinic M_1 receptors
Muscarinic M_2 receptors
Muscarinic M_3 receptors
Muscarinic M_4 receptors
Muscarinic M_5 receptors
Ion channels[b]
$K_V7.2$
$K_V7.3$
TASK1 channel; $K_{2P}3.1$

LIGANDS
4-DAMP
AF-DX 384
Angiotensin II
Himbacine
MT3
MT7
Muscarine
Nicotine
Pilocarpine
VU0255035

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

The chromaffin cells of the adrenal medulla, which originate from the same neural crest as sympathetic ganglion cells (Donoghue *et al.*, 2008), function as endocrine cells. Sympathetic ganglion cells and chromaffin cells in mammals are innervated by sympathetic preganglionic nerve fibres. The mechanisms of synaptic transmission in chromaffin cells, however, have not been elucidated to the same extent as those in sympathetic ganglion cells. In the latter, ACh mediates fast and slow EPSPs through nicotinic ACh receptors (nAChRs) and muscarinic ACh receptors (muscarinic receptors) respectively (Brown, 1967; 2013; Jan and Jan, 1982). ACh-mediated slow EPSPs have been shown to occur due to the inhibition of M channels (Adams *et al.*, 1982), which mainly comprise the $K_V7.2$ and $K_V7.3$ subunits (Brown and Passmore, 2009). On the contrary, due to the morphological constraint of the adrenal medulla, synaptic transmission mediated by ACh has not been sufficiently explored. In slices of rat adrenal medullae, field stimulation produces fast EPSPs via nAChRs, but not slow EPSPs via muscarinic receptors (Kajiwara *et al.*, 1997). However, the expression of muscarinic receptors has been detected biochemically and/or functionally in chromaffin cells of almost every, if not all, mammalian animals (Olivos and Artalejo, 2008; Harada *et al.*, 2011). We have recently reported that catecholamine secretion electrically evoked in perfused guinea pig adrenal medullae is partly blocked by retrograde application of hexamethonium, a nAChR blocker, and the remaining component is abolished by atropine, a muscarinic receptor antagonist (Inoue *et al.*, 2012), indicating that muscarinic receptors are involved in synaptic transmission at least in guinea pig chromaffin cells. To further elucidate the physiological functions of muscarinic receptors in chromaffin cells, it would be necessary to identify the muscarinic receptor subtypes involved in catecholamine secretion. Displacement experiments of ligand binding by muscarinic reagents have suggested the involvement of the M_1 and M_2 receptor subtypes in canine (Tobin *et al.*, 1992) and cat chromaffin cells (Ballesta *et al.*, 1989) respectively. Our experiments where immunocytochemical exploration was combined with functional analyses with muscarinic agonists suggested that M_5 receptors are involved in secretion in rat chromaffin cells (Harada *et al.*, 2011), whereas M_1 and M_3 (Asano *et al.*, 1995) or M_4 receptors (Inoue and Imanaga, 1995) appear to be involved in guinea pig cells by functional analysis. Each of these studies, however, lacks decisive evidence.

Genetic deletion of proteins is a powerful technique for the identification of receptors involved in a signalling cascade. This technique, however, is generally restricted to mice. Furthermore, the effects of deletion of a certain receptor subtype might be compensated for by other subtypes (Zhou *et al.*, 2013). Therefore, the results obtained from the deletion experiments should be interpreted with caution. Furthermore, as an important trait responsible for function is preserved beyond species, whether or not muscarinic receptor subtypes involved in catecholamine secretion are preserved in chromaffin cells of other species cannot be explored with knockout mice alone. The present study was undertaken to identify the muscarinic receptor subtypes involved in catecholamine secretion. For this purpose, pharmacological tools and various strains of mice where one or several muscarinic receptor subtypes are genetically deleted were used (Matsui *et al.*, 2004). The results unambiguously indicated that the M_1 receptor alone mediates catecholamine secretion in response to muscarine and the involvement of M_1 receptors is preserved in chromaffin cells of mice and rats.

Methods

All animal care and experimental procedures were approved by the IACUC of the University of Occupational and Environmental Health. Studies involving animals were reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 87 animals were used in the experiments described here. Male Wistar rats and male C57BL/6 mice, two to six months old, were obtained from

Kyudo (Tosu, Saga, Japan). The animals were allowed free access to food and water and maintained on a 12 h light/dark cycle with controlled temperature (22 ± 2°C) and humidity (55 ± 5%).

Whole-cell recording

The animals were killed by cervical dislocation, and adrenal glands were excised and immediately placed into ice-cold Ca^{2+}-deficient saline in which 1.8 mM $CaCl_2$ was omitted from standard saline (composition; 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.53 mM $NaHPO_4$, 5 mM D-glucose, 5 mM HEPES and 4 mM NaOH; pH 7.4). The adrenal gland was cut in half, and the adrenal cortex, which was yellowish in colour, was removed from the whitish adrenal medulla using microscissors and forceps under stereoscopic observation. The adrenal medullae were treated with 7 mg·mL^{-1} collagenase in Ca^{2+}-deficient saline for 20 min while they were gently stirred with bubbles of O_2 gas. This enzyme treatment was repeated twice with a fresh enzyme solution, and then the preparation was kept in Ca^{2+}-deficient saline at 5°C for up to 4 h until the experiment commenced.

The whole-cell current was recorded in an isolated chromaffin cell using the nystatin perforated patch method, as described elsewhere (Inoue and Imanaga, 1995; Inoue *et al.*, 2008). Briefly, one or two pieces of adrenal medulla preparations were placed in a bath set on the stage of an inverted microscope, and chromaffin cells were dissociated mechanically with fine needles. The standard pipette solution contained 120 mM potassium isethionate, 20 mM KCl, 10 mM NaCl, 10 mM HEPES and 2.6 mM KOH (pH 7.2). On the day of the experiment, nystatin dissolved in dimethyl sulfoxide (5 mg in 100 μL) was added to the pipette solution at a final concentration of 100 μg·mL^{-1}. Currents were recorded with a patch clamp amplifier (Axopatch 200A; Axon Instruments, Foster City, CA, USA) and then fed into a thermal recorder after loss-pass filtering at 15 Hz and into a digital audio tape (DAT) recorder (RD-120TE; TEAC, Tama, Tokyo, Japan). All chemicals were applied to the bath. The membrane potential was corrected for a liquid junction potential of −3 mV between the standard pipette solution and saline.

To examine the concentration dependence for muscarine-induced currents, a range of concentrations of muscarine were randomly applied to reduce the effects of time-dependent changes in these currents. For the analysis of the dose-dependence of the muscarine-induced current (I), Sigma plot (10.0; SPSS Inc., Chicago, IL, USA) was used to fit a peak value of I to a rectangular hyperbola $I = (I_{max} \times [A]) / (K_B + [A])$, where I_{max} is the maximum value of I, $[A]$ is the concentration of muscarine and K_B is a constant equal to the concentration of muscarine causing half the maximal response (EC_{50}). I was expressed relative to the current caused by 30 μM muscarine in the same cell. The approximation of control dose-dependence of the current with the hyperbola was constrained by I = 1 at 30 μM of muscarine. The muscarinic antagonists were assumed to act competitively; their dissociation constants (K_D) were estimated with $K_D = [B] \times K_B'/(K_B'' - K_B')$, where $[B]$ is the antagonist concentration and K_B' and K_B'' are the muscarine EC_{50} values in the absence and presence of the antagonist. This expression is a transformation of the Gaddum–Schild equation (Arunlakshana and Schild, 1959). Experiments were carried out at 26 ± 2°C.

Amperometry

Catecholamine secretion from single or clustered chromaffin cells was measured using amperometry, as described elsewhere (Inoue *et al.*, 1998). A carbon fibre electrode (ProCFE; Dagan Corporation, Minneapolis, MN, USA) was carefully placed on a chromaffin cell and +600 mV was applied to the electrode under voltage clamp conditions. The current due to the oxidation of catecholamine at the tip of the electrode was recorded using an Axopatch 200A amplifier. Data were stored on a DAT data recorder and fed to a thermal recorder after low-pass filtering at 15 Hz.

Immunocytochemistry

Immunostaining of dissociated chromaffin cells, which had been fixed in 4% paraformaldehyde-containing phosphate buffer, was performed as previously described (Inoue *et al.*, 2000; Harada *et al.*, 2011). For indirect immunofluorescence studies, cells were treated overnight with rabbit anti-M_1 receptor antibody (Frontier Institute, Ishikari, Japan), mouse anti-M_2 receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-M_4 receptor antibody (Chemicon, Temecula, CA, USA) or rabbit anti-M_5 receptor antibody (Abcam, Tokyo, Japan). Immunocytochemical specificity of the antibodies against the rat muscarinic receptor subtypes was confirmed in our earlier work (Harada *et al.*, 2011). After incubation, the cells were washed three times with phosphate buffer saline and then treated with a respective secondary antibody conjugated with Alexa 488 or 546 (Molecular Probe, Eugene, OR, USA). The fluorescence was observed using a laser scanning confocal microscope (LSM5 Pascal; Carl Zeiss, Tokyo, Japan). The objective lens was an oil-immersion lens with a magnification of 63×, and fluorescence was observed with appropriate laser lines and filter sets.

Cell culture and transfection

PC12 (provided by K. Mizuno) cells were cultured in DMEM (Invitrogen, Tokyo, Japan) supplemented with 10% FBS (Nichirei, Tokyo, Japan) and transfected as described elsewhere (Harada *et al.*, 2011). Briefly, the LipofectAMINE 2000 reagent (Invitrogen) was used to transfect PC12 cells with expression vectors for haemagglutinin (HA)-tagged human M_1 or M_5 muscarinic receptors or for GFP-TWIK-related acid-sensitive K^+ channels (TASK)1 (Matsuoka *et al.*, 2013) and constitutively active human M_1 or M_5 receptors (Missouri S&T cDNA Resource Center, Rolla, MO, USA), according to the manufacturer's instructions. The transfected cells were placed onto glass coverslips coated with collagen type I (BD Biosciences, San Jose, CA, USA) and then cultured for 24 h. After fixation with 4% paraformaldehyde and permeabilization with 0.1% Triton X-100, the cells were treated with anti-M_1 receptor antibody, anti-M_5 receptor antibody or anti-HA antibody (Santa Cruz Biotechnology) and then with the secondary antibody.

Data analysis

Data are shown as the means ± SEM, and the statistical analysis was evaluated by Student's t-test. A $P < 0.05$ was considered to be statistically significant.

Materials

Muscarine chloride, himbacine, and pilocarpine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA);

PD 102807, 4-DAMP and AF-DX 384 were from Tocris (Bristol, UK); MT3, MT7 and angiotensin II were from Peptide Institute (Osaka, Japan); nicotine was from Nacalai (Kyoto, Japan); collagenase was from Yakult (Tokyo, Japan); and McN-A-303 was from RBI (Natick, MA, USA).

Results

Muscarinic antagonists in rats

Different efficacies in muscarinic agonists suggest the involvement of M_5 receptors in catecholamine secretion in rat chromaffin cells (Harada *et al.*, 2011). Thus, muscarinic antagonists were used to explore whether or not the M_5 receptors were involved in our experimental system. The antagonists used here were himbacine, AF-DX384, PD102807 and 4-DAMP: the binding affinity values of the first two reagents against M_5 receptors are two orders lower than those against M_4 receptors, and the affinity of the last against M_2 receptors is one order lower than that against the others (Dörje *et al.*, 1991; Caulfield and Birdsall, 1998; Böhme *et al.*, 2002). As previously reported (Inoue *et al.*, 2008), bath application of muscarine induced an inward current via inhibiting TASK1 channels in a dose-dependent manner in rat chromaffin cells (Figure 1A and B). The peak amplitudes of the muscarine-induced currents were well approximated by a rectangular hyperbola with an EC_{50} of 12.7 μM. The dose–response curve was shifted in a parallel manner to the right in the presence of 0.1 μM himbacine, 0.1 μM AF-DX 384, 1 μM PD102807 or 0.01 μM 4-DAMP (Figure 1A and C). The apparent affinity value of each antagonist was calculated from a change in EC_{50} in the presence of the antagonist, and plotted against published values for binding affinity (Dörje *et al.*, 1991; Böhme *et al.*, 2002) (Figure 2). The functional affinity values showed better agreement with the binding affinity estimates for M_1, M_2 and M_3 receptors rather than those for M_4 and M_5 receptors.

Mouse chromaffin cells

Single or several muscarinic receptor subtypes have been genetically deleted in mice (Matsui *et al.*, 2004). Thus, these knockout (KO) mice were used for the identification of muscarinic subtypes. The amperometric method was used to measure catecholamine secretion in response to stimuli. Bath application of 30 μM muscarine induced secretion in 26% of isolated chromaffin cells of wild-type mice, which showed a robust secretion in response to 30 μM nicotine (Figure 3). This incidence of muscarinic secretion was the same as that in double KO mice lacking M_4 and M_5 receptor subtypes (Figure 3C; Table 1), and 64% (n = 7) and 60% (n = 6) of the cells responding to muscarine in the double KO mice also showed catecholamine secretion in response to two different muscarinic agonists McN-A-363 and pilocarpine (Richards

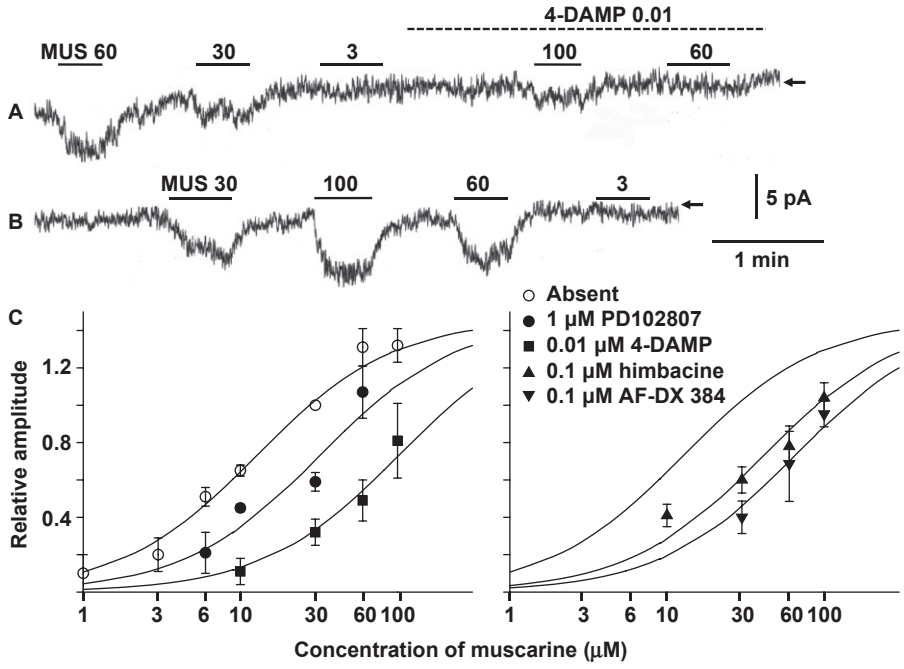

Figure 1

Effects of antagonists on dose-dependent production of an inward current in response to muscarine in rat chromaffin cells. (A, B) Experimental traces of whole-cell currents in response to a range of concentrations of muscarine, with and without 0.01 μM 4-DAMP. The whole-cell currents were recorded at −50 mV with the perforated patch clamp method in rat isolated chromaffin cells. Muscarine (MUS) and 4-DAMP were applied to the bath during the indicated periods (bars for muscarine; interrupted lines for 4-DAMP). Arrows indicate zero current level. A and B are different cells. (C) Dose–response curves for muscarine-induced currents in the absence and the presence of 1 μM PD102807, 0.01 μM 4-DAMP, 0.1 μM himbacine or 0.1 μM AF-DX 384. Each point represents mean ± SEM of 2–26 observations for control, 3–6 for PD102807, 4–7 for 4-DAMP, 3–8 for himbacine and 3 for AF-DX 384. The data were approximated by rectangular hyperbolae.

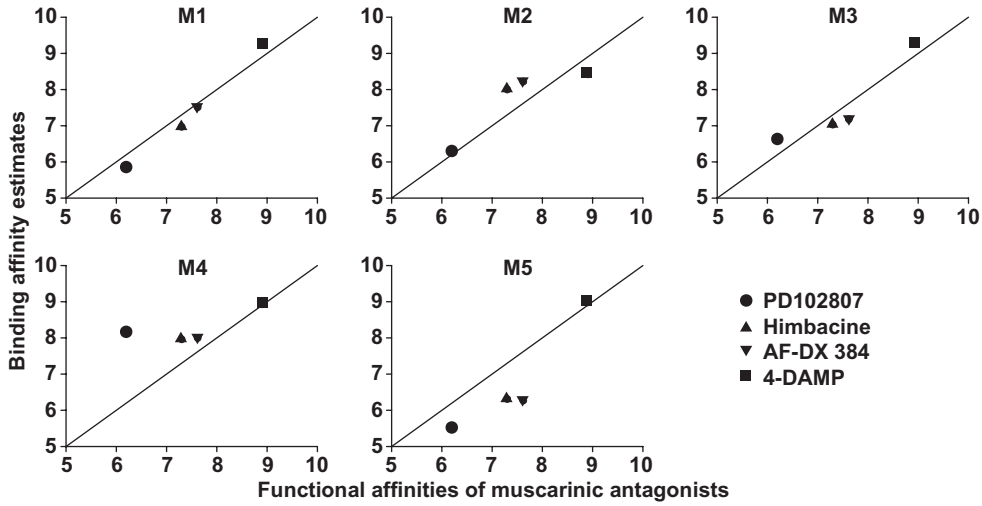

Figure 2

Comparison of functional affinities with binding affinity estimates. The negative logarithms of functional affinities (abscissa) of the muscarinic antagonists obtained in electrophysiological analyses are plotted against those of binding affinity estimates (ordinate). The data represent negative logarithms of means of functional affinities (means ± SEM in nM: 655 ± 195 for PD102807; 44.6 ± 97.6 for himbacine; 24.1 ± 66.1 for AF-DX 384; 1.43 ± 32.9 for 4-DAMP) and means ± SEMs of negative logarithms of binding affinity estimates [those of antagonists except PD102807 are from Dörje *et al.* (1991) and mean of PD102807 is from Böhme *et al.* (2002)]. All SEMs are within the symbols. The straight lines represent $y = x$. The correlation coefficients between the straight line and the data points for M_1, M_2, M_3, M_4 and M_5 receptors are 0.9708, 0.7927, 0.9325, 0.3118 and 0.7245 respectively.

Table 1

Muscarinic receptor-induced secretion in chromaffin cells from wild-type and muscarinic receptor KO mice

	Number of mice	% of cells responding to muscarine	% of cells suppressed by MT7
Wild-type	11	14/54 = 26%	5/5 = 100%
M_1KO	2	0/27 = 0%	–
M_3KO	2	1/18 = 6%	1/1 = 100%
M_4M_5KO	3	14/50 = 28%	7/7 = 100%
M_1M_4KO	2	0/20 = 0%	–
$M_1M_2M_4$KO	3	0/24 = 0%	–

and van Giersbergen, 1995) respectively (Figure 3C). Furthermore, catecholamines were secreted in response to muscarine in 1 of 18 chromaffin cells from M_3 KO mice (Table 1). On the contrary, muscarine did not induce secretion in any of the chromaffin cells examined from single (M_1), double (M_1 and M_4) and triple (M_1, M_2, and M_4) KO mice (Figure 3D and E; Table 1). These results suggest that only the M_1 receptor was involved in muscarinic agonist-induced secretion in mouse chromaffin cells. However, the failure of muscarine to induce secretion in chromaffin cells of M_1 KO mice might have been ascribed to a defect in signalling downstream of M_1 receptors. To explore this possibility, the effects of angiotensin II were examined. Angiotensin AT_1 receptors, whose stimulation leads to catecholamine secretion (Teschemacher and Seward, 2000), are coupled to PLC via G_q (De Gasparo *et al.*, 2000), as is the M_1 receptorsubgroup comprising M_1, M_3 and M_5 receptors. Thus, we examined the effects of genetic deletion of muscarinic receptors on angiotensin II-induced secretion. All the chromaffin cells examined (n = 8, n = 9 and n = 12 in wild-type, M_1M_4 KO, and $M_1M_2M_4$ KO mice respectively) secreted catecholamine in response to 1 μM angiotensin II (Figure 3B, D and E). These results indicate that G_q-PLC signalling was not altered by M_1 receptor ablation. Furthermore, a decrease in the external pH to 6.8 induced secretion, probably via inhibition of TASK channel activity (Inoue *et al.*, 2008; 2012), in 38% (n = 3), 38% (n = 6) and 60% (n = 18) of the cells examined in wild-type, M_1M_4KO and $M_1M_2M_4$ KO mice respectively (Figure 3B, D and E). These results suggest that the expression of TASK1 channels was not affected by the lack of M_1 receptors.

Muscarinic toxins

The results with the KO mice clearly indicated the involvement of the M_1 receptor subtype in secretion. This notion was further examined with MT7, a specific peptide inhibitor for M_1 receptors (Servent and Fruchart-Gaillard, 2009). To our surprise, catecholamine secretion in response to muscarine in wild-type mouse chromaffin cells was reversibly abolished by 10 nM MT7 (Figure 3A) (Krajewski *et al.*, 2001). A similar suppression of muscarinic secretion occurred in chromaffin cells from M_3 single KO and M_4M_5 double KO mice (Table 1). This reversible suppression of secretion prompted us to examine, in greater detail, the effects of MT7 on muscarine-induced currents in mouse chromaffin cells. As shown in Figure 4A and D, exposure to MT7 in mouse cells resulted in a time-dependent diminution of an inward current in response to

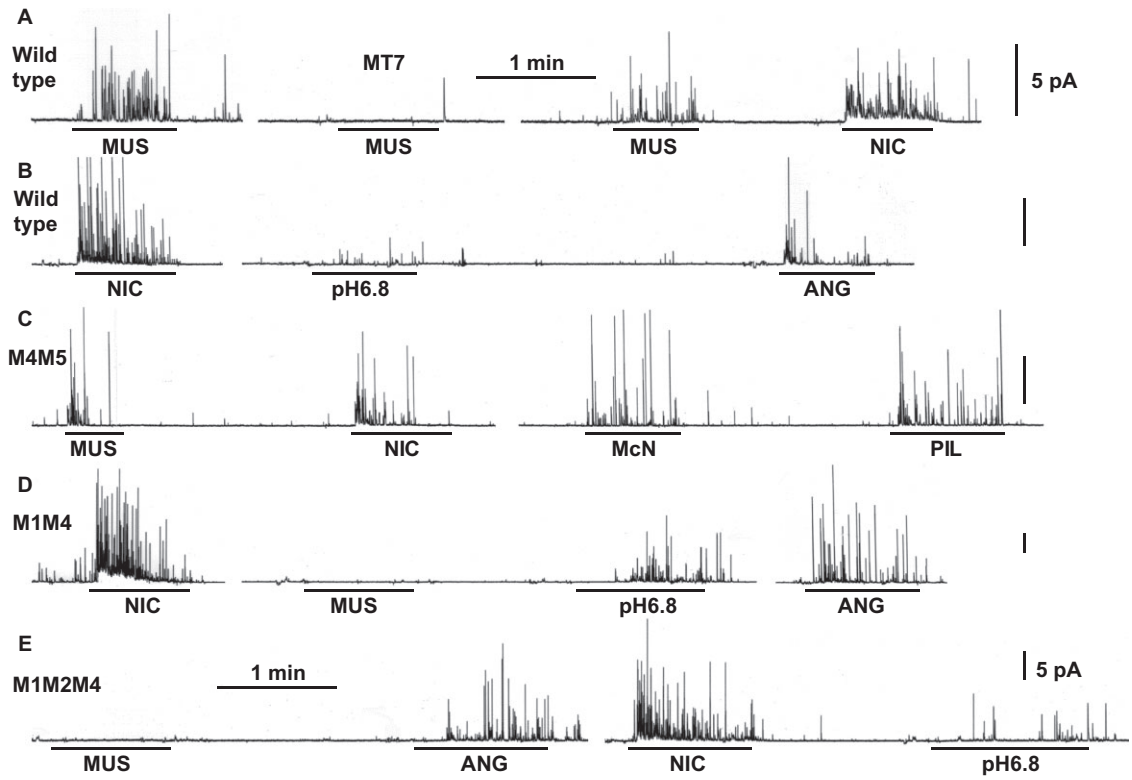

Figure 3

Catecholamine secretion in chromaffin cells of mice with or without genetic deletion of muscarinic receptors. Each row represents traces of amperometric recordings of catecholamine secretion in the same isolated chromaffin cell. Chemicals (MUS, 30 μM muscarine; NIC, 30 μM nicotine; ANG, 1 μM angiotensin II; PIL, 30 μM pilocarpine; McN, 30 μM McN-A-343; MT7, 0.01 μM MT7) were applied to the bath. (A) Muscarine-induced secretion was reversibly suppressed by MT7 in wild-type mice. (B) Catecholamine secretion evoked by nicotine, angiotensin II and a decrease in external pH to 6.8 (pH 6.8) in wild-type mice. (C) Catecholamine secretion induced by different muscarinic agonists (muscarine, McN-A-343 and pilocarpine) in M_4M_5 double KO mice. (D, E) catecholamine secretion occurred in response to nicotine, pH 6.8, and angiotensin II, but not to muscarine in M_1M_4 double KO and $M_1M_2M_4$ triple KO respectively.

muscarine. After washout of the inhibitor, the muscarinic current was restored. Also in rat chromaffin cells (Figure 4B), application of MT7 produced a reversible inhibition of the muscarine-induced current. In contrast to the potent inhibition by MT7, exposure to 100 nM MT3, a muscarinic toxin that is selective to M_4 receptors (Servent and Fruchart-Gaillard, 2009), resulted in no change in 30 μM muscarine-induced currents in rat chromaffin cells (Figure 4C and D). These results strongly suggest that MT7 suppressed the muscarine-induced currents by inhibiting M_1 receptors.

VU0255035

A novel selective antagonist of M_1 receptor, VU0255035, has recently been described by Sheffler *et al.*, (2009). As shown in Figure 5A, muscarine-induced currents were reversibly suppressed by 0.03 μM VU0255035. This antagonist shifted the dose–response curve for muscarine rightward, in a concentration-dependent manner (Figure 5B). The Schild plot, which was constructed from fold changes in dose–response curve in the presence of 0.03, 0.1 and 0.3 μM VU0255035, provided a K_D value of 14.8 nM (confidence interval, 6.7–32.8 nM). This value was identical to that (14.5 nM) obtained from binding experiments with M_1 receptors (Sheffler *et al.*, 2009).

Immunocytochemistry

Immunocytochemical techniques were also used to explore which subtypes of muscarinic receptors are expressed in mouse chromaffin cells. A portion of the M_1-like immunoreactivity (IR) was present at the cell periphery, but the majority was in the cytoplasm (Figure 6A). Genetic deletion of M_1 receptors clearly suppressed such IR (Figure 6B and C). Consistent with the results from rat cells, mouse chromaffin cells were not stained with an anti-M_2 receptor antibody (Figure 6A), which has been shown to be effective in immunostaining (Harada *et al.*, 2011). In contrast to M_1-like IR, M_4-like immunoreactions were mainly localized at the cell periphery in wild-type chromaffin cells, and the genetic deletion of the M4 receptor subtype abolished most of them (Figure 6B). M_5-like immunoreactive materials were exclusively present at the cell periphery, and again, genetic deletion of M_5 receptors resulted in the abolition of its IR (Figure 6B and C). It is worth noting that the immunostaining for M_5 receptors was not obviously altered in chromaffin cells lacking M_1 and M_4 receptor subtypes (Figure 6B).

Constitutively active muscarinic receptors

Immunocytochemistry clearly revealed that mouse chromaffin cells express M_1, M_4 and M_5 receptors. As M_1 and M_5

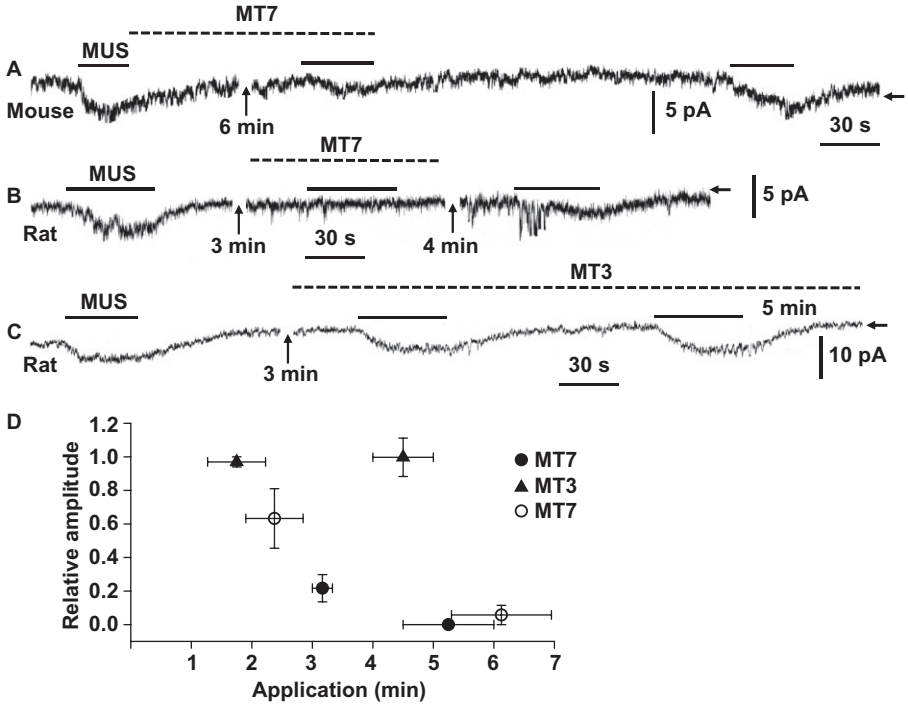

Figure 4

Suppression of muscarine (MUS)-induced currents by MT7 in mouse and rat chromaffin cells. (A) 30 μM muscarine-induced inward current is reversibly suppressed by 0.01 μM MT7 in a mouse cell. (B, C) muscarine-induced current is suppressed by MT7, but not by 0.1 μM MT3, in rat cells respectively. The whole-cell currents in A, B and C were recorded at −50, −55 and −50 mV with the perforated patch clamp method respectively. Arrows indicate zero current level. The current recordings were interrupted for the indicated times. (D) Summary of muscarine-induced currents in the presence of MT7 and MT3 in rat and MT7 in mouse chromaffin cells. Muscarine-induced currents in the presence of toxins were expressed relative to those in its absence. The data represent the mean ± SEM of four to six and four observations for MT7 and MT3 in rat, respectively, and three to four in mice.

receptors are both known to be coupled with PLC via a *Pertussis* toxin-insensitive G protein, was not clear why M_5 receptors were not able to mediate muscarine-induced secretion in these cells. One possibility is that M_1 and M_5 receptors are present in different membrane domains, and M_1, but not M_5, receptors are efficiently coupled with the signalling pathway responsible for catecholamine secretion. To address this issue, constitutively active muscarinic receptors were expressed in PC12 cells, an immortalized cell line of rat chromaffin cells (Greene and Tischler, 1976). Activation of muscarinic receptors in rat chromaffin cells results in an inhibition of TASK1 channels (Inoue *et al.*, 2008), and this inhibition is, at least in part, ascribed to internalization of channels (H. Matsuoka and M. Inoue, unpubl. obs.; see Matsuoka *et al.*, 2013). Therefore, the extent of channel internalization was quantified in PC12 cells expressing constitutively active M_1 or M_5 receptors (Figure 7). When GFP-TASK1 channels were expressed in PC12 cells, almost all of GFP-TASK1 proteins were localized at the cell periphery (Figure 7B and C), probably in the cell membrane. When GFP-TASK1 channels were expressed together with constitutively active M_1 receptors, almost all of the GFP-TASK1 proteins were present in the cytoplasm (Figure 7A and C). In contrast to M_1 receptors, expression of constitutively active M_5 receptors resulted in only partial internalization of GFP-TASK1- and half of the channels remained at the cell periphery (Figure 7A and C). This difference in internalization might have been accounted for by the expression levels of muscarinic receptors. This possibility, however, was not likely because the amounts of M_5 receptors immunologically detected did not obviously differ from those of M_1 receptors (Figure 7A). These results indicate that the low potency of constitutively active M_5 receptors to internalize TASK1 channels is more likely to be due to a poor coupling with the downstream signalling pathway involved in channel internalization than to a low expression level of these receptors.

Discussion and conclusion

Identification of muscarinic receptors

Immunocytochemical studies revealed the expression of several muscarinic receptor subtypes in rat (Harada *et al.*, 2011) and mouse chromaffin cells. In addition to M_4 and M_5 receptors that are predominantly expressed in rat chromaffin cells (Harada *et al.*, 2011), M_1-like IRs were detected in mouse cells and the majority of them were present in the cytoplasm. We do not know if there is a similar distribution of M_1-like IR in rat chromaffin cells. One of the possible causes for overlooking M_1-like IR is that these receptors are predominantly present in the cytoplasm. Because of this

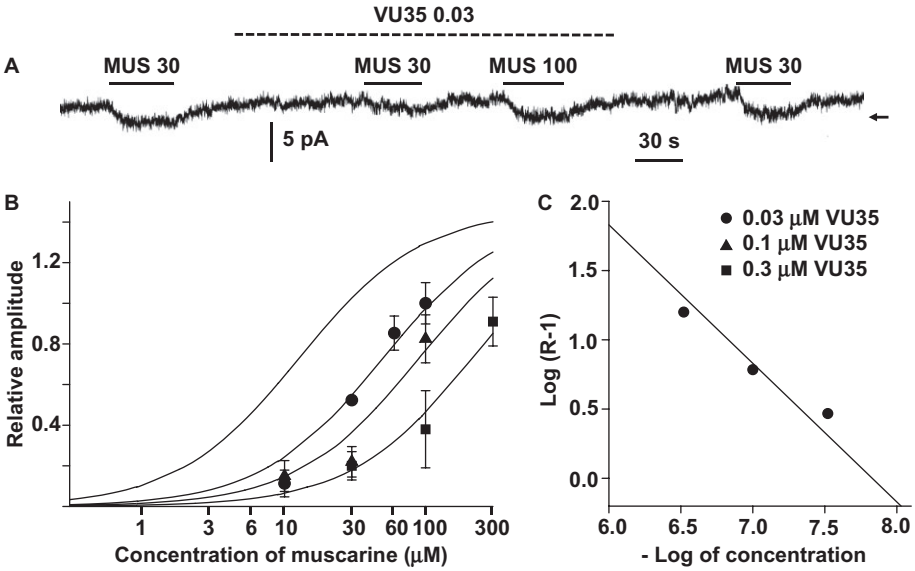

Figure 5

Effects of VU0255035 on dose-dependent production of an inward current in response to muscarine (MUS) in rat chromaffin cells. (A) Trace of whole-cell current in response to a range of concentrations of muscarine with or without 0.03 μM VU0255035. The whole-cell current was recorded at –40 mV with the perforated patch clamp method in an isolated rat chromaffin cell. MUS and VU0255035 (VU35) were applied to the bath during the indicated periods (bars for muscarine; interrupted lines for VU35). Arrow indicates zero current level. (B) Dose–response curves for muscarine-induced currents in the absence (without symbol) and the presence of 0.03, 0.1 and 0.3 μM VU35. Each point represents the mean ± SEM of four to six, three to four and five to six observations for 0.03, 0.1 and 0.3 μM VU35 respectively. The control dose–response curve is the same as that in Figure 1. The data were approximated by rectangular hyperbolae. (C) Schild plot. *R* represents the ratio of EC_{50} in the presence of VU35 to EC_{50} in the absence of VU35. Logarithms of $R - 1$ were plotted against negative logarithms of VU35 concentrations. $Y = 7.83 - x$ was fitted to data points with a least-squares method (correlation coefficient, 0.9253).

staining characteristic, it might have been thought to be non-specific. Genetic deletion of the M_1 receptor subtype resulted in a marked decrease in M_1-like IR in the cytoplasm as well as at the periphery, and M_4 and M_5-like IRs in the cell membrane also disappeared with the knockout of the M_4 and M_5 receptor genes. Therefore, immunostaining in chromaffin cells of wild-type and KO mice clearly indicate the expression of M_1, M_4 and M_5 receptor subtypes. The M_1 receptors are mainly present in the cytoplasm, whereas M_4 and M_5 receptors are at the cell periphery, probably in the cell membrane.

Muscarinic M_1 and M_5 receptors are coupled with PLC via a *Pertussis* toxin-insensitive G protein, whereas M_4 receptors are associated with adenylyl cyclase via a G_i protein. When M_1 or M_5 receptors are expressed in HEK293 cells together with M channels, stimulation of either of the receptors induces a similar extent of M channel inhibition (Guo and Schofield, 2003). Therefore, the finding that genetic deletion of the M_1 receptor alone was enough to abolish catecholamine secretion in response to muscarine was unexpected. The M_5 receptor, whose expression at the cell periphery was not impaired with the genetic deletion of M_1 receptors, was not able to substitute for the lack of M_1 receptors in terms of catecholamine secretion. Muscarine-induced secretion was not obviously affected by knockout of M_4 or M_5 receptors. However, muscarine did induce secretion in 1 of 18 chromaffin cells of the M_3KO mice. This incidence of the muscarine-induced secretion was lower than those in wild-type and M_4M_5 KO mice. One possibility for the low incidence in M_3 KO cells is that M_3 receptors play a facilitatory role for M_1 receptor-mediated secretion, as noted in guinea pig ileal smooth muscle (Zholos and Bolton, 1997). The results with the KO mice unambiguously indicate that the M_1 receptor subtype alone is responsible for muscarine-induced secretion. This notion is further supported by the abolition of muscarinic secretion in all the cells of wild-type, M_3 KO and M_4M_5 double KO mice by MT7, a specific inhibitor of M_1 receptors (Servent and Fruchart-Gaillard, 2009). The M_1 receptor is also involved in muscarinic excitation in rat chromaffin cells. Firstly, exposure to MT7, but not to MT3, even at concentrations 10 times higher, resulted in the suppression of membrane current responses to muscarine in rat cells. Secondly, the functional affinity value of VU0255035 for the inhibition of muscarine-induced currents completely accords with that against M_1 receptors obtained from binding experiments and differs by 50- to 160-fold from those against the other muscarinic receptor subtypes (Sheffler *et al.*, 2009). On the contrary, conventional antagonists were not sufficiently selective to identify muscarinic subtypes involved in muscarinic excitation.

Finally, it is worth considering why M_5 receptors cannot substitute for M_1 receptors in mediating muscarine-induced secretion in M_1 KO mice. One possible explanation would be that M_1 and M_5 receptors, which can both be coupled to PLC (Caulfield and Birdsall, 1998; Guo and Schofield, 2003), are localized in different membrane domains in chromaffin cells. This intracellular separation of receptors is possible as different receptor subtypes are known to be present at different

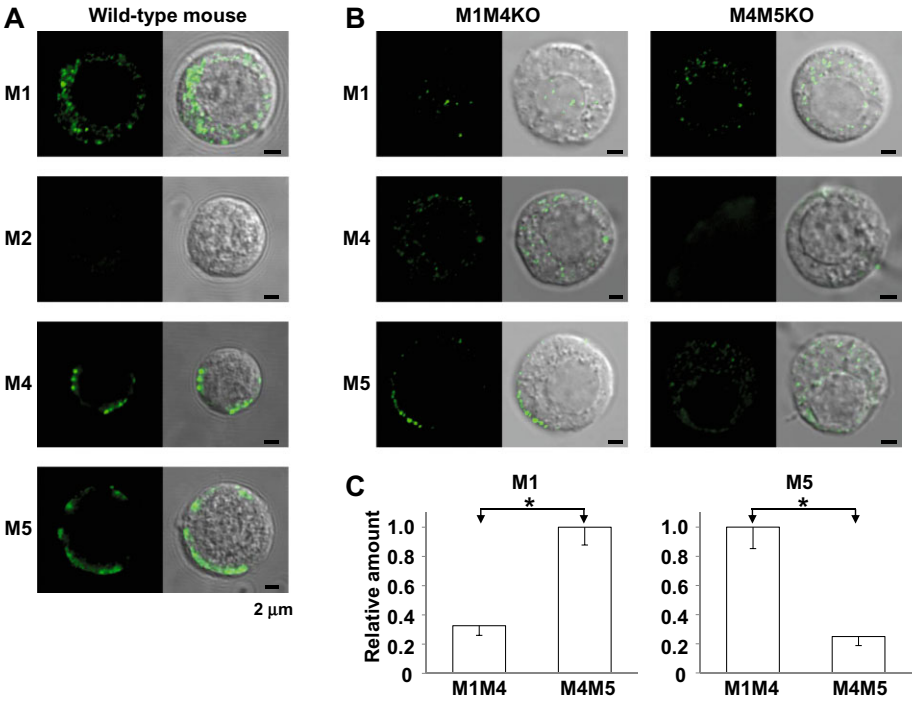

Figure 6

Immunocytochemical staining for muscarinic receptors in isolated chromaffin cells of wild-type and muscarinic receptor KO mice. (A) Immunostaining for M_1, M_2, M_4 and M_5 receptors in wild-type mice. First and second columns are confocal fluorescent images of M_1 ($n = 10$), M_2 ($n = 3$), M_4 ($n = 14$) and M_5 ($n = 9$)-like IRs and merge of fluorescent images and direct interference contrast (DIC) images. (B) Immunostaining for M_1, M_4 and M_5-like IRs in M_1M_4 and M_4M_5 double KO mice. First and second columns, confocal fluorescent images of M_1 (M_1M_4: $n = 10$; M_4M_5: $n = 11$), M_4 (M_1M_4: $n = 8$; M_4M_5: $n = 9$) and M_5 (M_4M_5: $n = 19$; M_4M_5: $n = 16$)-like IRs and merge of fluorescent images and DIC images. (C) Summary of amount of M_1- and M_5-like IRs in M_1M_4 and M_4M_5 double KO mice. Data represent the mean ± SEM. *$P < 0.001$, significantly different as indicated.

membrane sites in the same cell. Thus, β_1-adrenoceptors in rat cardiac myocytes were exclusively detected in raft membrane fractions, whereas β_2-adrenoceptors were present in raft and non-raft fractions (Rybin *et al.*, 2000). Furthermore, although stimulation of M_3 receptors endogenously present in HEK293 cells induced Ca^{2+} mobilization from store sites, it failed to suppress exogenously expressed M channels (Oldfield *et al.*, 2009). The activation of M_1 receptors expressed exogenously in the cells, however, resulted in M channel inhibition (Oldfield *et al.*, 2009). Fractionation analyses indicated that both M_1 receptors and M channels were enriched in raft membranes, which are known to be a platform for various forms of neurotransmitter signalling (Allen *et al.*, 2007). Based upon these results, the failure of endogenous M_3 receptors to inhibit M channels in HEK293 cells was assumed to be due to their presence in non-raft membranes. Similar arguments might apply to the findings that M_1, but not M_5, receptors were involved in catecholamine secretion, although both were expressed at the cell periphery. The majority of M_1-like immunoreactions in mouse chromaffin cells were detected in the cytoplasm, but some of them were present at the cell periphery, probably the cell membrane. Because of the suppression of the catecholamine secretion and membrane current responses by the peptide inhibitor MT7, this minority of M_1 receptors present at the cell membrane may be involved in the signalling responses to muscarinic agonists.

Phylogeny

It is worth considering the phylogeny of chromaffin cells in terms of receptors and ion channels as they are important traits mediating cellular functions. Catecholamine secretion from adrenal chromaffin cells in lower vertebrates, such as agnathans, is not under neuronal control, and with advancement in phylogeny, neuronal regulation of secretion becomes increasingly important (Perry and Capalda, 2011). Nerve terminals on chromaffin cells in archaic frogs are infrequent but become more abundant in advanced frogs, such as *Rana* (Milano and Accordi, 1983). In the adrenal chromaffin cells of the toad, ACh induces catecholamine secretion via nAChRs and not via muscarinic receptors (Nassar-Gentina *et al.*, 1991). On the contrary, muscarinic stimulation results in the generation of slow EPSPs by inhibiting M channels in the sympathetic ganglion cells of such frogs (Brown and Adams, 1980; Jan and Jan, 1982). In birds (Knight and Baker, 1986) and mammals (Wakade and Wakade, 1983), stimulation of muscarinic receptors in chromaffin cells induced catecholamine secretion. This phylogenic difference in nAChR- and muscarinic receptor-mediated secretion is reproduced in the ontogeny of chromaffin cells in rats (Oomori *et al.*, 1998).

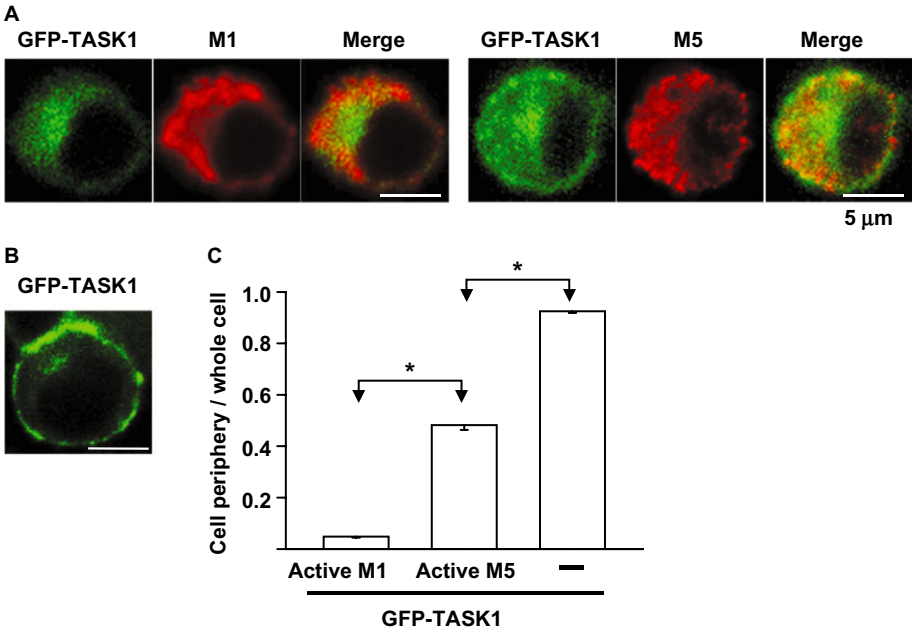

Figure 7

Effects of constitutively active muscarinic receptors on the distribution of GFP-TASK1 channels in PC12 cells. (A) Confocal fluorescent images of GFP-TASK1 and M_1- or M_5-like IRs in PC12 cells. PC12 cells were transfected with expression vectors for GFP-TASK1 and constitutively active M_1 or M_5. First, second and third columns, green fluorescence for GFP-TASK1, red fluorescence for M_1 or M_5 and merge of the first and the second. (B) Confocal fluorescent image of GFP-TASK1 in a PC12 cell. Green fluorescence. (C) Summary of the extents of GFP-TASK1 internalization in cells with or without (–) expression of constitutively active M_1 or M_5 receptors. The amounts of GFP-TASK1 at the cell periphery and in the cytoplasm were separately measured using Metamorph, and then the extent of channel internalization was expressed as fractions of GFP-TASK1 at the cell periphery to the total of GFP-TASK1 at the cell periphery and in the cytoplasm. Data represent the mean ± SEM of $n = 21$, $n = 24$ and $n = 5$ for active M_1, active M_5 and (–), respectively. *$P < 0.001$, significantly different as indicated.

Embryonic rat chromaffin cells, just before birth, exhibited an increase in intracellular Ca^{2+} concentration in response to stimulation of nAChRs, but not of muscarinic receptors, whereas cells after birth responded to muscarinic receptor stimulation as well. Muscarinic receptors are responsible for part of neuronal transmission in guinea pig chromaffin cells (Inoue *et al.*, 2012; Warashina and Inoue, 2012). Taken together with phylogenic and ontogenic considerations, this result suggests that muscarinic signalling in chromaffin cells develops to help animals cope with life-threatening stress. As has been elegantly demonstrated in canine sympathetic ganglion cells (Brown, 1967), muscarinic receptors may increase reliability in neuronal transmission under severely stressful conditions where nAChR-mediated transmission would be expected to be markedly impaired due to desensitization.

The different appearances of muscarinic receptors in sympathetic ganglion cells and adrenal chromaffin cells in phylogeny suggest that adrenal chromaffin cells may not differentiate from sympathetic ganglion cells. Rather, chromaffin cells and sympathetic ganglion cells originate from two distinct cells that are separated from a common cell at an early stage of development (Huber, 2006). This notion would be supported by the fact that the resting membrane potential in rat sympathetic ganglion cells (Lamas *et al.*, 2002; Brown, 2013) and chromaffin cells (Inoue *et al.*, 2008) is at least in part contributed by M channels and TASK channels respectively. Thus, it would be interesting that the M_1 receptor alone is responsible for muscarinic excitation in both chromaffin cells and sympathetic ganglion neurons (Bernheim *et al.*, 1992; Hamilton *et al.*, 1997).

In summary, although M_1, M_4 and M_5 receptors were expressed in mouse chromaffin cells, only the M_1 receptors were involved in muscarine-induced excitation, with consequent secretion of catecholamines.

Acknowledgements

This work was supported in part by JSPS KAKENHI (25460306 to K. H. and 24790228 to H. M.). VU0255035 was a gift from Jeffrey Conn (Vanderbilt Center for Neuroscience Drug Discovery, Nashville, TN, USA). Thanks are also due to K. Mizuno (Tohoku University, Sendai, Japan) for providing PC12 cells. We are grateful to Takeshi Asakawa (Information Administration Center UOEH) for consultation about statistics and Kyoko Tashiro for the technical assistance.

Author contributions

M. I., K. H. and H. M. performed experiments; M. I., K. H. and H. M. analysed data; M. M. and H. M. made and maintained KO mice; M. I., K. H. and H. M. were involved in conception and design of research; M. I. wrote the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

Adams PR, Brown DA, Constanti A (1982). Pharmacological inhibition of the M-current. J Physiol 332: 223–262.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al*. (2013a). The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors. Br J Pharmacol 170: 1459–1581.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Catterall WA *et al*. (2013b). The Concise Guide to PHARMACOLOGY 2013/14: Ion Channels. Br J Pharmacol 170: 1607–1651.

Allen JA, Halverson-Tamboli RA, Rasenick MM (2007). Lipid raft microdomains and neurotransmitter signalling. Nat Rev Neurosci 8: 128–140.

Arunlakshana O, Schild HO (1959). Some quantitative uses of drug antagonists. Br J Pharmacol Chemother 14: 48–58.

Asano T, Ohta T, Otsuguro K-I, Ito S, Nakazato Y (1995). Muscarinic receptor subtypes mediating catecholamine secretion and increase in intracellular Ca^{2+} concentration in adrenal chromaffin cells of the guinea pig. J Vet Med Sci 57: 1067–1071.

Ballesta JJ, Borges R, García AG, Hidalgo MJ (1989). Secretory and radioligand binding studies on muscarinic receptors in bovine and feline chromaffin cells. J Physiol 418: 411–426.

Bernheim L, Mathie A, Hille B (1992). Characterization of muscarinic receptor subtypes inhibiting Ca^{2+} current and M current in rat sympathetic neurons. Proc Natl Acad Sci U S A 89: 9544–9548.

Böhme TM, Augelli-Szafran CE, Hallak H, Pugsley T, Serpa K, Schwarz RD (2002). Synthesis and pharmacology of benzoxazines as highly selective antagonists at M_4 muscarinic receptors. J Med Chem 45: 3094–3102.

Brown AM (1967). Cardiac sympathetic adrenergic pathways in which synaptic transmission is blocked by atropine sulphate. J Physiol 191: 271–288.

Brown DA (2013). The other (muscarinic) acetylcholine receptors in sympathetic ganglia: actions and mechanisms. Neurophysiology 45: 60–66.

Brown DA, Adams PR (1980). Muscarinic suppression of a novel voltage-sensitive K^+ current in a vertebrate neurone. Nature 283: 673–676.

Brown DA, Passmore GM (2009). Neural *KCNQ* (Kv7) channels. Br J Pharmacol 156: 1185–1195.

Caulfield M, Birdsall NJM (1998). International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50: 279–290.

De Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T (2000). International Union of Pharmacology. XXIII. The angiotensin II receptors. Pharmacol Rev 52: 415–472.

Donoghue PC, Graham A, Kelsh RN (2008). The origin and evolution of the neural crest. Bioessays 30: 530–541.

Dörje F, Wess J, Lambrecht G, Tacke R, Mutshler E, Brann MR (1991). Antagonist binding profiles of five cloned human muscarinic receptor subtypes. J Pharmacol Exp Ther 256: 727–733.

Greene LA, Tischler AS (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci U S A 73: 2424–2428.

Guo J, Schofield GG (2003). Activation of muscarinic m5 receptors inhibits recombinant KCNQ2/KCNQ3 K^+ channels expressed in HEK293T cells. Eur J Pharmacol 462: 25–32.

Hamilton SE, Loose MD, Qi M, Levey A, Hille B, McKnight GS *et al*. (1997). Disruption of the m1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. Proc Natl Acad Sci U S A 94: 13311–13316.

Harada K, Matsuoka H, Sata T, Warashina A, Inoue M (2011). Identification and role of muscarinic receptor subtypes expressed in rat adrenal medullary cells. J Pharmacol Sci 117: 253–264.

Huber K (2006). The sympathoadrenal cell lineage: specification, diversification, and new perspectives. Dev Biol 298: 335–343.

Inoue M, Imanaga I (1995). Mechanism of activation of nonselective cation channels by putative M_4 muscarinic receptor in guinea-pig chromaffin cells. Br J Pharmacol 114: 419–427.

Inoue M, Fujishiro N, Imanaga I (1998). Hypoxia and cyanide induce depolarization and catecholamine release in dispersed guinea-pig chromaffin cells. J Physiol 507: 807–818.

Inoue M, Fujishiro N, Ogawa K, Muroi M, Sakamoto Y, Imanaga I *et al*. (2000). Pituitary adenylate cyclase-activating polypeptide may function as a neuromodulator in guinea-pig adrenal medulla. J Physiol 528: 473–487.

Inoue M, Harada K, Matsuoka H, Sata T, Warashina A (2008). Inhibition of TASK1-like channels by muscarinic receptor stimulation in rat adrenal medullary cells. J Neurochem 106: 1804–1814.

Inoue M, Harada K, Matsuoka H, Nakamura J, Warashina A (2012). Mechanisms and roles of muscarinic activation in guinea-pig adrenal medullary cells. Am J Physiol Cell Physiol 303: C635–C644.

Jan LY, Jan YN (1982). Peptidergic transmission in sympathetic ganglia of the frog. J Physiol 327: 219–246.

Kajiwara R, Sand O, Kidokoro Y, Barish ME, Iijima T (1997). Functional organization of chromaffin cells and cholinergic synaptic transmission in rat adrenal medulla. Jpn J Physiol 47: 449–464.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Knight DE, Baker PF (1986). Observations on the muscarinic activation of catecholamine secretion in the chicken adrenal. Neuroscience 19: 357–366.

Krajewski JL, Dickerson IM, Potter LT (2001). Site-directed mutagenesis of m1-Toxin1: two amino acids responsible for stable toxin binding to M_1 muscarinic receptors. Mol Pharmacol 60: 725–731.

Lamas JA, Reboreda A, Codesido V (2002). Ionic basis of the resting membrane potential in cultured rat sympathetic neurons. Neuroreport 13: 585–591.

Matsui M, Yamada S, Oki T, Manabe T, Taketo MM, Ehlert FJ (2004). Functional analysis of muscarinic acetylcholine receptors using knockout mice. Life Sci 75: 2971–2981.

Matsuoka H, Harada K, Nakamura J, Inoue M (2013). Nerve growth factor-induced endocytosis of TWIK-related acid-sensitive K^+ 1 channels in adrenal medullary cells and PC12 cells. Pflügers Arch 465: 1051–1064.

McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Milano EG, Accordi F (1983). Comparative morphology of the adrenal gland of anuran Amphibia. J Anat 136: 165–174.

Nassar-Gentina V, Luxoro M, Urbina N (1991). Cholinergic receptors and catecholamine secretion from adrenal chromaffin cells of the toad. Comp Biochem Physiol 100C: 495–500.

Oldfield S, Hancock J, Mason A, Hobson SA, Wynick D, Kelly E *et al*. (2009). Receptor-mediated suppression of potassium currents requires colocalization within lipid rafts. Mol Pharmacol 76: 1279–1289.

Olivos L, Artalejo AR (2008). Muscarinic excitation-secretion coupling in chromaffin cells. Acta Physiol 192: 213–220.

Oomori Y, Habara Y, Kanno T (1998). Muscarinic and nicotinic receptor-mediated Ca^{2+} dynamics in rat adrenal chromaffin cells during development. Cell Tissue Res 294: 109–123.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al.*; NC-IUPHAR (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. Nucl. Acids Res 42 (Database Issue): D1098–1106.

Perry SF, Capalda A (2011). The autonomic nervous system and chromaffin tissue: neuroendocrine regulation of catecholamine secretion in non-mammalian vertebrates. Auton Neurosci 165: 54–66.

Richards MH, van Giersbergen PL (1995). Human muscarinic receptors expressed in A9L and CHO cells: activation by full and partial agonists. Br J Pharmacol 114: 1241–1249.

Rybin VO, Xu X, Lisanti MP, Steinberg SF (2000). Differential targeting of β-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae: a mechanism to functionally regulate the cAMP signaling pathway. J Biol Chem 275: 41447–41457.

Servent D, Fruchart-Gaillard C (2009). Muscarinic toxins: tools for the study of the pharmacological and functional properties of muscarinic receptors. J Neurochem 109: 1193–1202.

Sheffler DJ, Williams R, Bridges TM, Xiang Z, Kane AS, Byun NE *et al.* (2009). A novel selective muscarinic acetylcholine receptor subtype 1 antagonist reduces seizures without impairing hippocampus-dependent learning. Mol Pharmacol 76: 358–368.

Teschemacher AG, Seward EP (2000). Bidirectional modulation of exocytosis by angiotensin II involves multiple G-protein-regulated transduction pathways in chromaffin cells. J Neurosci 20: 4776–4785.

Tobin JR, Breslow MJ, Traystman RJ (1992). Muscarinic cholinergic receptors in canine adrenal gland. Am J Physiol 263: H1208–H1212.

Wakade AR, Wakade TD (1983). Contribution of nicotinic and muscarinic receptors in the secretion of catecholamines evoked by endogenous and exogenous acetylcholine. Neuroscience 10: 973–978.

Warashina A, Inoue M (2012). Ca^{2+} imaging in perfused adrenal medullae. J UOEH 34: 163–173.

Zholos AV, Bolton TB (1997). Muscarinic receptor subtypes controlling the cationic current in guinea-pig ileal smooth muscle. Br J Pharmacol 122: 885–893.

Zhou C, Huang Z, Ding L, Deel ME, Arain FM, Murray CR *et al.* (2013). Altered cortical $GABA_A$ receptor composition, physiology, and endocytosis in a mouse model of a human genetic absence epilepsy syndrome. J Biol Chem 288: 21458–21472.