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ORIGINAL ARTICLE

The guinea pig ileum lacks the direct, high-potency, M_2-muscarinic, contractile mechanism characteristic of the mouse ileum

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Abstract We explored whether the M_2 muscarinic receptor in the guinea pig ileum elicits a highly potent, direct-contractile response, like that from the M_3 muscarinic receptor knockout mouse. First, we characterized the irreversible receptor-blocking activity of 4-DAMP mustard in ileum from muscarinic receptor knockout mice to verify its M_3 selectivity. Then, we used 4-DAMP mustard to inactivate M_3 responses in the guinea pig ileum to attempt to reveal direct, M_2 receptor-mediated contractions. The muscarinic agonist, oxotremorine-M, elicited potent contractions in ileum from wild-type, M_2 receptor knockout, and M_3 receptor knockout mice characterized by negative log EC_{50} (pEC_{50}) values ± SEM of 6.75±0.03, 6.26±0.05, and 6.99±0.08, respectively. The corresponding E_{max} values in wild-type and M_2 receptor knockout mice were approximately the same, but that in the M_3 receptor knockout mouse was only 36% of wild type. Following 4-DAMP mustard treatment, the concentration–response curve of oxotremorine-M in wild-type ileum resembled that of the M_3 knockout mouse in terms of its pEC_{50}, E_{max}, and inhibition by selective muscarinic antagonists. Thus, 4-DAMP mustard treatment appears to inactivate M_3 responses selectively and renders the muscarinic contractile behavior of the wild-type ileum similar to that of the M_3 knockout mouse. Following 4-DAMP mustard treatment, the contractile response of the guinea pig ileum to oxotremorine-M exhibited low potency and a competitive-antagonism profile consistent with an M_3 response. The guinea pig ileum, therefore, lacks a direct, highly potent, M_2-contractile component but may have a direct, lower potency M_2 component.

Keywords Ileum · Guinea pig · Muscarinic receptor knockout mice · 4-DAMP mustard · M_2 muscarinic receptor · M_3 muscarinic receptor

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Introduction

Subtype selective antagonists inhibit muscarinic contractions of gastrointestinal and urinary bladder smooth muscle in a manner that agrees with an M_3 receptor mechanism (Eglen et al. 1996). This behavior is consistent with the known coupling of the M_3 receptor to $G_{q/11}$ (Noronha-Blob et al. 1989; Candell et al. 1990; Roffel et al. 1990), which is often involved in Ca^{2+} mobilization. The details of how M_3-$G_{q/11}$ signaling leads to contraction are unclear, however, because contraction of the guinea pig ileum depends mainly on an extracellular source of Ca^{2+} (Bolger et al. 1983).

The M_2 muscarinic receptor is also expressed in smooth muscle, and it outnumbers the M_3 by a factor of at least four (Eglen et al. 1996). The apparent lack of a role of the

M_2 receptor in contraction can be explained by the nature of its known signaling mechanisms in smooth muscle (see Table 1). Stimulation of the M_2 receptor activates a nonselective cation conductance; however, the conductance depends on Ca^{2+} (Bolton 1979; Inoue 1991; Sakamoto et al. 2007). This explains why there is little increase in conductance unless both the M_2 and the Ca^{2+}-mobilizing M_3 receptor are activated simultaneously. The M_2 receptor is also known to inhibit Ca^{2+}-activated K^+ channels, which release smooth muscle from inhibitory K^+ currents and enhance contraction (Kotlikoff et al. 1992). Like the cation conductance, however, this mechanism also requires Ca^{2+} mobilization by another receptor to activate the K^+ current before the M_2 receptor can inhibit it. Finally, the M_2 receptor inhibits adenylate cyclase in smooth muscle. This inhibition opposes the relaxant effect of receptors that increase cyclic adenosine monophosphate (cAMP; e.g., β-adrenoceptor; Thomas et al. 1993; Thomas and Ehlert 1996; Sawyer and Ehlert 1998; Ehlert et al. 2005), and this effect requires Ca^{2+} mobilization in the first place; otherwise, there is no contraction to be relaxed by the β-adrenoceptor and no β-adrenoceptor response for the M_2 receptor to inhibit. In trachea, M_2 receptor activation opposes forskolin- but not isoproterenol-induced relaxation, which is consistent with the postulate that the β_2-adrenoceptor mediates relaxation through a non-cAMP mechanism in the trachea (Ostrom and Ehlert 1998; 1999).

It may seem that these M_2 effects would be manifest in standard pharmacological antagonism studies. However, we have shown that the competitive antagonism of a response mediated through M_2–M_3 receptor interactions resembles the profile of the directly acting receptor (i.e., the M_3) and not that of the conditionally acting receptor (i.e., the M_2; Ehlert 2003). Thus, the M_3 antagonism profile of standard muscarinic contractions of the ileum and bladder is not inconsistent with the postulate that both M_2 and M_3 receptors interact to elicit contraction.

Studies on the mouse uterus are consistent with a conditional role for the M_2 receptor in contraction (Kitazawa et al. 2008). The competitive antagonism of the muscarinic contractile response of wild-type uterus resembles an M_3 profile, but the E_{max} for contraction is inhibited by about 50% in uterus from the M_2 knockout (KO) mouse. In the M_3 KO mouse, muscarinic contractions are absent. Thus, the M_2 receptor is unable to elicit direct contraction of the mouse uterus, but is able to enhance M_3 receptor-mediated contractions.

Following inactivation of M_3 receptors in guinea pig ileum and colon, we have identified two types of muscarinic contractile responses for the M_2 receptor. One is a highly potent M_2 receptor-mediated inhibition of forskolin- and β-adrenoceptor-mediated relaxation, and the other is a less potent M_2 receptor-mediated enhancement of M_3 receptor contractile signaling (Ehlert 2003). Circumstantial evidence suggests that the latter is involved in heterologous desensitization, which requires activation of both M_2 and M_3 receptors and is potently antagonized by M_2 selective antagonists (Griffin et al. 2004).

Studies on muscarinic receptor knockout mice are consistent with these observations but have revealed an additional, highly potent, direct-contractile mechanism for the M_2 receptor. In M_3 KO mice, the M_2 receptor elicits a highly potent contractile response in ileum and trachea, although the maximum of this response is only about 40% that of the muscarinic contraction in wild-type and M_2 KO tissue (Matsui et al. 2000; Matsui et al. 2002).

Table 1 Summary of the types of contractions elicited by M_2 and M_3 muscarinic receptors in smooth muscle and their putative mechanisms

Receptor	Type of contraction	Tissue and species	Putative mechanism
M_2	Direct contraction	Mouse: ileum, trachea, and urinary bladder	Unknown, G_i mobilization of extracellular Ca^{2+}
	Conditional inhibition of cAMP-mediatied relaxation	Mouse: ileum, trachea, and urinary bladder Guinea pig: ileum, trachea, colon, and esophagus	G_i-mediated inhibition of adenylate cyclase
	Conditional enhancement of M_3-receptor-mediated contraction	Mouse: ileum, urinary bladder, and uterus Guinea pig: colon and ileum	G_i stimulation of I_{cat}; G_i inhibition of BK_{Ca}
M_3	Direct contraction	Widespread in guinea pig and mouse smooth muscle	Major: unknown G_q-mediated influx of extracellular Ca^{2+} Minor: G_q-mediated phosphoinositide hydrolysis and release of intracellular Ca^{2+}

Contraction is defined as *direct* if activation of the indicated receptor by itself is sufficient to cause contraction. If activation of the receptor subtype by itself has no effect on contraction but elicits or enhances contraction when other receptors are activated, then the muscarinic contraction is defined as *conditional*. Further details are described in the text.

I_{cat} nonselective cation conductance, BK_{Ca} Ca^{2+}-activated potassium channel

In the present study, we have investigated whether a similar, highly potent, direct-M_2-receptor-mediated contraction occurs in the guinea pig ileum. We show that treatment of the wild-type mouse ileum with 4-DAMP mustard (*N*-2-chloroethyl-4-piperidinyl diphenylacetate) uncovers a highly potent, direct-M_2-contractile mechanism and converts its pharmacological behavior into that of the M_3 KO mouse. In contrast, treatment of the guinea pig ileum with 4-DAMP mustard caused a large, 56-fold reduction in agonist potency, and the residual muscarinic response exhibited an M_3-pharmacological profile. Thus, the guinea pig ileum appears to lack the highly potent direct-contractile-M_2 mechanism observed in the mouse. Our data also illustrate that ileal smooth muscle from whole-body M_3 KO mice accurately displays the contractile activity of the M_2 receptor in wild-type mice and that 4-DAMP mustard is a useful tool for inactivating the M_3 responses selectively.

Methods

Animals M_2 muscarinic receptor knockout (M_2−/−; M_2 KO) and M_3 muscarinic receptor knockout (M_3−/−; M_3 KO) mice were generated as described by Matsui et al. (2002).

Contractile assays in isolated ileal tissue Contractile measurements were made on ileum from male Hartley guinea pigs (300–400 g) and C57Bl-6 mice (25–30 g) as described previously (Griffin et al. 2004). The medium was Krebs–Ringer bicarbonate (KRB) buffer (124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose) containing indomethacin (1 μM) and maintained at 37°C and gassed with O_2/CO_2 (19:1). Tissues were allowed to incubate for at least an hour and were subsequently challenged with KCl (50 mM) three times, followed by the measurement of a cumulative concentration–response curve to oxotremorine-M. This was done to speed up the equilibration of the ileum, which undergoes a time-dependent increase in contractile activity. After appropriate washing, the ileum was incubated for another 30 min prior to the collection of the data presented under "Results". Contractile responses to oxotremorine-M were measured using a cumulative technique. The E_{max} value of oxotremorine-M increased with an increase in age or body weight of each strain of mouse. In addition, the ileal contraction to KCl (50 mM) increased with an increase in body weight but was similar in mice of equivalent body weights across the different strains. All contractions to oxotremorine-M, therefore, were normalized relative to that elicited by KCl (50 mM).

4-DAMP mustard treatment A solution of 4-DAMP mustard was first cyclized in 10 mM phosphate buffer, pH 7.4, for 30 min at 37°C to allow formation of the aziridinium ion (Thomas et al. 1992). The solution was placed on ice and used as soon as possible. Ilea were incubated with 4-DAMP mustard (40 nM) in combination with the M_2 selective antagonist AF-DX 116 (4 μM; [[2-2(diethyl-amino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3b][1,4] benzodiazepine-6-one) for one or two 1-h time periods, in a final volume of 50 ml of KRB buffer. This minimum volume of medium is essential because 4-DAMP mustard is inactivated by tissue nucleophiles, particularly in substantial tissues like the guinea-pig ileum. After these incubations, the tissue was washed three times and incubated for 30 min prior to contractile measurements.

Analysis of concentration–response curves Concentration–response curves were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) using the variable slope concentration–response curve function. The negative log dissociation constants of antagonists (pK_B) were estimated from experiments in which their ability to shift the agonist concentration–response curve to the right was measured (Arunlakshana and Schild 1959):

$$pK_B = -Log\left(\frac{[I]}{shift - 1}\right)$$

In this equation, [*I*] denotes the molar concentration of antagonist, and shift denotes EC_{50} value of the agonist measured in the presence of the antagonist divided by that measured in its absence. The Log shift and pK_B values were determined for individual experiments and averaged. The significance of differences were evaluated using the unpaired Student's *t* test with the overly conservative Bonferroni adjustment of the critical value of *P* where appropriate.

Drugs and chemicals The reagents used in this study were obtained from the following sources: AF-DX 116, Boehringer Ingelheim Pharmaceutical, Ridgefield, CT, USA; oxotremorine-M and indomethacin, Sigma RBI, Natick, MA, USA; 4-DAMP was synthesized using a method similar to that described by Barlow et al. (1976), and 4-DAMP mustard was synthesized as described previously (Thomas et al. 1992).

Results

Contractile activity of oxotremorine-M in guinea pig and mouse ileum Oxotremorine-M potently elicited contractions in ilea from both the mouse and guinea pig. The average negative log EC_{50} (pEC_{50}) ± SEM and E_{max} ±

SEM values of oxotremorine-M were both greater in guinea pig (7.48±0.05 and 58.2±4.5 mN) than in wild-type mouse (6.75±0.03 and 12.2±0.5 mN). The average responses and their associated SEM values in guinea pig and wild-type mouse were normalized relative to the average E_{max} value of its respective group (i.e., guinea pig or wild-type mouse), and the normalized data are plotted in Fig. 1. The higher potency of oxotremorine-M in the guinea pig is readily apparent from the figure. The contractile activity of oxotremorine-M was also investigated in ilea from M_2 and M_3 KO mice. The responses in each mouse ileum were first normalized relative to the contraction elicited by KCl (50 mM) as described under "Methods." The mean contractile responses and their respective SEM values were then normalized relative to the E_{max} value of the wild-type mouse. The average E_{max} value ± SEM of oxotremorine-M in the M_2 KO mouse ileum (101.2±7.5%) was similar to that of wild type, whereas that measured in the M_3 KO mouse was substantially smaller (35.7±3.5%). Normalization of the muscarinic responses in the mouse to that of KCl did not significantly change the relationship among the E_{max} values in the M_2 KO and M_3 KO strains relative to wild type but did cause a modest reduction in the variance of the mean estimates in wild-type and M_2 KO mice. The potency of oxotremorine-M in the M_2 KO mouse (pEC_{50}=6.26±0.05) was about one third that of the wild-type mouse, whereas that in the M_3 KO mouse (pEC_{50}, 6.99±0.08) was 1.7-fold greater than wild type. These differences in pEC_{50} were significant as indicated in the summary of these data in the legend to Table 2. Since prior reports have shown that the ileum from the M_2/M_3 double KO mouse lacks a muscarinic contractile response, the data in Fig. 1 are consistent with the postulate that the muscarinic contractile response in the wild-type mouse ileum includes a major M_3-receptor component as well as a minor, but more potent, M_2-receptor component. Matsui et al. (2000; 2002) have reached a similar conclusion .

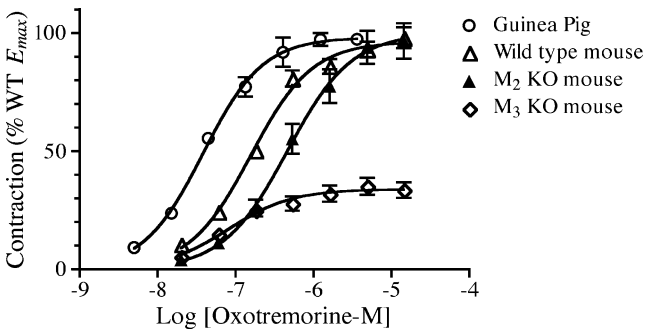

Fig. 1 Contractile activity of oxotremorine-M in ilea from the guinea pig and from wild type, M_2 KO, and M_3 KO mice. The data represent the mean values ± SEM from experiments on 13 guinea pigs, 39 wild-type mice, 25 M_2 KO mice, and 25 M_3 KO mice. The responses in the guinea-pig ileum have been normalized relative to E_{max} and those in mice to the E_{max} in wild type

Table 2 Contractile activity of oxotremorine-M in guinea pig ileum and in wild-type, M_2 KO, and M_3 KO mouse ileum

	pEC_{50}	E_{max}[a] (% wild type)	Hill slope
Guinea pig (13)	7.48±0.05[b]	100±7.7	1.38±0.20
Mouse			
Wild type (39)	6.75±0.03	100±4.1	1.14±0.06
M_2 KO (25)	6.26±0.05[c]	101±7.5	1.18±0.06
M_3 KO (25)	6.99±0.08[c]	35.7±3.5 [d]	1.18±0.18

The data are from Table 1 and represent the mean values ± SEM. The number of experiments is indicated in parentheses

[a] The E_{max} and SEM values have been normalized relative to the average wild-type E_{max} value for each species.

[b] Significantly different from the pEC_{50} value of wild-type mouse ileum ($P=4.3\times10^{-16}$)

[c] One-way analysis of variance showed highly significant differences among the pEC_{50} values measured in wild type, M_2 KO, and M_3 KO mouse ilea ($F_{2,86}=44.14$; $P=6.4\times10^{-14}$). Using the unpaired Student's t test, the P values for differences among the mean pEC_{50} values of wild type and M_2 KO ($P=3.3\times10^{-11}$), wild type and M_3 KO ($P=0.0054$), and M_2 KO and M_3 KO ($P=2.1\times10^{-10}$) were all less than Bonferroni's adjusted 0.05 value of P for three comparisons (0.0167).

[d] Significantly different from the E_{max} of wild-type ileum ($P=6.1\times10^{-19}$)

Antagonism of the muscarinic response in mouse ileum We investigated two muscarinic antagonists (AF-DX 116 and 4-DAMP (*N*,*N*-dimethyl-4-piperidinyl diphenylacetate)) with known selectivity for receptor subtypes to determine if their inhibitory action in the mouse ileum is consistent with the picture of M_2 and M_3 receptor function described above in connection with our studies on KO mice. The binding affinities (pK_D, negative log dissociation constant) of AF-DX 116 for the human M_2 and M_3 receptor subtypes are 7.27±0.05 and 6.10±0.06, and those of 4-DAMP are 7.87±0.03 and 8.81±0.05, respectively (Esqueda et al. 1996; Griffin et al. 2004). Thus, AF-DX 116 exhibits about 15-fold higher affinity for the M_2 receptor relative to M_3, whereas 4-DAMP exhibits an opposite tenfold selectivity. 4-DAMP actually exhibits high affinity for all subtypes of the muscarinic receptor except the M_2. We tested each antagonist at a concentration approximately equal to the greater of its two K_D values for M_2 and M_3 receptors. With this strategy, the M_2 selective AF-DX 116 and the M_3-selective 4-DAMP should only cause about twofold shifts in the concentration–response curve of an agonist for eliciting M_3 and M_2 responses, respectively, but much greater ten- to 15-fold shifts in responses mediated by the receptors for which they exhibit selectivity (i.e., M_2 and M_3, respectively).

The results of antagonism studies using AF-DX 116 (1 μM) in the mouse ileum from wild-type, M_2 KO, and M_3

KO mice are show in Fig. 2a–c. In ileum from the M_3 KO mouse (Fig. 2c), AF-DX 116 caused a 10.5-fold shift in the concentration response curve of oxotremorine-M, which yielded an estimated pK_B value of (6.97±0.03) similar to its binding affinity for the M_2 receptor (pK_D=7.27±0.05). In contrast, AF-DX 116 only caused 2.6- and 2.7-fold shifts in the concentration response curves of oxotremorine-M in wild-type and M_2 KO mice, respectively. 4-DAMP exhibited the opposite selectivity (Fig. 2a–c). It caused a 6.8-fold shift in the concentration–response curve of oxotremorine-M in the M_2 KO mouse (Fig. 2b), which yields an estimated pK_B value (8.74±0.19) similar to its binding affinity for the M_3 receptor (8.81±0.05, Griffin et al. 2004). A similar shift of 14-fold was observed in the wild-type mouse (Fig. 2a), whereas a much smaller shift (1.5-fold) was measured in ilea from the M_3 KO mouse (Fig. 2c). These results are summarized in Table 3 and are

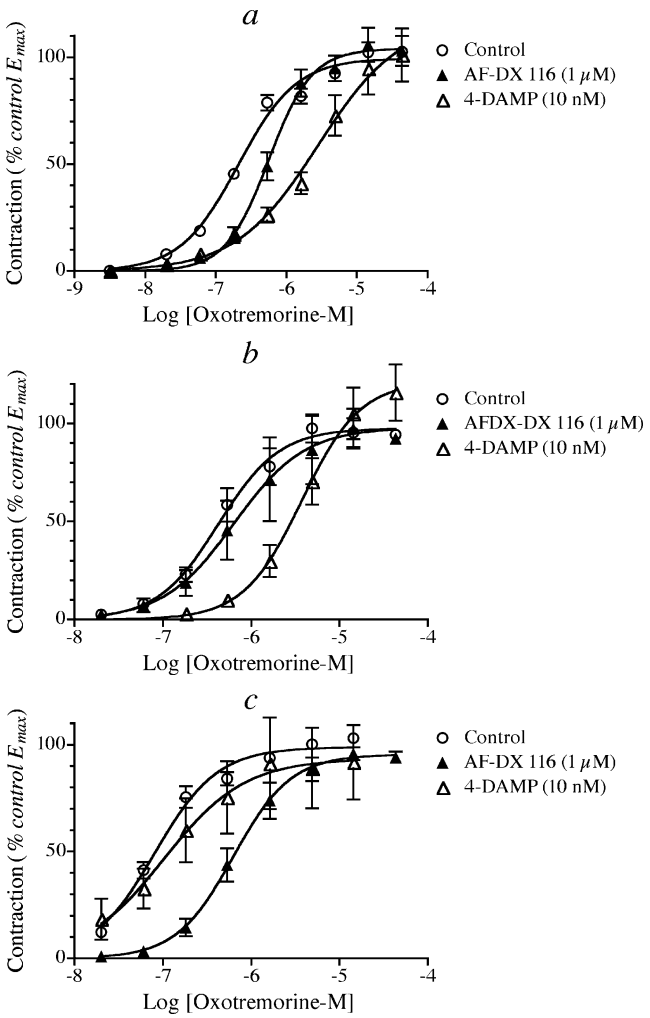

Fig. 2 Competitive antagonism of the response to oxotremorine-M by AF-DX 116 (1 μM) and 4-DAMP (10 nM) in ilea from wild type (**a**), M_2 KO (**b**), and M_3 KO (**c**) mice. The data are normalized relative to the E_{max} of control and represent the mean values ± SEM from four to seven experiments

consistent with the postulate mentioned above that the muscarinic contractile response in the mouse ileum includes major and minor, directly acting M_3 and M_2 components, respectively.

We also investigated the effects of 4-DAMP mustard on the muscarinic contractile response of the mouse ileum (Fig. 3). At neutral pH, 4-DAMP mustard forms an aziridinium ion that binds covalently to muscarinic receptors. When used at a concentration of 40 nM in the presence of AF-DX 116 (4 μM) for 1 h at 37°C, 4-DAMP mustard inactivates 96% of human M_3 receptors expressed in CHO cells but only 22% of human M_2 receptors (Griffin et al. 2003). Isolated ilea from wild-type, M_2 KO, and M_3 KO mice were incubated, with 4-DAMP mustard (40 nM) in combination with AF-DX 116 (4 μM) for a total time of 2 h, and washed extensively (see "Methods"). This treatment reduced the E_{max} value of oxotremorine-M in wild-type mouse ileum to only 43% of control while having little effect on EC_{50}. As shown in Fig. 3a, the residual response in wild-type ileum after 4-DAMP mustard treatment was nearly identical to that measured in the untreated ileum from the M_3 KO mouse. Similar results were obtained when the incubation with 4-DAMP mustard only lasted 1 h (Table 4). Treatment with 4-DAMP mustard (2 h) caused a large inhibition in the response to oxotremorine-M in the M_2 KO mouse ileum (Fig. 3b), but had little effect on the response in the M_3 KO ileum (Fig. 3c). The results are consistent with the postulate that 4-DAMP mustard treatment selectively inactivates M_3 responses over M_2, thereby converting the muscarinic behavior of the wild-type ileum into that of the M_3 KO ileum. These results are summarized in Table 4.

To obtain further support for this hypothesis, we characterized the pharmacological profile of the muscarinic response in 4-DAMP mustard-treated wild-type ileum using the competitive antagonists, 4-DAMP and AF-DX 116. After 4-DAMP mustard treatment, AF-DX 116 (1 μM) and 4-DAMP (10 nM) caused 9.8- and 3.0-fold shifts in the concentration response curve to oxotremorine-M in wild-type mouse ileum, yielding pK_B estimates of 6.90±0.13 and 8.30±0.28, respectively (Fig. 3d). This profile of antagonism is similar to that described above for the ileum from M_3 KO mice (Fig. 2c).

Characterization of the muscarinic contractile response in guinea pig ileum The effects of AF-DX 116 (1 μM) and 4-DAMP (10 nM) on the contractile response to oxotremorine-M in the guinea pig ileum are shown in Fig. 4a. These two antagonists caused shifts of 3.1- and tenfold, respectively, in the concentration–response curve. This behavior is consistent with the well-known M_3 profile of this tissue, yielding pK_B±SEM values of 6.28±0.10 and 9.00±0.06 for AF-DX 116 and 4-DAMP, respectively, in

Table 3 Effects of AF-DX 116 and 4-DAMP on the contractile response to oxotremorine-M in mouse ileum

	AF-DX 116 (1μM)		4-DAMP (10nM)	
	Log shift[a]	pK_B	Log shift[a]	pK_B
Wild type (7, 7)	0.42±0.06	6.17±0.14	1.16±0.13	9.12±.14
M_2 KO (4, 6)	0.43±0.18	5.93±0.42	0.83±0.19	8.74±.19
M_3 KO (7, 4)	1.02±0.02	6.97±0.03	0.18±0.17	n.d.[b]

The data are from Fig. 1 and represent the mean values ± SEM. The two numbers in parentheses beside each mouse strain denote the number of experiments done with AF-DX 116 and 4-DAMP, respectively

[a] The Log shift denotes the logarithm of the ratio of the EC_{50} value measured in the presence of the antagonist divided by that measured in its absence

[b] The pK_B was not determined because of the low log shift value

excellent agreement with the binding affinity for the human M_3 receptor. Treatment of the guinea pig ileum with 4-DAMP mustard (40 nM) in combination with AF-DX 116 (4 μM) for 2 h followed by washing caused a 56-fold dextral shift in the concentration–response curve to oxotremorine-M, with a small increase in its E_{max} value (Fig. 4b). This small effect can be attributed to time, since we observed time-dependent increases in E_{max} with repetitive measurement of concentration–response curves to oxotremorine-M. Unlike the behavior observed in wild-type mouse ileum, treatment of the guinea pig ileum with 4-DAMP mustard did not uncover a direct, highly potent contraction with a low E_{max} value. Following 4-DAMP mustard treatment, the effects of AF-DX 116 (1.3-fold dextral shift) and 4-DAMP (4.3-fold dextral shift) on the EC_{50} value of oxotremorine-M in the guinea pig ileum where qualitatively similar to those measured before 4-DAMP mustard treatment and, hence, suggestive of a direct M_3 mechanism. Control experiments showed that the potency of oxotremorine-M increased 1.45-fold 1 h after 4-DAMP mustard treatment, suggesting that the measured antagonist-induced shifts were underestimated. Correcting

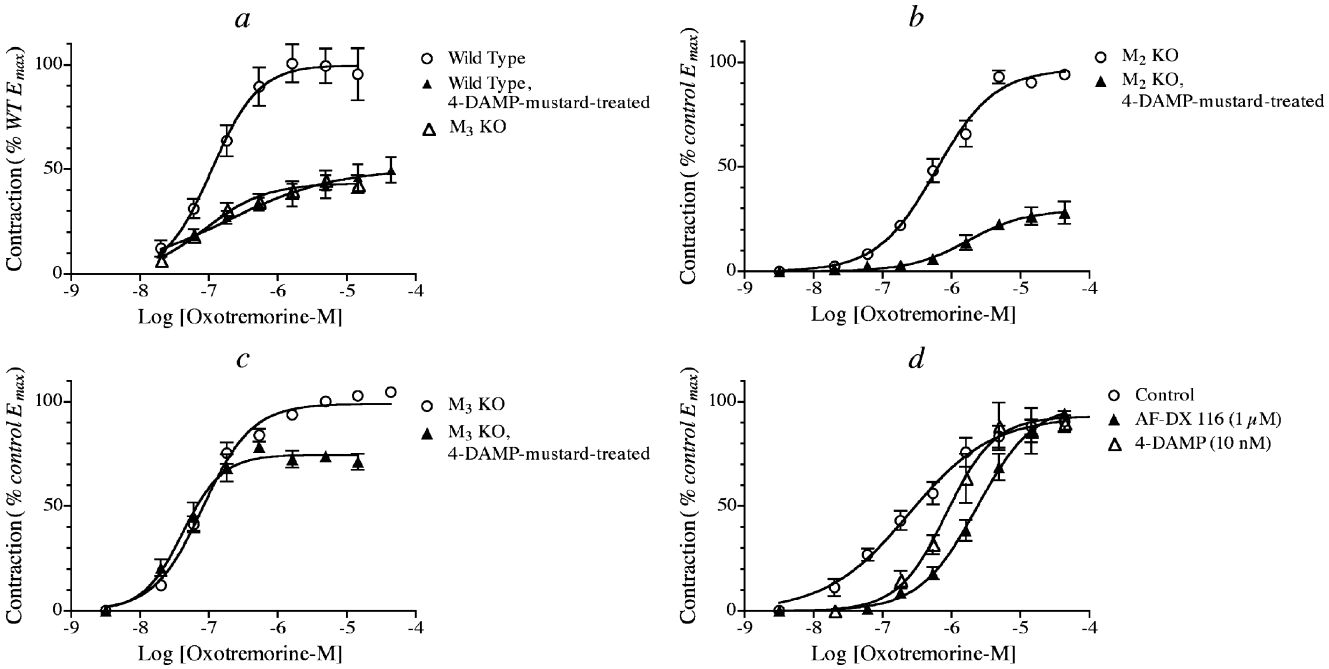

Fig. 3 Effects of 4-DAMP mustard treatment on contractions elicited to oxotremorine-M in mouse ileum. **a** Responses were measured in ilea from the M_3 KO mouse (*open triangles*) and from wild-type ileum before (*open circles*) and after (*closed triangles*) treatment with 4-DAMP mustard (40 nM) in combination with AF-DX 116 (4 μM) for 2 h followed by washing as described under "Methods." **b** Responses were measured in ilea from the M_2 KO mouse before (*open circles*) and after (*closed triangles*) treatment with 4-DAMP mustard as described in **a**. **c** Same as **b** except that responses were measured in ilea from the M_3 KO mouse. **d** All responses were measured in ilea from wild-type mice that had been treated with 4-DAMP mustard as described in **a**. After this treatment, responses were measured in the absence (*open circles*) and presence of AF-DX 116 (1 μM; *closed triangles*) or 4-DAMP (10 nM; *open triangles*). Mean values ± SEM from five to seven experiments are plotted in **a**–**d**

Table 4 Effect of 4-DAMP mustard treatment (40 nM) in combination with AF-DX 116 (4 μM) on the contractile activity of oxotremorine-M in mouse ileum

	Control	4-DAMP mustard	
	pEC_{50}	pEC_{50}	E_{max} (%)[a]
Wild type			
1 h treatment (9)	6.59±0.08	6.62±0.17	34±8
2 h treatment (7)	6.96±0.06	6.89±0.14	43±9
M_2 KO			
2 h treatment (6)	6.39±0.09	5.80±0.16	33±9
M_3 KO			
2 h treatment (5)	7.15±0.08	7.36±0.09	74±3

The data are from Fig. 2 and represent the mean values ± SEM. The numbers in parentheses beside each mouse strain denote the number of experiments. The E_{max} values in 4-DAMP mustard-treated ileum are normalized relative to that measured under control conditions

[a] E_{max} values and their SEM have been normalized relative to the average E_{max} value of control

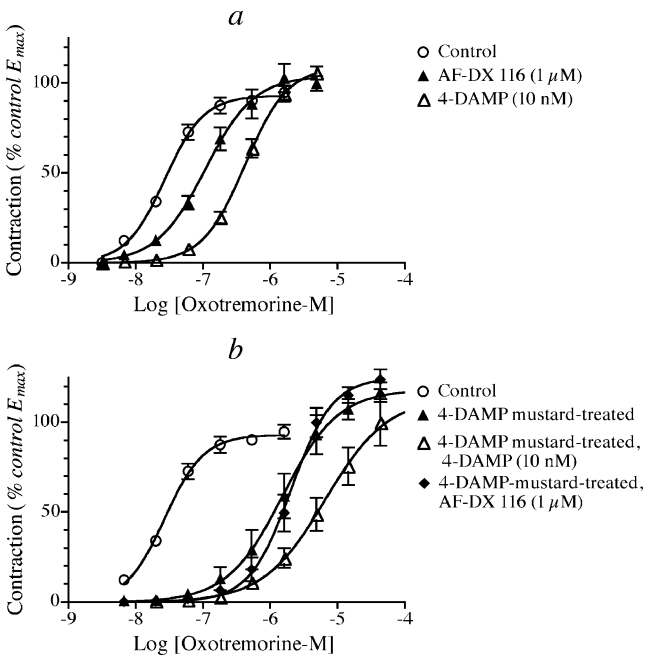

Fig. 4 Effects of AF-DX 116 (1 μM) and 4-DAMP (10 nM) before (**a**) and after (**b**) 4-DAMP mustard treatment on contractile responses to oxotremorine-M in the guinea pig ileum. **a** Responses were measured in the absence (*open symbols*) and presence of AF-DX 116 (*closed triangles*) or 4-DAMP (*open triangles*). **b** Responses were first measured under control conditions (*open circles*). After 4-DAMP mustard treatment and washing, responses were measured in the absence (*closed triangles*) and presence of AF-DX 116 (*closed diamonds*) or 4-DAMP (*open triangles*). Ilea were treated with 4-DAMP mustard (40 nM) for 1 h in the presence of AF-DX 116 (4 μM) and were then washed three times as described under "Methods." The data in **a** and **b** represent the mean response values ± SEM from ten to 13 experiments

these measured shifts by a factor of 1.45 yields theoretical shifts of 1.89 and 6.21 for AF-DX 116 (1 μM) and 4-DAMP (10 nM), respectively, which yield pK_B values of 5.94 and 8.72 for these antagonists.

Discussion

Muscarinic agonists elicit contraction in isolated ileum, trachea, and urinary bladder from many mammals, including the mouse. This function undergoes small, large, and complete losses in the M_2 KO, M_3 KO, and M_2/M_3 double KO mice, respectively (Matsui et al. 2000; Matsui et al. 2002), showing that M_2 and M_3 receptors account for contraction and that, in the absence of other agents, the latter contributes more to the response than the former. Contractions to efficacious muscarinic agonists are insensitive to tetrodotoxin, indicating that the relevant M_2 and M_3 receptors are located postjunctionally (Unno et al. 2005). Muscarinic agonists display high potency for eliciting contraction through the M_2 receptor in smooth muscle from the M_3 KO mouse, yet compared to that measured in wild-type tissue, their E_{max} values are only 10% in urinary bladder and 30–40% in ileum and trachea (Matsui et al. 2000). Direct M_2 receptor-mediated contractions in the ileum from the M_3 KO mouse have been reported to be evanescent (Unno et al. 2005), although we have found them to be reasonably stable over the time required to measure data for a cumulative, concentration–response curve. These contractions are pertussis toxin-sensitive and inhibited completely by the voltage-dependent Ca^{2+} antagonist, nicardipine (Unno et al. 2005). In contrast, M_3 receptor-mediated contractions are pertussis toxin-insensitive and are partially inhibited by nicardipine in mouse (Unno et al. 2005) but nearly completely inhibited by voltage-sensitive Ca^{2+} channel blockers in guinea pig (Bolger et al. 1983).

The first report (Matsui et al. 2000) of a directly mediated M_2 contraction in the M_3 KO mouse was surprising because prior studies on the guinea pig had not uncovered such a role, although clear evidence for conditional M_2 responses—that is, those dependent on other receptors—had been observed. It might be argued that the direct M_2 effect had gone unnoticed in guinea pigs because the antagonists used to characterize contraction lacked the requisite selectivity for muscarinic receptor subtypes to detect a small M_2 effect. This raises the question of whether the direct contractile role of the M_2 receptor was missed in the guinea pig or whether guinea pigs simply differ from mice in their lack of this potent M_2 function. For these reasons, we investigated whether it is possible to convert the muscarinic response of the ileum

from the wild-type mouse into that of the M_3 KO using 4-DAMP mustard and, if so, whether this treatment reveals a direct M_2 receptor-mediated contraction in the guinea pig.

The compound, 4-DAMP mustard, is a nitrogen mustard derivative that cyclizes spontaneously into a reactive aziridinium ion nearly identical to the competitive muscarinic antagonist 4-DAMP except for its lack of two hydrogen atoms (Barlow et al. 1990). The latter compound only exhibits about tenfold higher affinity for the M_3 receptor over the M_2. At 100% receptor occupancy, 4-DAMP mustard alkylates M_2 and M_3 receptors at a similar rate (rate constant, 0.1 min^{-1}; half time, 7 min), but selectivity for the M_3 receptor can be achieved at the cost of a slower rate of alkylation by using a lower concentration of the aziridinium ion or by adding a competitive, M_2-selective antagonist (e.g., AF-DX 116) to the incubation (Thomas et al. 1992). Using 4-DAMP mustard (40 nM) in combination with AF-DX 116 (4 μM) for 1 h, we showed that it is possible to alkylate 96% of a population of the human M_3 receptor expressed in CHO cells, while only inactivating 22% of human M_2 receptors (Griffin et al. 2003).

Treatment of the wild-type mouse ileum with 4-DAMP mustard reduced the E_{max} of the contractile response to oxotremorine-M by about 60% while having little effect on EC_{50}. The residual concentration–response curve resembled that measured in the M_3 KO mouse, in terms of its EC_{50}, E_{max}, and antagonism by AF-DX 116 and 4-DAMP. These compounds had pK_B values of 6.90 and 8.30, respectively, that differed by only about 0.4 log units from their binding affinities (pK_D) for human M_2 receptors (7.27 and 7.87 (Esqueda et al. 1996; Griffin et al. 2004)). The difference may be ascribed to recycling of muscarinic receptors after 4-DAMP mustard treatment as discussed below. In contrast, the pK_B values of the same compounds in wild-type mouse ileum (6.17 and 9.12) are similar to their respective binding affinities (pK_D) for the M_3 receptor (6.10 and 8.81 (Esqueda et al. 1996; Griffin et al. 2004)). The direct M_2-component of contraction in the wild-type mouse does not significantly perturb the antagonism profile of the wild-type response from that expected for a pure M_3 response, illustrating the inability of these antagonists to resolve a minor receptor component of the response. 4-DAMP mustard treatment had little effect on muscarinic contractions in the M_3 KO mouse. Our data suggest that 4-DAMP mustard treatment selectively inactivated M_3 receptors in the wild-type ileum to unmask direct M_2-receptor-mediated contractions that behaved similarly to those of the M_3 KO mouse.

In contrast, 4-DAMP mustard treatment completely eliminated the high-potency response of the guinea pig ileum to oxotremorine-M. Only low-potency contractions to oxotremorine-M remained after 4-DAMP mustard treatment, for the concentration–response curve shifted to the right about 56-fold with no decline in E_{max}. These low-potency contractions were antagonized by AF-DX 116 and 4-DAMP in a manner qualitatively resembling that expected for an M_3 response. The shift in the concentration–response curve caused by the M_2-selective AF-DX 116 was only one fortieth of that expected for an M_2 response, and that caused by the M_3-selective 4-DAMP was threefold greater than expected for an M_2 response. Both shifts, however, were about threefold smaller than that expected for an M_3 response. This decrement in antagonism may be explained, in part, by the trafficking of new muscarinic receptors to the plasma membrane after 4-DAMP mustard treatment because control experiments showed about a 1.5-fold increase in the potency of oxotremorine-M during the same time period.

The guinea pig ileum is exquisitely sensitive to muscarinic agonists, and only a fraction of 1% of the muscarinic receptor population is required for the response at EC_{50} (Ringdahl 1984). A recovery of such a small amount of receptors seems plausible after 4-DAMP mustard treatment and before the response to oxotremorine-M was measured in the presence of antagonist (45–60 min). This time was used for washing residual agonist from the tissue and incubating with antagonist. In tissue homogenates, there is no recovery of muscarinic receptor binding after a few hours following 4-DAMP mustard treatment (Thomas et al. 1992), although the error in this measurement is at the same level as that capable of causing a small leftward shift in the concentration–response curve (i.e., about 2% of the receptor population).

In contrast to that of the guinea pig, the M_3 response of the mouse ileum is much less sensitive to oxotremorine-M. Based on our prior work, it requires approximately 30% receptor occupancy by oxotremorine-M to elicit a 50% contractile response (Tran et al. 2009). This difference in the sensitivities of the mouse and guinea pig ileum can explain why it was possible to reduce the E_{max} value of oxotremorine-M in both the wild-type and M_2 KO ileum, while the same treatment did not affect the E_{max} in the guinea pig ileum.

Our inability to detect direct, M_2-receptor-mediated contractions in the guinea pig ileum does not rule them out; our point is that if they exist, they must be mediated by oxotremorine-M with much less potency than in the mouse or that it requires an agonist with much greater efficacy than oxotremorine-M to detect them. Since relative efficacy of oxotremorine-M is similar to or greater than that of acetylcholine at the M_2 receptor (Ehlert 1985; Tran et al. 2009), our data show that highly potent, direct M_2-receptor-mediated contractions are not mediated by acetylcholine physiologically. Thus, although the M_2 receptor of the guinea pig ileum mediates a high potency inhibition of relaxation and a low potency enhancement of M_3 receptor-mediated contractions (Ehlert 2003), it does not mediate a high potency direct contraction like that of the mouse ileum.

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