British Journal of Pharmacology (2005) 145, 1153–1159
www.nature.com/bjp

Distinct mixtures of muscarinic receptor subtypes mediate inhibition of noradrenaline release in different mouse peripheral tissues, as studied with receptor knockout mice

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1 The muscarinic heteroreceptors modulating noradrenaline release in atria, urinary bladder and vas deferens were previously studied in mice in which the M_2 or the M_4 muscarinic receptor genes had been disrupted. These experiments showed that these tissues possessed both M_2 and non-M_2 heteroreceptors. The analysis was now extended to mice in which either the M_3, both the M_2 and the M_3, or both the M_2 and the M_4 genes had been disrupted (M_3-knockout, $M_{2/3}$-knockout and $M_{2/4}$-knockout). Tissues were preincubated with ^{3}H-noradrenaline and then stimulated electrically (20 pulses per 50 Hz).

2 In wild-type atria, carbachol (0.01–100 μM) decreased the electrically evoked tritium overflow by maximally 60–78%. The maximum inhibition of carbachol was reduced to 57% in M_3-knockout and to 23% in $M_{2/4}$-knockout atria. Strikingly, the effect of carbachol was abolished in $M_{2/3}$-knockout atria.

3 In wild-type bladder, carbachol (0.01–100 μM) reduced the evoked tritium overflow by maximally 57–71%. This effect remained unchanged in the M_3-knockout, but was abolished in the $M_{2/4}$-knockout bladder.

4 In wild-type vas deferens, carbachol (0.01–100 μM) reduced the evoked tritium overflow by maximally 34–48%. The maximum inhibition of carbachol was reduced to 40% in the M_3-knockout and to 18% in the $M_{2/4}$-knockout vas deferens.

5 We conclude that the postganglionic sympathetic axons of mouse atria possess M_2 and M_3, those of the urinary bladder M_2 and M_4, and those of the vas deferens M_2, M_3 and M_4 release-inhibiting muscarinic receptors.
British Journal of Pharmacology (2005) **145,** 1153–1159. doi:10.1038/sj.bjp.0706297;
published online 20 June 2005

Keywords: Presynaptic muscarinic receptors; sympathetic axon terminals; ^{3}H-noradrenaline; M_3-knockout; $M_{2/3}$-knockout; $M_{2/4}$-knockout; mouse atria; mouse urinary bladder; mouse vas deferens; heterogeneity

Abbreviations: PCR, polymerase chain reaction

Introduction

Ever since the discovery of the M_1–M_5 muscarinic receptor subtypes, their role in the function of specific cells has been the subject of intense research. Among these cells are postganglionic sympathetic neurons where presynaptic muscarinic receptors (often called muscarinic heteroreceptors) either facilitate or inhibit action potential-evoked release of noradrenaline (see Fuder & Muscholl, 1995). Initial studies based on the use of antagonists endowed with a limited degree of receptor subtype selectivity suggested that the noradrenaline release-facilitating receptors may represent M_1 receptors (Fuder & Muscholl, 1995). The more prominent muscarinic receptors mediating inhibition of transmitter release, however, often escaped clear identification, although M_2 receptors seemed to play a predominant role (Fuder & Muscholl, 1995). The main reason for this uncertainty was the lack of muscarinic agonists or antagonists endowed with a high degree of receptor subtype selectivity (Caulfield & Birdsall, 1998; Wess, 2004).

Genetically engineered mice in which specific muscarinic receptor genes (M_1–M_5) have been disrupted represent valuable novel tools for identifying the muscarinic receptor subtype(s) involved in specific physiological functions (see Wess, 2004). We have recently used a set of muscarinic receptor mutant mice to identify the presynaptic muscarinic heteroreceptors present on postganglionic sympathetic neurons (Trendelenburg *et al*., 2003a). Using mice that lacked either the M_2 or the M_4 receptor gene (M_2-knockout and M_4-knockout, respectively), we found that the muscarinic heteroreceptors displayed an unexpected heterogeneity in the heart atria, urinary bladder and vas deferens. In all the three tissues, the sympathetic axons possessed both M_2 and non-M_2 release-inhibiting muscarinic receptors. The non-M_2 heteroreceptors in the atria and bladder remained unknown, whereas those in

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the vas deferens were tentatively identified as M_4 (Trendelenburg *et al*., 2003a).

We have now extended this analysis to mice lacking either M_3 (M_3-knockout; Yamada *et al*., 2001), both M_2 and M_3 ($M_{2/3}$-knockout; Struckmann *et al*., 2003) or both M_2 and M_4 muscarinic receptors ($M_{2/4}$-knockout; Zhang *et al*., 2002a). The new findings derived from the present study allow a more accurate identification of the muscarinic heteroreceptors involved in inhibiting noradrenaline release in different peripheral tissues of the mouse.

Methods

Tissues and superfusion

The generation of M_3-knockout mice (genetic background: 129SvEv × CF1) as well as $M_{2/3}$- and $M_{2/4}$-knockout mice (genetic background: 129J1 × 129SvEv × CF1) has been described previously (Yamada *et al*., 2001; Zhang *et al*., 2002a; Struckmann *et al*., 2003). Age- and sex-matched wild-type mice of the corresponding genetic background were used as controls. Genotyping was carried out by PCR analysis of mouse tail DNA. The mice (male) were killed by cervical dislocation when aged >2 months. From each animal 6–8 pieces of the atria, 12–15 pieces of the urinary bladder or 8–12 pieces of the vas deferens were obtained. Tissue pieces were preincubated in 1 ml medium (see below) containing 0.2 μM ^{3}H-noradrenaline for 30 min at 37°C and then placed in 12 superfusion chambers between platinum electrodes, one piece per chamber, where they were superfused with ^{3}H-noradrenaline-free medium at a rate of 1.2 ml min^{-1}. Successive 2-min samples of the superfusate were collected from $t = 50$ min onwards ($t = 0$ min being the start of superfusion). At the end of experiments, tissues were dissolved and tritium was determined in superfusate samples and tissues.

The superfusion medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03 and desipramine 0.001. The medium for preincubation with ^{3}H-noradrenaline contained no desipramine and only 0.2 mM $CaCl_2$ (Limberger *et al*., 1992).

Protocol

There were seven periods of electrical stimulation. Each stimulation period consisted of rectangular pulses of 1 ms width and 47 V cm^{-1} voltage drop between the electrodes of each chamber, yielding a current strength of 80 mA. The first stimulation period (180 pulses at 3 Hz) was delivered at $t = 30$ min and was not used for determination of tritium overflow. The subsequent stimulation periods (S_1–S_6) were applied at $t = 54$, 72, 90, 108, 126 and 144 min and consisted of 20 pulses at 50 Hz. Carbachol was introduced at increasing concentrations after S_1, 12 min before S_2, S_3, S_4, S_5 and S_6. The muscarinic receptor antagonists methoctramine and pirenzepine were present throughout superfusion at a fixed concentration (M_3-knockout vas deferens only).

Evaluation

The outflow of tritium was calculated as a fraction of the tritium content of the tissue at the onset of the respective collection period (fractional rate; min^{-1}). The overflow elicited by electrical stimulation was calculated as the difference 'total tritium outflow during and after stimulation' minus 'basal outflow', and was then expressed as a percentage of the tritium content of the tissue at the time of stimulation (see Trendelenburg *et al*., 1997).

For further evaluation, S_n/S_1 overflow ratios were calculated. Overflow ratios obtained in the presence of carbachol, added after S_1, were also calculated as a percentage of the corresponding ratio in controls in which no drug was added after S_1. Effects of drugs on basal tritium outflow were evaluated similarly (Trendelenburg *et al*., 1997).

Concentration–response data for carbachol given alone were evaluated by sigmoid curve fitting (Equation (25) of Waud, 1976). This yielded the E_{max} (maximal effect) of carbachol and its EC_{50} (concentration causing a half-maximal effect) in the absence of antagonist. Sigmoids could not be fitted to concentration–response data for carbachol in the presence of antagonists (M_3-knockout vas deferens). Therefore, for determination of apparent antagonist pK_d values (negative logarithms of the apparent K_d), carbachol EC_{50} values in the presence of antagonist were interpolated from the nearest points of the respective concentration–response curves, assuming that the E_{max} of the agonist had not changed; the pK_d value was then calculated from the EC_{50} increase (Equation (4) of Furchgott, 1972). The pK_d values are apparent because only one antagonist concentration was used and the competitive character of the interaction was not verified.

Results are expressed as arithmetic means ± s.e.m. (estimates ± s.e. defined by Waud (1976), in the case of E_{max} values of carbachol). Groups were tested for significant differences with the Mann–Whitney test with Bonferroni correction. $P < 0.05$ was taken the as limit of statistical difference. n represents the number of tissue pieces.

Drugs

The following drugs were used: (−)-[ring-2,5,6-^{3}H]noradrenaline, specific activity 51.8–70.7 Ci mmol^{-1} (NEN, Köln, Germany), carbachol chloride, desipramine HCl, methoctramine 4 HCl and pirenzepine 2 HCl (Sigma, Deisenhofen, Germany). Drugs were dissolved in distilled water.

Results

In a previous study, we used M_2 and M_4 receptor single-knockout mice to characterize the presynaptic muscarinic heteroreceptors on sympathetic nerve endings of segments from atria, urinary bladder and vas deferens (Trendelenburg *et al*., 2003a). In the present study, we extended this analysis by carrying out analogous experiments with atria, urinary bladders and vasa deferentia from M_3- and $M_{2/4}$-knockout mice as well as atria from $M_{2/3}$-knockout mice and their corresponding wild-type controls. Vesicular noradrenaline stores were labelled with ^{3}H-noradrenaline. Short bursts of 20 pulses at 50 Hz were used for stimulation, conditions with little, if any, development of α_2-autoinhibition and optimal for the characterization of presynaptic heteroreceptors (see Starke, 1987; Schlicker & Göthert, 1998; Trendelenburg *et al*., 2003a). In the present study, both basal efflux and evoked tritium overflow (S_1) were similar to values obtained previously in

NMRI, M_2-knockout, M_4-knockout and their corresponding wild-type control mice (data not shown; Wahl *et al*., 1996; Trendelenburg *et al*., 1999; 2003a). In control experiments without carbachol the overflow peaks were similar from S_1 to S_6, giving S_n/S_1 ratios close to unity (data not shown; compare Wahl *et al*., 1996; Trendelenburg *et al*., 1999; 2003a). The antagonists pirenzepine and methoctramine, when present throughout superfusion, did not change the stimulation-evoked overflow of tritium (S_1; M_3-knockout vas deferens). Neither carbachol nor the antagonists had any effect on basal tritium outflow (data not shown).

Carbachol reduced the evoked overflow of tritium with very similar concentration–response curves in atria from M_3-wild-type, $M_{2/4}$-wild-type and $M_{2/3}$-wild-type mice (Figure 1), and these curves were also very similar to our previous curves in atria from NMRI, M_2-wild-type and M_4-wild-type mice (Trendelenburg *et al*., 2003a) with values from 60 to 78% inhibition of transmitter release (Trendelenburg *et al*. (2003a) and present study taken together). The same was true for urinary bladders (E_{max} values from 57 to 71% inhibition) and vasa deferentia (E_{max} values from 34 to 48% inhibition; Figures 2 and 3; Trendelenburg *et al*., 2003a).

Atria

We showed previously that deletion of the M_4 receptor had no significant effect on the release-inhibitory activity of carbachol in mouse atria, whereas deletion of the M_2 receptor reduced the maximum inhibitory effect of carbachol from 68 to 26%, indicating the coexistence of predominant M_2 and non-M_2 muscarinic heteroreceptors in mouse atria (Trendelenburg *et al*., 2003a). In the present study, combined knockout of both the M_2 and the M_4 genes ($M_{2/4}$-knockout) attenuated the maximum inhibition by carbachol from $69\pm3\%$ ($M_{2/4}$-wild type) to $23\pm4\%$ ($M_{2/4}$-knockout; Figure 1b), an effect similar to that observed after disruption of the M_2 receptor gene alone. Deletion of the M_3 receptor also attenuated the maximum inhibitory effect of carbachol from $76\pm9\%$ (M_3-wild type) to $57\pm4\%$ (M_3-knockout; Figure 1a). Strikingly, deletion of both the M_2 and M_3 receptor genes ($M_{2/3}$-knockout) abolished the release-inhibiting effect of carbachol (Figure 1c), indicating that the non-M_2 presynaptic muscarinic heteroreceptors present in mouse atria represent M_3 receptors.

Urinary bladder

We demonstrated previously that deletion of the M_4 receptor had no significant effect on the release-inhibiting effects of carbachol in mouse urinary bladder segments, whereas deletion of the M_2 receptor reduced the maximum inhibitory effect of carbachol from 69 to 19%, indicating the coexistence of predominant M_2 and non-M_2 muscarinic heteroreceptors on

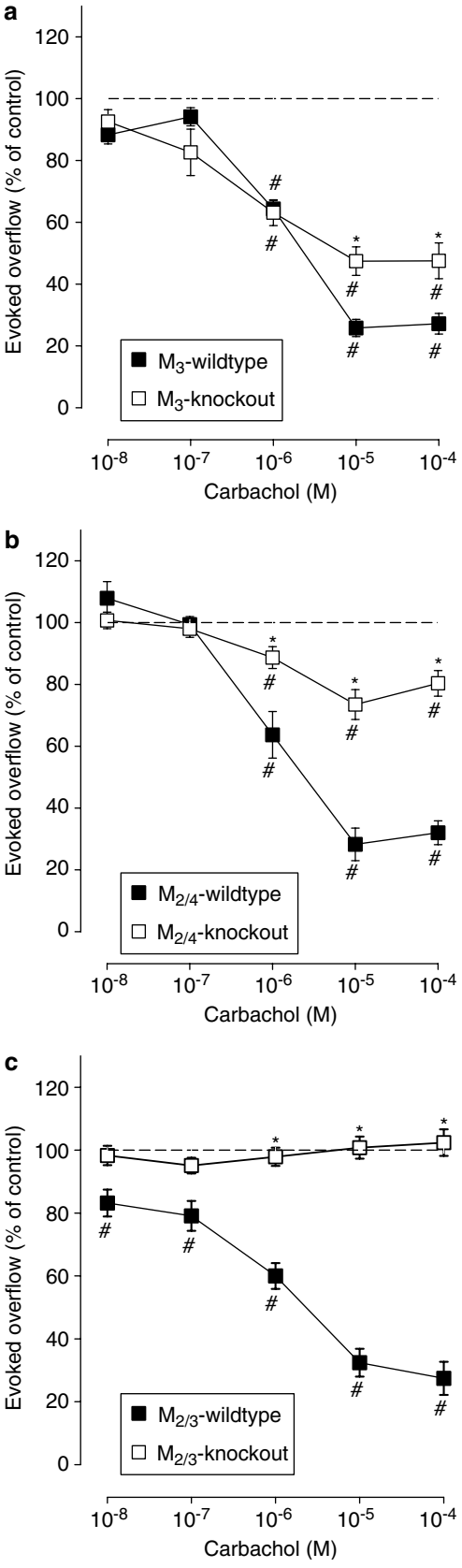

Figure 1 Effect of carbachol on the evoked overflow of tritium from atria of M_3-wild-type or M_3-knockout (a), $M_{2/4}$-wild-type or $M_{2/4}$-knockout (b), and $M_{2/3}$-wild-type or $M_{2/3}$-knockout (c) mice. After preincubation with ^{3}H-noradrenaline, tissues were superfused and stimulated six times by 20 pulses at 50 Hz (S_1–S_6). Carbachol was added at increasing concentrations (abscissae) before S_2–S_6. Ordinates, evoked overflow of tritium, calculated from S_n/S_1 ratios and expressed as a percentage of the corresponding control (no carbachol). Means$\pm$s.e.m. from $n=6$–14 tissue pieces. Significant differences from corresponding control (no carbachol): $^{\#}P<0.05$. Significant differences from corresponding wild type: $^{*}P<0.05$.

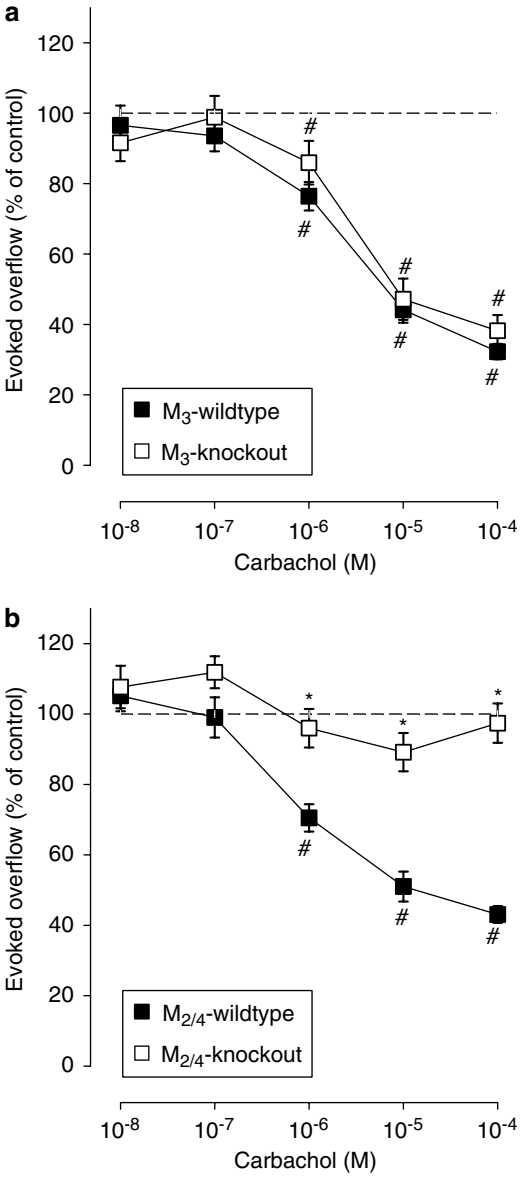

Figure 2 Effect of carbachol on the evoked overflow of tritium from urinary bladder of M_3-wild-type or M_3-knockout (a) and $M_{2/4}$-wild-type or $M_{2/4}$-knockout (b) mice. After preincubation with ^{3}H-noradrenaline, tissues were superfused and stimulated six times by 20 pulses at 50 Hz (S_1–S_6). Carbachol was added at increasing concentrations (abscissae) before S_2–S_6. Ordinates, evoked overflow of tritium, calculated from S_n/S_1 ratios and expressed as a percentage of the corresponding control (no carbachol). Means±s.e.m. from $n=9$–14 tissue pieces. Significant differences from corresponding control (no carbachol): $^{\#}P<0.05$. Significant differences from corresponding wild type: $^{*}P<0.05$.

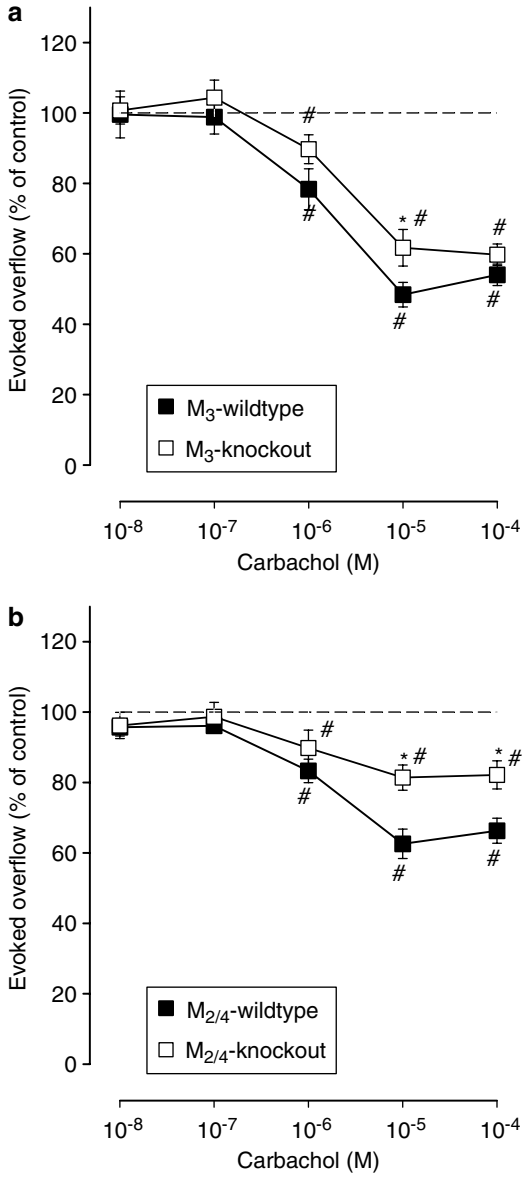

Figure 3 Effect of carbachol on the evoked overflow of tritium from vas deferens of M_3-wild-type or M_3-knockout (a) and $M_{2/4}$-wild-type or $M_{2/4}$-knockout (b) mice. After preincubation with ^{3}H-noradrenaline, tissues were superfused and stimulated six times by 20 pulses at 50 Hz (S_1–S_6). Carbachol was added at increasing concentrations (abscissae) before S_2–S_6. Ordinates, evoked overflow of tritium, calculated from S_n/S_1 ratios and expressed as a percentage of the corresponding control (no carbachol). Means±s.e.m. from $n=6$–15 tissue pieces. Significant differences from corresponding control (no carbachol): $^{*}P<0.05$. Significant differences from corresponding wild type: $^{\#}P<0.05$.

sympathetic nerve endings in the urinary bladder (Trendelenburg *et al*., 2003a). In the present study, disruption of the M_3 receptor gene had no significant effect on the release-inhibitory activity of carbachol in the urinary bladder (Figure 2a). In contrast, combined deletion of the M_4 and M_2 receptors abolished this activity. As shown in Figure 2b, no carbachol-mediated inhibition of transmitter release was observed in bladder preparations from $M_{2/4}$-knockout mice, indicating that the non-M_2 muscarinic heteroreceptors present in this tissue represent M_4 receptors.

Vas deferens

As shown previously, deletion of the M_4 receptor reduced the maximum inhibitory effect of carbachol on transmitter release from 46 to 33%, and deletion of the M_2 receptor decreased this effect from 34 to 20% inhibition, indicating the coexistence of M_2 and M_4 heteroreceptors on sympathetic nerve terminal in the mouse vas deferens (Trendelenburg *et al*., 2003a). In the present study, combined knockout of both the M_2 and M_4 receptor genes attenuated the maximum inhibition

by carbachol from 37±5% ($M_{2/4}$-wild type) to 18±2% ($M_{2/4}$-knockout; Figure 3b), an effect similar to that seen after disruption of the M_2 or M_4 receptor genes alone. The carbachol-mediated inhibition remaining in the $M_{2/4}$-knockout preparations is consistent with the presence of at least one additional muscarinic heteroreceptor in the mouse vas deferens. Interestingly, deletion of the M_3 receptor also attenuated the maximum inhibitory response of carbachol in the vas deferens, from 48±2% (M_3-wild type) to 40±2% (M_3-knockout; Figure 3a), suggesting that M_3 receptors, in addition to the M_2 and M_4 receptor subtypes, may function as muscarinic heteroreceptors in the mouse vas deferens.

To further test the hypothesis that M_3 heteroreceptors are present on sympathetic nerves innervating the mouse vas deferens, we carried out additional experiments using the two muscarinic antagonists, methoctramine and pirenzepine. A previous study using vas deferens preparations from NMRI, M_2-wild-type and M_4-wild-type mice showed that carbachol-mediated inhibition of noradrenaline release was antagonized with a potency order pirenzepine (pK_d 6.3–6.8) $\geqslant$methoctramine ($pK_d < 6$–6.1), which is the characteristic affinity profile of these two antagonists at M_3, M_4 and M_5 receptors (see Introduction of Trendelenburg *et al*., 2003a). In vas deferens preparations from M_4-knockout mice, this order was reversed to methoctramine (pK_d 7.2) >pirenzepine ($pK_d < 6$), a pattern characteristic for M_2 receptors (see Introduction of Trendelenburg *et al*., 2003a), suggesting that the pharmacology of the mixed population of muscarinic heteroreceptors present in the vas deferens of wild-type mice is dominated by the presence of M_4 receptors. In the present study, deletion of the M_3 receptor resulted in an antagonist affinity order similar to that observed with vas deferens preparations from M_4-knockout mice (methoctramine (pK_d 7.8)>pirenzepine (pK_d 6.4)) (Figure 4), indicating the presence of M_3 heteroreceptors which, like the M_4 receptors, also appear to be required for the observation that the mixed population of muscarinic heteroreceptors present in the vas deferens of wild-type mice displays an $M_{3/4/5}$ pharmacology.

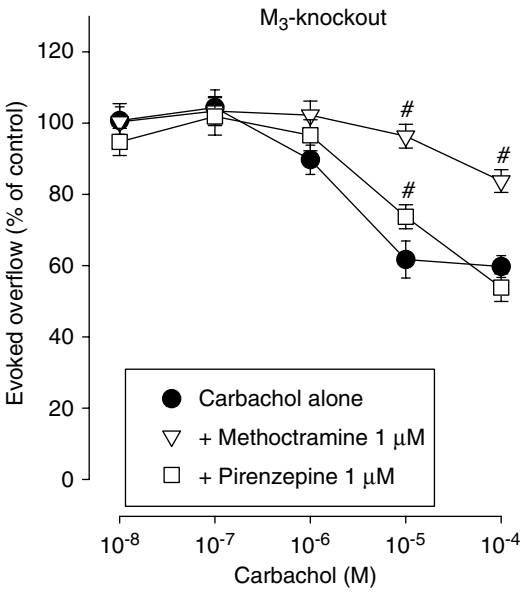

Figure 4 Interaction of the muscarinic antagonists methoctramine and pirenzepine with carbachol on the evoked overflow of tritium from vas deferens of M_3-knockout mice. After preincubation with ^{3}H-noradrenaline, tissues were superfused and stimulated six times by 20 pulses at 50 Hz (S_1–S_6). Carbachol was added at increasing concentrations (abscissae) before S_2–S_6. Carbachol was given either alone or combined with the indicated antagonists which were present throughout superfusion. Ordinates, evoked overflow of tritium, calculated from S_n/S_1 ratios and expressed as a percentage of the corresponding control (no carbachol). Means±s.e.m. from $n=15$–19 tissue pieces. Significant differences from carbachol alone: $^{\#}P<0.05$.

Discussion

The present results confirm the heterogeneity of presynaptic muscarinic heteroreceptors at postganglionic sympathetic axons. Moreover, our findings indicate that different peripheral tissues are endowed with distinct populations (mixtures) of presynaptic muscarinic heteroreceptors.

We have recently shown that sympathetic axons of mouse atria and urinary bladder possess both M_2 and non-M_2 muscarinic heteroreceptors (Trendelenburg *et al*., 2003a). In the present study, we observed that carbachol had no significant inhibitory effect on transmitter release from sympathetic neurons innervating the atria of $M_{2/3}$-double knockout mice, indicating that the non-M_2 atrial heteroreceptors represent M_3 receptors (Figure 1c). Analogously, carbachol had no significant effect on noradrenaline release from urinary bladder segments from $M_{2/4}$-double-knockout mice, strongly suggesting that the non-M_2 heteroreceptors represent M_4 receptors in the urinary bladder (Figure 2b).

Previously, we detected both M_2 and M_4 presynaptic muscarinic heteroreceptors in the mouse vas deferens (Trendelenburg *et al*., 2003a). We therefore considered it likely that the release-inhibitory activity of carbachol would be absent in vas deferens preparations from $M_{2/4}$-double-knockout mice. However, carbachol still retained some activity in vasa deferentia from $M_{2/4}$-double-knockout mice (Figure 3b), indicating the presence of at least one additional muscarinic heteroreceptor in this tissue. Two observations suggest that M_3 heteroreceptors are present in the mouse vas deferens. First, deletion of the M_3 receptor led to a small but significant reduction of the release-inhibitory effect of carbachol (Figure 3a). Second, the lack of M_3 receptors changed the pharmacology of the overall presynaptic muscarinic receptor population in the mouse vas deferens. In all wild-type strains, the order of antagonist potencies in blocking carbachol-mediated inhibition of transmitter release was pirenzepine $\geqslant$methoctramine, typical for the pharmacology of M_3, M_4 and M_5 receptors. Similar to the pharmacology of vas deferens preparations from M_4 receptor knockout mice (Trendelenburg *et al*., 2003a), deletion of the M_3 receptor changed the order of antagonist potencies in blocking carbachol-mediated inhibition of noradrenaline release to methoctramine>pirenzepine, typical of M_2 receptors (Trendelenburg *et al*., 2003a). The presence of both M_3 and M_4 receptors is therefore required to confer an $M_{3/4/5}$ receptor-like antagonist pharmacology on the mixed population of muscarinic heteroreceptors in the wild-type vas deferens.

The identification of the release-inhibiting muscarinic heteroreceptors in mouse atria and urinary bladder can be considered clear-cut, because carbachol was devoid of any effect on sympathetic transmitter release in preparations from

$M_{2/3}$- and $M_{2/4}$-double-knockout-mice, respectively. On the other hand, our data do not rule out the possibility that additional muscarinic receptor subtypes, besides the M_2, M_3 and M_4 receptors, contribute to the mixture of muscarinic heteroreceptors present in the mouse vas deferens.

As is generally the case with gene knockout mouse strains, we cannot completely rule out the possibility that compensatory changes in the expression levels or subcellular distribution of individual muscarinic receptor subtypes may have occurred in the different mutant mouse strains used in the present study. However, all studies that have addressed this issue so far suggest that such changes are unlikely to occur (Gautam *et al*., 2004; 2005; Wess, 2004). Moreover, we cannot completely rule out the possibility that formation of muscarinic heterodimers (Novi *et al*., 2005) may have affected the outcome of the functional studies.

It has been suggested that postganglionic sympathetic axons in several tissues including mouse atria (Costa & Majewski, 1991) possess release-facilitating M_1 receptors. However, under our experimental conditions, we have never observed facilitation of noradrenaline release by carbachol in any of the three tissues investigated, even after genetic deletion of the release-inhibitory muscarinic heteroreceptors.

Studies on genetically modified mice have revealed heterogeneous receptor populations also for other presynaptic receptor systems. *In vitro* neurotransmitter release studies showed that the muscarinic autoreceptors inhibiting the release of acetylcholine in mouse brain cortex and hippocampus are predominantly M_2, whereas in the corpus striatum they are predominantly M_4 (Zhang *et al*., 2002a). However, *in vivo* microdialysis studies have shown that M_4 autoreceptors may also contribute to the regulation of acetylcholine release in the hippocampus (Tzavara *et al*., 2003). As to the peripheral parasympathetic system, mouse atria possess both M_4 and non-M_4 (probably M_2) muscarinic autoreceptors, whereas the muscarinic autoreceptors exclusively represent M_4 receptors in the mouse urinary bladder (Zhou *et al*., 2002). In the corpus striatum, presynaptic M_5 heteroreceptors enhance the release of dopamine (Zhang *et al*., 2002b). Early work suggested that the α_2 autoreceptors of noradrenergic neurons mainly consist of α_{2A} receptors (see Starke, 2001). However, recent studies with adrenergic receptor knockout mice indicated that the α_{2C} and α_{2B} adrenoceptors also function as autoreceptors (see Hein *et al*., 1999; Trendelenburg *et al*., 2003b). In the corpus striatum, both presynaptic α_{2A} and α_{2B} heteroreceptors depress the release of dopamine (Bücheler *et al*., 2002).

In neurons possessing multiple presynaptic muscarinic receptors or α_2 adrenoceptors, deletion of only one receptor subtype gene often has no significant effect on agonist responses. Disruption of only the α_{2C} adrenoceptor gene, for example, did not diminish the inhibitory effect of α_2 adrenoceptor agonists on the release of noradrenaline in the vas deferens (Altman *et al*., 1999), and disruption of only the M_4 muscarinic receptor gene did not diminish the inhibitory effect of carbachol on the release of noradrenaline in the urinary bladder (Trendelenburg *et al*., 2003a; see also Bücheler *et al*., 2002; Trendelenburg *et al*., 2003b; Tzavara *et al*., 2003). It is likely that in these cases the remaining receptor subtypes are able to induce a signal that is sufficiently large to mediate full agonist responses.

The M_2 and M_4 muscarinic receptor subtypes are preferentially coupled to G proteins of the $G_{i/o}$ family (Caulfield & Birdsall, 1998), similar to many other presynaptic inhibitory receptors such as the α_2 adrenoceptors, opioid receptors and cannabinoid CB_1 receptors. The activation of this class of G proteins has been shown to mediate the inhibition of fast, voltage-sensitive N- and P/Q-type Ca^{2+} channels, leading to reduced transmitter release (see Shapiro *et al*., 1999; Starke, 2001; Zhang *et al*., 2002a). In contrast, the M_1, M_3 and M_5 receptor subtypes selectively couple to G proteins of the G_q family (Caulfield & Birdsall, 1998). It has been suggested, based on the use of subtype-'preferring' muscarinic antagonists, that M_3 receptors mediate the inhibition of noradrenaline release in guinea-pig atria (Olmez *et al*., 1995) and cat femoral artery (Fernandes *et al*., 1991) or acetylcholine release in rat stomach (Yokotani *et al*., 1993). However, the present study provides the first piece of evidence for the existence of release-inhibiting M_3 receptors that does not rely on the use of pharmacological tools of limited selectivity. Electrophysiological studies on muscarinic receptors expressed in mouse superior cervical ganglia suggest that G_q-mediated presynaptic inhibition of neurotransmitter release may involve voltage-independent inhibition of Ca^{2+} channels (Shapiro *et al*., 1999).

Immunologic and mRNA expression studies have shown that sympathetic neurons express M_1, M_2 and M_4 muscarinic receptors (see Doerje *et al*., 1991; Ludlam *et al*., 1994; Shapiro *et al*., 1999). In contrast, neither M_3 nor M_5 receptors could be detected by the use of these techniques, most likely due to very low receptor expression levels.

In conclusion, our study involving the use of genetically modified mice clearly shows that postganglionic sympathetic neurons use different mixtures of muscarinic receptor subtypes as presynaptic release-inhibiting heteroreceptors. In atria, this mixture is composed of M_2 and M_3, in the urinary bladder of M_2 and M_4, and in vasa deferentia of at least three receptor subtypes, M_2, M_3 and M_4. The mechanism by which G_q-coupled M_3 receptors can mediate presynaptic inhibition of neurotransmitter release remains to be investigated.

This work was supported by the Deutsche Forschungsgemeinschaft Grant SFB 505 (to A.U. Trendelenburg, K. Starke).

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(Received February 14, 2005
Revised April 7, 2005
Accepted May 6, 2005
Published online 20 June 2005)