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RESEARCH PAPER

Quantitative analysis of the loss of muscarinic receptors in various peripheral tissues in M_1–M_5 receptor single knockout mice

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Background and purpose: To compare loss in binding to muscarinic receptor (mAChR) subtypes with their known functions, the total density of muscarinic receptors was measured in peripheral tissues from wild type (WT) and mAChR knockout (KO) mice.
Experimental approach: Binding parameters of [N-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) were determined in 10 peripheral tissues of WT and M_1–M_5 receptor KO mice. Competition between [^{3}H]NMS and darifenacin (selective M_3 receptor antagonist) was also measured
Key results: There was an extensive loss of [^{3}H]NMS-binding sites (maximal number of binding sites, B_{max}) in heart and smooth muscle from M_2KO mice, compared with WT mice. Smooth muscle from M_3KO mice also showed a moderate loss of B_{max}. B_{max} fell in pancreas and bladder of M_4KO mice and in prostate in M_1KO and M_3KO mice. There was a large loss of B_{max} in exocrine and endocrine glands of M_3KO mice with a moderate decrease in M_2KO mice. Darifenacin inhibited specific [^{3}H]NMS binding in submandibular gland and bladder of WT, M_2KO and M_3KO mice. K_i (inhibition constant) values for darifenacin in the submandibular gland were the same in WT and M_2KO mice but increased in M_3KO mice. However, K_i values in bladder were decreased in M_2KO mice and increased in M_3KO mice.
Conclusions and implications: Single mAChR KO mice exhibit a loss of mAChR in peripheral tissues that generally paralleled the reported loss of function. Quantitative analysis of data, however, also suggested that, in some instances, normal expression of a receptor subtype depended on expression of other subtypes.
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Keywords: peripheral muscarinic receptor subtype; distribution; knockout mice

Abbreviations: [^{3}H]NMS, [N-methyl-^{3}H]scopolamine methyl chloride; B_{max}, maximal number of binding sites; K_d, apparent dissociation constant; K_i, inhibition constant; KO, knockout; mAChR, muscarinic acetylcholine receptor; WT, wild type

Introduction

The family of muscarinic acetylcholine receptors (mAChRs) consists of five molecularly distinct subtypes (M_1–M_5). Based on their G protein-coupling properties, the five receptors can be subdivided into major functional classes. M_1, M_3 and M_5 receptors are usually coupled to the $G_{q/11}$ protein that activates phospholipase C, whereas the M_2 and M_4 subtypes are mainly coupled to the $G_{i/o}$ protein that inhibits adenylate cyclase activity (Caulfield, 1993).

Muscarinic acetylcholine receptors are distributed throughout peripheral tissues. The actions mediated by peripheral mAChRs are closely related to parasympathetic functions including reductions in heart rate and the stimulation of glandular secretion and smooth muscle contraction (Bymaster *et al.*, 2003). The distribution of mAChR subtypes in peripheral tissues has been investigated mainly by pharmacological studies with subtype-selective agents and also by molecular and immuno-precipitation studies with measurements of levels of mRNA and protein (Doods *et al.*, 1987;

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Peralta *et al.*, 1987; Maeda *et al.*, 1988; Dörje *et al.*, 1991; Levey, 1993; Shida *et al.*, 1993; Boschero *et al.*, 1995; Krejčí and Tuček, 2002; Bymaster *et al.*, 2003). However, identification of the regional distribution of pharmacologically relevant mAChR subtypes has proven a difficult task, primarily due to the lack of highly selective ligands and to the fact that most tissues or organs express multiple mAChRs. One promising approach is to utilize mice deficient in specific mAChR receptor genes (M_1–M_5) generated by gene-targeting technology (Wess *et al.*, 2003; Matsui *et al.*, 2004).

In the present study, we have measured specific [N-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) binding in 10 peripheral tissues of wild type (WT) and mAChR subtype (M_1–M_5) knockout (KO) mice. The advantage of our technique is that it allows the direct estimation of physiologically or pharmacologically relevant receptors and can be adapted to investigate the interaction of acetylcholine (ACh) and selective agonists and antagonists with mAChR subtypes, as revealed by our current quantitation of central nervous system mAChR (Oki *et al.*, 2005). The decreases in the density of [^{3}H]NMS-binding sites in tissues of single mAChR KO mice would represent the net effect of a loss of the receptor together with any negative or positive compensatory changes. Our data have confirmed that there may be a significant tissue-based difference in the distribution of mAChR subtypes in the parasympathetic nervous system.

Methods

Animals

This study was conducted in accordance with the guide for care and use of laboratory animals as adopted by the US National Institutes of Health. Mice were housed with a 12 h light–dark cycle and fed laboratory food and water *ad libitum*. The generation and characterization of WT, M_1KO, M_2KO, M_3KO, M_4KO and M_5KO mice was described previously (Matsui *et al.*, 2004). The genetic background of the mice used in this study was a mixture of the 129/SvJ and C57BL/6J strains. Most of the animals used were at least N7th generation C57BL/6JJcl mice (CLEA, Japan). Accordingly, the WT mice used here are reasonably similar in genetic background to all five of the mutant lines.

Tissue preparation

Male (3–8 months of age) WT, M_1KO, M_2KO, M_3KO, M_4KO and M_5KO mice were exsanguinated by taking the blood from the descending aorta after an intraperitoneal administration of pentobarbitone (161 μmol·kg^{-1}), and the tissues were perfused with cold saline from the aorta. Then the submandibular gland, sublingual gland, lung, heart, stomach, pancreas, ileum, colon, bladder and prostate were excised, and fat and blood vessels were removed by trimming. The entire tissue was used for the measurement of mAChR subtypes. The tissues from three mice were pooled for a single determination, because of very low tissue weights. The tissues were minced with scissors and homogenized by a Kinematica Polytron homogenizer (Model K with a PTA10TS shaft) at 10 000 rpm in 19 volumes of ice-cold 30 mmol·L^{-1} Na$^+$/HEPES buffer (pH 7.5) for 1 min, and the homogenates were then centrifuged at 40 000× *g* for 20 min. The resulting pellet was suspended in buffer for the binding assay. Protein concentrations were measured by the method of Lowry *et al.* (1951).

Muscarinic receptor-binding assay

The binding assay for mAChR was performed by using [^{3}H]NMS as previously described (Ehlert and Tran, 1990; Oki *et al.*, 2005). The tissue weight (3–10 mg per assay) and protein concentration (34–1250 μg protein per assay) used for the assay differed among tissues, taking account of the amount of specific [^{3}H]NMS binding and on overall tissue weight. The ratios of protein to tissue weight in WT tissues were 0.06 (submandibular gland), 0.06 (sublingual gland), 0.04 (lung), 0.11 (heart), 0.07 (stomach), 0.13 (pancreas), 0.05 (ileum), 0.03 (colon), 0.06 (bladder) and prostate (0.04). The ratio in each tissue did not differ significantly between WT mice and each of the KO mice.

The crude membrane fractions of mouse tissues were incubated with different concentrations (0.06–1.0 nmol·L^{-1}) of [^{3}H]NMS in 30 mmol·L^{-1} Na$^+$/HEPES buffer (pH 7.5). Incubation was carried out for 60 min at 25°C. The reaction was terminated by rapid filtration (Cell Harvester, Brandel Co., Gaithersburg, MD, USA) through Whatman GF/B glass fibre filters, and the filters were then rinsed two times with 3 mL of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters overnight by immersion in scintillation fluid (2 L toluene, 1 L Triton X-100, 15 g 2,5-diphenyloxazole and 0.3 g 1,4-*bis*[2-(5-phenyloxazolyl)]benzene), and the radioactivity was measured with a liquid scintillation counter. Specific [^{3}H]NMS binding was determined experimentally from the difference between counts in the absence and presence of 1 μmol·L^{-1} atropine. All assays were conducted in duplicate.

In the competitive inhibition experiments with darifenacin, the inhibitory effects of five or six different concentrations (0.3 nmol·L^{-1}–3 μmol·L^{-1}) of this agent on the specific binding of [^{3}H]NMS (submandibular gland: 0.1 nmol·L^{-1}, bladder: 0.2 nmol·L^{-1}) in the submandibular gland and bladder of WT, M_2KO and M_3KO mice were examined.

Data analysis

[N-methyl-^{3}H]scopolamine methyl chloride binding data was subjected to a non-linear regression analysis by using Graph Pad PRISM (version 4, Graph Pad Software, San Diego, CA, USA). The apparent dissociation constant (K_d) and maximal number of binding sites (B_{max}) for [^{3}H]NMS were estimated. The ability of darifenacin to inhibit the specific binding of [^{3}H]NMS was estimated from the IC_{50}, which is the molar concentration of darifenacin necessary to displace 50% of the specific binding of [^{3}H]NMS (determined by Graph Pad PRISM). The inhibition constant, K_i, was calculated from the equation, $K_i = IC_{50}/(1 + L/K_d)$, where L equals the concentration of [^{3}H]NMS.

Statistical analysis of the data was performed by a one-way analysis of variance, followed by Dunnett's test for multiple comparison. Statistical significance was accepted at $P < 0.05$.

Materials

[N-methyl-^{3}H]scopolamine methyl chloride (3.03 TBq·mmol^{-1}) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). All other chemicals were purchased from commercial sources.

Results

[^{3}H]NMS-binding characteristics in mAChR KO mice

The specific binding of [^{3}H]NMS (0.06–1.0 nmol·L^{-1}) was saturable in crude membrane fractions of 10 peripheral tissues (submandibular gland, sublingual gland, lung, heart, stomach, pancreas, ileum, colon, bladder and prostate) of WT and each (M_1–M_5) mAChR KO mice. Table 1 shows pK_d and B_{max} values for specific [^{3}H]NMS binding in these tissues. The binding parameters in each tissue of M_1–M_5 subtype KO mice were compared with those of WT mice.

In the submandibular gland, sublingual gland and pancreas, the B_{max} values for [^{3}H]NMS binding were markedly (70–80%) lower in M_3KO mice than in WT mice. Similarly, in M_2KO mice, there were significant (40%) decreases in B_{max} values in the submandibular gland and pancreas and a tendency for decrease (29%) in the sublingual gland. In the latter case, it is considered that there may be changes, but the low number of experiments did not allow us to detect the difference. In other words, the statistical power of the data was insufficient to detect a 29% difference. The M_4KO mice showed a slight (20%) loss of [^{3}H]NMS-binding sites only in the pancreas.

There was a significant (90%) decrease in B_{max} values for [^{3}H]NMS binding in the heart of M_2KO mice compared with WT mice. The B_{max} value for cardiac [^{3}H]NMS binding was not significantly altered in the other mice relative to WT mice.

In the lung and ileum, there were significant (30–90%) decreases in B_{max} values for [^{3}H]NMS binding in M_2KO and M_3KO mice compared with WT mice. The decrease was greatest (90%) in the lung and ileum of M_2KO mice and moderate (30% and 40% respectively) in M_3KO mice. The B_{max} values for [^{3}H]NMS binding in the stomach and colon were markedly (90% and 70% respectively) lower in M_2KO mice than WT mice. Also, there was a significant (30%) decrease in the lungs of M_5KO mice but a non-significant decrease in the stomach and colon of M_3KO mice.

In the bladder, the greatest (80%) decrease in B_{max} values for [^{3}H]NMS binding was in M_2KO mice, with moderate (40%, 30% and 30% respectively) decreases in M_3KO, M_4KO and M_5KO mice. These decreases were statistically significant. The binding in the bladder of M_1KO mice was similar to that in WT mice.

There were significant (60% and 40% respectively) decreases in B_{max} values for [^{3}H]NMS binding in the prostate of M_1KO and M_3KO mice with a tendency for lower (18%) B_{max} values in M_2KO mice, compared with WT mice.

There was little change in the pK_d values for [^{3}H]NMS binding in peripheral tissues of any of the KO mice compared with WT mice, except in the lung, ileum, colon and bladder that showed significant (2.3-, 5.4-, 2.4- and 3.1-fold respectively) increases in M_2KO mice.

Table 1 K_d and B_{max} for specific [N-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) binding in the peripheral tissues of wild type (WT) and muscarinic acetylcholine receptor (mAChR) subtype (M_1–M_5) knockout (KO) mice

Tissues	*KO type*	*pK_d*	*B_{max} (fmol·mg·protein^{-1})*
Submandibular gland	WT	9.88 ± 0.02	174 ± 9 (1.0)
	M_1KO	9.86 ± 0.04	169 ± 20
	M_2KO	9.93 ± 0.02	112 ± 8 (0.6)**
	M_3KO	9.96 ± 0.05	37 ± 3 (0.2)**
	M_4KO	9.90 ± 0.03	160 ± 12
	M_5KO	9.88 ± 0.03	147 ± 11
Sublingual gland	WT	9.85 ± 0.05	183 ± 11 (1.0)
	M_1KO	9.84 ± 0.04	170 ± 23
	M_2KO	9.87 ± 0.04	130 ± 4
	M_3KO	9.94 ± 0.06	51 ± 6 (0.3)**
	M_4KO	9.83 ± 0.07	172 ± 15
	M_5KO	9.82 ± 0.04	151 ± 16
Lung	WT	9.67 ± 0.04	72 ± 5 (1.0)
	M_1KO	9.68 ± 0.03	80 ± 7
	M_2KO	10.03 ± 0.02**	6.9 ± 1.2 (0.1)**
	M_3KO	9.62 ± 0.03	49 ± 4 (0.7)*
	M_4KO	9.68 ± 0.04	55 ± 6
	M_5KO	9.75 ± 0.03	50 ± 3 (0.7)*
Heart	WT	9.44 ± 0.04	29 ± 1 (1.0)
	M_1KO	9.44 ± 0.07	32 ± 3
	M_2KO	9.29 ± 0.15	2 ± 1 (0.1)**
	M_3KO	9.45 ± 0.06	25 ± 3
	M_4KO	9.46 ± 0.03	28 ± 4
	M_5KO	9.43 ± 0.04	28 ± 1
Stomach	WT	9.54 ± 0.05	192 ± 25 (1.0)
	M_1KO	9.52 ± 0.04	194 ± 11
	M_2KO	9.80 ± 0.12	12 ± 2 (0.1)**
	M_3KO	9.50 ± 0.11	144 ± 34
	M_4KO	9.46 ± 0.05	199 ± 11
	M_5KO	9.43 ± 0.08	164 ± 18
Pancreas	WT	9.61 ± 0.08	19 ± 1 (1.0)
	M_1KO	9.76 ± 0.03	16 ± 1
	M_2KO	9.76 ± 0.07	12 ± 2 (0.6)**
	M_3KO	9.75 ± 0.08	5 ± 1 (0.3)**
	M_4KO	9.75 ± 0.10	15 ± 1 (0.8)*
	M_5KO	9.67 ± 0.05	17 ± 1
Ileum	WT	9.38 ± 0.06	366 ± 43(1.0)
	M_1KO	9.23 ± 0.06	506 ± 59
	M_2KO	10.11 ± 0.07**	39 ± 8 (0.1)**
	M_3KO	9.37 ± 0.13	232 ± 27 (0.6)*
	M_4KO	9.36 ± 0.07	278 ± 40
	M_5KO	9.28 ± 0.03	327 ± 26
Colon	WT	9.57 ± 0.08	234 ± 25 (1.0)
	M_1KO	9.59 ± 0.06	273 ± 29
	M_2KO	9.92 ± 0.17*	65 ± 9 (0.3)**
	M_3KO	9.62 ± 0.10	190 ± 23
	M_4KO	9.48 ± 0.04	233 ± 19
	M_5KO	9.49 ± 0.06	222 ± 40
Bladder	WT	9.58 ± 0.03	128 ± 6 (1.0)
	M_1KO	9.49 ± 0.04	136 ± 15
	M_2KO	10.07 ± 0.05**	23 ± 5 (0.2)**
	M_3KO	9.49 ± 0.11	77 ± 10 (0.6)*
	M_4KO	9.40 ± 0.09	83 ± 7 (0.7)*
	M_5KO	9.55 ± 0.11	84 ± 15 (0.7)*
Prostate	WT	9.91 ± 0.03	158 ± 7 (1.0)
	M_1KO	9.74 ± 0.08	60 ± 10 (0.4)**
	M_2KO	9.82 ± 0.04	130 ± 29
	M_3KO	9.91 ± 0.11	95 ± 21 (0.6)*
	M_4KO	9.77 ± 0.02	153 ± 10
	M_5KO	9.88 ± 0.02	177 ± 28

The specific binding of [^{3}H]NMS (0.06–1.0 nmol·L^{-1}) was measured in peripheral tissues taken from WT and mAChR KO mice. The ages (in weeks) of these mice were 13–39 (WT), 14–46 (M_1KO), 13–31 (M_2KO), 12–51 (M_3KO), 11–26 (M_4KO) and 11–40 (M_5KO). Each tissue was derived from the same animals. In addition, there was little difference in the age of each mouse used in determining [^{3}H]NMS-binding parameters (K_d, B_{max}). Values in parentheses represent the B_{max} relative to WT (controls). Values represent the mean ± SEM of three to eight determinations.

Asterisks show a significant difference from the values in WT mice, *$P < 0.05$, **$P < 0.01$.

Table 2 Competitive inhibition by darifenacin of specific [N-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) binding in the submandibular gland and bladder of wild type (WT), M_2 and M_3 knockout (KO) mice

	Submandibular gland		*Bladder*		K_i *(submandibular gland)/*K_i *(bladder)*
	K_i *(nmol·L^{-1})*	*nH*	K_i *(nmol·L^{-1})*	*nH*	
WT	3.0 ± 0.6*	0.8 ± 0.1	24 ± 7	0.6 ± 0.1	0.1
M_2KO	4.3 ± 0.9$^{\#\#}$	1.0 ± 0.1†	2.5 ± 0.7$^{\dagger,\#}$	1.0 ± 0.2†	1.7
M_3KO	19 ± 2*,††	1.1 ± 0.1†	43 ± 10†	0.9 ± 0.1†	0.4

The submandibular gland and bladder were taken from WT and muscarinic acetylcholine receptor (M_2, M_3) KO mice. Specific [^{3}H]NMS binding in the submandibular gland (0.1 nmol·L^{-1}) and bladder (0.2 nmol·L^{-1}) was measured in the absence and presence of five to six different concentrations (0.3 nmol·L^{-1}–3 μmol·L^{-1}) of darifenacin, and the values of K_i and Hill coefficients (nH) were estimated. Values represent the mean ± SEM of three to six determinations.
Symbols show a significant difference from the values for bladder in the same group of mice (*$P < 0.05$), a significant difference from the values for the same tissue in WT mice ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.001$) and a significant difference from the values for the same tissue in M_3KO mice ($^{\#}P < 0.05$, $^{\#\#}P < 0.001$).

Table 3 Semiquantitative localization of muscarinic acetylcholine receptor (mAChR) subtypes (M_1–M_5) in various tissues of mice

Tissues	*Loss of mAChR sites in muscarinic knockout mice*	*mRNA expression*	*Antibody binding*
Submandibular gland	$M_3 > M_2$	M_3 (Shida *et al.*, 1993) M_1, M_3 (Gautam *et al.*, 2004)	M_3 (42%), M_1 (36%), M_2 (12%), M_2 (7%) (Dörje *et al.* 1991)
Sublingual gland	M_3		
Lung	$M_2 > M_3$, M_5	M_1, M_2, M_3 (Struckmann *et al.*, 2003)	M_4 (41%), M_2 (40%) (Dörje *et al.*, 1991)
Heart	M_2	M_2 (Maeda *et al.*, 1988) M_2 (Peralta *et al.*, 1987) M_2 (Levey, 1993) M_2 (>90%) (Krejčí and Tuček, 2002)	M_2 (88%) (Krejčí and Tuček, 2002)
Stomach	M_2	M_1–M_5 (Aihara *et al.*, 2005)	
Pancreas	$M_3 > M_2$, M_4	M_4 (Peralta *et al.*, 1987) M_1, M_3 (Boschero *et al.*, 1995)	
Ileum	$M_2 > M_3$		M_2 (69%), M_4 (12%), M_3 (4%), M_1 (3%) (Dörje *et al.*, 1991) M_2, M_4 (Takeuchi *et al.*, 2002)
Colon	M_2		
Bladder	$M_2 > M_3$, M_4, M_5	M_2, M_3 (Maeda *et al.*, 1988)	M_1–M_5 (Giglio *et al.*, 2005)
Prostate	$M_1 > M_3$	M_3 (Kim *et al.*, 2005)	M_2 (Ruggieri *et al.*, 1995) M_2, M_3 (Pontari *et al.*, 1998) M_1 (>70%) (Ruggieri *et al.*, 1995)

The rank order is based on the loss of [N-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS)-binding sites measured in each single muscarinic receptor knockout mice (M_1–M_5), in comparison with wild type, as shown in Table 1. The distribution of mRNA and antibody binding was taken from the references shown.

Competitive inhibition by darifenacin of specific [^{3}H]NMS binding in the submandibular gland and bladder of WT, M_2KO and M_3KO mice

We examined the competitive inhibition of specific [^{3}H]NMS binding by darifenacin, a highly selective antagonist of the M_3 receptor subtype, in the submandibular gland and bladder from WT, M_2KO and M_3KO mice. Darifenacin (0.3 nmol·L^{-1}–3 μmol·L^{-1}) inhibited the binding of [^{3}H]NMS in these tissues in a concentration-dependent manner. As shown in Table 2, the K_i value of darifenacin for the competitive inhibition of specific [^{3}H]NMS binding in the submandibular gland was not changed in the M_2KO mice (4.3 nmol·L^{-1}) compared with WT mice (3.0 nmol·L^{-1}) and was significantly (6.3-fold) increased in M_3KO mice (19 nmol·L^{-1}). On the other hand, the K_i value of darifenacin in the bladder was markedly decreased in the M_2KO mice (2.5 nmol·L^{-1}) compared with WT mice (24 nmol·L^{-1}) and was significantly increased in the M_3KO mice. Thus, the ratios of K_i (submandibular gland)/K_i (bladder) were 0.1 (WT), 1.7 (M_2KO) and 0.4 (M_3KO). Further, the Hill coefficients for the competition by this agent in both tissues were significantly increased, being close to unity in M_2KO and M_3KO mice compared with WT mice. These results are consistent with the idea that the M_3 receptor is predominant in the submandibular gland while the M_2 receptor is dominant in the bladder.

Discussion

The binding parameters of the five mAChRs were compared in peripheral tissues of WT and mAChR subtype (M_1–M_5) KO mice. Table 3 summarizes the relative loss of mAChR-binding sites in tissues of mAChR KO mice based on the data in Table 1, with other information such as mRNA expression and antibody binding of mAChR subtypes from previously published work. Functional and immunological studies have consistently shown that the M_3 receptor subtype plays a key role in the parasympathetic control of salivation (Caulfield, 1993; Levey, 1993; Moriya *et al.*, 1999). In fact, severe hyposalivation that led to growth failure due to eating difficulties was previously demonstrated in M_3KO mice (Matsui *et al.*, 2000; 2004). The M_3 receptor may account for at least 70% of all

mAChRs present in the submandibular and sublingual glands, as estimated by the loss of exocrine [^{3}H]NMS-binding sites in M_3KO mice compared with WT mice. Also, the mRNA for the M_3 receptor subtype has been found in rat exocrine glands (Maeda *et al.*, 1988). In M_2KO mice, there was moderate loss of [^{3}H]NMS-binding sites in exocrine glands. Although there is little published to explain the physiological significance of exocrine M_2 receptors, *in situ* hybridization by using oligonucleotide probes has shown that the mRNA for the M_2 receptor subtype is expressed in the rat salivary gland (Shida *et al.*, 1993). Takeuchi *et al.* (2002) found, using M_5KO mice, that this subtype may be involved in the slow secretory process of pilocarpine-induced salivation, and our data in Table 1 disclose a slight loss of [^{3}H]NMS-binding sites in both submandibular and sublingual glands of the M_5KO mice.

A previous functional study on our mAChR KO mice had shown that the acinar cells of the submandibular glands expressed lower levels of M_1 receptors than M_3 receptors (Gautam *et al.*, 2004; Nakamura *et al.*, 2004). In the current study, however, the submandibular glands of M_1KO mice showed no significant reduction of [^{3}H]NMS binding compared with those of WT mice (Table 1). This may indicate that the numbers of M_1 receptors in the submandibular glands of WT mice were so small that the difference between the genotypes was below the level detectable by our receptor-binding assay. Alternatively, the expression of residual subtypes might be up-regulated to compensate for the loss of M_1 receptors in the M_1KO mice.

The pancreas showed a significant (40% and 70% respectively) loss of mAChR-binding sites in M_2KO and M_3KO mice, compared with WT mice. The predominance of M_3 receptors in the pancreas has provided convincing evidence for a functional role of this subtype in cholinergic secretory responses of insulin in pancreatic islets (Boschero *et al.*, 1995; Zawalich Zawalich *et al.*, 2004) and of amylase in acinar cells (Gautam *et al.*, 2005). Further, the mRNA for the M_3 receptor subtype was detected in rat pancreatic islet cells (Boschero *et al.*, 1995). Consistent with previous observations (Dörje *et al.*, 1991; Levey, 1993), the density of mAChR M_2 receptors in cardiac and smooth muscular tissues was confirmed to be high, by using M_1–M_5 receptor KO mice. It is known that cardiac responses to cholinergic agonists are exclusively mediated through stimulation of the M_2 receptor subtype (Caulfield, 1993; Stengel *et al.*, 2000). The present data obtained with KO mice have shown M_2 receptors to predominate in the mouse heart. This observation is fully consistent with the quantitative data for M_2 receptor mRNA representing more than 90% of all mAChR mRNA in rat atria and ventricles (Krejčí and Tuček, 2002). The predominance of M_2 receptor mRNA in the heart was shown also by others (Peralta *et al.*, 1987; Maeda *et al.*, 1988; Levey, 1993).

The mRNA for the M_2 receptor subtype was expressed in smooth muscular tissues (Maeda *et al.*, 1988; Levey, 1993). In the lung, stomach, ileum, colon and bladder, the M_2 receptor predominated. Also, there were moderate amounts of the M_3 receptor in the lung, ileum and bladder. These subtypes are involved in mechanical responses of smooth muscles to cholinergic agonists (Matsui *et al.*, 2000; 2004; Struckmann *et al.*, 2003; Wess *et al.*, 2003). The lung mAChR is involved in bronchial parasympathetic control. The measurement of lung mAChR revealed the M_2 receptor subtype to be predominant with moderate levels of the M_3 and M_5 receptors. The coexistence of M_2 and M_3 receptors in the lung has been suggested previously (Esqueda *et al.*, 1996; Myers and Undem, 1996; Struckmann *et al.*, 2003). Notably, the videomicroscopy and digital imaging of lung slices has shown that muscarine-mediated bronchoconstriction was diminished partly in M_3KO mouse lung and completely abolished in M_2/M_3 double KO mouse lung, suggesting a concerted action of both subtypes in the cholinergic constriction of murine airways (Struckmann *et al.*, 2003).

Interestingly, in the bladder, there might be significant loss of mAChR-binding sites in M_4KO and M_5KO mice in addition to M_2KO and M_3KO mice. A recent immunohistochemical study by Giglio *et al.* (2005) showed all subtypes of mAChR to be present in rat urinary bladder with a prominent increase in the expression of the M_5 receptor subtype in both smooth muscle and urothelium from cyclophosphamide-treated rats. Hence, the M_5 receptor may be significantly involved in the pathogenesis of urinary bladder disorders such as interstitial cystitis. Also, it should be noted that the negative feedback mechanism inhibiting the release of ACh in the urinary bladder may be mediated by prejunctional M_4 receptors (D'Agostino *et al.*, 1997; Zhou *et al.*, 2002).

The K_i values (nmol·L^{-1}) for the competitive inhibition by darifenacin of specific [^{3}H]NMS binding in CHO-K1 cell lines expressing human mAChR subtypes were 31 (M_1), 57 (M_2), 2.5 (M_3), 16 (M_4) and 9.6 (M_5) (Maruyama *et al.*, 2006). In this case, the K_i value was significantly smaller for the M_3 receptors than the M_1 and M_2 receptor subtypes. The K_i values in the submandibular gland of WT mice (3.0 nmol·L^{-1}) and M_2KO mice (4.3 nmol·L^{-1}) and in the bladder of M_2KO mice (2.5 nmol·L^{-1}) (Table 2) were close to the K_i value for the human M_3 receptor. The K_i values in the bladder of M_2KO (2.5 nmol·L^{-1}) and M_3KO (43 nmol·L^{-1}) mice seem to reflect the affinity of darifenacin mainly for the M_3 and M_2 receptor subtypes respectively. These results are consistent with the major subtype of mAChR being M_3 in the submandibular gland and M_2 in the bladder.

Ruggieri *et al.* (1995) showed the localization of the M_2 receptor in rat prostate using an antibody, whereas receptor binding and pharmacological studies with selective antagonists suggested the presence of functional M_3 receptors (Latifpour *et al.*, 1991; Yazawa and Honda, 1993; Lau and Pennefather, 1998). In agreement with pharmacological observations, there was significant loss of mAChR-binding sites in the prostate of M_3KO mice compared with WT mice. The greater decrease of mAChR density was also observed in this tissue in M_1KO mice. To our knowledge, the existence of the M_1 receptor subtype in the murine prostate is little reported, but, interestingly, an antibody study in human prostate revealed a major (>70%) presence of M_1 receptors in the glandular epithelial cells (Ruggieri *et al.*, 1995).

The M_5 receptor was shown to be expressed ubiquitously throughout the brain and in non-neuronal tissues such as skin fibroblasts and keratinocytes, endothelial cells and smooth muscle of the neurovasculature and lymphocytes (Ndoye *et al.*, 1998; Buchli *et al.*, 1999). The M_5 receptor has been the hardest mAChR subtype to study for at least two reasons: no selective ligands for the M_5 receptor have been

found, and no tissues have been found where M_5 receptors are in higher concentrations than all other mAChRs. In M_5KO mice, compared with WT mice, there were moderate decreases in B_{max} values for [^{3}H]NMS binding in the lung and bladder (Table 1).

There were significant increases in pK_d values for [^{3}H]NMS binding in the lung, ileum, colon and bladder of M_2KO mice. It is reported that [^{3}H]NMS exhibits higher affinity for M_1, M_3 and M_4 receptors than M_2 receptors as shown in K_d values of 120 pmol·L^{-1} (M_1, NB-OK1 cells), 500 pmol·L^{-1} (M_2, rat heart), 120 pmol·L^{-1} (M_3, rat pancreas) and 50 pmol·L^{-1} (M_4, rat striatum) (Waelbroeck *et al.*, 1990). Therefore, the significant increases in pK_d values in these tissues in M_2KO mice may be due to the enhanced affinity of [^{3}H]NMS for the residual receptors, following the marked loss of the M_2 receptors.

The sum of the loss in mAChR-binding sites in the single KO mice greatly exceeds the binding sites in WT, indicating that in some instances, the expression of a given receptor subtype depends on the expression of the other subtypes. The sum of the reduction in all the individual B_{max} values in the lung and bladder of each of the KO mice relative to WT mice was 1.5- and 1.8-fold greater, respectively, than B_{max} values of WT mice (Table 1). This suggests that the loss of some mAChR subtypes causes a decrease, and not a compensatory *increase*, in the residual mAChR-binding sites. Although the precise mechanism of this unexpected phenomenon is unclear at present, it should be noted that exposure to chronically increased release of ACh may cause a down-regulation of mAChR expression (Yamada *et al.*, 1983a,b). A similar consideration was noted in the corpus striatum and pons-medulla oblongata of mAChR subtype KO mice relative to WT mice (Oki *et al.*, 2005). M_2 and M_4 receptors are located at the nerve terminals as autoreceptors to inhibit ACh release, and these prejunctional autoreceptors may function in the lung and bladder (Kilbinger *et al.*, 1995; Myers and Undem, 1996; D'Agostino *et al.*, 1997; Zhou *et al.*, 2002; Bymaster *et al.*, 2003). Hence, in M_2KO and M_4KO mice, ACh levels in the cholinergic synapses of lung and bladder are presumably elevated. Taken together, it is conceivable that the decreases in the [^{3}H]NMS-binding sites in these tissues of M_2KO and M_4KO mice reflect not only loss of these subtypes but also desensitization of the other receptor subtypes caused by a hyper-cholinergic status. Alternatively, 'cross-talk' might occur among mAChR subtypes, resulting in compensatory changes, as has been assumed for β_3 adrenoceptor KO mice (Susulic *et al.*, 1995).

In conclusion, the comparative measurement of mAChRs in KO mouse tissues has confirmed the pharmacologically relevant M_1–M_5 subtypes are widely distributed throughout peripheral tissues but with significant variation. The quantitative distribution of mAChR subtypes may contribute not only to further understanding of cholinergic function but to the development of novel muscarinic therapeutic drugs, such as selective antagonists of the M_4 and M_5 receptors.

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Conflict of interest

The authors state no conflict of interest.

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