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# Heterogeneity of presynaptic muscarinic receptors mediating inhibition of sympathetic transmitter release: a study with $M_2$- and $M_4$-receptor-deficient mice

*,[1,3]Anne-Ulrike Trendelenburg, [2]Jesus Gomeza, [1]Werner Klebroff, [1]Hongxia Zhou & [2]Jürgen Wess

[1]Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albertstrasse 25, D-79104 Freiburg, Germany and [2]Laboratory of Bioorganic Chemistry, NIH-NIDDK, 8 Center Drive MSC 0810, Bethesda, Maryland, MD 20892-0810, U.S.A.

**1** Presynaptic muscarinic receptors modulate sympathetic transmitter release. The goal of the present study was to identify the muscarinic receptor subtype(s) mediating inhibition of sympathetic transmitter release in mouse atria, urinary bladder and vas deferens. To address this question, electrically evoked noradrenaline release was assessed using tissue preparations from NMRI, $M_2$- and $M_4$-knockout, and the corresponding $M_2$- and $M_4$-wildtype mice, after preincubation with $^3$H-noradrenaline.

**2** The muscarinic agonist carbachol decreased evoked tritium overflow (20 pulses/50 Hz) in each tissue and strain investigated. After deletion of the $M_2$-receptor the maximal inhibition by carbachol was significantly reduced (by 41–72%), but not abolished, in all tissues. After deletion of the $M_4$-receptor a moderate and significant reduction of the maximal inhibition by carbachol (by 28%) was observed only in the vas deferens.

**3** Experiments with the muscarinic antagonists methoctramine and pirenzepine confirmed that the presynaptic muscarinic receptors were predominantly $M_2$ in atria and bladder and probably a mixture of $M_2$ and $M_4$ in the vas deferens.

**4** Experiments in the urinary bladder with the cholinesterase inhibitor physostigmine and the muscarinic antagonist ipratropium demonstrated that endogenously released acetylcholine predominantly acted through $M_2$-receptors to inhibit noradrenaline release. However, the results do not exclude a minor contribution of $M_4$-receptors to this endogenous inhibition.

**5** In conclusion, our results clearly indicate that the release-inhibiting muscarinic receptors on postganglionic sympathetic axons in mouse atria, bladder and vas deferens represent mixtures of $M_2$- and non-$M_2$-receptors. The non-$M_2$-receptors remain unknown in atria and the bladder, and may represent primarily $M_4$-receptors in the vas deferens. These results reveal an unexpected heterogeneity among the muscarinic receptors mediating inhibition of noradrenaline release.
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**Keywords:** Presynaptic muscarinic receptors; muscarinic receptor subtypes; sympathetic axon terminals; $^3$H-noradrenaline; $M_2$-knockout; $M_4$-knockout; mouse atria; mouse urinary bladder; mouse vas deferens; heterogeneity

**Abbreviations:** NMRI, Naval Medical Research Institute; PCR, polymerase chain reaction

## Introduction

Presynaptic release-modulating muscarinic receptors on postganglionic sympathetic axon terminals, first described in 1967 (Löffelholz, 1967; Lindmar *et al*., 1968), contribute to the interplay between the sympathetic and parasympathetic nervous systems (reviewed by Fuder & Muscholl, 1995). In general, these receptors mediate an inhibition of sympathetic transmitter release, but enhancement has also been reported, at least in some tissues (Fuder & Muscholl, 1995).

There are five molecularly distinct subtypes of muscarinic receptors, $M_1$ to $M_5$ (Caulfield & Birdsall, 1998; Alexander *et al*., 2001). Probably due to the lack of highly subtype-selective drugs, pharmacological studies aimed at identifying the presynaptic muscarinic receptors at sympathetic axons have often led to conflicting results. Release-facilitating receptors were suggested to be $M_1$-receptors across tissues and species (e.g. mouse atria, Costa & Majewski, 1991; see Fuder & Muscholl, 1995). The more prominent release-inhibiting receptors, on the other hand, were proposed to represent $M_2$-receptors in the majority of tissues and species (e.g. mouse atria, Costa & Majewski, 1991). However, the existence of release-inhibiting $M_1$-receptors (e.g. rabbit vas deferens; Grimm *et al*., 1994) and $M_3$-receptors (e.g. guinea-pig atria; Olmez *et al*., 1995) has also been reported, and in a substantial number of studies the identity of the presynaptic muscarinic heteroreceptors has remained unclear (e.g. rat vas deferens; Miranda *et al*., 1994).

A new approach to receptor classification that circumvents the difficulties associated with pharmacological tools of limited subtype selectivity involves the use of mutant mice

*Author for correspondence;
E-mail: anne-ulrike.trendelenburg@pharma.novartis.com
[3]Current address: Novartis Ophthalmics Research AG, WSJ-386.7.46, P.O. Box, CH-4002 Basel, Switzerland

in which specific receptor genes have been disrupted by molecular genetic techniques. This strategy has shown, for example, that postganglionic sympathetic neurons and central noradrenergic neurons possess release-inhibiting presynaptic $\alpha_{2A}$-(which predominate) and $\alpha_{2C}$-adrenoceptors (autoreceptors; Altman *et al*., 1999; Hein *et al*., 1999; Trendelenburg *et al*., 1999; 2001). The use of mutant mice has also shown that parasympathetic cholinergic neurons possess release-inhibiting muscarinic autoreceptors which are exclusively $M_4$ in the urinary bladder but $M_4$ plus non-$M_4$ (probably $M_2$) in atria (Zhou *et al*., 2002).

In the present study, we used mice lacking either the $M_2$-receptor ($M_2$-knockout; Gomeza *et al*., 1999a) or the $M_4$-receptor ($M_4$-knockout; Gomeza *et al*., 1999b) to investigate presynaptic muscarinic receptors mediating inhibition of noradrenaline release. The corresponding $M_2$-wildtype and $M_4$-wildtype mice served as controls. In addition, NMRI mice were included as a commonly used laboratory strain. In the first part of the study, we identified and characterized presynaptic muscarinic receptors in heart atria, urinary bladder, and vas deferens. The agonist carbachol and the antagonists ipratropium (high-affinity, non-selective), methoctramine and pirenzepine were used as pharmacological tools. The latter two antagonists were chosen because they allow a limited distinction between muscarinic receptor subtypes: the rank order methoctramine > pirenzepine is typical for $M_2$-receptors, whereas pirenzepine $\geqslant$ methoctramine is typical for $M_3$-, $M_4$- and $M_5$-receptors and pirenzepine > methoctramine for $M_1$-receptors (Lazareno *et al*., 1990; Dörje *et al*., 1991; Lazareno & Birdsall, 1993; Eglen *et al*., 1996; Caulfield & Birdsall, 1998). In the second part, we examined through which receptor subtype(s) endogenously released acetylcholine modulates noradrenaline release in the urinary bladder. Neurotransmitter release experiments were carried out using isolated tissue segments after labelling with $^3$H-noradrenaline.

## Methods

### *Tissues and superfusion*

The generation of $M_2$-knockout (genetic background: 129J1 × CF1) and $M_4$-knockout (genetic background: 129SvEv × CF1) mice has been described previously (Gomeza *et al*., 1999a, b). In all experiments, aged-matched wildtype mice of the corresponding genetic background were used as controls ($M_2$-wildtype, 129J1 × CF1; $M_4$-wildtype, 129SvEv × CF1). Mouse genotyping was carried out by PCR analysis of mouse tail DNA. Male NMRI (Naval Medical Research Institute; bred in the local animal facility), $M_2$-wildtype, $M_4$-wildtype, $M_2$-knockout and $M_4$-knockout mice aged > 2 months were killed by cervical dislocation. From each animal six to eight pieces of the atria, 12 to 15 pieces of the urinary bladder or eight to 12 pieces of the vas deferens were obtained. Tissue pieces were preincubated in 1 ml medium (see below) containing 0.2 $\mu$M $^3$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).

### *Protocols*

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/3 Hz) was delivered at $t = 30$ min and was not used for determination of tritium overflow. The subsequent stimulation periods ($S_1$ to $S_6$) were applied at $t = 54$, 72, 90, 108, 126 and 144 min and differed, depending on the type of experiment.

In experiments designed to detect and characterize presynaptic muscarinic receptors in atria, the urinary bladder and the vas deferens by means of carbachol and different muscarinic antagonists, $S_1$ to $S_6$ each consisted of 20 pulses/50 Hz. Carbachol was introduced at increasing concentrations after $S_1$, 12 min before $S_2$, $S_3$, $S_4$, $S_5$ and $S_6$. Antagonists were present throughout superfusion at a fixed concentration.

In experiments designed to study the potential modulation of noradrenaline release by endogenously released acetylcholine in the urinary bladder, the following stimulations were applied: $S_1$ and $S_4$ consisted of 20 pulses/50 Hz; $S_2$ and $S_5$ consisted of 120 pulses/3 Hz, and $S_3$ and $S_6$ consisted of 360 pulses/3 Hz. Drugs were present either throughout superfusion or introduced after $S_3$, 12 min before $S_4$.

### *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, overflow ratios were calculated: $S_n/S_1$ in experiments with identical stimulation patterns at $S_1$ to $S_6$, and $S_n/S_{n-3}$ in experiments with different stimulation patterns (note that stimulation patterns at $S_n$ and $S_{n-3}$ were identical). Overflow ratios obtained in the presence of drugs added after $S_1$ or $S_3$ were also calculated as a percentage of the corresponding ratio in controls in which no drug was added after $S_1$ or $S_3$. 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 (eq. 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. In most cases sigmoid curves could not be fitted to concentration-response data for carbachol in

the presence of antagonists. 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 increase in $EC_{50}$ values (equation no. 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.mean (estimates ± s.e. defined by Waud, 1976, in the case of $E_{max}$ and $EC_{50}$ values of carbachol; s.e. of $pK_d$ values were calculated by means of the Gaussian law of error propagation). Groups were tested for significant differences with the Mann-Whitney test with Bonferroni correction. $P<0.05$ was taken as limit of statistical difference. *n* represents the number of tissue pieces.

### *Drugs*

Drugs were (−)-[ring-2,5,6-$^3$H]-noradrenaline, specific activity 51.8–70.7 Ci/mmol (NEN, Köln, Germany), carbachol chloride, desipramine HCl, physostigmine hemisulphate, ipratropium bromide, methoctramine 4 HCl, pirenzepine 2 HCl, rauwolscine HCl (Sigma, Deisenhofen, Germany) and phentolamine methanesulfonate (Ciba-Geigy, Basel, Switzerland). Drugs were dissolved in distilled water.

## Results

All experiments were done with tissue segments prepared from atria, urinary bladder and vas deferens from either NMRI, $M_2$-wildtype, $M_4$-wildtype, $M_2$-knockout or $M_4$-knockout mice after preincubation with $^3$H-noradrenaline to label vesicular noradrenaline pools. Electrical stimulation was applied to elicit the release of $^3$H-noradrenaline measured as tritium overflow.

### *Detection and characterization of presynaptic muscarinic heteroreceptors in atria, urinary bladder and vas deferens*

In this series of experiments, tissues were stimulated by short bursts of 20 pulses/50 Hz. As shown in Figure 1 for the urinary bladder, which has not been studied in this manner previously, electrical stimulation led to clear peaks of tritium overflow. In control experiments without carbachol the magnitude of these peaks was similar from $S_1$ to $S_6$, giving $S_n/S_1$ ratios close to unity (Figure 1). Similar observations were made in atria and the vas deferens (not shown; see also atria: Wahl *et al*., 1996; vas deferens: Trendelenburg *et al*., 1999). The overflow of tritium evoked by $S_1$ amounted to 0.39 ± 0.03% of tissue tritium in atria, 0.21 ± 0.02% in bladder, and 0.30 ± 0.01% in vas deferens from NMRI mice ($n = 16–29$). Similar values were observed in tissues from $M_2$-wildtype, $M_4$-wildtype, $M_2$-knockout and $M_4$-knockout mice (data not shown). The overflow values in atria and vas deferens from NMRI mice were similar to previous studies (Trendelenburg *et al*., 1999; 2000).

We have shown previously that short bursts of 20 pulses/50 Hz led to little, if any, $\alpha_2$-autoinhibition of noradrenaline release in mouse atria and vas deferens (Trendelenburg *et al*., 1999; 2000). The same stimulation pattern (i.e. 20 pulses/50 Hz), also led to little $\alpha_2$-autoinhibition in the mouse urinary bladder as indicated by an only small facilitatory effect of the α-adrenoceptor antagonists phentolamine (1 μM) and rauwolscine (1 μM) on evoked tritium overflow (see

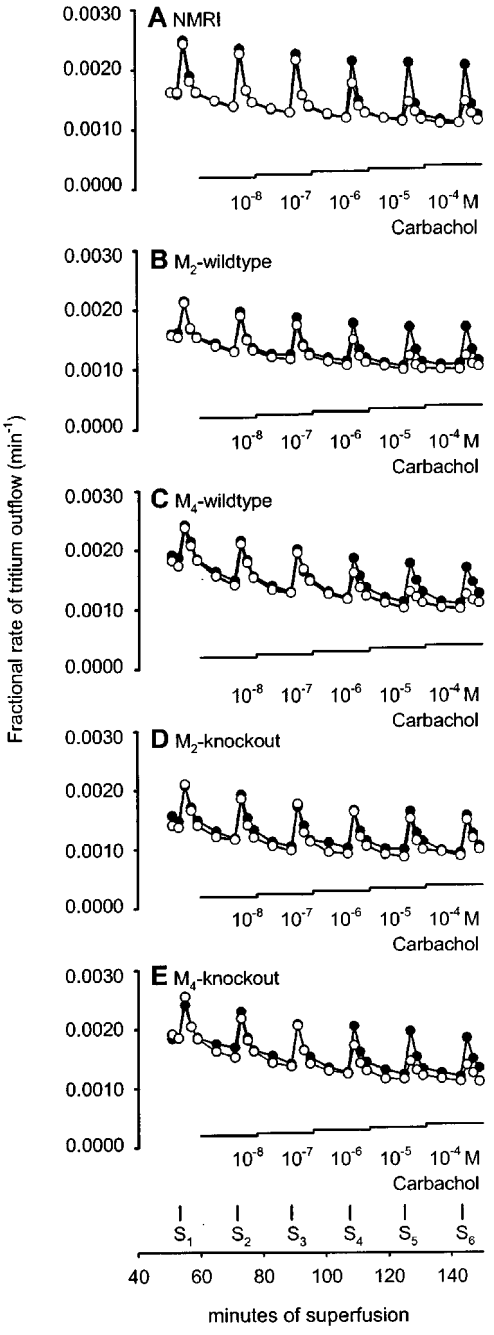


**Figure 1** Outflow of tritium from urinary bladder pieces taken from NMRI (A), $M_2$-wildtype (B), $M_4$-wildtype (C), $M_2$-knockout (D) or $M_4$-knockout mice (E): effects of electrical stimulation and carbachol. After preincubation with $^3$H-noradrenaline tissues were superfused and stimulated electrically six times by 20 pulses/50 Hz ($S_1$ to $S_6$). Filled circles, controls (no carbachol). Empty circles, carbachol was added at increasing concentrations as indicated. Each line represents the mean of three to 13 bladder pieces.

section below on 'Inhibition of noradrenaline release by endogenous acetylcholine in the urinary bladder'). These stimulation conditions with no or little $\alpha_2$-autoinhibition were chosen in order to provide optimal conditions for the detection and characterization of presynaptic modulation of noradrenaline release by exogenous muscarinic agonists (see Starke, 1987; Schlicker & Göthert, 1998).

The muscarinic agonist carbachol (10 nM to 100 $\mu$M) reduced the evoked overflow of tritium in all three tissues of all five mouse strains studied. This is shown as efflux-versus-time curves in Figure 1 and as concentration-response curves in Figure 2 (NMRI), Figure 3 ($M_2$-wildtype and $M_2$-knockout) and Figure 4 ($M_4$-wildtype and $M_4$-knockout). The $E_{max}$ of carbachol (expressed as per cent inhibition of stimulation-evoked tritium overflow compared to controls without carbachol), obtained from logistic curve fitting, amounted to $65\pm1\%$ in NMRI heart atria, $56\pm1\%$ in NMRI urinary bladder, and $43\pm1\%$ in NMRI vas deferens. The carbachol $EC_{50}$ values were $1.9\pm0.4$ $\mu$M in NMRI atria, $1.0\pm0.6$ $\mu$M in NMRI bladder and $1.1\pm1.3$ $\mu$M in NMRI vas deferens. The concentration-response curves of carbachol in $M_2$-wildtype tissues (Figure 3) and $M_4$-wildtype tissues (Figure 4) did not differ from the NMRI curves (Figure 2).

Strikingly, deletion of the $M_2$-receptor led to a marked reduction ($P<0.05$) in the maximum inhibition of stimulation-evoked overflow of tritium in all three tissues investigated. Logistic curve fitting showed that the $E_{max}$ of carbachol was reduced from $68\pm4\%$ ($M_2$-wildtype) to $26\pm10\%$ ($M_2$-knockout) in atria (Figure 3A), from $69\pm8\%$ ($M_2$-wildtype) to $19\pm4\%$ ($M_2$-knockout) in bladder (Figure 3B) and from $34\pm4\%$ ($M_2$-wildtype) to $20\pm3\%$ ($M_2$-knockout) in vas deferens (Figure 3C). In all three tissues, statistically significant inhibitory carbachol responses remained in the absence of $M_2$-receptors (Figure 3). Lack of

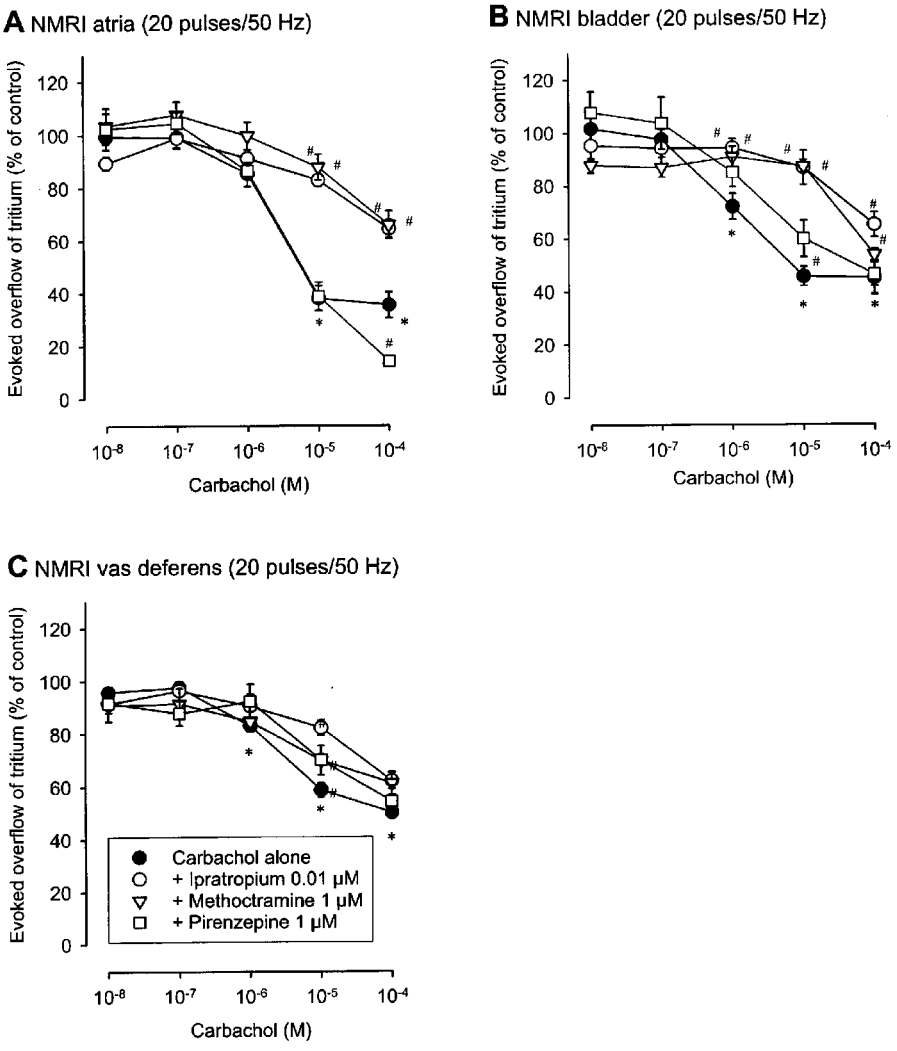


**Figure 2** Interaction of muscarinic antagonists with carbachol on the evoked overflow of tritium from atria (A), urinary bladder (B) and vas deferens (C) of NMRI mice. After preincubation with $^3$H-noradrenaline tissues were superfused and stimulated electrically six times by 20 pulses/50 Hz ($S_1$ to $S_6$). Carbachol was added at increasing concentrations (abscissae) before $S_2$ to $S_6$. 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$\pm$s.e.mean from $n=6$ to 16 tissue pieces. Significant differences from corresponding control (no carbachol): $^*P<0.05$. Significant differences from carbachol alone: $\#P<0.05$.

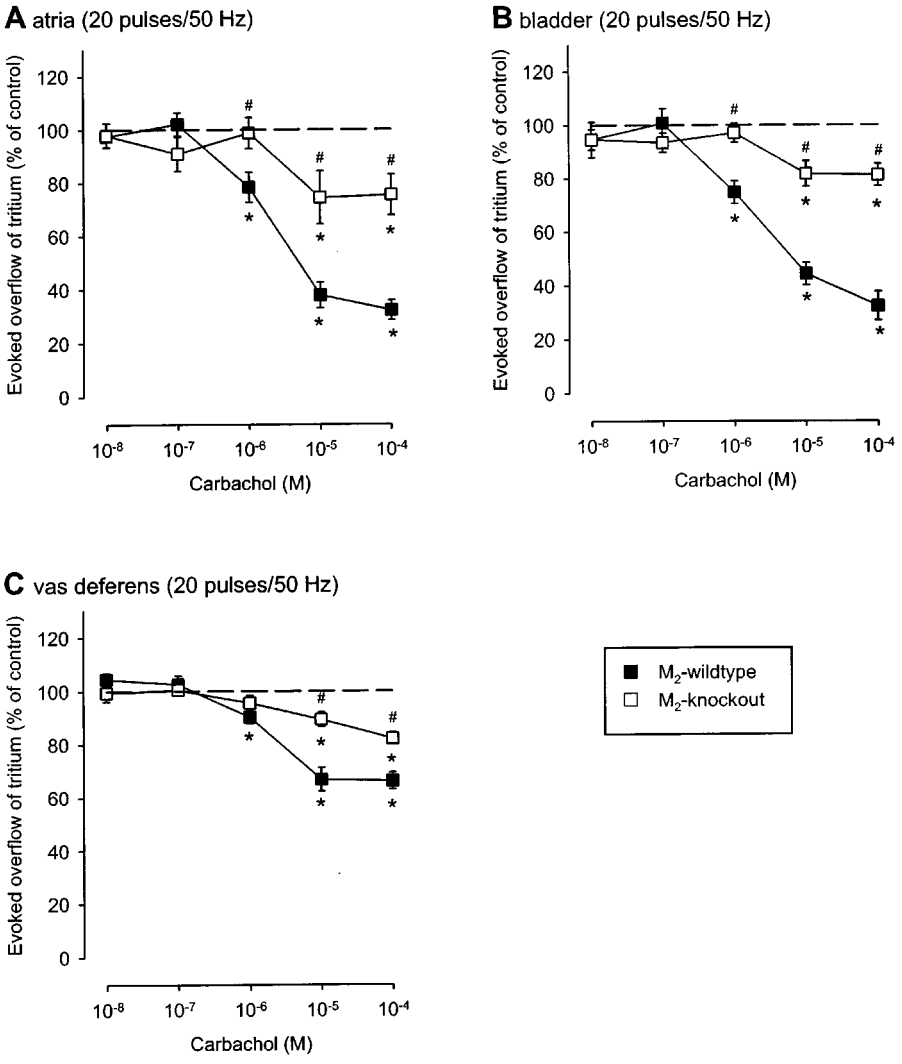


**Figure 3** Effect of carbachol on the evoked overflow of tritium from atria (A), bladder (B) and vas deferens (C) of $M_2$-wildtype or $M_2$-knockout mice. After preincubation with $^3$H-noradrenaline tissues were superfused and stimulated electrically six times by 20 pulses/50 Hz ($S_1$ to $S_6$). Carbachol was added at increasing concentrations (abscissae) before $S_2$ to $S_6$. 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.mean from $n=5$ to 16 tissue pieces. Significant differences from corresponding control (no carbachol): $^*P<0.05$. Significant differences from $M_2$-wildtype: $\#P<0.05$.

$M_4$-receptors, in contrast, had no significant effect on the inhibition by carbachol in atria and urinary bladder (Figure 4A,B) although it moderately reduced the maximum inhibition in the vas deferens: the fitted $E_{max}$ values for carbachol were $46\pm9\%$ ($M_4$-wildtype) and $33\pm6\%$ ($M_4$-knockout), respectively.

The receptors mediating the inhibition of noradrenaline release were characterized further by means of the antagonists ipratropium (high-affinity, non-selective; used in NMRI tissues only) as well as methoctramine and pirenzepine which allow a limited distinction between muscarinic receptor subtypes (see Introduction). Neither ipratropium (0.01 $\mu$M) nor pirenzepine (1 $\mu$M), when present throughout superfusion, changed the stimulation-evoked overflow of tritium ($S_1$), whereas methoctramine (1 $\mu$M) increased the evoked overflow of tritium ($S_1$) from atria, bladder and vas deferens of several mouse strains by up to 35% (data not shown; compare Casado *et al*., 1992). In control experiments without carbachol, $S_n/S_1$ ratios were close to unity also in the presence of the antagonists. None of the muscarinic receptor ligands used had any effect on basal tritium outflow (data not shown).

In all three tissues from NMRI mice, ipratropium (0.01 $\mu$M), methoctramine (1 $\mu$M) and pirenzepine (1 $\mu$M) shifted the carbachol concentration-response curve to the right (Figure 2), except for pirenzepine in atria (Figure 2A). The apparent antagonist $pK_d$ values calculated from the shifts are given in Table 1. The rank order of antagonist potency was ipratropium > methoctramine > pirenzepine in atria and bladder but ipratropium > pirenzepine $\geqslant$ methoctramine in vas deferens (Figure 2; Table 1).

Methoctramine (1 $\mu$M) and pirenzepine (1 $\mu$M) were also studied as antagonists against carbachol in preparations from $M_2$- and $M_4$-knockout mice and the two corresponding wildtype control strains. As indicated by the $pK_d$ values summarized in Table 1, the results obtained in $M_2$- and $M_4$-

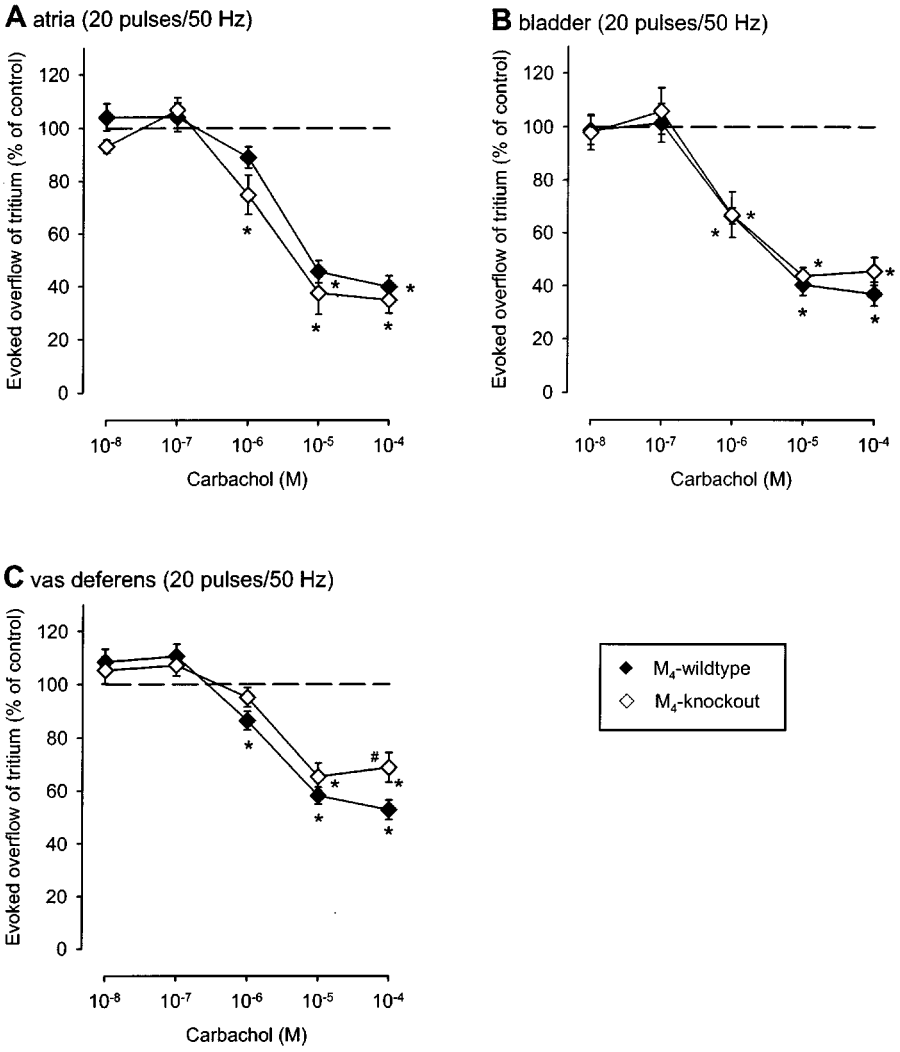


**Figure 4** Effect of carbachol on the evoked overflow of tritium from atria (A), bladder (B) and vas deferens (C) of $M_4$-wildtype or $M_4$-knockout mice. After preincubation with $^3$H-noradrenaline tissues were superfused and stimulated electrically six times by 20 pulses/50 Hz ($S_1$ to $S_6$). Carbachol was added at increasing concentrations (abscissae) before $S_2$ to $S_6$. 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.mean from $n=5$ to 16 tissue pieces. Significant differences from corresponding control (no carbachol): $^*P<0.05$. Significant differences from $M_4$-wildtype: $\#P<0.05$.

wildtype tissues agreed well with those obtained in NMRI tissues: in $M_2$- and $M_4$- wildtype atria and bladder, the antagonist potency order again was methoctramine > pirenzepine, whereas in $M_2$- and $M_4$-wildtype vas deferens, the potency order again was pirenzepine ≥ methoctramine. The interaction of methoctramine and pirenzepine with carbachol in the $M_4$-wildtype vas deferens is shown in Figure 5A. Comparison with Figure 2C shows the similarity to the NMRI vas deferens.

In $M_2$-knockout atria, bladder and vas deferens, the concentration-response curves of carbachol were flat, with a small maximum (Figure 3). For this reason, although antagonism by methoctramine and pirenzepine was evident, antagonist $pK_d$ values could not be quantified with a sufficient degree of certainty (data not shown).

In $M_4$-knockout tissues, the concentration-response curves of carbachol were sufficiently steep (Figure 4) for quantification of antagonist $pK_d$ values for methoctramine and pirenzepine. The $pK_d$ values are summarized in Table 1. In $M_4$-knockout atria and bladder, the values were very close to the corresponding NMRI, $M_2$-wildtype and $M_4$-wildtype values, yielding again the potency order methoctramine > pirenzepine. In contrast, a different pattern was observed in the vas deferens. Whereas the order of antagonist potencies was pirenzepine ≥ methoctramine in vasa deferentia from NMRI mice and the two wildtype strains, the order was reversed to methoctramine > pirenzepine in the $M_4$-knockout vas deferens (Table 1 and Figure 5).

### *Inhibition of noradrenaline release by endogenous acetylcholine in the urinary bladder*

In the next series of experiments, we investigated whether the presynaptic muscarinic receptors mediating inhibition of noradrenaline release could also be activated by endogenously released acetylcholine, rather than being exclusively

**Table 1** $pK_d$ values of antagonists at presynaptic muscarinic receptors in NMRI, $M_2$-wildtype, $M_4$-wildtype and $M_4$-knockout mice

| *Antagonist at concentration tested* | *Heart atria* | | | | *Urinary bladder* | | | | *Vas deferens* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *NMRI* | *$M_2$-wildtype* | *$M_4$-wildtype* | *$M_4$-knockout* | *NMRI* | *$M_2$-wildtype* | *$M_4$-wildtype* | *$M_4$-knockout* | *NMRI* | *$M_2$-wildtype* | *$M_4$-wildtype* | *$M_4$-knockout* |
| Ipratropium 0.01 $\mu$M | $9.4\pm0.2^{23}$ | n.d. | n.d. | n.d. | $9.6\pm0.4^{123}$ | n.d. | n.d. | n.d. | $9.1\pm0.1^{23}$ | n.d. | n.d. | n.d. |
| Methoctramine 1 $\mu$M | $7.4\pm0.3^{23}$ | $7.3\pm0.4^{123}$ | $7.5\pm0.3^{23}$ | $7.6\pm0.4^{23}$ | $7.5\pm0.2^{123}$ | $7.6\pm0.3^{123}$ | $7.5\pm0.2^{123}$ | $7.6\pm0.4^{23}$ | $6.0\pm0.5^{2}$ | $<6$ | $6.1\pm0.6^{2}$ | $7.2\pm0.2^{2}$ |
| Pirenzepine 1 $\mu$M | $<6$ | $<6$ | $<6$ | $<6$ | $6.4\pm0.5^{2}$ | $<6$ | $6.4\pm0.4^{1}$ | $<6$ | $6.3\pm0.2^{2}$ | $6.8\pm0.2^{12}$ | $6.6\pm0.2^{12}$ | $<6$ |

After preincubation with $^3$H-noradrenaline tissues were superfused and stimulated electrically six times by 20 pulses/50 Hz ($S_1$ to $S_6$). Carbachol was added at increasing concentrations before $S_2$ to $S_6$. Antagonists were present throughout superfusion. $pK_d$ values were calculated from the rightward shifts of carbachol concentration-response curves by antagonists. Antagonist $pK_d$ values were calculated only if the antagonist attenuated the effect of carbachol significantly ($P<0.05$; for carbachol 1 $\mu M^1$, 10 $\mu M^2$ or 100 $\mu M^3$ as indicated). Each $pK_d$ value (mean$\pm$s.e.) is based on five to 16 preparations (preparations that received agonist only were not included). n.d. not determined.

sites of action of exogenous drugs. These experiments were carried out using the mouse urinary bladder as a model system. Each bladder preparation was stimulated by 20 pulses/50 Hz ($S_1$ and $S_4$), 120 pulses/3 Hz ($S_2$ and $S_5$) and 360 pulses/3 Hz ($S_3$ and $S_6$). The long pulse trains were used to increase the chance that endogenous muscarinic inhibition of noradrenaline release developed. We noted that a considerable $\alpha_2$-adrenergic autoinhibition of noradrenaline release occurred during the long pulse trains but not during the short bursts in bladder segments of NMRI mice, as indicated by the effects of a combined administration of phentolamine (1 $\mu$M) and rauwolscine (1 $\mu$M) after $S_3$: the overflow elicited by 20 pulses/50 Hz was increased only by $28\pm12\%$, whereas the overflow elicited by 120 pulses/3 Hz and 360 pulses/3 Hz was markedly increased by $244\pm25$ and $212\pm19\%$, respectively ($n=6$). Thus, in order to prevent $\alpha_2$-adrenergic autoinhibition of noradrenaline release, $\alpha_2$-autoreceptors were blocked by adding phentolamine (1 $\mu$M) and rauwolscine (1 $\mu$M) throughout superfusion in all subsequent experiments. In addition, the cholinesterase inhibitor, physostigmine (1 $\mu$M), was added throughout superfusion in some experiments in order to raise synaptic acetylcholine levels.

The overflow of tritium elicited by stimulation with 20 pulses/50 Hz was not significantly changed by deletion of either the $M_2$- or the $M_4$-receptor gene or by addition of physostigmine (1 $\mu$M) or ipratropium (1 $\mu$M; data not shown). Thus, only the results obtained with 120 pulses and 360 pulses/3 Hz are presented in the following. The stimulation-evoked overflow of tritium obtained under these conditions ($S_2$ and $S_3$) is shown in Table 2. Physostigmine (1 $\mu$M), when present throughout superfusion, significantly reduced (by 24 and 36%; respectively) the overflow elicited by 120 pulses/3 Hz ($S_2$) and 360 pulses/3 Hz ($S_3$) in bladder preparations from NMRI mice and tended to reduce the overflow in bladder segments from $M_2$- and $M_4$-wildtype mice (Table 2). Strikingly, deletion of either $M_2$- or $M_4$-receptors led to a significant increase (by 28 to 52%) of tritium overflow elicited by 120 ($S_2$) or 360 pulses/3 Hz ($S_3$) in the presence of physostigmine, as compared to the values obtained with the corresponding wildtype control mice (Table 2).

To further investigate muscarinic inhibition of noradrenaline release by endogenous acetylcholine in the mouse bladder, we next administered physostigmine (1 $\mu$M) and ipratropium (1 $\mu$M) after $S_3$. Physostigmine, when administered after $S_3$ to bladder preparations from NMRI mice not previously treated with physostigmine, reduced the evoked overflow of tritium by 24 to 38% (Figure 6). Under the same conditions, ipratropium had no significant effect on transmitter release (Figure 6). However, ipratropium increased the evoked overflow of tritium by 40 to 52% when added to bladder preparations superfused throughout the experiment with physostigmine (Figure 6).

Finally, analogous experiments were carried out using bladder preparations from $M_2$- and $M_4$-knockout mice and the two corresponding wildtype control strains to detect the muscarinic receptor subtype(s) responsible for the inhibition of noradrenaline release by endogenously released acetylcholine. The responses observed with bladder preparations from $M_2$- and $M_4$-wildtype mice closely resembled those found with NMRI mice (Figure 7A,B). Physostigmine (1 $\mu$M), when administered after $S_3$, reduced the evoked overflow by 15 to 26% (Figure 7A,B). On the other hand, ipratropium (1 $\mu$M),

**Table 2** Electrically evoked tritium overflow from bladder preparations of adult NMRI, $M_2$-wildtype, $M_4$-wildtype, $M_2$-knockout or $M_2$-knockout mice

| | *Evoked tritium overflow (% of tissue tritium)* | | | |
|---|---|---|---|---|
| | *120 pulses/3 HZ* ($S_2$) | | *360 pulses/3 HZ* ($S_3$) | |
| *Mouse strain* | *No physostigmine* | *Physostigmine 1 μM* | *No physostigmine* | *Physostigmine 1 μM* |
| NMRI | 1.61±0.08 | 1.23±0.14* | 3.80±0.21 | 2.43±0.16* |
| $M_2$-wildtype | 1.53±0.12 | 1.42±0.10 | 3.53±0.34 | 3.15±0.28 |
| $M_4$-wildtype | 1.48±0.15 | 1.21±0.14 | 3.69±0.41 | 2.93±0.38 |
| $M_2$-knockout | 1.71±0.10 | 1.82±0.09# | 3.88±0.26 | 4.35±0.23# |
| $M_4$-knockout | 1.80±0.13 | 1.78±0.14# | 4.43±0.33 | 4.44±0.35# |

Bladder pieces were stimulated by 120 pulses/3 Hz ($S_2$) and 360 pulses/3 Hz ($S_3$). Phentolamine (1 μM) and rauwolscine (1 μM) were present throughout superfusion. Where indicated physostigmine (1 μM) was also added throughout superfusion. Data are means±s.e.mean from 15 to 28 tissue pieces. Significant differences from corresponding experiments without physostigmine: *$P<0.05$. Significant differences from corresponding wildtype: #$P<0.05$.

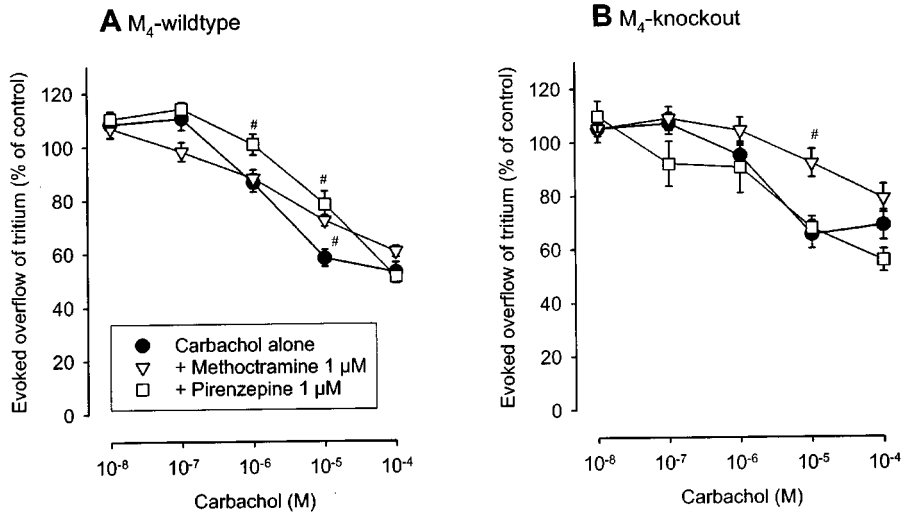


**Figure 5** Interaction of muscarinic antagonists with carbachol on the evoked overflow of tritium from vas deferens of $M_4$-wildtype (A) and $M_4$-knockout (B) mice. After preincubation with $^3$H-noradrenaline tissues were superfused and stimulated electrically six times by 20 pulses/50 Hz ($S_1$ to $S_6$). Carbachol was added at increasing concentrations (abscissae) before $S_2$ to $S_6$. 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.mean from $n=5$ to 16 tissue pieces. The concentration-inhibition curves of carbachol given alone in both $M_4$-wildtype and $M_4$-knockout are identical with those in Figure 4C. Significant differences from carbachol alone: #$P<0.05$.

when added to bladder preparations pre-exposed to physostigmine, increased the evoked overflow by 22 to 38% (Figure 7A,B).

Deletion of $M_2$-receptors abolished both the inhibitory effect of physostigmine in previously physostigmine-free tissues and the facilitatory effect of ipratropium in tissues pre-exposed to physostigmine (Figure 7C). The small remaining effects in $M_2$-knockout preparations were not statistically significant (Figure 7C). Deletion of the $M_4$-receptor, in contrast, did not significantly change the effects of physostigmine and ipratropium. However, there was a trend towards reduced physostigmine and ipratropium effects, as compared to the corresponding values from $M_4$-wildtype mice (compare Figure 7D with 7B).

## Discussion

In this study, we initially demonstrated that mouse atria, urinary bladder, and vas deferens possess presynaptic release-inhibiting muscarinic receptors at their sympathetic fibres. In the mouse, muscarinic heteroreceptors on sympathetic axons have been described previously in atria (Costa & Majewski, 1991) and cultured sympathetic neurons (Göbel *et al*., 2000). This is the first report demonstrating their presence in mouse urinary bladder and vas deferens.

### *Identification of muscarinic receptor subtypes inhibiting noradrenaline release*

Previous work has shown that presynaptic muscarinic receptors at postganglionic sympathetic axons are not homogeneous (Fuder & Muscholl, 1995). It is well known that these receptors can mediate inhibition as well as facilitation of noradrenaline release (Fuder & Muscholl, 1995). The release-facilitating receptors are likely to represent $M_1$ receptors across tissues and species (Muscholl *et al*., 1989; Vizi *et al*., 1989; Habermeier-Muth *et al*., 1990; Costa & Majewski, 1991; Casado *et al*., 1992; Somogyi *et al*., 1996). In contrast, the majority of release-inhibiting receptors were suggested to be

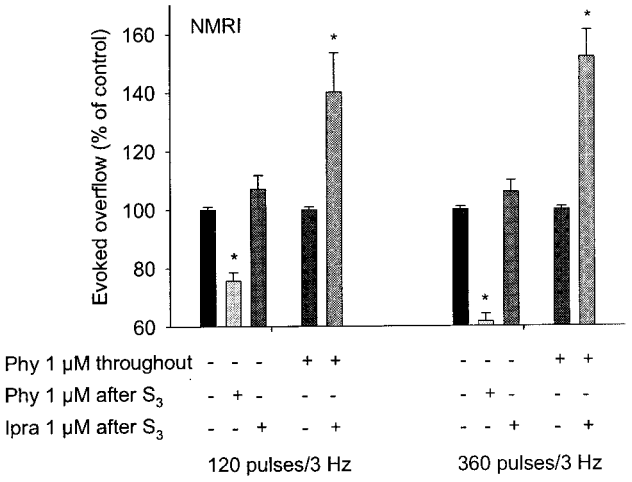


**Figure 6** Effects of physostigmine and ipratropium on the evoked overflow of tritium from NMRI bladder. After preincubation with $^3$H-noradrenaline tissues were superfused and stimulated electrically six times ($S_1$ to $S_6$). $S_1$ and $S_4$ consisted of 20 pulses/50 Hz (results not shown), $S_2$ and $S_5$ of 120 pulses/3 Hz, and $S_3$, and $S_6$ of 360 pulses/3 Hz. Phentolamine (1 $\mu$M) and rauwolscine (1 $\mu$M) were present throughout superfusion. Physostigmine (Phy) was present either throughout superfusion or was added after $S_3$, and ipratropium (Ipra) was added after $S_3$, as indicated. Ordinates, evoked overflow of tritium, calculated from $S_n/S_{n-3}$ ratios and expressed as a percentage of the corresponding control (no drug added after $S_3$). Means± s.e.mean from $n=4$ to 8 tissue pieces. Significant differences from corresponding control (no drug added after $S_3$): $^*P<0.05$.

$M_2$. However, the existence of release-inhibiting $M_1$- and $M_3$-receptors has also been proposed in certain tissues, and in some studies the identity of the presynaptic muscarinic receptors remained unclear (see Introduction). In most cases, these classifications were based on the use of muscarinic antagonists with a limited degree of subtype selectivity, which may be responsible for at least some of the discrepant results (see Introduction; Fuder & Muscholl, 1995).

Our knockout approach confirms the heterogeneity of presynaptic muscarinic heteroreceptors at postganglionic sympathetic axons and reveals an additional, hitherto unsuspected heterogeneity: in all three tissues, the sympathetic nerve terminals are endowed with at least two distinct muscarinic receptor subtypes mediating inhibition of transmitter release. The carbachol responses and antagonist potencies determined with preparations from $M_2$-wildtype and $M_4$-wildtype mice were generally similar to those obtained with NMRI mice (Figures 2 to 5; Table 1). Therefore, the presynaptic muscarinic heteroreceptors are essentially the same in the three wildtype mouse strains.

Deletion of $M_2$-receptors significantly reduced the maximal inhibitory effect of carbachol on noradrenaline release in all tissues: by 62% in atria, by 72% in the urinary bladder, and by 41% in the vas deferens (Figure 3). These observations show that $M_2$-receptors play a key role as release-inhibiting heteroreceptors in each of the three tissues. However, in all tissues lacking $M_2$-receptors, a significant inhibition by carbachol remained, clearly indicating the additional involvement of non-$M_2$-receptors in each of the three tissues. This complexity remained undetected in functional studies using different muscarinic antagonists in wildtype mice, probably due to the limited subtype selectivity of the antagonists.

What, then, is the identity of the non-$M_2$-heteroreceptors? We observed that deletion of the $M_4$-receptors did not significantly affect the release-inhibiting effect of carbachol in atria and urinary bladder (Figure 4A,B) but moderately reduced (by 28%; $P<0.05$) the maximal inhibitory effect of carbachol in the vas deferens (Figure 4C). This observation may support the view that $M_4$-heteroreceptors are also present in the vas deferens. It also suggests that the non-$M_2$-receptors in atria and bladder may not be $M_4$. However, the possibility exists that a contribution of presynaptic $M_4$-receptors to inhibition of noradrenaline release in atria and bladder remained undetected due to the predominance of the $M_2$ pathway which may be sufficient to allow maximal inhibition. In fact, such a pattern has been found previously for $\alpha_{2A}$- and $\alpha_{2C}$-autoreceptors: although both co-exist on sympathetic fibres, only deletion of the $\alpha_{2A}$-adrenoceptor but not deletion of the $\alpha_{2C}$-adrenoceptor reduced the release-inhibiting effect of $\alpha_2$-adrenoceptor agonists; a contribution of the $\alpha_{2C}$-autoreceptor became manifest only as inhibition remaining in the $\alpha_{2A}$-receptor knockout mice (Altman *et al*., 1999; Hein *et al*., 1999; Trendelenburg *et al*., 1999).

In order to gain additional insight into the identity of the muscarinic heteroreceptors mediating inhibition of noradrenaline release, we also tried to determine antagonist potencies of methoctramine and pirenzepine against carbachol in all tissues and mouse strains. In atria and bladder from NMRI, $M_2$-wildtype, $M_4$-wildtype and $M_4$-knockout mice, the potency order was methoctramine > pirenzepine, which is the $M_2$ order (see Introduction), in accord with the view that in both tissues of the four strains the $M_2$-heteroreceptor detected by the $M_2$-knockout experiment determined the pharmacological properties of the overall $M_2$-plus non-$M_2$-heteroreceptor population. In contrast, in the vas deferens of NMRI, $M_2$-wildtype and $M_4$-wildtype mice the potency order was pirenzepine ⩾ methoctramine, the $M_{3/4/5}$ order: thus, it seems that the non-$M_2$-heteroreceptors determined the pharmacological properties of the overall heteroreceptor population in the vas deferens of wildtype mice.

Unfortunately, the residual effect of carbachol in tissues from $M_2$-knockout was too small in size (Figure 3) to permit an acceptable quantification of antagonist potencies. The antagonist experiments are therefore not useful to classify the non-$M_2$-heteroreceptors in the atria and bladder. In contrast, the antagonist potencies determined in $M_4$-knockout preparations support the idea that non-$M_2$-heteroreceptors in the vas deferens may be $M_4$, as mentioned above: the potency order in the vas deferens of wildtype mice was pirenzepine ⩾ methoctramine, in accord with a pharmacological predominance of non-$M_2$-receptors. Deletion of the $M_4$-receptor reversed the order to methoctramine > pirenzepine, indicating that the remaining $M_2$-heteroreceptors became dominant in the absence of $M_4$-receptors (Figure 5). These findings support the concept that $M_4$-heteroreceptors co-exist with $M_2$-heteroreceptors in the vas deferens.

### *Activation of muscarinic heteroreceptors by released acetylcholine in the urinary bladder*

An early study in rabbit atria (Löffelholz & Muscholl, 1970) showed that electrical stimulation of the vagus nerves reduced the release of noradrenaline elicited by stimulation of the accelerans nerves, suggesting that presynaptic muscarinic

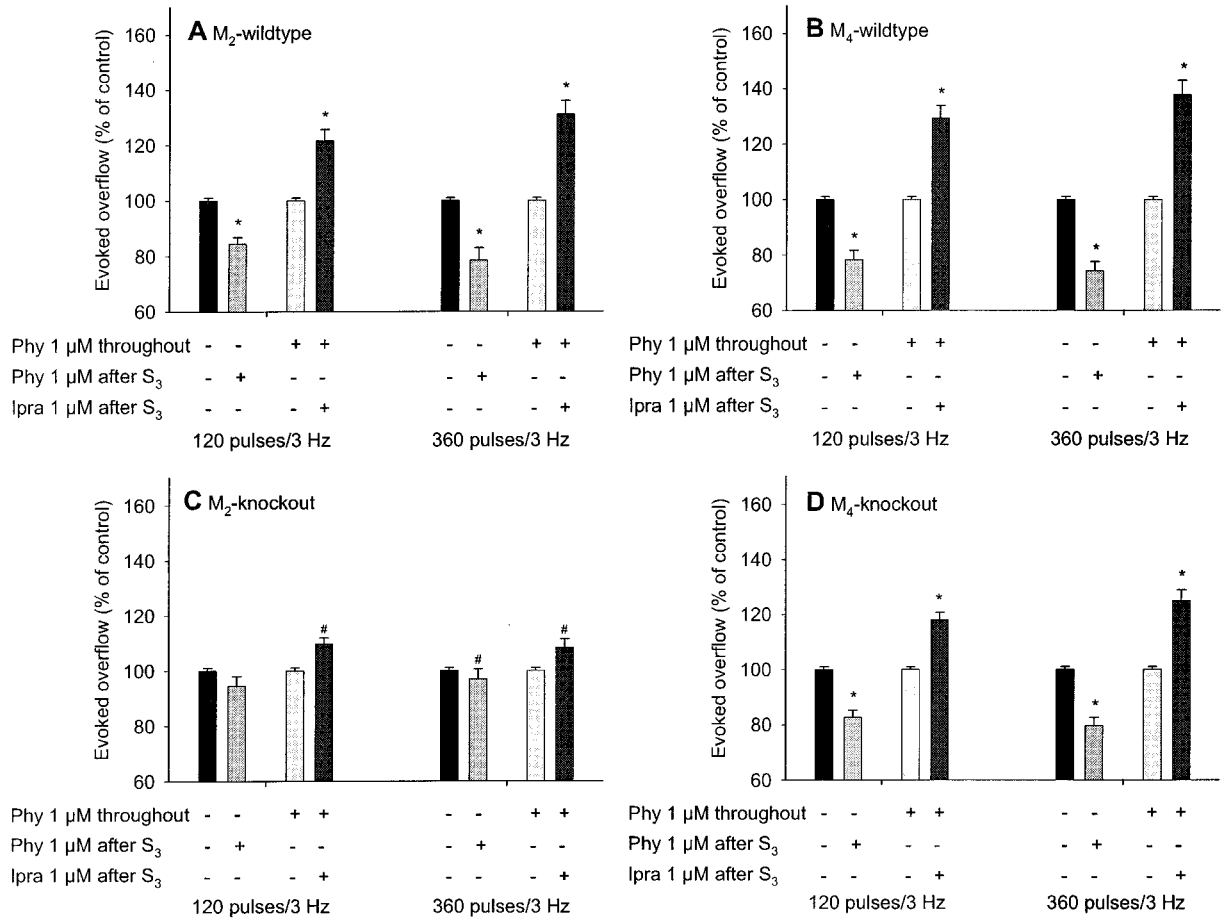


**Figure 7** Effects of physostigmine (Phy) and ipratropium (Ipra) on the evoked overflow of tritium from $M_2$-wildtype (A), $M_4$-wildtype (B), $M_2$-knockout (C) and $M_4$-knockout bladder (D). For details, see legend to Figure 6. Means ± s.e.mean from $n = 5$ to 13 tissue pieces. Significant differences from corresponding control (no drug added after $S_3$): $^*P < 0.05$. Significant differences from corresponding wildtype: $\#P < 0.05$. The tendencies of an inhibitory effect of physostigmine and a facilitatory effect of ipratropium, added after S3, are not significant (C).

receptors were operative physiologically. Consistent with this observation, subsequent studies with simultaneous field stimulation of sympathetic and parasympathetic axons showed, for example, that atropine increased the release of noradrenaline in mouse atria and rat urinary bladder, at least under certain experimental conditions (Somogyi & de Groat, 1990; Costa & Majewski, 1991). To study whether endogenously released acetylcholine could trigger muscarinic receptor-mediated inhibition of noradrenaline release in the urinary bladder, and if so through which subtype(s), we carried out systematic release studies using bladder preparations from NMRI as well as $M_2$- and $M_4$-knockout mice and the two corresponding wildtype control strains. In preparations from NMRI and $M_2$- and $M_4$-wildtype mice, physostigmine (1 $\mu$M) reduced, whereas ipratropium (1 $\mu$M), if added in the presence of physostigmine, increased the stimulated release of noradrenaline, consistent with the concept that endogenous acetylcholine activates release-inhibiting presynaptic muscarinic receptors. To reveal this effect, the pulse trains applied to stimulate transmitter release had to be relatively long, presumably to release sufficient amounts of acetylcholine and to leave time for muscarinic inhibition to develop. When trains of 120 or 360 pulses/3 Hz were used, physostigmine reduced the release of noradrenaline both when present throughout superfusion (Table 2; this effect was significant only in bladder pieces from NMRI mice) and when added after $S_3$ (Figures 6 and 7A,B). The release-facilitating effect of ipratropium became evident only in the presence of physostigmine, suggesting that high synaptic levels of acetylcholine were required to reveal this effect (Figure 6).

The release-inhibiting effects of endogenously released acetylcholine in mouse urinary bladder are mediated mainly by $M_2$-receptors. This notion is supported primarily by the following two observations. First, in bladder preparations from $M_2$-knockout mice, noradrenaline release in the presence of physostigmine throughout superfusion was increased, as compared to the corresponding responses in $M_2$-wildtype mice (Table 2), suggesting that activation of $M_2$-receptors by endogenously released acetylcholine caused inhibition of noradrenaline release. Second, both the release-inhibitory effect of physostigmine and the release-facilitatory effect of ipratropium, when added in the presence of physostigmine after $S_3$, were virtually abolished in bladder preparations from $M_2$-knockout mice (Figure 7C). As shown in Table 2, deletion of the $M_4$-receptor also led to a

significant increase in the release of noradrenaline in physostigmine-treated bladder preparations. This observation is consistent with a minor contribution of $M_4$-receptors, besides the predominant $M_2$-receptors, to the release-inhibitory effects of endogenously released acetylcholine in mouse urinary bladder.

In conclusion, presynaptic release-inhibiting muscarinic receptors occur on the sympathetic axons innervating the heart atria, urinary bladder and vas deferens of mice. In all three tissues, these receptors represent mixtures of $M_2$- and non-$M_2$-receptors. The non-$M_2$-receptors remain unknown in atria and the bladder, and appear to represent primarily $M_4$-receptors in the vas deferens. This is the first report demonstrating the co-existence of multiple inhibitory presynaptic muscarinic receptor subtypes in one and the same tissue. Our results underscore the usefulness of muscarinic receptor knockout mice to reveal the molecular identity of muscarinic heteroreceptors mediating inhibition of noradrenaline release. These results should contribute to a better understanding of the molecular mechanisms governing the interplay between the sympathetic and parasympathetic nervous systems under physiological and pathophysiological conditions.

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