The M_2 Muscarinic Receptor Mediates Contraction through Indirect Mechanisms in Mouse Urinary Bladder [a]

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Abbreviations: AF-DX 116, [[2-[(diethylamino) methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3b][1,4]-benzodiazepine-6-one; 4-DAMP, N,N-dimethyl-4-piperidinyl diphenylacetate; 4-DAMP mustard, N-2-chloroethyl-4-piperidinyl diphenylacetate; EC_{50}, concentration of agonist eliciting half-maximal response; E_{max}, maximal response; K_B, antagonist dissociation constant measured by functional antagonism; K_D, antagonist dissociation constant measured by ligand binding; KO, knockout; KRB, Krebs Ringer Bicarbonate.

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ABSTRACT

We investigated the contractile role of M_2 muscarinic receptors in mouse urinary bladder. When measured in the absence of other agents, contractions elicited to the muscarinic agonist oxotremorine-M exhibited properties consistent with that expected for an M_3 response in urinary bladder from wild type and M_2 knockout mice. Evidence for a minor, M_2 receptor-mediated contraction was revealed by comparison of responses in M_3 knockout and M_2/M_3 double knockout mice. Treatment of wild type and M_2 knockout urinary bladder with 4-DAMP mustard caused a large inhibition of the muscarinic contractile response. The residual contractions were much smaller in M_2 knockout bladder as compared to wild type, suggesting that M_2 receptors rescue the muscarinic contractile response in wild type bladder following inactivation of M_3 receptors with 4-DAMP mustard. When measured in the presence of $PGF_{2\alpha}$ and isoproterenol or forskolin, oxotremorine-M mediated a potent contractile response in urinary bladder from M_3 KO mice. This response exhibited an M_2 profile in competitive antagonism studies, and was completely absent in M_2/M_3 KO mice. Following 4-DAMP mustard-treatment, oxotremorine-M elicited a contractile response in wild type urinary bladder in the presence of KCl and isoproterenol or forskolin, and this response was diminished in M_2 KO mice. Our results show that the M_2 receptor mediates contractions indirectly in the urinary bladder by enhancing M_3 receptor-mediated contractions and by inhibiting relaxation. We also show that it is difficult to detect M_2 receptor function in competitive antagonism studies under conditions where a simultaneous activation of M_2 and M_3 receptors occurs.

Micturition is mediated through the actions of several neurotransmitters. Among those that directly influence the tone of urinary bladder smooth muscle, acetylcholine is important in contracting the reservoir and relaxing the outlet through activation of muscarinic receptors (de Groat and Yoshimura, 2001). Most evidence shows that it is the M_3 subtype of the muscarinic receptor that mediates the direct contractile response to acetylcholine in the urinary bladder. For example, the contractile response to muscarinic agonists exhibits an M_3 profile in competitive antagonism studies (Noronha-Blob et al., 1989; Longhurst et al., 1995; Choppin and Eglen, 2001), and these contractions are nearly absent in urinary bladder from M_3 muscarinic receptor knockout (M_3 KO) mice (Matsui et al., 2000). Male M_3 KO mice exhibit prominent urinary bladder distension *in vivo*, demonstrating the essential role of the M_3 receptor in micturition (Matsui et al., 2000). The small, direct contractile response that persists in urinary bladder from M_3 KO mice is completely lost in mice lacking both M_2 and M_3 muscarinic receptors (M_2/M_3 KO mice), demonstrating that the M_2 receptor is capable of mediating very small contractions, and that muscarinic receptors other than M_2 and M_3 do not appear to mediate direct contraction of the urinary bladder (Matsui et al., 2002).

The signaling mechanisms of M_2 and M_3 muscarinic receptors in smooth muscle are consistent with their respective roles in contraction. The M_3 receptor interacts with $G_{q/11}$ to mediate phosphoinositide hydrolysis (Noronha-Blob et al., 1989; Candell et al., 1990; Roffel et al., 1990; Zhang and Buxton, 1991) and Ca^{2+} mobilization, which is essential for contraction, whereas the M_2 receptor interacts with $G_{i/o}$ to mediate responses that are ultimately contingent upon activation of other Ca^{2+} mobilizing receptors, like the M_3. For example, M_2 receptors mediate an inhibition of adenylyl cyclase (Noronha-Blob et al., 1989; Candell et al., 1990; Yang et al., 1991; Zhang and Buxton, 1991). In smooth muscle, cAMP causes relaxation (Conti and Adelstein, 1980; Kerrick and Hoar, 1981; Ruegg et al., 1981). Thus, activation of the M_2 receptor has the potential to mediate an inhibition of the relaxant effects of forskolin or ß-adrenoceptors on contractions elicited through activation of a G_q linked receptor. We refer to this type of contractile mechanism as indirect, because it represents an inhibition of relaxation

and not a direct mediation of contraction. The M_2 receptor has also been shown to mediate an inhibition of Ca^{2+} activated K^+ (BK_{Ca}) channels (Cole et al., 1989; Kume et al., 1992; Wade and Sims, 1993). Through this mechanism, the M_2 receptor would be expected to diminish the inhibitory effects of BK_{Ca} channels on the contraction mediated by other Ca^{2+} mobilizing receptors. This potential muscarinic mechanism is also indirect, because M_2 receptor activation is expected to have little effect by itself, but nonetheless, enhance the effect of other contractile receptors. It has been demonstrated through mathematical modeling that a response mediated through an interaction between directly and indirectly acting receptors has a tendency to display the pharmacological profile of the directly acting receptor (i.e., M_3) in competitive antagonism studies and not that of the indirectly acting receptor (i.e., M_2) (Sawyer and Ehlert, 1999a; Ehlert, 2003b). This model explains why it is difficult, if not impossible, to detect a role for the M_2 receptor using competitive antagonists in experiments where a simultaneous activation of both M_2 and M_3 receptors occurs. This rationale also explains the large loss of the muscarinic contractile function in smooth muscle from M_3 KO mice. If the action of the M_2 receptor is contingent upon M_3 receptor activation, then the M_2 response will also be lost in the M_3 KO mouse.

Because of these limitations, a method was developed to isolate the indirect contractile response of the M_2 receptor from the direct response of the M_3 in competitive antagonism experiments on isolated smooth muscle (Thomas et al., 1993). The method involves first inactivating M_3 receptors with a selective nitrogen mustard derivative (4-DAMP mustard) and then measuring the contractile response to a muscarinic agonist in the presence of heterologous contractile (e.g., histamine) and relaxant (forskolin) agents. Using this approach, it has been demonstrated that M_2 receptors mediate an inhibition of the relaxant effect of forskolin, and in some instances isoproterenol, on histamine-induced contractions of the colon (Sawyer and Ehlert, 1998; Sawyer and Ehlert, 1999b), ileum (Thomas et al., 1993; Thomas and Ehlert, 1994), esophagus (Eglen et al., 1996) and trachea (Thomas and Ehlert, 1996; Ostrom and Ehlert, 1998; Ostrom and Ehlert, 1999) and also on KCl-induced contractions of rat urinary bladder (Hegde et

al., 1997). However, no indirect mechanism for the M_2 receptor was detected in mouse urinary bladder using the same approach (Choppin and Eglen, 2001).

In the present report, we have investigated an indirect role for the M_2 receptor in mouse urinary bladder using tissue from wild type, M_2 KO, M_3 KO and M_2/M_3 KO mice. Our results are consistent with the postulate that the M_2 receptor mediates an inhibition of the relaxant effects of isoproterenol and forskolin on contractions mediated by FP and M_3 receptors. We also obtained evidence that M_2 receptors enhance the contractile response to M_3 receptor activation in the absence of relaxant agents. Our results are consistent with a recent report demonstrating that the relaxant effects of isoproterenol and forskolin against muscarinic agonist-induced contractions are enhanced in urinary bladder from M_2 KO mice (Matsui et al., 2003).

MATERIALS AND METHODS

Isolated urinary bladder: M_2 muscarinic receptor knockout (M_2–/–; M_2 KO), M_3 muscarinic receptor knockout (M_3–/–; M_3 KO), and M_2/M_3 muscarinic receptor double knockout (M_2–/–, M_3–/–; M_2/M_3 KO) mice were generated in a mixed background between 129/SvJ and C57BL/6 as described in Matsui et al. (2002) and Matsui et al. (2000), respectively. These hybrid lines were backcrossed with C57BL/6 mice to yield an N4 generation of M_2 KO mice, an N8 generation of M_3 KO mice and an N2 generation of M_2/M_3 KO mice, which were used in the pharmacological studies described in this report. Only male knockout mice were used as well as male wild type (M_2+/+, M_3 +/+) C57BL/6 mice. The mice were euthanized by CO_2 asphyxiation, and the whole urinary bladder was excised and used in contractile studies.

In most experiments, the whole urinary bladder was mounted longitudinally in tissue baths (50 ml capacity) with silk thread attached at the apex and at the outlet of the urinary bladder. In some experiments, the bladder was cut in half, and each half was mounted longitudinally in a similar fashion. The tissues were bathed at 37°C in 50 ml of Krebs Ringer Bicarbonate (124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, and 10 mM glucose; KRB) buffer containing indomethacin (1 μM) and were connected to force-displacement transducers. Isometric tension was recorded using a PowerLab (ADInstruments, Grand Junction, CO) recording system. Resting tension was adjusted to that generated by a mass of 1 g for the whole urinary bladder and to 0.5 g for the half urinary bladder strip. The tissues were allowed to equilibrate for at least 60 min prior to measurement of contractile responses. Three test doses of KCl (50 mM) were applied to the tissues first, and subsequent contractile measurements were normalized relative to the third KCl test dose. Concentration-response curves to the muscarinic agonist oxotremorine-M were measured using a cumulative technique, essentially the same as that described previously (Matsui et al., 2003). The bladder was allowed to rest for at least 30 min between consecutive measurements of the concentration-response curve to oxotremorine-M. When present, competitive antagonists were

incubated with the bladder for 30 min prior to the measurement of contractile responses. In experiments where the irreversible muscarinic antagonist 4–DAMP mustard was used, the compound was first incubated at 37°C for 30 min in 10 mM sodium-potassium phosphate buffer, pH 7.4, to allow formation of the reactive aziridinium ion, essentially as described previously (Thomas et al., 1992). Solutions of cyclized 4-DAMP mustard were kept on ice and used as soon as possible. Following treatment of the bladder with a combination of 4–DAMP mustard and AF-DX 116, the tissue was washed four times over a period of 30 min to remove AF-DX 116 and the transformation products of 4-DAMP mustard.

Calculations: An increasing logistic equation was fitted to the oxotremorine-M concentration-response curve by nonlinear regression analysis to estimate the maximal response (E_{max}), the concentration of oxotremorine–M eliciting a half-maximal response (EC_{50}) and the Hill slope as described previously (Candell et al., 1990). The dissociation constant (K_B) of competitive antagonists were estimated in contractile studies using the following equation (Arunlakshana and Schild, 1959):

$$K_B = \frac{[B]}{CR - 1} \qquad 1$$

in which B denotes the concentration of the antagonist, and CR denotes the EC_{50} value of oxotremorine-M measured in the presence of the antagonist divided by that measured in its absence. The K_B and EC_{50} values were estimated in molar units and then converted to negative logarithms before statistical analysis. In most instances, a two-tailed, students t test was used to determine the statistical significance of differences between parameter estimates.

Drugs and chemicals: The reagents used in this study were obtained from the following sources: AF-DX 116, Boehringer Ingelheim Pharmaceutical, Ridgefield, CT; oxotremorine-M, Sigma RBI, Natick, MA; atropine, isoproterenol and $PGF_{2\alpha}$, Sigma Chemical Company, St. Louis, MO; forskolin, Calbiochem, 4-DAMP was synthesized in our laboratory 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 and $PGF_{2\alpha}$: Oxotremorine-M elicited contractions with high potency (pEC_{50} = 6.54) in urinary bladder from wild type mice (Figure 1*a*). When expressed relative to the contraction elicited to KCl (50 mM), the E_{max} value was calculated to be 326%. Oxotremorine-M exhibited similar contractile activity in urinary bladder from M_2 KO mice, although the potency and E_{max} were both slightly lower (1.7-fold increase in EC_{50} (P = 0.012) and a nonsignificant 11% decrease in E_{max} (P = 0.64)). In contrast, the contractile response to oxotremorine-M was greatly inhibited in urinary bladder from M_3 KO mice. This effect was characterized by a highly significant ($P = 4.3 \times 10^{-6}$) 85% reduction in the E_{max} without a significant change in EC_{50}. It was impossible to detect contractile responses to oxotremorine-M in urinary bladder from M_2/M_3 KO mice. The results in Figure 1*a* are summarized in Table 1.

We also investigated the effects of $PGF_{2\alpha}$ in the isolated urinary bladder (see Figure 1*b*). In contrast to oxotremorine-M, $PGF_{2\alpha}$ was much less potent in urinary bladder from wild type (pEC_{50} = 5.48) and M_2 KO (pEC_{50} = 5.23) mice (see Figure 1*b*). Although we did not investigate high concentrations of $PGF_{2\alpha}$ that elicited clear maximal responses, the estimates of E_{max} by regression analysis in wild type and M_2 KO mice were 89 and 86%, respectively. In urinary bladder from mice lacking M_3 receptors, $PGF_{2\alpha}$ was much more active. The pEC_{50} and E_{max} values of $PGF_{2\alpha}$ in M_3 KO mice were 6.39 and 186%, respectively, and the corresponding values in M_2/M_3 KO mice were 6.60 and 138%. Thus, the loss of M_3 receptor function in the urinary bladder from male mice appears to trigger an increase in sensitivity to $PGF_{2\alpha}$. These results are summarized in Table 2.

Competitive antagonism: We investigated the ability of M_2 selective (AF-DX 116) and M_3 selective (4-DAMP) muscarinic antagonists to inhibit the contractile response to oxotremorine-M in urinary bladder from wild type and muscarinic receptor KO mice. At a concentration of 1

μM, AF-DX 116 only caused 3.0- and 2.1-fold shifts in the concentration-response curve to oxotremorine-M in urinary bladder from wild type and M_2 KO mice. These data yield pK_B estimates of 6.28 and 6.01, respectively. In contrast, the same concentration of AF-DX 116 caused an 11-fold shift in the oxotremorine-M concentration response in urinary bladder from M_3 KO mice, yielding a pK_B estimate of 6.97. At a concentration of 10 nM, 4-DAMP shifted the oxotremorine-M concentration response curve to the right 12- and 16-fold in urinary bladder from wild type and M_2 KO mice, respectively, which yields pK_B estimates of 9.03 and 9.19. These data are summarized in Table 3 together with the binding affinities of AF-DX 116 and 4-DAMP at recombinant human M_2 and M_3 receptors. The pK_B values estimated in wild type and M_2 KO mice agree with the binding affinity measured at M_3 receptors. In contrast, the pK_B value measured for AF-DX 16 in the M_3 KO mouse agrees with the binding affinity at M_2 receptors.

4-DAMP mustard-treatment: It has been shown that treatment of cell lines and native tissues with the aziridinium ion of 4-DAMP mustard in combination with AF-DX 116 causes a selective, irreversible alkylation of the recognition site of M_3 receptors while having little effect on M_2 receptors (Thomas et al., 1992; Thomas et al., 1993; Griffin et al., 2003). By itself, 4-DAMP mustard exhibits moderate selectivity for M_3 receptors over M_2, although it is has the capacity to alkylate both receptors, depending upon its concentration and length of incubation. By carrying out the incubation in the presence of the competitive, M_2-selective antagonist AF-DX 116, it is possible to increase the apparent selectivity of 4-DAMP mustard for M_3 receptors by protecting the M_2 with AF-DX 116. Consequently, we were interested in examining the influence of 4-DAMP mustard-treatment on the muscarinic contractile response in urinary bladder from wild type and muscarinic receptor KO mice. Because of the instability of the aziridinium ion, urinary bladders were given two, consecutive one-hour treatments with 4-DAMP mustard (10 nM) in combination with AF-DX 116 (1 μM), with fresh drug solutions being used for the second treatment. The contractile activity of oxotremorine-M was assessed after each one-hour treatment. Treatment of urinary bladder from wild type mice with 4-DAMP

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mustard caused a large shift to the right in the oxotremorine-M concentration-response curve after one hour and a further shift to the right and decrease in E_{max} after two hours of treatment (see Figure 2). Similar effects were observed in experiments on urinary bladder from M_2 KO mice except that the inhibitory effects of 4-DAMP mustard were greater and were characterized by a larger depression in the E_{max} of oxotremorine-M. In contrast, 4-DAMP mustard-treatment had little influence on the contractile response to oxotremorine-M in the urinary bladder from M_3 KO mice. We did measure 2.4- and 2.7-fold shifts to the right in the oxotremorine-M concentration-response curve in these experiments after one- and two-hr treatment with 4-DAMP mustard, respectively. However, control experiments showed that incubation of the mouse urinary bladder from M_3 KO mice with AF-DX 116 (1 μM) only for one and two hr followed by four washes over 30 min cause similar 3.3- and 4.7-fold shifts, respectively, in the concentration-response curves. In contrast, incubating the urinary bladder from M_3 KO mice in the absence of any drugs for several hr caused no change in its sensitivity to oxotremorine-M. Consequently, the small shift in the concentration-response curve to oxotremorine-M noted in urinary bladder from M_3 KO mice after 4-DAMP mustard-treatment can be attributed to residual AF-DX 116 in the tissue and not to inactivation of muscarinic receptors with 4-DAMP mustard. The results in Figure 2 are consistent with the postulate that the M_3 receptor mediates most of the direct contractile response in the urinary bladder from wild type and M_2 KO mice, whereas the 4-DAMP mustard-insensitive M_2 receptor mediates contraction in the M_3 KO mouse urinary bladder. These results are summarized in Table 4.

Although the contractile response to oxotremorine-M was greatly inhibited by 4-DAMP mustard in urinary bladder from both wild type and M_2 KO mice, the response in M_2 KO mice was inhibited more. Figure 3*a* shows a plot of the concentration-response curves to oxotremorine-M in urinary bladder from wild type and M_2 KO mice after one- and two-hour treatment with 4-DAMP mustard. The larger size of the responses in wild type bladder relative to those measured in M_2 KO bladder is readily apparent. These results suggest that the M_2 receptor partially rescues contraction in the urinary bladder from wild type mice after most of the

M_3 receptors have been inactivated with 4-DAMP mustard. To investigate the nature of the interaction between M_2 and M_3 receptors, we measured the competitive antagonism of contraction in wild type urinary bladder after two-hour 4-DAMP mustard-treatment. In these experiments, urinary bladder was first treated with 4-DAMP mustard for two hours, washed, and then a concentration-response curve to oxotremorine-M was measured. The tissue was washed, incubated with AF-DX 116 or 4-DAMP for 30 min, and contractions were measured again in the presence of the antagonist. However, control experiments without the antagonist showed that there was some small recovery from 4-DAMP mustard-treatment during the 30 min after measurement of the first concentration-response curve - the pEC_{50} value of oxotremorine-M increased by 0.17 ± 0.071 log units (1.5-fold increase in potency) and the E_{max} increased by 33 ± 7%. Thus, in these experiments, the antagonist induced-shifts where corrected for the slow recovery from 4-DAMP mustard blockade. Following 4-DAMP mustard-treatment, AF-DX 116 (1 μM) caused a 1.6-fold shift in the oxotremorine-M concentration response curve, whereas 4-DAMP (10 nM) caused a 8.5-fold shift (see Figure 3*b* and *c*). When these shifts were corrected for recovery of the oxotremorine-M response, the calculated pK_B values of AF-DX 116 and 4-DAMP were 6.09 ± 0.098 and 9.06 ± 0.047, respectively. These values are in close agreement with the binding affinities of AF-DX 116 (pK_D = 6.10) and 4-DAMP (pK_D = 8.81) for recombinant human M_3 receptors, but not M_2 receptors (i.e., 7.27 and 7.87, respectively). Thus, competitive antagonism experiments in wild type urinary bladder after inactivation of a majority of the M_3 receptors with 4-DAMP mustard provides no evidence for a role of the M_2 receptor. Nevertheless, as described under "Introduction" and also under "Discussion" the antagonist profile for a response mediated through a directly acting M_3 receptor and an indirectly acting M_2 receptor has a tendency to resemble the profile of the M_3 receptor and not that of the M_2 receptor.

Effects of isoproterenol and forskolin: As described under "Introduction" it is possible to measure indirect, M_2 receptor mediated contractions in guinea pig smooth muscle by first

inactivating M_3 receptors with 4-DAMP mustard and then measuring contraction to a muscarinic agonist in the presence of both a contractile (e.g., histamine) and a cAMP generating, relaxant agent (isoproterenol). Presumably, under these conditions, M_2 receptors mediate an inhibition of relaxation, thereby allowing histamine to elicit contraction. Consequently, we were interested in using urinary bladder from M_3 KO mice in this experimental paradigm. M_3 KO mice are devoid of M_3 receptors; thus, it should be possible to measure contractions mediated indirectly by the M_2 receptor without interference from the M_3 and without the necessity of 4-DAMP mustard-treatment. The latter is never completely effective in eliminating the contractile response of the M_3 receptor. In these experiments, isolated urinary bladder from M_3 KO mice was first contracted with $PGF_{2\alpha}$ (1 μM). After approximately one min, the contractile response to $PGF_{2\alpha}$ reached a stable plateau. At this time, isoproterenol (1 μM) or forskolin (10 μM) was added, which causes a complete relaxation of the $PGF_{2\alpha}$-induced contraction. Then, in the continued presence of $PGF_{2\alpha}$ and the relaxant agent, a cumulative concentration-response curve to oxotremorine-M was measured. Figure 4 shows the results of these experiments. In the presence of $PGF_{2\alpha}$ (1 μM) and isoproterenol (1 μM), oxotremorine-M elicits a potent contractile response characterized by mean pEC_{50} and E_{max} values ± SEM of 6.87 ± 0.10 and 114 ± 10%, respectively (Figure 4*a*). When forskolin was used as the relaxant agent, the pEC_{50} and E_{max} values of oxotremorine-M were 6.58 ± 0.061 and 99 ± 18%, respectively. The E_{max} value of oxotremorine-M under these conditions was much greater than the E_{max} value of oxotremorine-M measured in the absence of $PGF_{2\alpha}$ and the relaxant agents (about 30 – 50%, see Figures 1*a* and 2*c*). Thus, most of the contraction in these experiments represents an oxotremorine-M-mediated inhibition of the relaxant effects of forskolin and isoproterenol on $PGF_{2\alpha}$-induced contractions, and not simply a direct, M_2 receptor-mediated contraction. Oxotremorine-M was without effect in urinary bladder from M_2/M_3 KO mice under these conditions, indicating that the indirect contractile response in M_3 KO mouse urinary bladder was mediated by the M_2 receptor.

Since the foregoing data demonstrate that the M_2 receptor in urinary bladder from M_3 KO mice mediates a substantial contraction, we predicted that M_2 selective antagonists should inhibit

this response with high potency. To test this postulate, we measured the ability of AF-DX 116 (1 μM) to inhibit the contractile response to oxotremorine-M in the presence of $PGF_{2\alpha}$ (0.6 – 1.0 μM) and either isoproterenol (0.6 μM) or forskolin (10 μM). When isoproterenol was used, AF-DX 116 caused a 6.9-fold shift in the concentration-response curve, whereas a 11.2-fold shift was noted when forskolin was present (see Figure 5). These shifts yield mean pK_B estimates ± SEM of 6.77 ± 0.05 and 7.01 ± 0.076 for AF-DX 116 in experiments with isoproterenol and forskolin, respectively. These values are in closer agreement with the binding affinity of AF-DX 116 at M_2 muscarinic receptors (pK_D = 7.27) as compared to that of M_3 receptors (pK_D = 6.10).

We also investigated whether the M_2 receptor could mediate contractions under similar conditions in urinary bladder from wild type mice that had been treated with 4-DAMP mustard to inactivate M_3 receptors. In these experiments, urinary bladder was incubated with 4-DAMP mustard (10 nM) in combination with AF-DX 116 (1 μM) for a total of two hr as described under "Material and Methods." Contractions to oxotremorine-M were measured subsequently in the presence of KCl and either isoproterenol (1 μM) (Figure 6*a*) or forskolin (10 μM) (Figure 6*b*). Since $PGF_{2\alpha}$ exhibited low activity in urinary bladder from wild type mice (see Figure 1*b*), we used KCl as the heterologous contractile agent in these experiments. We found that isoproterenol and forskolin caused a complete inhibition of the KCl response when KCl was present at a concentration of 37.5 mM, but no higher. Consequently, we used KCl at a concentration of 37.5 mM for these experiments. When measured after 4-DAMP mustard-treatment and in the presence of KCl and isoproterenol, oxotremorine-M elicited contractions in urinary bladder from wild type mice that were characterized by mean pEC_{50} and E_{max} values ± SEM of 6.19 ± 0.15 and 311 ± 21%, respectively. The corresponding values in urinary bladder from M_2 KO mice were smaller (5.57 ± 0.22 and 189 ± 45%, respectively). The reduction in agonist potency (4.2-fold increase in EC_{50}) in the M_2 KO mouse was statistically significant (P = 0.032), whereas the 39% decrease in E_{max} was not quite significant (P = 0.067). When measured under similar conditions in wild type mice using forskolin as the relaxant agent, the mean pEC_{50} and E_{max} values ± SEM of oxotremorine-M were 5.89 ± 0.16 and 285 ± 37%, respectively. The

corresponding values in urinary bladder from M_2 KO mice were smaller (5.55 ± 0.21 and 105 ± 27%, respectively). In these experiments, the 63% reduction in E_{max} in the M_2 KO mouse was statistically significant (P = 0.017), whereas the reduction in agonist potency (2.2-fold increase in EC_{50}) was not (P = 0.26).

It is important to note that with regard to the data in Figure 6, the E_{max} values of oxotremorine-M measured in urinary bladder from wild type mice greatly exceeded the initial contraction elicited by 37.5 mM KCl. This latter concentration of KCl elicited a contraction that was approximately 69 ± 8.7% of that elicited by the standard test concentration of KCl (50 mM). Thus, the oxotremorine-M-induced contractions in wild type bladder shown in Figure 6 cannot only be attributed to a simple reversal of relaxation, but must also involve a substantial direct muscarinic contractile component, probably mediated, in part, through residual M_3 receptors not inactivated by 4-DAMP mustard.

We also measured the ability of AF-DX 116 and 4-DAMP to antagonize the contractile response to oxotremorine-M in wild type mice under the conditions of the experiments shown in Figure 6. When measured after 4-DAMP mustard-treatment and in the presence of KCl and isoproterenol, AF-DX 116 (1 μM) and 4-DAMP (10 nM) shifted the oxotremorine-M concentration-response curve to the right 3.7- and 3.5-fold, respectively (see Figure 7*a*). These values represent shifts that were corrected for the time-dependent recovery of contraction after 4-DAMP mustard-treatment as described above in connection with the data shown in Figure 3*b* and *c*. The corresponding mean pK_B estimates ± SEM of AF-DX 116 and 4-DAMP were 6.43 ± 0.10 and 8.40 ± 0.060, respectively. When these experiments were repeated under the same conditions, but with isoproterenol replaced with forskolin (see Figure 7*b*), the mean pK_B values ± SEM of AF-DX 116 and 4-DAMP were 6.45 ± 0.21 and 8.74 ± 0.090, respectively. The pK_B values of AF-DX 116 (approximately 6.4) and 4-DAMP (approximately 8.6) in these experiments were in closer agreement with their respective binding affinities (pK_D's) for M_3 receptors (6.10 and 8.81) as compared to M_2 receptors (7.27 and 7.87). Thus, although the data in Figure 6 demonstrate a substantial loss of contractile function in the urinary bladder from M_2

KO mice, the pharmacological antagonism experiments provide little evidence for an M_2 response. As described under "Discussion," these results can be rationalized by the nature of the interaction between M_2 and M_3 receptors.

DISCUSSION

Our results on the contractile effects of oxotremorine-M in urinary bladder are consistent with previous reports showing little loss of muscarinic contractile function in M_2 KO mice (Stengel et al., 2000; Matsui et al., 2002), a very large loss of function in M_3 KO mice (Matsui et al., 2000) and a complete loss of function in M_2/M_3 KO mice (Matsui et al., 2002). We also noted that male mice lacking M_3 muscarinic receptors exhibited a large increase in sensitivity to the contractile effects of $PGF_{2\alpha}$ in urinary bladder. Perhaps this increase in sensitivity may partially compensate for the substantial urinary bladder distension that occurs in male M_3 KO and M_2/M_3 KO mice (Matsui et al., 2000; Matsui et al., 2002). In contrast, female mice lacking M_3 receptors do not exhibit urinary bladder distension (Matsui et al., 2000), nor do they exhibit an increased sensitivity to $PGF_{2\alpha}$ (data not shown). We have previously observed a modest increase in sensitivity to $PGF_{2\alpha}$ in ileum from M_2 KO and M_3 KO mice (Matsui et al., 2003).

As reported by others, we found that competitive antagonists inhibited the muscarinic contractile response in urinary bladder from wild type and M_2 KO mice in a manner consistent with an M_3 response, whereas behavior consistent with an M_2 response was observed in tissue from the M_3 KO mouse (Matsui et al., 2000; Stengel et al., 2000). We also noted that the contractile response to oxotremorine-M in urinary bladder from wild type and M_2 KO mice was greatly inhibited by 4-DAMP mustard-treatment, whereas that observed in the M_3 KO mouse was unaffected. These results are consistent with the postulate that the M_3 receptor is the major muscarinic subtype generating the direct contractile response in wild type and M_2 KO mice, because 4-DAMP mustard-treatment (i.e., 10 nM 4-DAMP and 1 μM AF-DX 116) has been shown to inactivate M_3 receptors selectively, while having little effect on M_2 receptors (Griffin et al., 2003).

Although 4-DAMP mustard-treatment is effective in alkylating M_3 receptors with high selectivity, it is difficult to inactivate M_3 receptors completely with this agent. Furchgott analysis (Furchgott, 1966) of the contractile measurements in urinary bladder from M_2 KO mice after

one- and two-hr of 4-DAMP mustard-treatment yields estimates of 8.5 and 1.5%, respectively, for the fraction of residual M_3 receptors mediating contraction. It may be impossible to inactive M_3 receptors much beyond this level using our two-hour treatment paradigm because even a very small percentage of new receptors being transported to the sarcolemma during the wash period (30 – 40 min) could restore contractile function to the levels that we observe.

When measured after 4-DAMP mustard-treatment, the contractile responses to oxotremorine-M were much greater in wild type than in M_2 KO mice, particularly when oxotremorine-M was used in the concentration range of 0.05 to 1.5 μM (see Figure 3*a*). This difference in contractile function between wild type and M_2 KO mice amounted to 78 to 162% of the response to KCl (50 mM). It follows that the M_2 component of contraction in wild type mice under these conditions is equivalent to 78 to 162% of the KCl-induced contraction. However, this M_2 component is much greater than the E_{max} value of oxotremorine-M in urinary bladder from M_3 KO mice, which was only 30 to 50% of the KCl response. Thus, the contractile mechanism of the M_2 receptor in wild type urinary bladder appears much greater than the direct M_2 receptor-mediated contractions observed in M_3 KO mice. This situation suggests that the M_2 component in wild type urinary bladder is not a direct contraction, but rather, an M_2 receptor-mediated enhancement in the contractile response of the M_3 receptor. If this M_2 mechanism is less potent than the direct contractile mechanism of the M_3 receptor, this condition could explain why this M_2 mechanism is more apparent after M_3 receptors in wild type urinary bladder have been inactivated with 4-DAMP mustard. A related phenomenon has been reported in guinea pig colon. The muscarinic contractile response of this tissue exhibits an M_3 profile in competitive antagonism studies and is insensitive to pertussis toxin, which uncouples M_2 receptor-mediated responses (Sawyer and Ehlert, 1998; Sawyer and Ehlert, 1999b). However, after extensive inactivation of M_3 receptors, the residual muscarinic contractile response is greatly inhibited by pertussis toxin-treatment, suggesting a role for the M_2 receptor. However, this residual contractile response exhibits an M_3 profile in competitive antagonism experiments, just like that of the wild type mouse urinary bladder after 4-DAMP mustard-treatment. As described above,

this behavior is consistent with a model in which the M_2 receptor acts indirectly to enhance the direct contractile response of the M_3 receptor. Mathematical modeling shows that this interaction has a tendency to exhibit an M_3 profile in competitive antagonism studies (Sawyer and Ehlert, 1999b; Ehlert, 2003b).

Previous studies have demonstrated that it is possible to measure relatively pure M_2 muscarinic contractile responses in wild type smooth muscle from guinea pigs, by first inactivating M_3 receptors with 4-DAMP mustard, and then, measuring muscarinic agonist-induced contractions in the presence of histamine and isoproterenol or forskolin (Thomas et al., 1993). These contractions are pertussis toxin-sensitive (Thomas and Ehlert, 1994) and exhibit an M_2 profile in competitive antagonism experiments (Ehlert and Thomas, 1995). A limitation in this experiment is that it is difficult to eliminate the contractile response of the M_3 receptor completely. Consequently, we were interested in exploring this paradigm in M_3 KO mouse bladder in which the problem of M_3 receptor-mediated contractions is obviously precluded. We found that oxotremorine-M mediated substantial contractions when measured in the presence of $PGF_{2\alpha}$ and either forskolin or isoproterenol in M_3 KO mouse urinary bladder. These contractions were completely eliminated in M_2/M_3 KO mouse bladder, indicating that they were mediated by the M_2 receptor. In addition, the M_2 selective antagonist, AF-DX 116, potently antagonized these contractions. The contractile mechanism probably involves an M_2 receptor-mediated inhibition of the relaxant effect of isoproterenol and forskolin on $PGF_{2\alpha}$-mediated contractions. This M_2 mechanism should act to diminish relaxant effects on contractile receptors other than those for $PGF_{2\alpha}$ (i.e., FP). We have shown that the relaxant effects of isoproterenol and forskolin against muscarinic agonist induced contractions are enhanced in M_2 KO mouse urinary bladder (Matsui et al., 2003). Collectively, these results demonstrate that muscarinic agonists activate both M_2 and M_3 receptors in urinary bladder and that activation of the M_2 receptor inhibits the relaxant effects of isoproterenol and forskolin on M_3 receptor-mediated contractions.

We were unable to demonstrate a role for the M_2 receptor in wild type mouse urinary bladder in competitive antagonism studies after 4-DAMP mustard-treatment even though there

was a large loss of contractile function in M_2 KO mouse urinary bladder under the same conditions. These results can be explained by the nature of the interaction between M_2 and M_3 receptors under these conditions. Even in the presence of KCl and relaxant agents (see Figure 6), most of the M_2 contractile mechanism in our experiments on wild type urinary bladder involves an enhancement in M_3 contractile function, either by inhibiting relaxation (Figure 6) or by directly enhancing M_3-mediated contractions (Figures 2*a* and 3*a*). We have previously shown that this type of interaction exhibits an M_3 profile in competitive antagonism experiments (Sawyer and Ehlert, 1999b; Ehlert, 2003b). The paradigm used for the experiments shown in Figure 6 has the capacity to reveal M_2 responses in competitive antagonism studies. However, to do so it is necessary to inactivate M_3 receptors effectively and to use a heterologous contractile agent that elicits a substantial contraction that is greatly inhibited by the relaxant agent. In our experiments, we were unable to generate sizable contractions to KCl that were sensitive to isoproterenol or forskolin. Moreover, we could not overcome this limitation through more effective inactivation of M_3 receptors. Our results show the utility of muscarinic KO mice for addressing muscarinic function in this situation.

Studies on human urinary bladder have shown an abundance of M_2 muscarinic receptors (Kories et al., 2003) and a large cholinergic component to the contractile response to electrical field stimulation (Sibley, 1984), suggesting a role for M_2 mechanisms in contraction of human urinary bladder. Nevertheless, several investigators have suggested that cholinergic contractions are mediated exclusively by M_3 receptors because the pharmacological antagonism of the muscarinic contractile response in human urinary bladder exhibits an M_3 profile (Fetscher et al., 2002). If one considers the possibility of two receptors, then the data are also not inconsistent with an $M_2 - M_3$ interaction, in which the M_2 receptor enhances the direct contractile action of the M_3 receptor through a mechanism contingent upon M_3 receptor activation. As mentioned above, this model exhibits an M_3 profile in competitive antagonism studies (Ehlert, 2003b; Ehlert, 2003a). Thus, our results showing a substantial indirect role for the M_2 receptor in mediating contraction of the urinary bladder suggest that muscarinic antagonists with high

affinity for both M_2 and M_3 receptors may be more useful in the treatment of urinary incontinence in humans than antagonists with selectivity for the M_3 receptor only.

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FOOTNOTES

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Legend to Figures

Figure 1: *The contractile effects of oxotremorine-M (a) and $PGF_{2\alpha}$ (b) in urinary bladder from wild type, M_2 KO, M_3 KO and M_2/M_3 KO mice.* The contractile responses are normalized relative to that elicited by KCl (50 mM). The data represent mean values ± SEM. The number of replicates for each experiment is indicated in Tables 1 and 2.

Figure 2: *Effect of 4-DAMP mustard-treatment on the contractile response to oxotremorine-M in urinary bladder from wild type (a), M_2 KO (b) and M_3 KO (c) mice.* Urinary bladder was treated with 4-DAMP mustard (10 nM) in combination with AF-DX 116 (1 μM) for one or two hr, and the tissue was washed repeatedly as described under "Materials and Methods." The contractile responses are normalized relative to that elicited by KCl (50 mM). The data represent mean values ± SEM. The number of replicates for each experiment is indicated in Table 4.

Figure 3: *Effect of 4-DAMP mustard-treatment on the contractile response to oxotremorine-M in urinary bladder from wild type and M_2 KO mice*. *a*: The contractile response to oxotremorine-M was measured in urinary bladder from wild type and M_2 KO mice after one and two hours of 4-DAMP mustard-treatment. The data are from Figure 3. *b*: The competitive antagonism of the contractile response to oxotremorine-M in wild type mouse urinary bladder by AF-DX 116 (1 μM) was measured after two hr of 4-DAMP mustard-treatment. The data represent the mean contractile values ± SEM from seven experiments, each done on urinary bladder from a different mouse. *c*: The competitive antagonism of the contractile response to oxotremorine-M in wild type mouse urinary bladder by 4-DAMP (10 nM) was measured after two hr of 4-DAMP mustard-treatment. The data represent the mean contractile values ± SEM from four experiments, each done on urinary bladder from a different mouse. The contractile responses are normalized relative to that elicited by KCl (50 mM). For these experiments, 4-DAMP mustard-

treatment was accomplished by incubating urinary bladder with 4-DAMP mustard (10 nM) in combination with AF-DX 116 (1 μM) for the indicated times.

Figure 4: *Contractile effects of oxotremorine-M in the presence of $PGF_{2\alpha}$ and isoproterenol (a) and forskolin (b) in urinary bladder from M_3 KO and M_2/M_3 KO mice*. The data represent the mean contractile values ± SEM from five experiments on wild type and three experiments on M_2/M_3 KO mice, each done on urinary bladder from a different mouse. The contractile responses are normalized relative to that elicited by KCl (50 mM).

Figure 5: *Competitive antagonism of the contractile response to oxotremorine-M in M_3 KO mouse urinary bladder by AF-DX 116 when measured in the presence of $PGF_{2\alpha}$ and isoproterenol (a) or forskolin (b)*. The data represent the mean contractile values ± SEM from four experiments with isoproterenol and three experiments with forskolin, each done on urinary bladder from a different mouse. The contractile responses are normalized relative to that elicited by KCl (50 mM).

Figure 6: *Contractile effects of oxotremorine-M in the presence of KCl and isoproterenol (a) and forskolin (b) in urinary bladder from wild type and M_2 KO mice following two-hour 4-DAMP mustard-treatment*. The data represent the mean contractile values ± SEM from three experiments, each done on urinary bladder from a different mouse. The contractile responses are normalized relative to that elicited by KCl (50 mM). For these experiments, 4-DAMP mustard-treatment was accomplished by incubating urinary bladder with 4-DAMP mustard (10 nM) in combination with AF-DX 116 (1 μM) for two, one-hour time periods.

Figure 7: *Competitive antagonism of the contractile response to oxotremorine-M in wild type mouse urinary bladder by AF-DX 116 and 4-DAMP when measured following two-hr 4-DAMP mustard-treatment in the presence of KCl and isoproterenol (a) or forskolin (b)*. The data represent the mean contractile values ± SEM from three experiments, each done on urinary bladder from a different mouse. The contractile responses are normalized relative to that elicited by KCl (50 mM). For these experiments, 4-DAMP mustard-

treatment was accomplished by incubating urinary bladder with 4-DAMP mustard (10 nM) in combination with AF-DX 116 (1 μM) for two, one-hour time periods.

Table 1: Contractile activity of oxotremorine-M in urinary bladder from wild type, M_2 KO and M_3 KO mice. [a]

Mouse strain	pEC_{50}	E_{max} *(% KCl)*
Wild type (n = 6)	6.54 ± 0.041	326 ± 27%
M_2 KO (n = 8)	6.31 ± 0.066 [b]	290 ± 67%
M_3 KO (n = 10)	6.60 ± 0.13	48 ± 5.4% [c]

[a] The data were calculated from the experiments shown in Figure 1*a*. Mean values ± SEM are shown. The number of experiments is indicated in parentheses. Each experiment was done on urinary bladder from a different mouse.

[b] Significantly different from the corresponding value for wild type, P = 0.012.

[c] Significantly different from the corresponding value for wild type, P = 4.3 x 10^{-6}.

Table 2: Contractile activity of $PGF_{2\alpha}$ in urinary bladder from wild type, M_2 KO, M_3 KO and M_2/M_3 KO mice. [a]

Mouse strain	pEC_{50}	E_{max} *(% KCl)*
Wild type (n = 4)	5.48 ± 0.036	89 ± 18%
M_2 KO (n = 6)	5.22 ± 0.17	86 ± 19%
M_3 KO (n = 10)	6.39 ± 0.092 [b]	186 ± 14% [c]
M_2/M_3 KO (n = 2)	6.60 ± 0.15 [d]	137 ± 19

[a] The data were calculated from the experiments shown in Figure 1*b*. Mean values ± SEM are shown. The number of experiments is indicated in parentheses. Each experiment was done on urinary bladder from a different mouse.

[b] Significantly different from the corresponding value for wild type, $P = 7.5 \times 10^{-6}$.

[c] Significantly different from the corresponding value for wild type, P = 0.0029.

[d] Significantly different from the corresponding value for wild type, P = 0.0039.

Table 3: Competitive antagonism of the contractile response to oxotremorine-M in urinary bladder from wild type, M_2 KO and M_3 KO mice. [a]

Tissue or cell line	*AF-DX 116* -Log dissociation constant	*4-DAMP* -Log dissociation constant
Urinary bladder, wild type (n = 5)	6.28 ± 0.048	9.03 ± 0.030%
Urinary bladder, M_2 KO (n = 4)	6.01 ± 0.072	9.19 ± 0.023
Urinary bladder, M_3 KO (n = 10)	6.97 ± 0.096	-
CHO M_2	7.27 ± 0.05 [b]	7.87 ± 0.031 [c]
CHO M_3	6.10 ± 0.06 [b]	8.81 ± 0.052 [c]

[a] Mean values ± SEM are shown. The number of experiments is indicated in parentheses. Each experiment was done on urinary bladder from a different mouse.

[b] The binding affinity values for AF-DX 116 are from Esqueda et al. (1996).

[c] The binding affinity values for 4-DAMP are from Griffin et al. (2004).

Table 4: Effects of 4-DAMP mustard-treatment on the contractile response to oxotremorine-M in urinary bladder from wild type, M_2 KO and M_3 KO mice. [a]

	1 hr 4-DAMP mustard-treatment		*2 hr 4-DAMP mustard-treatment*	
	EC_{50} shift [b]	*E_{max} inhibition*	*EC_{50} shift* [b]	*E_{max} inhibition*
Wild type (n = 7)	18-fold (1.25 ± 0.036)	- 22 ± 9%	23-fold (1.35 ± 0.038)	20 ± 8%
M_2 KO (n = 4)	13-fold (1.12 ± 0.071)	42 ± 14% [c]	22-fold (1.34 ± 0.18)	70 ± 10% [d]
M_3 KO (n = 3)	2.1-fold (0.33 ± 0.075)	-29 ± 18%	2.7-fold (0.43 ± 0.11)	-21 ± 14%

[a] The data were calculated from the experiments shown in Figure 2. 4-DAMP mustard-treatment was accomplished by incubating urinary bladder with 4-DAMP mustard (10 nM) in combination with AF-DX 116 (1 μM) for the indicated times. The number of experiments is indicated in parentheses in the column on the left. Each experiment was done on urinary bladder from a different mouse.

[b] Denotes the EC_{50} value of oxotremorine-M measured after 4-DAMP mustard –treatment divided by that measured before treatment. The values in parentheses beneath each estimate denote the mean log shift ± SEM.

[c] Significantly different from the corresponding value for wild type, P = 0.0041.

[d] Significantly different from the corresponding value for wild type, P = 0.0039.

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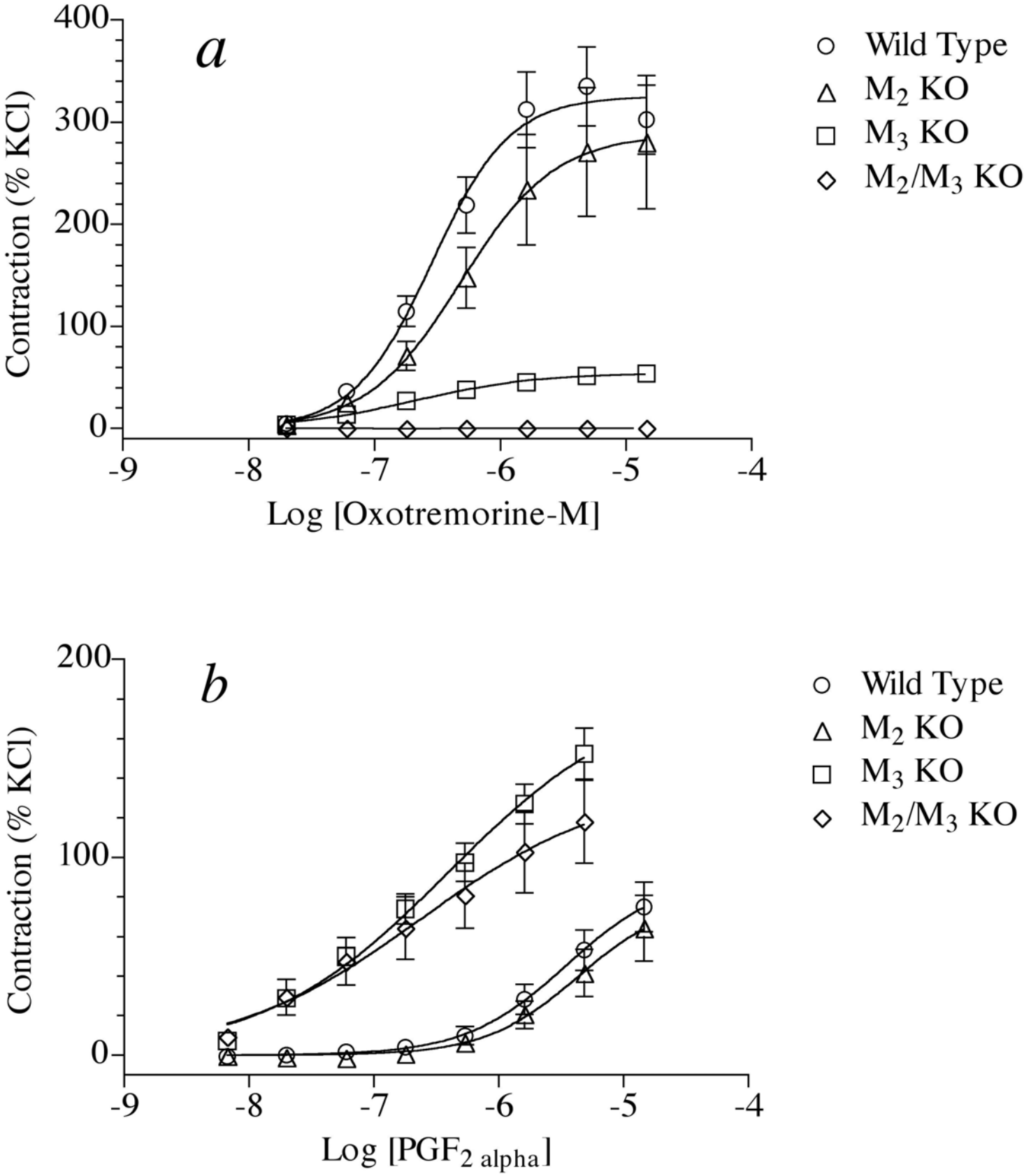

Figure 1

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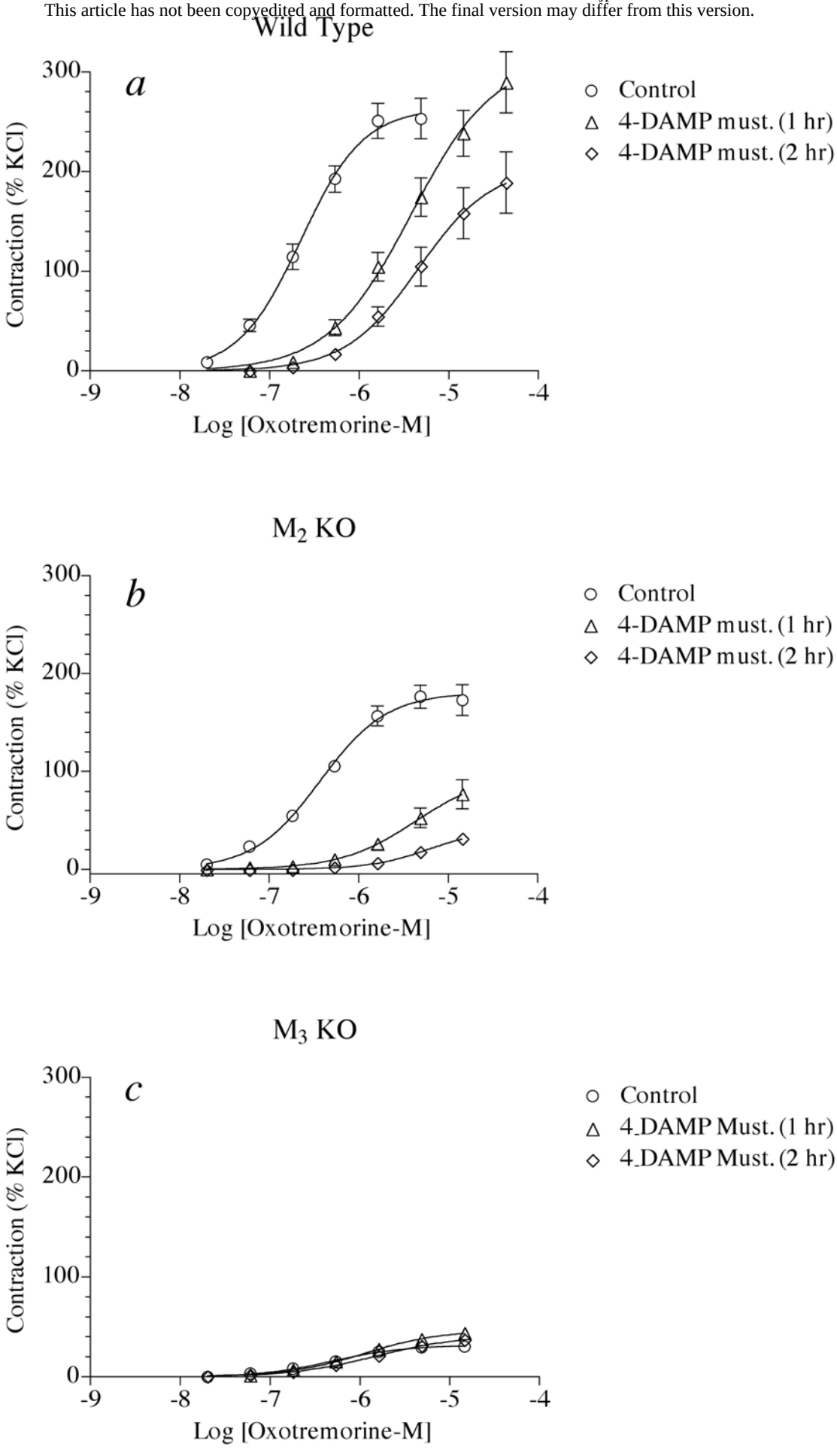

Figure 2

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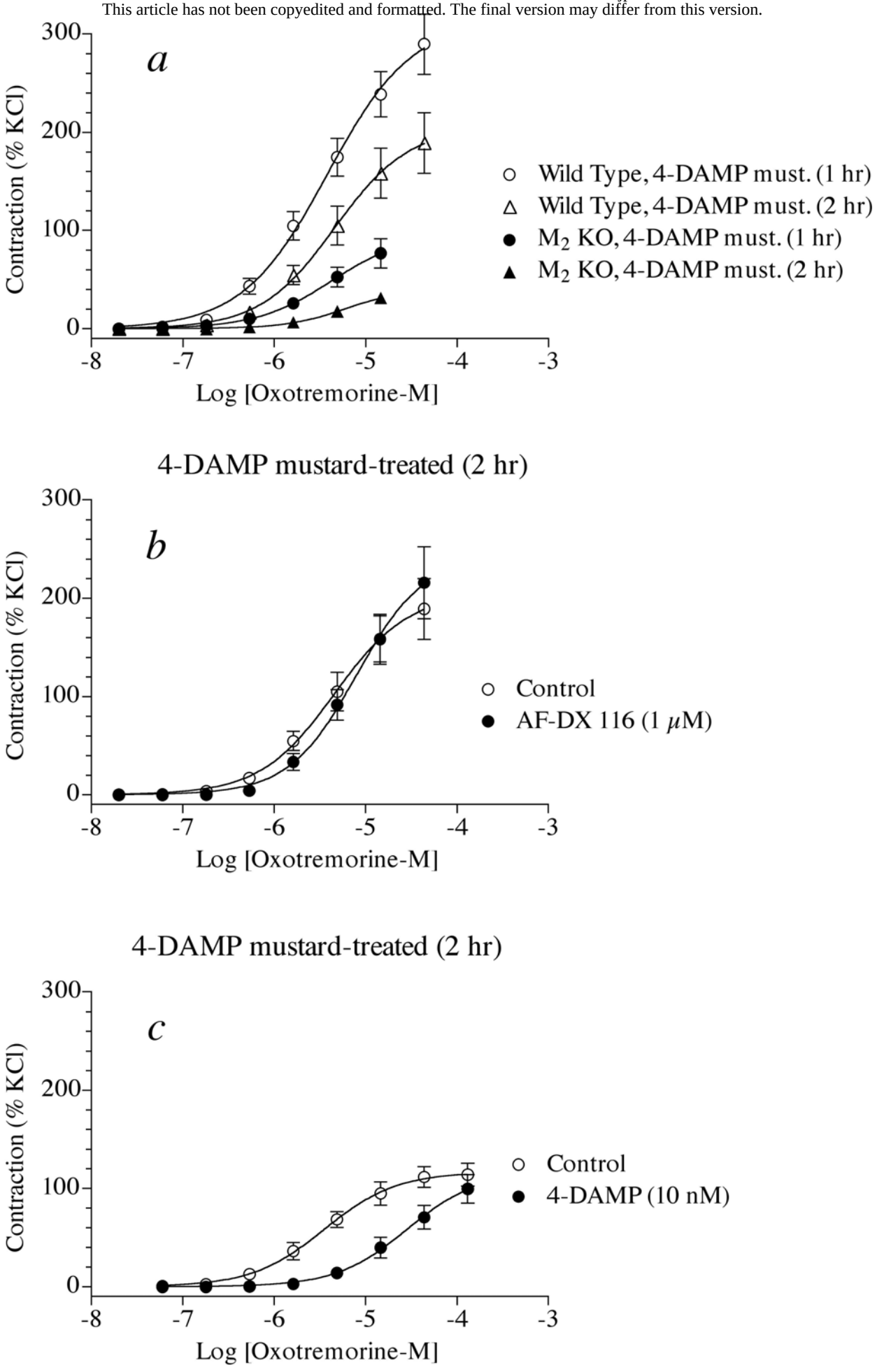

Figure 3

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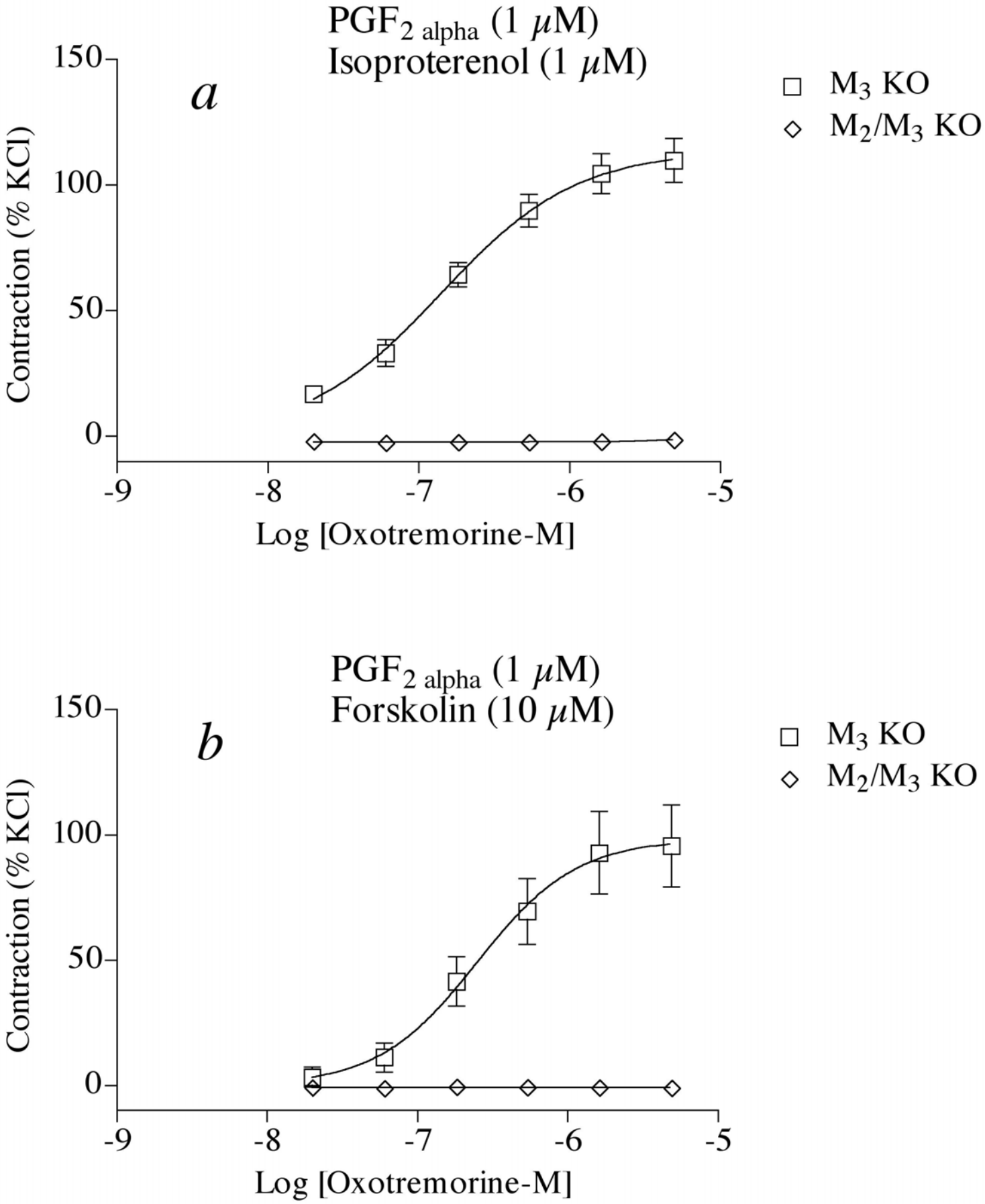

Figure 4

JPET Fast Forward. Published on December 17, 2004 as DOI: 10.1124/jpet.104.077909
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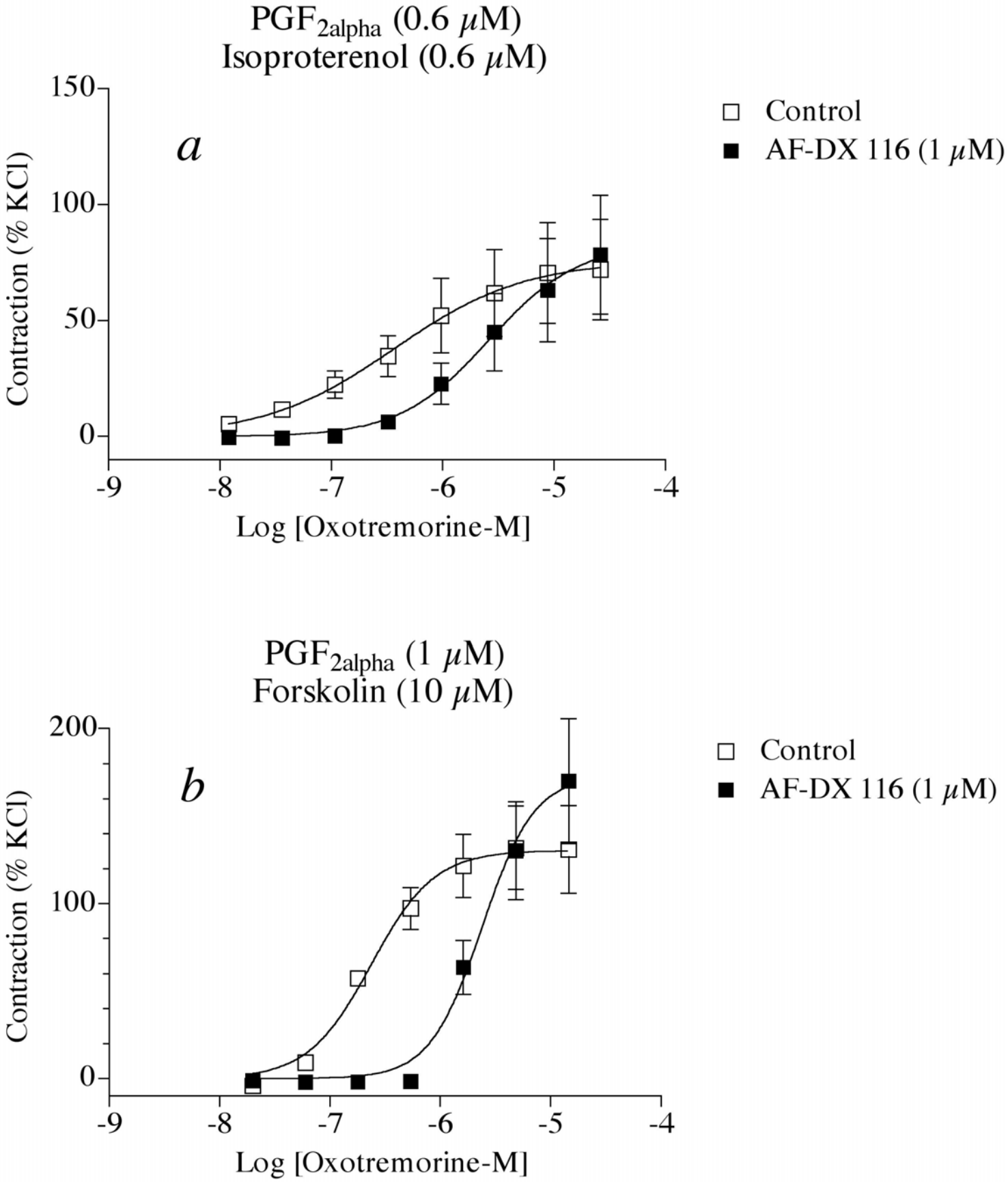

Figure 5

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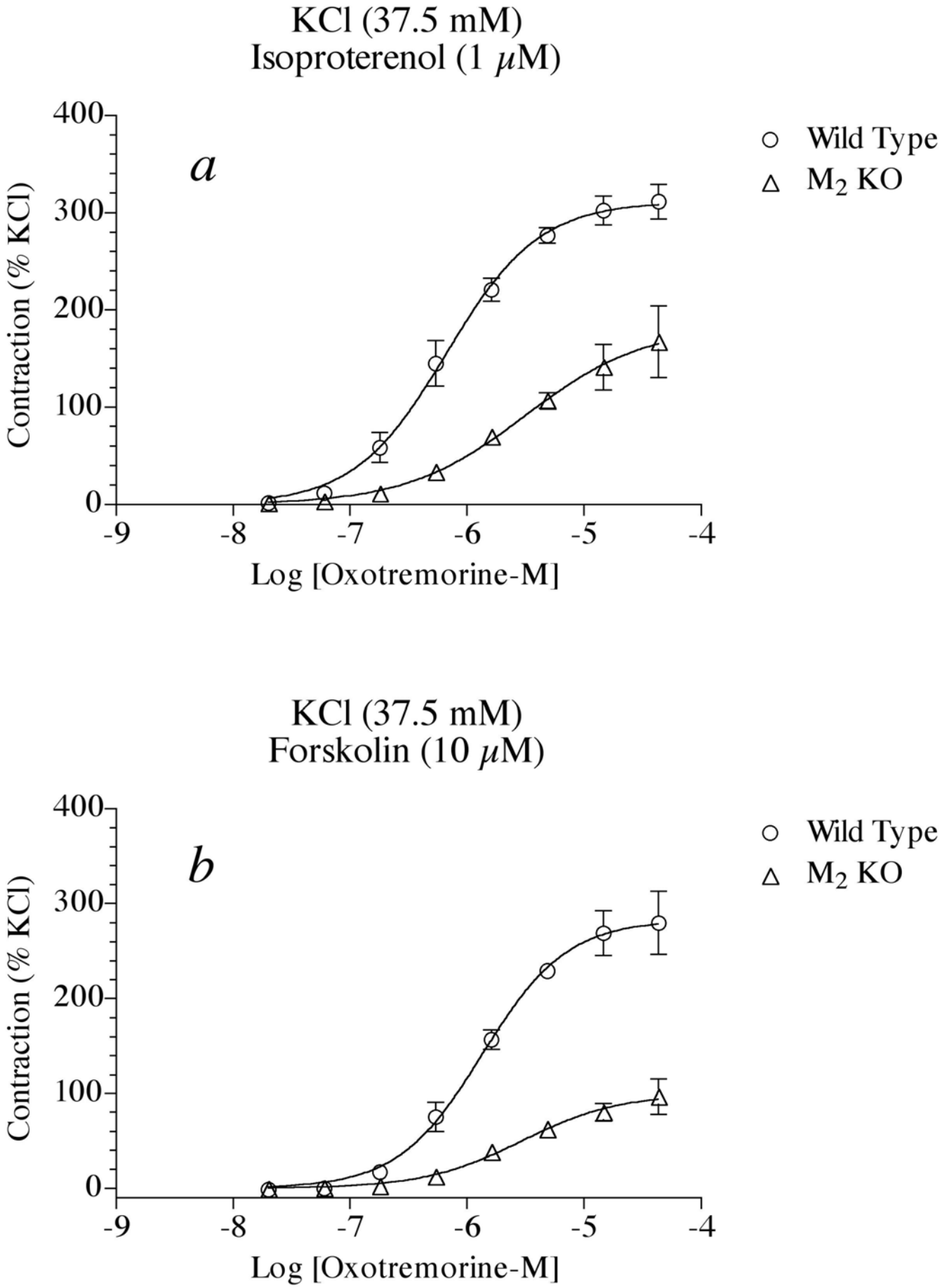

Figure 6

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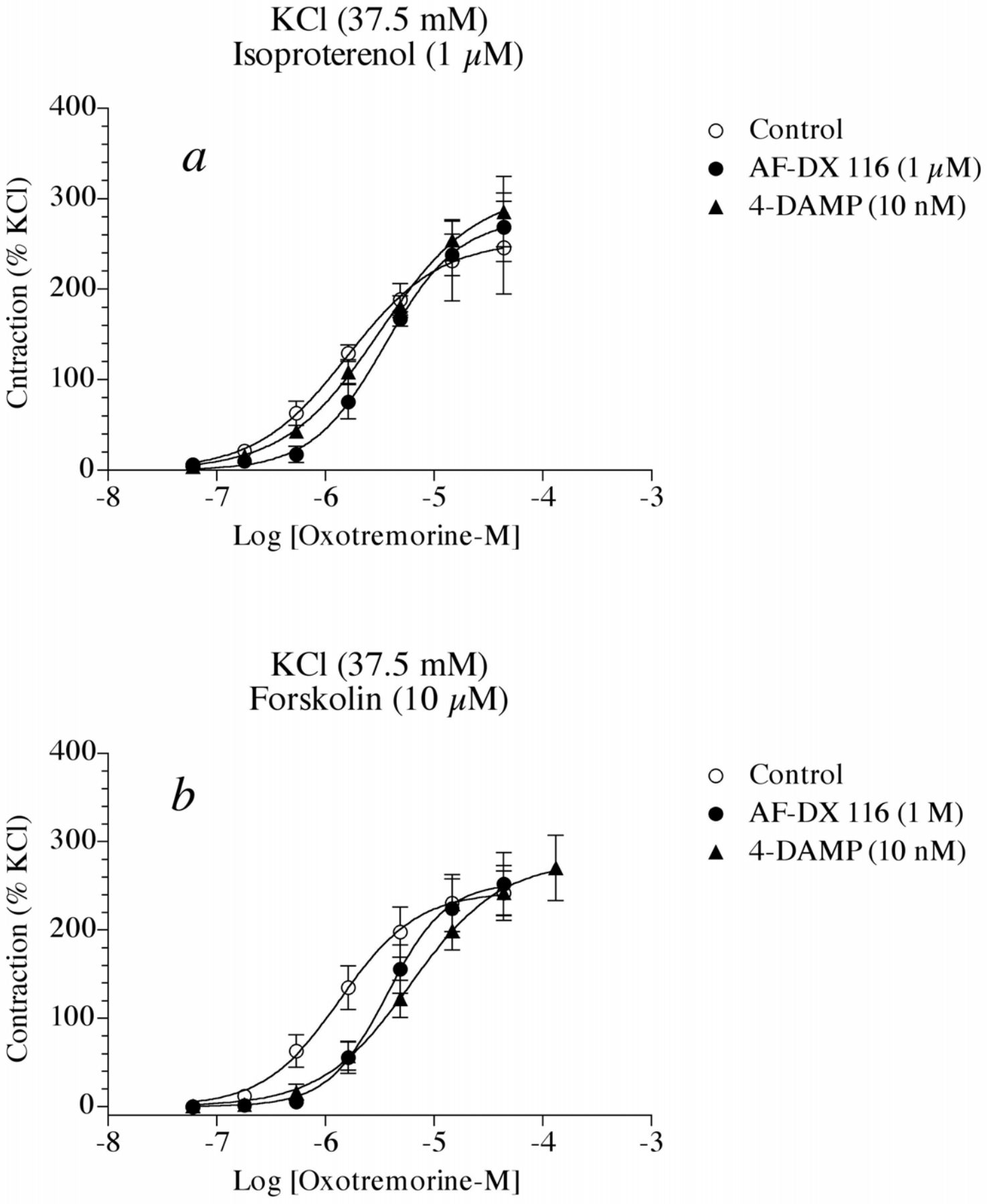

Figure 7