

# Impaired $M_3$ and enhanced $M_2$ muscarinic receptor contractile function in a streptozotocin model of mouse diabetic urinary bladder

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**Abstract** We investigated the contractile roles of  $M_2$  and  $M_3$  muscarinic receptors in urinary bladder from streptozotocin-treated mice. Wild-type and  $M_2$  muscarinic receptor knockout ( $M_2$  KO) mice were given a single injection of vehicle or streptozotocin ( $125 \text{ mg kg}^{-1}$ ) 2–24 weeks prior to bladder assays. The effect of forskolin on contractions elicited to the muscarinic agonist, oxotremorine-M, was measured in isolated urinary bladder (intact or denuded of urothelium). Denuded urinary bladder from vehicle-treated wild-type and  $M_2$  KO mice exhibited similar contractile responses to oxotremorine-M, when contraction was normalized relative to that elicited by KCl (50 mM). Eight to 9 weeks after streptozotocin treatment, the  $EC_{50}$  value of oxotremorine-M increased 3.1-fold in urinary bladder from the  $M_2$  KO mouse ( $N=5$ ) compared to wild type ( $N=6$ ;  $P<0.001$ ). Analogous changes were observed in intact bladder. In denuded urinary bladder from vehicle-treated mice, forskolin ( $5 \mu\text{M}$ ) caused a much greater inhibition of contraction in  $M_2$  KO bladder compared to wild type. Following streptozotocin treatment, this forskolin effect increased 1.6-fold ( $P=0.032$ ). At the

20- to 24-week time point, the forskolin effect increased 1.7-fold for denuded as well as intact bladders ( $P=0.036$ , 0.01, respectively). Although streptozotocin treatment inhibits  $M_3$  receptor-mediated contraction in denuded urinary bladder, muscarinic contractile function is maintained in wild-type bladder by enhanced  $M_2$  contractile function.  $M_2$  receptor activation opposes forskolin-induced relaxation of the urinary bladder, and this  $M_2$  function is enhanced following streptozotocin treatment.

**Keywords**  $M_2$  muscarinic receptor · Urinary bladder · Contraction · Streptozotocin · Diabetes · Smooth muscle

## Introduction

Diabetes is associated with an alteration in the contractility of urinary bladder smooth muscle in addition to the well-known increase in urinary frequency (Longhurst and Belis 1986; Latifpour et al. 1989; Michel and Barendrecht 2008). The two main neurotransmitters eliciting contraction in human and rodent urinary bladder are adenosine triphosphate and acetylcholine, which act on a ligand-gated ion channel (P2X receptor) and two G-protein-coupled receptors ( $M_2$  and  $M_3$  muscarinic), respectively (de Groat and Yoshimura 2001). A common approach for studying how the functions of these receptors change in diabetes mellitus is to examine their contractile roles in isolated urinary bladder from animals treated with streptozotocin (STZ).

Treatment with STZ causes a hypertrophy of the rat urinary bladder (Longhurst and Belis 1986; Latifpour et al. 1989) and an upregulation in  $M_2$  and  $M_3$  muscarinic receptors in the urothelium and smooth muscle (Tong and Cheng 2002; Tong et al. 2006; Cheng et al. 2007). Not surprisingly, the contractile response to muscarinic agonists

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in whole urinary bladder increases following STZ treatment (Kolta et al. 1985; Nsabimana and Ojewole 2008). To control for the STZ-induced hypertrophy, several investigators have normalized contraction relative to the cross-sectional area of the muscle strip or have measured contractions in strips of urinary bladder having a similar cross-sectional area. These studies have yielded conflicting reports, however, including an increase (Latifpour et al. 1989), a decrease (Longhurst and Belis 1986), and no change (Lincoln et al. 1984) in muscarinic agonist-induced contraction. Whether there are specific changes in the contractile functions of  $M_2$  and  $M_3$  muscarinic receptors in urinary bladder from STZ-treated rodents is unknown.

In smooth muscle from the urinary bladder and several other organs, the  $M_2$  receptor outnumbers the  $M_3$  by a ratio that is often at least four to one, yet its function at the level of contraction is less than obvious (Ehlert 2003a, b). For example, it has long been known that subtype-selective muscarinic antagonists inhibit contraction of the urinary bladder with a profile consistent with that of an  $M_3$  mechanism (Hegde and Eglén 1999). In addition, there is little loss of muscarinic agonist-induced contraction in isolated urinary bladder from the  $M_2$  muscarinic receptor knockout out ( $M_2$  KO) mouse (Matsui et al. 2000, 2002). In smooth muscle from the  $M_3$  KO mouse, muscarinic agonists elicit contraction, and this response is lost in the  $M_2/M_3$  double KO mouse, indicating that the  $M_2$  receptor can mediate contraction. Although the potency of this response is high, its  $E_{\max}$  expressed relative to that of wild type is only 10% in urinary bladder and about 30% in ileum and the fundus of the stomach (Matsui et al. 2000; Stengel et al. 2002). Thus, experiments on the  $M_3$  KO mouse have revealed a small role for the  $M_2$  receptor in contraction. In guinea pig ileum, this high-potency  $M_2$  mechanism is absent (Griffin et al. 2009).

Part of the reason why the  $M_2$  receptor appears to have little role in the experiments described above can be explained by the nature of some of the  $G_i$ -signaling pathways of the  $M_2$  receptor in smooth muscle. Muscarinic activation of smooth muscle elicits a  $Ca^{2+}$ -dependent nonselective cation conductance ( $I_{\text{cat}}$ ; Bolton 1979; Inoue 1991). This conductance is pertussis toxin-sensitive (Inoue and Isenberg 1990), and it is greatly inhibited in intestinal smooth muscle from either the  $M_2$  KO or the  $M_3$  KO mouse (Sakamoto et al. 2007). Muscarinic receptor activation indirectly stimulates a  $Ca^{2+}$ -activated  $K^+$  ( $BK_{Ca}$ ) channel in smooth muscle from guinea pig trachea (Wade and Sims 1993) and many other organs, presumably through mobilization of  $Ca^{2+}$  by the  $M_3$  receptor. This conductance may represent an inhibitory-feedback mechanism to suppress excessive excitation of smooth muscle. Muscarinic receptor activation in canine colon

(Cole and Sanders 1989) and many other types of smooth muscle also directly inhibits the  $BK_{Ca}$  channel, and this response is pertussis toxin-sensitive (Kume et al. 1995; Nakamura et al. 2002), which suggests a role for the  $M_2$  receptor. In rat urinary bladder, pertussis toxin-treatment and  $M_2$  selective antagonists inhibit the direct muscarinic suppression of  $BK_{Ca}$  (Nakamura et al. 2002). Both the  $M_2$  inhibition of the  $BK_{Ca}$  channel and the  $M_2$  stimulation of  $I_{\text{cat}}$  are expected to be lost in the  $M_3$  KO mouse, because they are contingent upon  $Ca^{2+}$  mobilization triggered by  $M_3$  receptor activation. Perhaps this accounts for the near complete loss of muscarinic contractile function in urinary bladder from the  $M_3$  KO mouse. Obviously, this  $M_2$ – $M_3$  interaction mechanism cannot account for the residual  $M_2$  receptor-mediated contraction in the ileum and fundus of the stomach from the  $M_3$  KO mouse.

One might expect some decrease in muscarinic agonist-induced contraction in smooth muscle from the  $M_2$  KO mouse or from pertussis toxin-treated animals because of the loss of  $M_2$  conductance mechanisms just described. In most instances, however, this is not observed (Thomas and Ehlert 1994; Sawyer and Ehlert 1999; Ehlert 2003a). We have previously shown that the potency of the  $M_2$  enhancement mechanism in intestinal (Sawyer and Ehlert 1999; Ehlert 2003a) and urinary bladder (Ehlert et al. 2005, 2007) smooth muscle is less than that of the  $M_3$  contractile mechanism so that muscarinic contractions are already maximal at an agonist concentration that is too low to trigger  $M_2$  mechanisms. One exception is the mouse uterus where muscarinic agonist-induced contraction is greatly inhibited in the  $M_2$  KO mouse (Kitazawa et al. 2008).

In smooth muscle, cAMP mediates relaxation, and activation of the  $M_2$  receptor inhibits adenylate cyclase and the relaxation elicited by forskolin and activation of  $\beta$ -adrenoceptors in gastrointestinal and urinary bladder smooth muscle (Ehlert 2003b). The potency of muscarinic agonists for eliciting this response in the guinea pig ileum is actually greater than that for eliciting a direct contraction through the  $M_3$  receptor (Ostrom and Ehlert 1997). In bovine and guinea pig trachea,  $M_2$  receptor activation inhibits forskolin- but not isoproterenol-mediated relaxation, suggesting that the  $\beta$ -adrenoceptor mediates relaxation through a non-cAMP mechanism (Ostrom and Ehlert 1998).

The  $M_3$  receptor interacts with  $G_q$  to mediate phosphoinositide hydrolysis and direct contraction in smooth muscle (Ehlert 2003b). Both the effect on phosphoinositide hydrolysis and contraction are pertussis toxin-insensitive (Ehlert 2003b). While many  $G_q$ -linked receptors elicit contraction in smooth muscle, the source of  $Ca^{2+}$  for  $M_3$  receptor-mediated contractions is mainly extracellular through voltage-sensitive  $Ca^{2+}$  channels (Bolger et al. 1983). How the  $M_3$  receptor triggers an influx of  $Ca^{2+}$  is unclear. Most of the older electrophysiological literature

suggests that the initial depolarization occurs through  $I_{cat}$  followed by an influx of  $Ca^{2+}$  through voltage-sensitive channels, but neither the activation of  $I_{cat}$  nor the inhibition of  $BK_{Ca}$  channels can explain the potent muscarinic agonist-induced contraction of isolated smooth muscle because the latter is insensitive to pertussis toxin (Ehlert 2003b), whereas the former two ionic mechanisms are pertussis toxin-sensitive (see above).

Given the importance of the STZ model of diabetes, we undertook a study to determine how  $M_2$  and  $M_3$  receptor-mediated contractions are altered in isolated urinary bladder from STZ-treated mice. We observed an increase in  $M_2$  and a decrease in  $M_3$  receptor-mediated contractions in urinary bladder smooth muscle from STZ-treated mice. We also observed a similar phenomenon in urinary bladder with intact urothelium, although the results are more complicated because of changes in muscarinic function in the urothelium with STZ treatment.

## Methods

### Animals

All procedures were approved by the Institutional Laboratory Animal Care and Use Committee.  $M_2$  muscarinic receptor knockout ( $M_2$  KO) mice were generated as previously described (Matsui et al. 2000, 2002). Male C57BL/6 wild-type (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) and  $M_2$  KO mice (2–3 months old, 18–30 g) were fasted for 4–6 h and then briefly anesthetized with isoflurane (Phoenix Pharmaceuticals, Inc., St. Joseph, MO, USA) before a single intraperitoneal injection with either vehicle (sodium citrate dihydrate, Fisher Scientific, Pittsburgh, PA, USA) or 125 mg  $kg^{-1}$  of STZ (Sigma Aldrich Chemical, St. Louis, MO, USA) 2 to 24 weeks prior to experiments on isolated bladder. Water containing 10% sucrose was given to animals 48 h after injection to prevent hypoglycemic shock. All mice were housed in a 12-h light/dark facility with free access to water and food. Blood glucose was measured using an Ascensia Contour glucometer (Bayer, Leverkusen, Germany), and diabetic ketoacidosis was assessed by Keto-Diastix (Bayer, Leverkusen, Germany). Heparinized saline was used to dilute and estimate blood glucose levels above the 600-mg/dL glucometer limit.

### Isolated urinary bladder

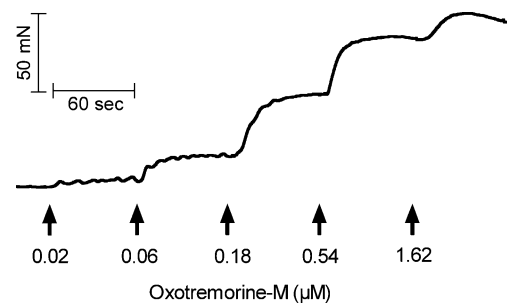
Whole mouse urinary bladder was removed and cut in half sagittally. The urothelium was carefully dissected from one of the strips, while the other was left intact. Half-bladder strips were mounted in an organ bath in a longitudinal orientation and connected to a force-displacement trans-

ducer using silk thread. Tissues were immersed in a Krebs–Ringer bicarbonate buffer (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) at 37°C and gassed with  $O_2/CO_2$  (19:1) as previously described (Ehlert et al. 2005). Resting tension was adjusted to a load of 1 g (9.8 mN) during an equilibration period of at least 1 h. Contractile responses were first generated by two test doses of KCl (50 mM). Bladder was washed and allowed to rest for 10 min after each test dose. The contractile response to KCl was calculated as the stable plateau value after a 3-min period minus the initial resting tension. Responses to other agents were either expressed in millinewton of force or normalized relative to the larger of the two responses to KCl, which were usually similar.

Contraction of the urinary bladder to the highly efficacious muscarinic agonist, oxotremorine-M, was measured using a cumulative technique. Each aliquot of the agonist was added to the bath, and the response was calculated as the stable plateau achieved within approximately 1 min minus the initial resting tension. Figure 1 shows a representative recording with the arrows indicating the final cumulative concentrations of agonist. After a complete concentration–response curve was measured, tissues were washed five to eight times over a period of 35–45 min before measurement of another curve. This initial concentration–response curve was not utilized but enabled the bladder to equilibrate and to achieve a maximal contractile state. Subsequent responses were recorded in a similar manner and are described under “Results” section.

### Analysis of oxotremorine-M concentration–response curves

The maximal response ( $E_{max}$ ) and concentration of agonist eliciting half-maximal response ( $EC_{50}$ ) were estimated from the concentration–response curve using nonlinear regression



**Fig. 1** Contractile response of denuded urinary bladder to oxotremorine-M measured using a cumulative technique. The urinary bladder was from a wild-type mouse that had been treated with streptozotocin approximately 20 weeks prior. Force of contraction of this representative experiment is plotted against time. The arrows indicate when aliquots of oxotremorine-M were added to the tissue bath. The final, cumulative concentration of oxotremorine-M is indicated beneath each arrow

analysis with Prism software (GraphPad Software, Inc., San Diego, CA, USA).

To estimate the potential loss of function in the M<sub>2</sub> KO mouse bladder relative to wild type, we fitted the concentration–response curves from wild-type and M<sub>2</sub> KO mice to Eqs. 1 and 2, respectively:

$$y = \frac{X^n E_{\max}}{X^n + EC_{50}^n} \quad (1)$$

$$y = \frac{X^n (E_{\max} + \delta)}{X^n + \gamma EC_{50}^n} \quad (2)$$

In both of these equations, EC<sub>50</sub> and E<sub>max</sub> represent the corresponding estimates for wild-type tissue, *y* represents the measured contraction, *X* represents the concentration of oxotremorine-M, and *n* represents the Hill slope. In Eq. 2,  $\delta$  represents the change in E<sub>max</sub>, and  $\gamma$  represents the scalar change in EC<sub>50</sub> between wild type and M<sub>2</sub> KO. For each treatment (i.e., vehicle or STZ), the concentration–response curves were fitted simultaneously to Eqs. 1 and 2 sharing the estimate of E<sub>max</sub> and EC<sub>50</sub> between wild type and M<sub>2</sub> KO and obtaining unique estimates of *n* for both wild type and M<sub>2</sub> KO and unique estimates of  $\delta$  and  $\gamma$  for the M<sub>2</sub>KO. To test for differences between bladders from vehicle- and STZ-treated animals, we determined the significance of the increase in residual variance when the estimate of  $\delta$  or  $\gamma$  was shared between the vehicle- and STZ-treated groups as described by Motulsky and Christopoulos (2003).

We used the operational model (Black and Leff 1983) to quantify the effect of forskolin on the concentration–response curve to oxotremorine-M:

$$y = \frac{X^m M_{\text{sys}}}{X^m + \frac{(X+K)^m}{\tau^m}} \quad (3)$$

In this equation, *y* represents the measured contraction, *X* represents the concentration of oxotremorine-M, M<sub>sys</sub> represents the maximum response of the system, *K* represents the observed dissociation constant, *m* represents the transducer slope factor, and  $\tau$  represents a parameter related to coupling efficiency. Specifically, the value of  $\tau$  is proportional to the intrinsic efficacy of the agonist ( $\varepsilon$ ), the amount of potentially active receptors (*R<sub>T</sub>*), and the sensitivity of the signaling cascade (1/*K<sub>E</sub>*). In our experiments, forskolin either caused a shift to the right or both a shift to the right and a decrease in the E<sub>max</sub> of the concentration–response curve. Both situations were adequately described by a reduction in the  $\tau$  parameter of the operational model. The effects of forskolin on the two disparate parameters, EC<sub>50</sub> and E<sub>max</sub>, could be adequately described, therefore, by a decrease in the value of a single parameter ( $\tau$ ) that accurately accounts for the

inhibitory effect of cAMP on the contractile elements (i.e., decrease in 1/*K<sub>E</sub>*).

To quantify this effect in bladder from the wild-type mouse, we analyzed the control concentration–response curve using Eq. 3 and that measured in the presence of forskolin using an identical equation, but with the parameter  $\tau$  replaced with the product,  $\alpha\tau$ :

$$y = \frac{X^m M_{\text{sys}}}{X^m + \frac{(X+K)^m}{\alpha\tau^m}} \quad (4)$$

The parameter  $\alpha$ , therefore, represents the factor by which forskolin reduces the sensitivity of the signaling pathway for contraction.

To investigate how the M<sub>2</sub> receptor opposes forskolin-induced relaxation, we compared the relaxant effect of forskolin in urinary bladder from wild-type and M<sub>2</sub> KO mice. The control concentration–response curve in the M<sub>2</sub> KO mouse was analyzed using Eq. 3 and that in the presence of forskolin was analyzed using an identical equation, but with the parameter  $\tau$  replaced with the product,  $\alpha\beta\tau$ :

$$y = \frac{X^m M_{\text{sys}}}{X^m + \frac{(X+K)^m}{\alpha\beta\tau^m}} \quad (5)$$

Here,  $\beta$  represents the relative difference, expressed as a factor, between the effect of forskolin in wild-type ( $\alpha$ ) and M<sub>2</sub> KO mice ( $\alpha\beta$ ). This parameter, therefore, is a measure of the role of the M<sub>2</sub> receptor in opposing forskolin-induced relaxation—the smaller the value of  $\beta$  (or alternatively, the larger the value of  $-\log \beta$ ), the greater the role of the M<sub>2</sub> receptor.

To estimate  $\beta$ , the concentration–response curves in the absence (control) and presence of forskolin and in both wild-type and M<sub>2</sub> KO mice were fitted simultaneously to Eqs. 3, 4, and 5 to obtain unique estimates of M<sub>sys</sub>, *m*,  $\alpha$ , and  $\beta$  (global nonlinear regression analysis). The estimate of  $\tau$  was shared among the curves and that of *K* was shared between those of control and forskolin conditions. Figure 2 shows a pictorial representation of our analysis, and the legend describes additional details.

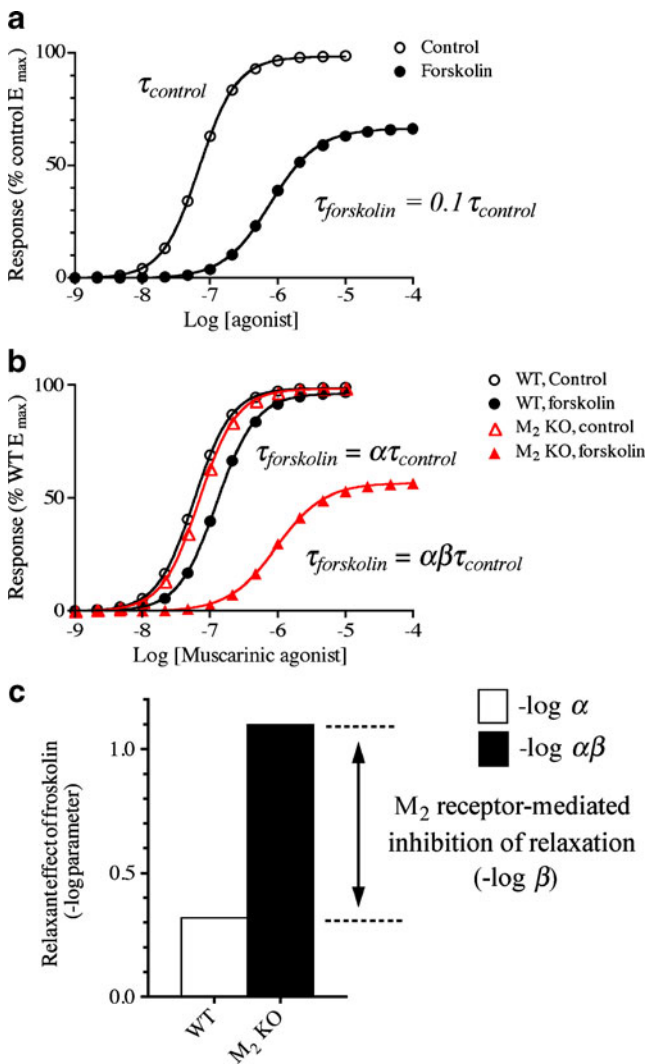
The statistical significance of differences between parameter estimates measured in urinary bladders from vehicle- and STZ-treated mice was determined as described above in connection with Eqs. 1 and 2.

## Results

### Effects of STZ on body weight and blood glucose

Wild-type and M<sub>2</sub> KO mice injected with a single dose (125 mg kg<sup>-1</sup>) of STZ exhibited significantly higher fasting





**Fig. 2** Determination of the role of the  $M_2$  receptor in opposing forskolin-induced relaxation. **a** The plot shows a simulation of a concentration–response curve to a muscarinic agonist in the absence (control) and presence of forskolin. The operational model (Eq. 3) was used to simulate the data. All of the parameters were the same for the two curves except that the parameter  $\tau$  for the curve measured in the presence of forskolin was reduced to one tenth that of the control curve. The values of the parameters in the simulation were  $M_{\text{sys}}=100$ ,  $m=1.65$ ,  $K=10^6$ , and  $\tau=15$ . **b** The simulation illustrates the effect of forskolin on the concentration–response curve to a muscarinic agonist in smooth muscle from wild-type and  $M_2$  KO animals. For the wild-type curves, the effect of forskolin has been simulated by reducing the value of  $\tau$  by the factor  $\alpha$ . In other words, Eqs. 3 and 4 were used to simulate the control and forskolin conditions, respectively, with the value of  $\alpha$  set to 0.48 in Eq. 4 and the values of all of the other parameters the same for the two equations. For the  $M_2$  KO curves, the effect of forskolin has been simulated by reducing  $\tau$  by the factor  $\alpha\beta$ , with  $\alpha=0.48$  as in wild type and  $\beta=0.16$ . It follows that the log difference in the effect of forskolin in wild type ( $\log \alpha$ ) and  $M_2$  KO ( $\log \alpha\beta$ ) is equivalent to  $\log \beta$ . The values of the parameters for the simulations were, wild-type control,  $M_{\text{sys}}=100$ ,  $m=1.65$ ,  $K=10^6$ , and  $\tau=18$ ; wild-type forskolin, same as control with  $\alpha=0.48$ ;  $M_2$  KO control, same as wild type but with  $\log \tau=15$ ;  $M_2$  KO forskolin, same as  $M_2$  KO but with  $\alpha\beta=0.078$ . **c** The plot shows that the role of the  $M_2$  receptor in opposing forskolin-induced relaxation ( $\log \beta$ ) is equivalent to the difference between the relaxant effect of forskolin in wild type ( $\log \alpha$ ) and  $M_2$  KO ( $\log \alpha\beta$ ). The values of  $\alpha$  and  $\beta$  are the same as those used in the simulation in (b)

blood glucose levels compared to control animals (Table 1). Body weight decreased significantly more in STZ-treated mice compared to those treated with vehicle with the exception of wild-type mice at the 2- to 3-week time point (Table 1). Although STZ significantly decreased the weight of  $M_2$  KO compared to wild-type mice at 8 to 9 weeks (Table 1), denuded bladder tissue from these animals did not exhibit a difference in KCl-induced contraction (data not shown).

#### Effects of STZ on denuded urinary bladder

**Muscarinic agonist-induced contractions** Two to 3 weeks after treatment with STZ, wild-type urinary bladder lacking urothelium (denuded) displayed a slight decrease in contractile responsiveness to oxotremorine-M as compared to tissue from vehicle-treated mice (Fig. 3a). At both the 8–9- and 20–24-week time points after STZ treatment, however, the  $E_{\text{max}}$  values of oxotremorine-M in denuded

bladders were markedly greater than those from vehicle-treated animals (Fig. 3d, g). These effects were manifest mainly as significant 58% and 61% increases in  $E_{\text{max}}$  when expressed in units of force (millinewton; Table 2). These data illustrate a time-dependent increase of contractile response in tissues from STZ-treated mice.

When normalized relative to the contraction elicited by KCl (50 mM), however, the concentration–response curves in bladder from wild-type mice showed little difference between vehicle and STZ groups at all time points (Fig. 3b, e, h and Table 2), suggesting that STZ treatment might cause smooth muscle hypertrophy or a general increase in contractility over time. In experiments on urinary bladder from  $M_2$  KO mice, there was little difference between vehicle and STZ groups at the early (2–3 weeks) and late (20–24 weeks) time points (Fig. 3c, i). At the intermediate time point (8–9 weeks), STZ treatment caused a significant loss of contractile function (Fig. 3f), which was manifest as a significant 2.3-fold increase in  $EC_{50}$  (Table 2).

To quantify the role of the  $M_2$  receptor, we compared the loss of contractile function in the  $M_2$  KO mouse relative to wild type. In vehicle-treated mice, there was little difference in the contractile activity of oxotremorine-M in urinary bladder from wild-type and  $M_2$  KO mice. Similarly, there was little difference in contractile activity between wild-type and  $M_2$  KO mice at the early (2–3 weeks) and late (20–24 weeks) time points after STZ treatment. At the intermediate time point (8–9 weeks), however, STZ

**Table 1** Body weight percent change and fasting blood glucose levels 2–24 weeks after injection

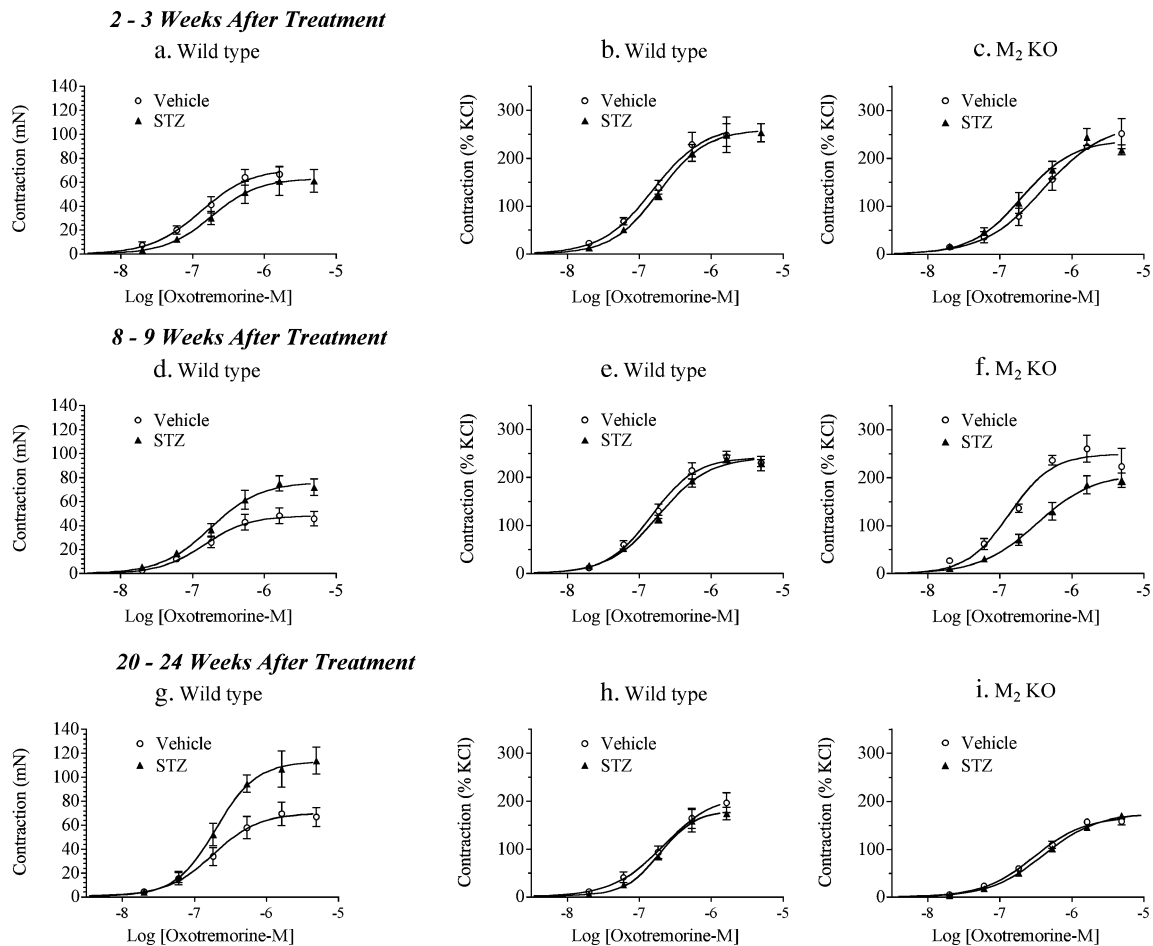
		Percent body weight change		Fasting blood glucose (mgdL <sup>-1</sup> )	
		Wild type	M <sub>2</sub> KO	Wild type	M <sub>2</sub> KO
2–3 weeks after treatment	Vehicle	5.9±2.7 (7)	1.2±1.2 (3)	111.1±14.7 (7)	113.7±18.5 (3)
	STZ	1.4±8.5 (3)	-9.2±1.0 (3) <sup>a</sup>	298.3±59.2 (3) <sup>a</sup>	436.0±39.7 (3) <sup>a</sup>
8–9 weeks after treatment	Vehicle	27.0±2.4 (6)	21.1±3.5 (4)	115.0±11.2 (6)	140.8±8.9 (4)
	STZ	14.3±2.4 (6) <sup>a</sup>	4.36±1.4 (5) <sup>a, b</sup>	431.3±61.3 (6) <sup>c</sup>	470.6±37.9 (5) <sup>c</sup>
20–24 weeks after treatment	Vehicle	48.2±6.7 (5)	41.2±1.8 (4)	133.0±5.3 (5)	127.5±6.3 (4)
	STZ	14.4±8.3 (3) <sup>a</sup>	8.9±4.2 (4) <sup>c</sup>	458.7±113.3 (3) <sup>a</sup>	513.7±36.3 (4) <sup>c</sup>

Vehicle (sodium citrate-buffered saline) and streptozotocin (STZ) were injected intraperitoneally (125 mg kg<sup>-1</sup>). Data are means ± SE (number of animals)

<sup>a</sup> Statistically significant difference from vehicle ( $P < 0.05$ )

<sup>b</sup> Significantly different from STZ-treated wild-type mice ( $P = 0.011$ )

<sup>c</sup> Statistically significant difference from vehicle ( $P < 0.001$ )



**Fig. 3** Effect of streptozotocin (STZ) treatment on oxotremorine-M-mediated contractions in urinary bladder lacking urothelium from wild-type and M<sub>2</sub> KO mice. Concentration–response curves were measured 2 to 3 weeks (a–c), 8 to 9 weeks (d–f), and 20 to 24 weeks (g–i) after STZ treatment in urinary bladder from wild-type (a, b, d, e,

g, h) and M<sub>2</sub> KO (c, f, i) mice. Contractions are expressed in units of force (millinewton; a, d, g) or relative to the contraction elicited by KCl (50 mM; b, c, e, f, h, i). Circles denote the vehicle-treated group; triangles denote the STZ-treated group. Mean ± SEM values from three to seven experiments are shown, each done on a separate bladder

**Table 2**  $E_{\max}$  and  $pEC_{50}$  values measured in denuded bladders from mice 2–24 weeks after injection

Denuded bladder	2–3 weeks			8–9 weeks			20–24 weeks		
	$E_{\max}$ (mN)	$E_{\max}$ (% KCl)	$pEC_{50}$	$E_{\max}$ (mN)	$E_{\max}$ (% KCl)	$pEC_{50}$	$E_{\max}$ (mN)	$E_{\max}$ (% KCl)	$pEC_{50}$
Vehicle	WT	268.10±30.43	6.81±0.14	48.17±3.76	242.20±8.82	6.83±0.052	70.27±6.15	215.60±26.79	6.66±0.14
	M <sub>2</sub> KO	272.60±26.26	6.39±0.13	–	249.50±15.35	6.86±0.088	–	168.30±6.51	6.53±0.051
STZ	WT	63.12±6.05	6.73±0.064	76.28±4.94 <sup>b</sup>	242.60±8.93	6.73±0.052	113.30±7.12 <sup>b</sup>	181.70±12.84	6.72±0.071
	M <sub>2</sub> KO	–	6.69±0.087	–	208.60±17.21	6.49±0.11 <sup>a</sup>	–	175.30±3.08	6.40±0.027

Data are means ± SE (number of animals)

<sup>a</sup> Statistically significant difference from M<sub>2</sub> KO vehicle ( $P<0.01$ )<sup>b</sup> Significantly different from wild-type vehicle ( $P<0.001$ )

induced a greater loss of function in urinary bladder from the M<sub>2</sub> KO mouse (Fig. 3f) compared to that from wild type (Fig. 3e), which was manifest as a 3.1-fold increase in  $EC_{50}$  (log shift in  $EC_{50}$ = $0.49\pm0.055$ ;  $F=21.30$ ,  $P<0.001$ ). Since muscarinic contractile function is absent in urinary bladder from M<sub>2</sub>/M<sub>3</sub> double KO mice (Matsui et al. 2002), these data indicate that STZ treatment inhibits M<sub>3</sub> receptor-mediated contraction and that this response is restored by the M<sub>2</sub> receptor.

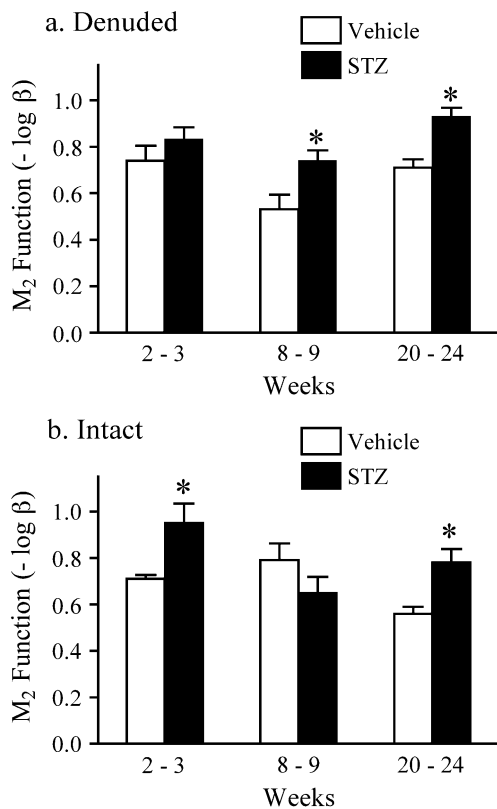
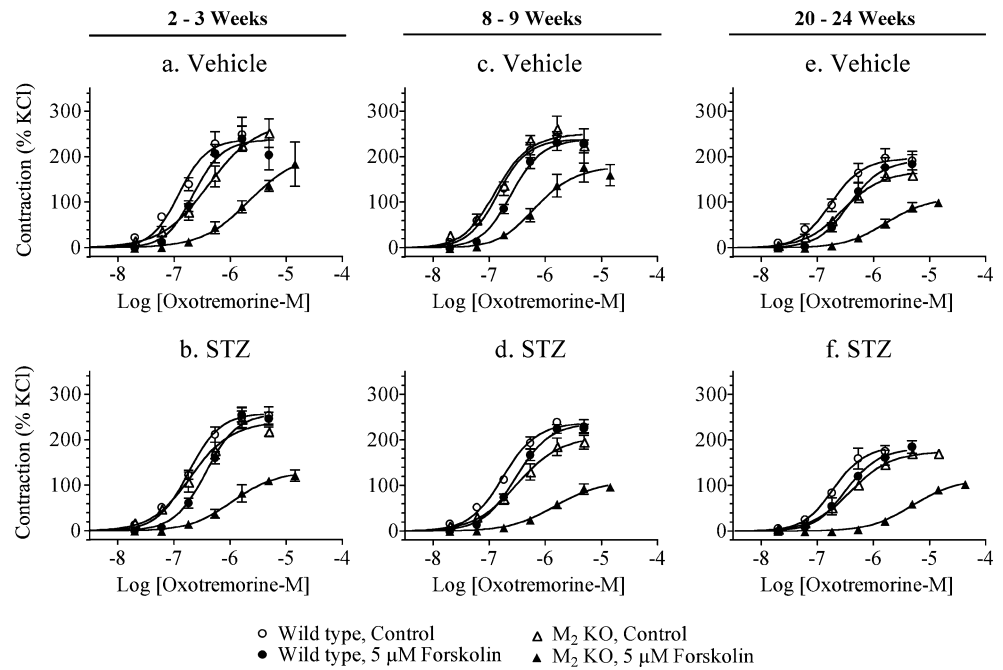
*M<sub>2</sub> muscarinic receptor-mediated inhibition of forskolin-induced relaxation* We investigated the effect of prior treatment with STZ on forskolin-induced inhibition of contraction to oxotremorine-M in denuded urinary bladder from wild-type and M<sub>2</sub> KO mice. Forskolin (5  $\mu$ M) had a small inhibitory effect on the concentration–response curve to oxotremorine-M in wild-type bladder from vehicle- and STZ-treated mice at all time points (Fig. 4). A much greater effect was observed in denuded bladder from M<sub>2</sub> KO mice, which is consistent with the role of the M<sub>2</sub> receptor in opposing adenylate cyclase-mediated inhibition of contraction.

We used the operational model (see “Methods” section) to estimate the relaxant effect of forskolin in urinary bladder from wild-type ( $-\log \alpha$ ) and M<sub>2</sub> KO ( $-\log \alpha/\beta$ ) mice. The difference between these two estimates ( $-\log \beta$ ) is a measure of the function of the M<sub>2</sub> receptor in opposing forskolin-induced relaxation. The  $-\log \beta$  values were estimated in urinary bladder from vehicle- and STZ-treated mice at the three time points, and the results are summarized in Fig. 5a. At the 8–9- and 20–24-week time points, STZ treatment caused a significant increase in the role of the M<sub>2</sub> receptor in opposing the relaxant effect of forskolin.

#### Effects of STZ on intact urinary bladder

*Muscarinic agonist-induced contractions* The presence of urothelium caused a modest change in the contractile activity of oxotremorine-M in the urinary bladder (Fig. 6). The overall average  $E_{\max}$  value of oxotremorine-M in intact, wild-type urinary bladder from all of the vehicle-treated groups ( $201.9\pm8.9\%$ , KCl-normalized contraction, Fig. 6b, e, h) was significantly smaller than the corresponding estimate for denuded urinary bladder ( $243.4\pm11.6\%$ , KCl-normalized contraction, Fig. 3b, e, h;  $F=8.82$ ;  $P=0.0033$ ). There was little or no difference in the contractile activity of oxotremorine-M in denuded bladder from vehicle-treated wild-type and M<sub>2</sub> KO mice at all time points (Fig. 3). In contrast, the overall average  $EC_{50}$  value of oxotremorine-M in intact, M<sub>2</sub> KO urinary bladder from all of the vehicle-treated groups ( $pEC_{50}=6.38\pm0.13$ ) was

**Fig. 4** Effect of streptozotocin (STZ) treatment on  $M_2$  receptor-mediated inhibition of relaxation in urinary bladder lacking urothelium. The effect of forskolin (5  $\mu$ M) on oxotremorine-M-mediated contractile responses was measured in wild-type and  $M_2$  KO bladders 2 to 3 weeks (a, b), 8 to 9 weeks (c–d), and 20 to 24 weeks (e–f) after STZ treatment. *Open circles* denote wild type, control; *closed circles* denote wild type in the presence of forskolin; *open triangles* denote  $M_2$  KO, control; *closed triangles* denote  $M_2$  KO in the presence of forskolin. Mean  $\pm$  SEM values from three to seven experiments are indicated, each done on a separate bladder



**Fig. 5** Effect of streptozotocin treatment on the role of the  $M_2$  receptor in opposing forskolin-induced relaxation in denuded (a) and intact (b) urinary bladder. The function of the  $M_2$  receptor is quantified as the parameter  $-\log \beta$ , which is defined as the difference between the effect of forskolin in urinary bladders from wild-type ( $-\log \alpha$ ) and  $M_2$  KO ( $-\log \alpha\beta$ ) mice as described under “Methods” section. The parameters were estimated from the data in Figs. 4 and 7. \*Significantly different from vehicle,  $P < 0.05$

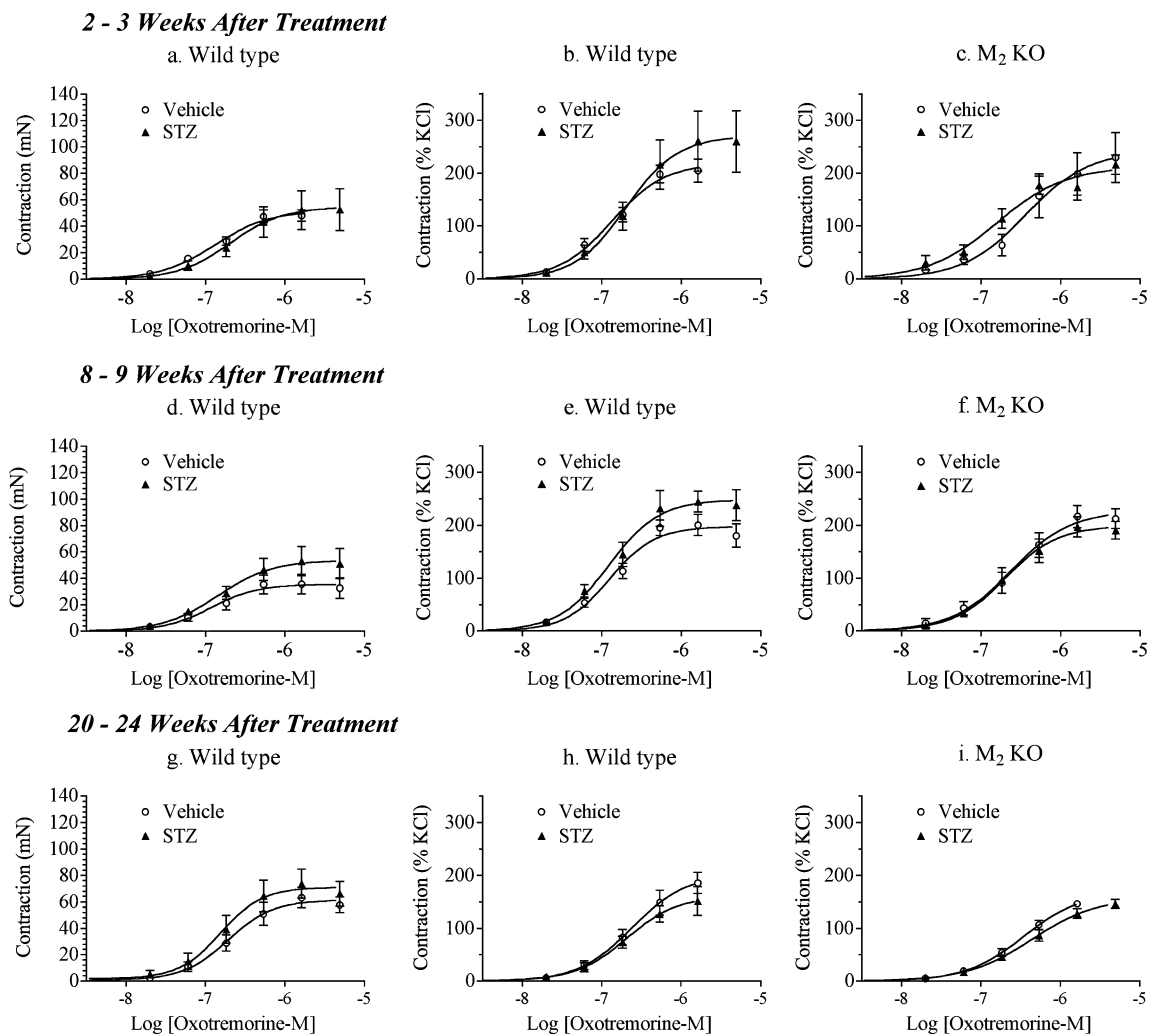
significantly greater (lower potency) than the corresponding estimate for wild-type bladder ( $pEC_{50} = 6.84 \pm 0.055$ ; Fig. 6;  $F = 16.029$ ,  $P < 0.001$ ).

Eight to 9 weeks after STZ treatment, the  $E_{max}$  value of oxotremorine-M, expressed in millinewton, was significantly greater by 51% in intact urinary bladder from STZ-treated wild-type mice compared to intact bladder from vehicle-treated wild-type animals (Fig. 6d and Table 3). When contraction was normalized relative to that elicited by KCl (50 mM; Fig. 6e), this difference persisted (Table 3). STZ treatment (8 to 9 weeks prior) had no effect on the contractile action of oxotremorine-M in intact urinary bladder from the  $M_2$  KO mouse (Fig. 6f). Nonetheless, when the activity of oxotremorine-M in the intact  $M_2$  KO bladder is compared to that of wild type, there is a greater reduction in  $E_{max}$  following STZ treatment ( $F = 5.11$ ,  $P = 0.026$ ).

These changes were not significant 2 to 3 weeks (Fig. 6a–c) or 20 to 24 weeks (Fig. 6g–i) after STZ treatment.

*$M_2$  muscarinic receptor-mediated inhibition of forskolin-induced relaxation* We also investigated  $M_2$  receptor-mediated inhibition of relaxation in urinary bladder with intact urothelium (Fig. 7). As observed in denuded bladder, the relaxant effect of forskolin on oxotremorine-M-induced contractions was greater in intact urinary bladder from the  $M_2$  KO mouse compared to that from wild type, illustrating the role of the  $M_2$  receptor in opposing the relaxant effect of forskolin. This action of the  $M_2$  receptor was significantly enhanced 2 to 3 weeks and 20 to 24 weeks after STZ treatment (Fig. 5b).





**Fig. 6** Effect of streptozotocin (STZ) treatment on oxotremorine-M-mediated contractions in urinary bladder with intact urothelium from wild-type and  $M_2$  KO mice. Concentration–response curves were measured 2 to 3 weeks (a–c), 8 to 9 weeks (d–f), and 20 to 24 weeks (g–i) after STZ treatment in urinary bladder from wild-type (a, b, d, e,

g, h) and  $M_2$  KO (c, f, i) mice. Contractions are expressed in units of force (millinewton; a, d, g) or relative to the contraction elicited by KCl (50 mM; b, c, e, f, h, i). Circles denote the vehicle-treated group; triangles denote the STZ-treated group. Mean  $\pm$  SEM values from three to seven experiments are shown, each done on a separate bladder

## Discussion and conclusions

In our study, we used a single injection of STZ ( $125 \text{ mg kg}^{-1}$ ) to induce diabetes. This dose was determined to yield maximal levels of hyperglycemia with minimal mortality (data not shown). High doses of STZ ( $\geq 200 \text{ mg kg}^{-1}$ ) promote nonspecific cytotoxicity and acute renal tubular damage (Tay et al. 2005). When moderate doses ( $100\text{--}125 \text{ mg kg}^{-1}$ ) are used on two consecutive days, nonspecific and acute renal cytotoxicity is markedly reduced, and mice develop renal injury biochemically and histologically similar (albeit less severe) to human nephropathy (Tesch and Nikolic-Paterson 2006; Tesch and Allen 2007), which can develop as a late diabetic complication after 10–20 years (Molitch et al. 2004). Our use of a single injection of STZ ( $125 \text{ mg kg}^{-1}$ ) probably caused even less STZ-induced

nonspecific and acute cytotoxicity. Also, it is unlikely that our mice suffered from diabetic ketoacidosis as urinalysis test strips showed zero, or very rarely, trace ( $5 \text{ mg dL}^{-1}$ ) amounts of acetoacetic acid in animals treated with STZ at all time points (data not shown). We found that  $M_3$  KO mice were very sensitive to STZ, and none survived long enough for experiments.  $M_3$  KO mice of a C57BL/6 genetic background exhibit impaired glucose tolerance (Gautam et al. 2006), which may contribute to their high sensitivity to STZ.

We observed that STZ inhibits  $M_2$  KO but not wild-type bladder contraction 8 to 9 weeks after treatment (Fig. 3e, f), suggesting that the  $M_2$  receptor rescues impaired  $M_3$  receptor-mediated contraction in denuded diabetic bladder. Mimata et al. (1995) observed a 30% increase in carbachol-stimulated phosphoinositide hydro-

**Table 3**  $E_{\max}$  and  $pEC_{50}$  values measured in intact bladders from mice 2–24 weeks after injection

Intact bladder	2–3 weeks			8–9 weeks			20–24 weeks			
	$E_{\max}$ (mN)	$E_{\max}$ (% KCl)	$pEC_{50}$	$E_{\max}$ (mN)	$E_{\max}$ (% KCl)	$pEC_{50}$	$E_{\max}$ (mN)	$E_{\max}$ (% KCl)	$pEC_{50}$	
Vehicle	WT	218.50±4.28	218.50±18.04	6.87±0.099	35.53±3.96	197.20±10.90	6.90±0.080	68.64±10.40	201.20±26.81	6.62±0.14
	M <sub>2</sub> KO	–	242.20±40.89	6.44±0.22	–	227.20±18.14	6.63±0.11	–	168.80±15.63	6.47±0.096
STZ	WT	54.48±8.48	269.70±31.94	6.69±0.16	53.63±6.14 <sup>a</sup>	248.60±16.69 <sup>a</sup>	6.91±0.10	71.21±6.17	161.40±21.21	6.69±0.14
	M <sub>2</sub> KO	–	212.30±19.14	6.80±0.14	–	199.40±14.93	6.70±0.10	–	157.90±11.02	6.36±0.091

Data are means ± SE (number of animals)

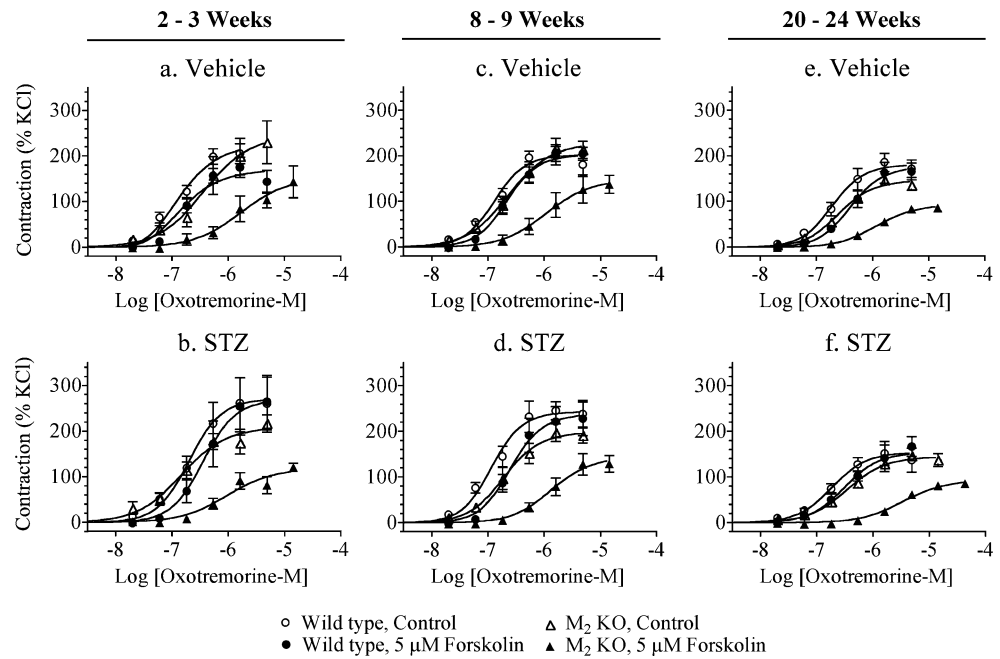
<sup>a</sup> Statistically significant difference from wild-type vehicle ( $P < 0.01$ )

lysis in [<sup>3</sup>H]inositol-labeled diabetic rat bladder when this response was expressed as the conversion of labeled [<sup>3</sup>H] phosphoinositides into [<sup>3</sup>H]inositol phosphates. Since the M<sub>3</sub> receptor is known to mediate phosphoinositide hydrolysis in mouse urinary bladder (Tran et al. 2006), the results of Mimata et al. (1995) suggest an increase in M<sub>3</sub> function in diabetic bladder. Perhaps this increase might contribute to the overall increase in muscarinic receptor-induced contractile force (millinewton) that we observed in wild-type mouse urinary bladder. Most of this increase in contraction can be attributed to the M<sub>2</sub> receptor, however, which does not signal through phosphoinositide hydrolysis in mouse urinary bladder (Tran et al. 2006). Also, the increase in muscarinic contractile force was absent when contractions were normalized relative to that elicited by KCl. Alternatively, there may be differences in the effects of STZ on urinary bladder from rats and mice.

Previous work from our lab has shown that the M<sub>2</sub> receptor enhances contractions elicited by the M<sub>3</sub> receptor in colon (Sawyer and Ehlert 1999) and urinary bladder (Ehlert et al. 2005) from normal guinea pig and mouse, respectively. This M<sub>2</sub> enhancement mechanism acts at a lower potency than that required for M<sub>3</sub>-mediated contractions, and oxotremorine-M elicits a maximal contraction through the M<sub>3</sub> receptor at a concentration that is too low to activate the M<sub>2</sub> enhancement function (Sawyer and Ehlert 1999; Ehlert et al. 2005). When a portion of the population of M<sub>3</sub> receptors is pharmacologically inactivated, oxotremorine-M is still able to elicit maximal contraction, albeit at a 20-fold lower potency. In these bladders, inhibition of M<sub>2</sub> receptor signaling by pertussis toxin (Sawyer and Ehlert 1999) or by gene deletion (Ehlert et al. 2005) induces a substantial loss of function in urinary bladder, revealing M<sub>2</sub> enhancement of contractions elicited by the M<sub>3</sub> receptor. Since the M<sub>3</sub> receptor can elicit a maximal contraction on its own, the normal physiological significance for the M<sub>2</sub> enhancement mechanism is unclear, but the observation in the current study that this mechanism rescues M<sub>3</sub> receptor-mediated contractions, which have been attenuated by STZ treatment, may suggest that the M<sub>2</sub> receptor has a crucial role in sustaining function in disease states such as diabetes.

A similar upregulation in M<sub>2</sub> receptor function occurs with denervation of the urinary bladder or obstruction of its outlet. In the rat model of these conditions, there is an upregulation of M<sub>2</sub> muscarinic receptor expression and mRNA, an increase in the potency of muscarinic agonists for eliciting contraction, and a change in the potency of antagonists for inhibiting contraction that is consistent with a greater role for the M<sub>2</sub> receptor (Braverman et al. 1998, 1999). This increase in M<sub>2</sub> function is caused by hypertrophy of the bladder because the increase in M<sub>2</sub> receptor

**Fig. 7** Effect of streptozotocin (STZ)-treatment on  $M_2$  receptor-mediated inhibition of relaxation in urinary bladder with intact urothelium. The effect of forskolin (5  $\mu$ M) on oxotremorine-M-mediated contractile responses was measured in wild-type and  $M_2$  KO bladders 2 to 3 weeks (a, b), 8 to 9 weeks (c, d), and 20 to 24 weeks (e, f) after STZ treatment. Open circles denote wild type, control; closed circles denote wild type in the presence of forskolin; open triangles denote  $M_2$  KO, control; closed triangles denote  $M_2$  KO in the presence of forskolin. Mean  $\pm$  SEM values from three to seven experiments are indicated, each done on a separate bladder



expression is prevented in rats that have had their ureters sutured to the colon to divert the flow of urine to the GI tract and prevent the denervation-induced distension and hypertrophy of the bladder (Braverman and Ruggieri 2003). An analogous increase in  $M_2$  function has been observed in human urinary bladder with reduced innervation as a result of spinal injury (Pontari et al. 2004; Ruggieri and Braverman 2006).

Another role of the  $M_2$  receptor involves inhibition of cAMP-mediated relaxation in smooth muscle from mouse (Matsui et al. 2003; Ehlert et al. 2005, 2007), guinea pig (Noronha-Blob et al. 1989; Thomas et al. 1993), and rat (Hegde et al. 1997). In the current study, we investigated how STZ treatment affects this  $M_2$  receptor-mediated disinhibition of contraction by comparing the degree of forskolin-induced inhibition of muscarinic contractions in  $M_2$  KO versus wild-type bladders. Forskolin had a greater effect on the concentration–response curve to oxotremorine-M (i.e., rightward shift) in bladder from  $M_2$  KO mice compared to that from wild-type animals, illustrating the ability of the  $M_2$  receptor to oppose the relaxant effect of forskolin. This  $M_2$  disinhibition of contraction was significantly greater at the intermediate (8–9 weeks) and long (20–24 weeks) time points after STZ treatment in denuded urinary bladders (Fig. 5a). These data are consistent with the observation that STZ treatment increases  $M_2$  receptor expression (Tong et al. 1999, 2002a, b, 2006; Stevens et al. 2006) in bladder smooth muscle.

Urothelium is known to release an inhibitory factor upon muscarinic stimulation (Hawthorn et al. 2000; Kosan et al.

2005). Our results are consistent with this phenomenon, because we found that oxotremorine-M exhibited a significantly greater  $E_{max}$  value for contraction in denuded wild type compared to intact wild type urinary bladder. We also found that while there was no difference in the contractile activity of oxotremorine-M in denuded bladder from wild-type and  $M_2$  KO mice, the agonist did exhibit 2.9-fold lower potency in intact  $M_2$  KO relative to intact wild-type urinary bladder. Previously, we reported a similar difference in intact bladder (Matsui et al. 2003). These results might suggest that urothelial  $M_2$  receptors mediate an inhibition of the release of the inhibitory factor, whose initial release is stimulated by a muscarinic subtype other than  $M_2$ . The human urothelium expresses mRNA for all muscarinic subtypes, with  $M_2$  mRNA greatly exceeding that of the other subtypes (Bschleipfer et al. 2007). Urothelial  $M_2$  (Tong et al. 2006) and  $M_3$  (Cheng et al. 2007) receptor expression is upregulated with STZ treatment. The urothelium is now known to be more involved in bladder function beyond its traditional role as a passive barrier to underlying smooth muscle (Birder and de Groat 2007).

The inhibitory effect of the urothelium on muscarinic contractility is abolished by STZ treatment in rats, suggesting a loss in release of the inhibitory factor (Kosan et al. 2005). We also confirmed that STZ treatment enhanced contractions in intact wild-type mouse urinary bladder (Fig. 6e), but had no effect on denuded wild-type bladder when contraction was normalized relative to KCl (Fig. 3e).

In denuded  $M_2$  KO bladder (Fig. 3f), we found that STZ treatment inhibited muscarinic contractile potency, whereas in intact  $M_2$  KO bladder (Fig. 6f), STZ treatment had no effect.

This seemingly conflicting result can be explained by the opposing effects of STZ treatment on muscarinic contractility in intact M<sub>2</sub> KO urinary bladder—an enhancement from the loss of urothelial inhibition and an inhibition from a loss of M<sub>3</sub> function. The loss of M<sub>3</sub> function was manifest in intact urinary bladder as a significant reduction in the  $E_{\max}$  of oxotremorine-M in M<sub>2</sub> KO relative to wild type 8 to 9 weeks after STZ treatment. This loss of function was significantly greater than that observed in intact bladder from vehicle-treated animals. Thus, our hypothesis that STZ treatment inhibits M<sub>3</sub> receptor contractile function in smooth muscle and that the smooth muscle M<sub>2</sub> receptor rescues contractile function is supported by our findings on both denuded and intact bladder 8 to 9 weeks after STZ treatment. This effect seems to be short-lived, however, because it is lost 20 to 24 weeks after STZ treatment.

Our experiments on the role of the M<sub>2</sub> receptor in opposing forskolin-induced relaxation in intact bladders showed an increase in this function following STZ treatment. We observed a significant increase in M<sub>2</sub> function at the early (2–3 weeks) and late (20–24 weeks) time points after STZ treatment, but not at the intermediate time point (8–9 weeks). In denuded bladder, significant differences were measured at the intermediate and late time points. We have no explanation for this difference, but experimental variation and changes in the effect of forskolin on the release of mediators from the urothelium after STZ treatment (Birder et al. 2002) may confound our results.

This temporal progression of differential changes in M<sub>2</sub> and M<sub>3</sub> contractile function in urinary bladder is likely caused by gradual STZ-induced alterations. Hyperglycemia-induced neuronal dysfunction and subsequent damage to the neuromuscular junction may alter contractile function in smooth muscle. Additionally, the rich innervation of the urothelium (Wakabayashi et al. 1995) and its high level of glucose metabolism may make urothelial tissue gradually susceptible to diabetic conditions. Our results provide a clearer understanding of the differential roles of M<sub>2</sub> and M<sub>3</sub> muscarinic receptors in STZ-induced diabetes and how the M<sub>2</sub> receptor maintains contractile function in this disease model.

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