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## Muscarinic regulation of neonatal rat bladder spontaneous contractions

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### Abstract

In vitro preparations of whole urinary bladders of neonatal rats exhibit prominent myogenic spontaneous contractions, the amplitude and frequency of which can be increased by muscarinic agonists. The muscarinic receptor subtype responsible for this facilitation was examined in the present experiments. Basal spontaneous contractions in bladders from 1- to 2-wk-old Sprague-Dawley rats were not affected by M<sub>2</sub> or M<sub>3</sub> receptor antagonists. However, administration of 0.5 μM physostigmine, an anticholinesterase agent that increases the levels of endogenous acetylcholine, or 50–100 nM carbachol, a cholinergic agonist at low concentrations, which did not cause tonic contractions, significantly augmented the frequency and amplitude of spontaneous contractions. Blockade of M<sub>2</sub> receptors with 0.1 μM AF-DX 116 or 1 μM methoctramine or blockade of M<sub>3</sub> receptors with 50 nM 4-diphenylacetoxy-*N*-methylpiperidine methiodide or 0.1 μM 4-diphenylacetoxy-*N*-(2-chloroethyl)piperidine hydrochloride (4-DAMP mustard) reversed the physostigmine and carbachol responses. M<sub>2</sub> and M<sub>3</sub> receptor blockade did not alter the facilitation of spontaneous contractions induced by 10 nM BAY K 8644, an L-type Ca<sup>2+</sup> channel opener, or 0.1 μM iberiotoxin, a large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel blocker. NS-1619 (30 μM), a large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel opener, decreased carbachol-augmented spontaneous contractions. These results suggest that spontaneous contractions in the neonatal rat bladder are enhanced by activation of M<sub>2</sub> and M<sub>3</sub> receptors by endogenous acetylcholine released in the presence of an anticholinesterase agent or a cholinergic receptor agonist.

### Keywords

M<sub>2</sub> receptors; M<sub>3</sub> receptors; overactive bladder; detrusor overactivity

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IN VITRO WHOLE ORGAN or muscle strip preparations of the neonatal rat bladder during postnatal *weeks 1* and *2* exhibit high-amplitude low-frequency spontaneous contractions, which, later in postnatal maturation, are converted to low-amplitude high-frequency contractions (32, 34). Although these spontaneous contractions are most likely myogenic in origin because they occur in the absence of neural stimulation (6, 19, 32, 33), they can be modulated by activation of various types of receptors (muscarinic, purinergic, and adrenergic) (9, 10). We previously showed that these contractions are markedly facilitated in the presence of carbachol, a muscarinic agonist (34). Interest in the cholinergic regulation of

spontaneous bladder contractions (11) was stimulated by the recent hypothesis that symptoms of overactive bladder may be due to enhancement of spontaneous contractions by leakage of small amounts of acetylcholine from intramural nerves during bladder filling (1).

The present experiments were undertaken to examine the types of receptors involved in the cholinergic modulation of spontaneous bladder activity. On the basis of the ratio of M<sub>2</sub> to M<sub>3</sub> receptors in the bladder (9:1 in rat and 3:1 in human) (25, 35), one would expect that M<sub>2</sub> receptors would be involved in cholinergically evoked bladder contractions. However, it is clear from studies using M<sub>2</sub>, M<sub>3</sub>, and M<sub>2</sub>/M<sub>3</sub> receptor-knockout mice (22–24) and pharmacological data (2, 29) that the M<sub>3</sub> receptor is primarily responsible for large-amplitude bladder contractions elicited by stimulation of cholinergic nerves (4, 30) and that the M<sub>2</sub> receptor works indirectly by potentiating the M<sub>3</sub> receptor-mediated contractions or by counteracting  $\beta$ -adrenergic receptor-mediated relaxation (8, 13, 24, 38).

In this study, we provide evidence that activation of M<sub>2</sub> as well as M<sub>3</sub> receptors by endogenous acetylcholine or exogenous carbachol can enhance spontaneous contractions in whole bladder preparations from normal 1- to 2-wk-old rats. The ability of muscarinic receptor mechanisms to modulate spontaneous bladder contractions raises the possibility that these mechanisms may play a role in the generation of symptoms in patients with detrusor overactivity.

## MATERIALS AND METHODS

### In vitro whole bladder preparation

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Adult (8 female and 1 male) and 1- to 2-wk-old Sprague-Dawley rats were anesthetized with 4% (vol/vol) isoflurane and killed by cervical dislocation. We used mostly 1- to 2-wk-old rats ( $n = 96$ , 48 male and 48 female), because we previously showed that the amplitude of spontaneous contractions at this age is maximal (34, 36); therefore, studies conducted at this age allowed for easier detection of changes elicited by muscarinic receptor activation. We modified our previous technique for whole bladder studies (32) by cannulating the urethra, rather than the dome, and not tying off the ureters, because leakage was not seen from the ureters during bladder filling. The bladder was exposed by a midline abdominal incision and removed from the abdomen by an incision at the bladder neck. A 26-gauge needle was inserted at the bladder neck and tied with 5-0 silk sutures. The needle was connected to an infusion pump and pressure transducer via polyethylene tubing and a three-way stopcock. The needle and tubing were filled with Krebs solution (in mM: 113 NaCl, 19.8 NaHCO<sub>3</sub>, 11.1 dextrose, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 4.7 KCl, 2.5 MgCl<sub>2</sub>, and 1.7 CaCl<sub>2</sub>). The bladder was placed between two platinum stimulating electrodes inside an organ bath (Radnoti Glass, Monrovia, CA) filled with 37°C Krebs solution and bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub>. Bladder pressure was recorded by WinDaq Acquisition software (version 2.13 for Windows, Akron, OH). After a 30-min equilibration period, the bladder was filled slowly with Krebs solution in 50- $\mu$ l increments during intermittent electrical field stimulation (50 V, 1.6-ms pulses at 32 Hz for 15–30 s) to determine the bladder volume necessary to produce maximal bladder contractions. Field stimulation was delivered by a Grass S88 stimulator (Quincy, MA). The distended bladder was washed three times with 15 ml of fresh Krebs solution and equilibrated for another 15 min, and then drug treatment was started. We used the last 5-min interval within the 15-min observation period to calculate the mean amplitude, frequency, and area under the curve (AUC) of the spontaneous contractions. The criteria proposed by Imai et al. (17) were used to define a single spontaneous contraction event: a response with an amplitude  $\geq 30\%$  of the peak spontaneous contraction during the 15-min observation period. Also, when a contraction was superimposed on the previous event before reaching baseline, the two contractions were

considered a single contraction event. The peak amplitude of the spontaneous contractions was normalized as a percentage of the maximal  $K^+$ -evoked contraction amplitude. The  $K^+$ -evoked contraction was induced at the end of the experiments by a bath solution containing 80 mM KCl. Frequency was determined by counting the number of contractions over a 5-min interval.

## Chemicals

All chemicals used for the bath solution in the *in vitro* whole bladder preparation studies were obtained from Sigma (St. Louis, MO). Recombinant iberiotoxin (IBTX) was obtained from Alomone Lab (Jerusalem, Israel). Carbachol, *S*-(-)-BAY K 8644, NS-1619, methoctramine tetrahydrochloride, 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-Damp), 4-diphenylacetoxy-*N*-(2-chloroethyl)piperidine hydrochloride (4-DAMP mustard), and physostigmine (eserine) were obtained from Sigma. AF-DX 116 was obtained from Tocris Cookson (Ellisville, MO). The  $M_3$  antagonist 4-DAMP was used as a competitive inhibitor and 4-DAMP mustard as a noncompetitive inhibitor in combination with 1  $\mu$ M AF-DX 116 to protect the  $M_2$  receptors during the irreversible alkylation of the  $M_3$  receptor caused by 0.1  $\mu$ M 4-DAMP mustard for 1 h (8). Then 4-DAMP mustard and AF-DX 116 were removed by extensive washes (20 ml) over a 15-min period.

## Statistical analysis

Values are means (SD);  $n$  is the number of bladders used for each set of experiments. Repeated-measures oneway ANOVA followed by Tukey's multiple posttest was used for multiple group comparison. Paired *t*-test was used for comparison between two groups. Statistics were calculated using Prism 4 (Windows version 4.02, GraphPad Software, San Diego, CA). Statistical significance was considered when a 95% confidence level ( $P \leq 0.05$ ) was reached.

## RESULTS

### Muscarinic regulation of basal spontaneous contractions

Spontaneous contractions (Fig. 1) appeared in 1- to 2-wk-old bladders after they were filled to 150–200  $\mu$ l, which was the volume required for maximum field stimulation-evoked contractions. The mean amplitude of the spontaneous contractions was 6.9 cmH<sub>2</sub>O (SD 4.2), which was 11.5% of  $K^+$  evoked contraction ( $n = 96$ ). The mean frequency was 3.2 contractions/min (SD 1.5) ( $n = 96$ ).

The nonselective muscarinic agonist carbachol enhanced spontaneous bladder contractions in a concentration-dependent manner (Fig. 1). Carbachol (0.05 and 0.1  $\mu$ M) significantly increased the amplitude, frequency, and AUC of the spontaneous contractions. A low concentration of carbachol (0.05  $\mu$ M) increased spontaneous contractions without causing a tonic contraction (i.e., an increase in baseline tone), whereas 0.1  $\mu$ M carbachol caused a 1- to 5-min transient rise in basal tone (Fig. 1A). Higher concentrations of carbachol (0.5–50  $\mu$ M) elicited large-amplitude tonic contractions (Fig. 1A) followed by high-amplitude, low-frequency spontaneous contractions (40–70 cmH<sub>2</sub>O) superimposed on an elevated basal tone ( $n = 4$ ).

The large conductance  $Ca^{2+}$ -activated  $K^+$  ( $BK_{Ca}$ ) channel blocker IBTX produced changes in spontaneous activity similar to those induced by carbachol, including a concentration-dependent rise in amplitude, frequency, and AUC of basal spontaneous contractions (Fig. 2). Conversely, the  $BK_{Ca}$  channel opener NS-1619 elicited a concentration-dependent decrease in amplitude, frequency, and AUC of carbachol-augmented spontaneous contractions (Fig.

3). The L-type  $\text{Ca}^{2+}$  channel opener *S*-(-)-BAY K 8644 also increased the amplitude and AUC of basal spontaneous contractions (Fig. 4A).

### Effects of muscarinic receptor antagonists

Muscarinic 2 ( $M_2$ ) receptor blockade with 0.1  $\mu\text{M}$  AF-DX 116 or  $M_3$  receptor blockade with 0.05  $\mu\text{M}$  4-DAMP mustard did not affect the amplitude or AUC of basal spontaneous contractions (Fig. 5). 4-DAMP mustard, but not AF-DX 116, decreased the frequency of basal spontaneous contractions (Fig. 5). The antagonists suppressed the effects of carbachol on spontaneous activity. The  $M_2$  selective antagonists methoctramine (Fig. 6) and AF-DX 116 (Fig. 7) blocked the carbachol facilitatory effects on amplitude and AUC. AF-DX 116, but not methoctramine, also suppressed the effect of carbachol on the frequency of contractions (Figs. 7C and 6B). After AF-DX 116 blockade of the carbachol response, BAY K 8644 (Figs. 4B and 7A) or IBTX (Figs. 4C and 7B) was still able to enhance spontaneous activity. 4-DAMP (0.05  $\mu\text{M}$ ) decreased the carbachol-augmented spontaneous contraction (Figs. 8A and 9A), but this effect could be overcome by a 2-log-unit increase in carbachol concentration (Figs. 8A and 9B). Similarly, the irreversible  $M_3$  antagonist 4-DAMP mustard blocked the effect of carbachol, and this blockade could also be reversed by higher concentrations of carbachol ( $\geq 10 \mu\text{M}$ ; Figs. 8B and 9C). AF-DX 116 reversed the effects of high concentrations of carbachol in preparations treated with 4-DAMP mustard (Fig. 10A). When  $M_3$  and  $M_2$  receptors were blocked (Fig. 10B), the  $\text{BK}_{\text{Ca}}$  channel blocker IBTX was still able to increase the amplitude, frequency, and AUC of spontaneous contractions.

### Physostigmine regulation of spontaneous contractions

The acetylcholinesterase inhibitor physostigmine (0.5  $\mu\text{M}$ ) increased the amplitude and AUC of the spontaneous contractions, producing responses similar to those induced by carbachol (Fig. 11A). This augmentation was reversed by  $M_2$  receptor blockade with AF-DX 116 (Fig. 11B). In addition,  $M_3$  receptor blockade by pretreatment with 4-DAMP mustard completely blocked the facilitatory effect on spontaneous contractions of a subsequent administration of physostigmine (Fig. 12).

### Adult rat bladder spontaneous contractions

The effects of combined  $M_2$  and  $M_3$  receptor blockade on muscarinic stimulation of spontaneous bladder contractions were also studied in bladders from adult rats. Consistent with previous results (34), the basal amplitude of spontaneous contractions was lower and the frequency was higher in adult animals than in neonates.  $M_2$  receptor antagonism with 1  $\mu\text{M}$  AF-DX 116 after 0.05  $\mu\text{M}$  carbachol decreased amplitude, frequency, and AUC, similar to neonatal bladders (data not shown). After  $M_3$  receptor blockade by pretreatment with 4-DAMP mustard, a high concentration of carbachol (2  $\mu\text{M}$ ) increased spontaneous contractions (Fig. 13). Addition of AF-DX 116 reduced the contractions to basal levels. As in neonatal bladders, IBTX increased spontaneous contractions after blockade of  $M_3$  and  $M_2$  receptors (Fig. 13).

## DISCUSSION

The present experiments revealed that spontaneous contractile activity in the *in vitro* whole bladder preparation of the neonatal rat is not dependent on activation of  $M_3$  and  $M_2$  receptors by endogenous acetylcholine. However, treatment of the preparations with physostigmine, an anticholinesterase agent that inhibits the metabolism of acetylcholine and increases the extracellular levels of the transmitter, markedly facilitated the spontaneous activity. Administration of carbachol, a cholinergic agonist, mimicked the effect of physostigmine. The effects of both agents were suppressed by  $M_3$  and  $M_2$  receptor

antagonists, indicating that spontaneous contractile activity in the neonatal bladder can be regulated by cholinergic muscarinic mechanisms.

Previous studies in neonatal rat bladder preparations demonstrated large-amplitude rhythmic contractions that persisted after block of nerve action potentials with tetrodotoxin and blockade of muscarinic receptors with atropine (21, 33). These data indicated that the spontaneous contractions were myogenic in origin. The present results confirm that spontaneous contractions in the untreated neonatal bladder are not dependent on activation of  $M_3$  and  $M_2$  receptors by cholinergic nerves. However, treatment with physostigmine produced a marked enhancement of spontaneous activity that was blocked by muscarinic receptor antagonists. These data indicate that, after acetylcholinesterase inhibition, endogenous acetylcholine can rise to levels sufficient to activate muscarinic receptors in the tissue. The source of the acetylcholine is uncertain, because acetylcholine is known to be released spontaneously from nerves and also from urothelium by bladder distension. However, spontaneous activity in muscle strips from neonatal rat bladders is reduced by atropine (34). This raises the possibility that preparation of muscle strips damages nerves or urothelium, triggering an increase in spontaneous release of acetylcholine.

In contrast to the predominant role of  $M_3$  receptors in large-amplitude, neurally evoked bladder contractions, the facilitation of spontaneous contractions in the neonatal bladder seems to depend on the activation of  $M_2$  and  $M_3$  receptors. The facilitatory effects of physostigmine could be inhibited by pretreatment with 4-DAMP mustard, an irreversible  $M_3$  receptor antagonist, and abolished by administration of AF-DX 116, an  $M_2$  receptor antagonist. These data indicate that endogenous acetylcholine activation of both types of receptors is required for enhancement of spontaneous activity. The facilitatory effect of carbachol also seems to be mediated by the activation of either type of receptor. However, activation of only one type of receptor by carbachol is sufficient to enhance the contractions. For example, although 4-DAMP or 4-DAMP mustard blocked the effect of low concentrations of carbachol (0.1  $\mu\text{M}$ ), increasing the concentration of carbachol to 1–10  $\mu\text{M}$  overcame the block, suggesting that activation of  $M_2$  receptors alone can facilitate the spontaneous activity.  $M_2$  receptor antagonists (AF-DX 116 or methoctramine) alone also reduced the effect of carbachol.

It is possible that the facilitatory effect of muscarinic receptor activation on spontaneous activity occurs by a mechanism different from that mediating the tonic contractions induced by muscarinic receptor activation (9, 10). As shown in Fig. 1, low concentrations of carbachol (0.05  $\mu\text{M}$ ) are sufficient to facilitate spontaneous activity without inducing a tonic contraction. Higher concentrations are needed to induce large-amplitude tonic contractions similar to those induced by nerve stimulation. Similarly, physostigmine facilitated spontaneous contractions without inducing large-amplitude tonic contractions (Fig. 11). The large-amplitude contractions may be mediated by  $M_3$  receptors, because they did not return to control levels after pretreatment with 4-DAMP mustard, even when the tissue was exposed to very high concentrations of carbachol (50  $\mu\text{M}$ ; Fig. 8B). These findings in neonatal rat bladders confirm previous results in adult guinea pig bladders (9) in which low doses of carbachol (0.1–0.3  $\mu\text{M}$ ) induced spontaneous contractions without eliciting tonic contractions. Recent data suggest that  $M_2$  receptors are responsible for inhibiting smooth muscle relaxation elicited by increased cytosolic cAMP (13, 38) and potentiating contractions initiated by  $M_3$  receptors in gastrointestinal, respiratory, and urinary tract smooth muscles (8). As reviewed by Ehlert (7), the contribution of the  $M_2$  receptors to tonic contractions depends on the presence of other direct contractile mechanisms, such as the  $M_3$  receptor mechanism.



Although the role of the  $M_2$  receptor is recognized in neuropathic bladders, defining its function in normal bladders is more difficult. The spinal cord injury model, in which  $M_2$  receptors play a much larger role than  $M_3$  receptors, is one in which there is both denervation and hypertrophy (2, 29). It is also possible that changes in tissue perfusion in pathological tissues could affect the local metabolism of acetylcholine and possibly account for some of the shift toward  $M_2$ , rather than  $M_3$ , receptor regulation of bladder contraction. The role of muscarinic regulation of bladder contractions appears to decrease with age (28), yet the level of stretch-induced, nonneuronal basal acetylcholine release is higher in the bladders of elderly individuals (39).

$M_3$  receptor antagonism appears to be more effective than  $M_2$  receptor antagonism in inhibiting spontaneous contractions. Methoctramine, when used at an  $M_2$  receptor-effective concentration of 0.1  $\mu\text{M}$ , decreased the amplitude of spontaneous contractions by only 50%, whereas at an  $M_3$  receptor-effective concentration of 1  $\mu\text{M}$  (13), it reduced the amplitude back to baseline, completely reversing the augmentation caused by carbachol (Fig. 6). After treatment with  $M_3$  receptor antagonists (4-DAMP and 4-DAMP mustard), the inhibition of spontaneous contractions could be reversed by an increase in the carbachol concentration by 2 log units. One of the problems in interpreting experiments in which muscarinic receptors are blocked is the possibility of incomplete blockade. It has been estimated that ~9% of  $M_3$  receptors are still available after 1 h of 4-DAMP mustard treatment (8). Although we would interpret the finding that higher concentrations of carbachol can reverse blockade of  $M_3$  receptors as evidence that carbachol is acting via the  $M_2$  receptor, it is possible that the effect of carbachol after  $M_3$  receptor blockade is due to persistent activation of  $M_3$  receptors. The finding that AF-DX 116 administered after 4-DAMP mustard and carbachol results in further blockade of spontaneous contractions in neonatal and adult bladders is additional evidence that carbachol after 4-DAMP mustard acts via the  $M_2$  receptors. Similarly, the partial blockade of carbachol effects by low  $M_2$  receptor-selective concentrations of methoctramine is evidence that  $M_2$  receptors are involved in the control of spontaneous contractions.

Basal spontaneous contractions, which were unaffected by  $M_2$  or  $M_3$  receptor blockade, were sensitive to  $\text{BK}_{\text{Ca}}$  channel and L-type  $\text{Ca}^{2+}$  channel regulation. This suggests that although the spontaneous contractions are “myogenic” in origin, the degree to which these contractions are expressed is dependent on properties of the bladder smooth muscle, which may be modulated by muscarinic signaling. After large-amplitude neurally evoked bladder contractions,  $\text{BK}_{\text{Ca}}$  channels act as a brake on muscle activity. Elevated intracellular  $\text{Ca}^{2+}$  levels after smooth muscle contraction open  $\text{BK}_{\text{Ca}}$  channels, repolarize the membrane potential, and inactivate L-type  $\text{Ca}^{2+}$  channels. We also found that  $\text{BK}_{\text{Ca}}$  channels are important in the postnatal downregulation of spontaneous bladder contractions during maturation (36). Spontaneous contractions in normal adult and diabetic bladders can be augmented by blocking  $\text{BK}_{\text{Ca}}$  channels with IBTX (3, 12, 15–17, 26). In this study, blocking  $\text{BK}_{\text{Ca}}$  channels with IBTX also enhanced spontaneous activity, whereas opening these channels with NS-1619 reversed the augmentation of spontaneous contractions by carbachol. Nakamura et al. (27) showed that activation of  $M_2$  receptors inhibits  $\text{BK}_{\text{Ca}}$  channels in rat bladder, but the signaling pathway by which  $M_2$  receptors achieve this inhibition is unknown. Our finding that closing large-conductance  $\text{K}^+$  channels after  $M_2$  and  $M_3$  receptor blockade (Figs. 7 and 10) results in an increase in spontaneous contractions suggests that cell depolarization, which regulates spontaneous contractions, occurs downstream of the muscarinic receptors.

Control over intracellular  $\text{Ca}^{2+}$  levels via the L-type  $\text{Ca}^{2+}$  channels is also important in the regulation of spontaneous contractions. Spontaneous contractions are always preceded by spontaneous action potentials (12, 18), the upstroke of which is due to influx of  $\text{Ca}^{2+}$

through the L-type channels (14). Blockade of L-type  $\text{Ca}^{2+}$  channels with nifedipine abolished spontaneous action potentials and spontaneous contractions in normal bladders (12, 14, 15, 18, 20), whereas activation of L-type  $\text{Ca}^{2+}$  channels with the dihydropyridine analog *S*-(-)-BAY K 8644 enhanced the amplitude and the frequency of spontaneous contractions in normal and diabetic adult rat bladder smooth muscle strips (5, 26). We confirm that enhanced  $\text{Ca}^{2+}$  influx by activation of L-type  $\text{Ca}^{2+}$  channels alone was sufficient to increase the spontaneous contractions in 1- to 2-wk-old bladders. In addition, the effect of BAY K 8644 after AF-DX 116 on the AUC of spontaneous contractions is similar to that of IBTX (Fig. 11, *B* and *C*). However, in contrast to IBTX, BAY K 8644 did not significantly increase the frequency of spontaneous contractions. Thus the frequency of spontaneous contractions appears to be regulated in a more selective manner than amplitude. Although IBTX, NS-1619, and carbachol affected frequency, BAY K 8644, methoctramine, and physostigmine did not.

In conclusion, endogenous acetylcholine or administration of a cholinergic agonist facilitated spontaneous contractions in the neonatal rat bladder by activation of  $\text{M}_2$  and  $\text{M}_3$  receptors. Muscarinic mechanisms that modulate spontaneous bladder contractions are of considerable interest, because antimuscarinic drugs are used clinically to treat the sensory and motor symptoms of overactive bladder. The potential clinical relevance of the present findings would be limited if muscarinic modulation of spontaneous activity were only present in neonatal bladders. Thus the finding that muscarinic receptor activation also unmasks spontaneous contractions in adult rat bladders is important, because it indicates that even though spontaneous bladder activity is downregulated during postnatal development, the muscarinic facilitatory mechanisms that regulate spontaneous activity persist in mature bladders.

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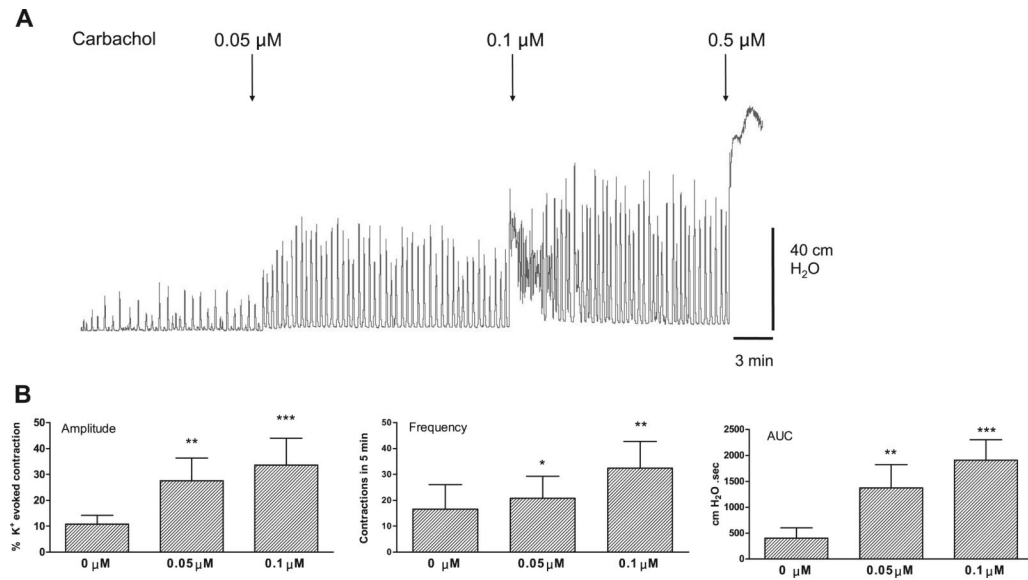
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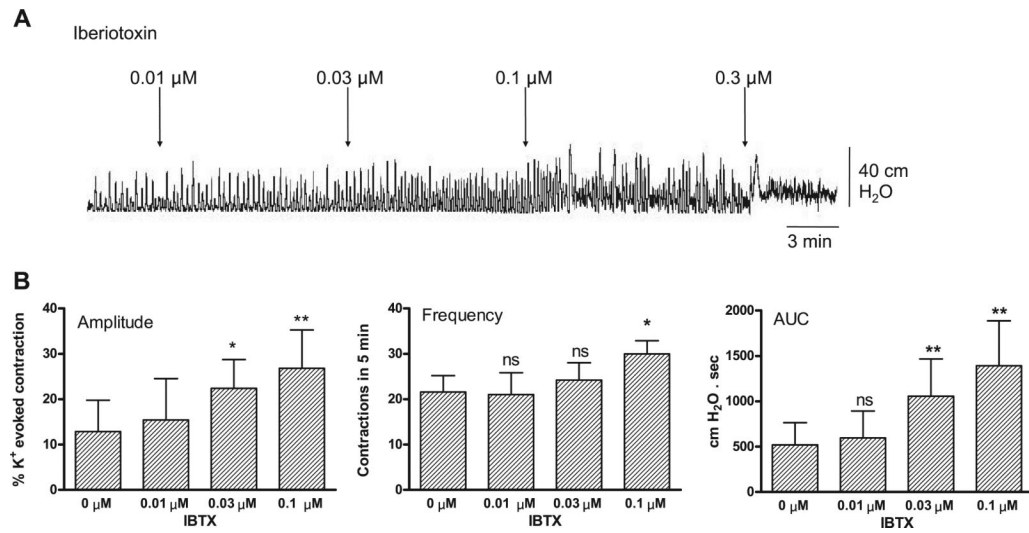
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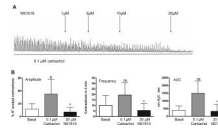
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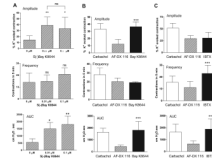
**Fig. 1.** Concentration-response study of the effect of carbachol on basal spontaneous contractions. Low concentrations of carbachol enhance spontaneous contractions without producing a tonic contraction; higher concentrations produce tonic contractions. AUC, area under the curve. Values are means (SD);  $n = 5$ . \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  vs. basal (0  $\mu\text{M}$ ).



**Fig. 2.** Concentration-response study of the effect of large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (BK<sub>Ca</sub>) channel blocker iberiotoxin (IBTX) on basal spontaneous contractions. ns, Not significant. Values are means (SD); *n* = 5. \**P* < 0.05; \*\**P* < 0.01 vs. basal.



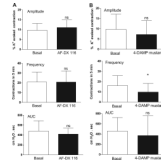
**Fig. 3.**  
 BK<sub>Ca</sub> channel opener NS-1619 reverses carbachol-enhanced spontaneous contractions.  
 Values are means (SD);  $n = 4$ . \* $P < 0.05$  vs. carbachol. # $P < 0.05$  vs. basal.



**Fig. 4.**

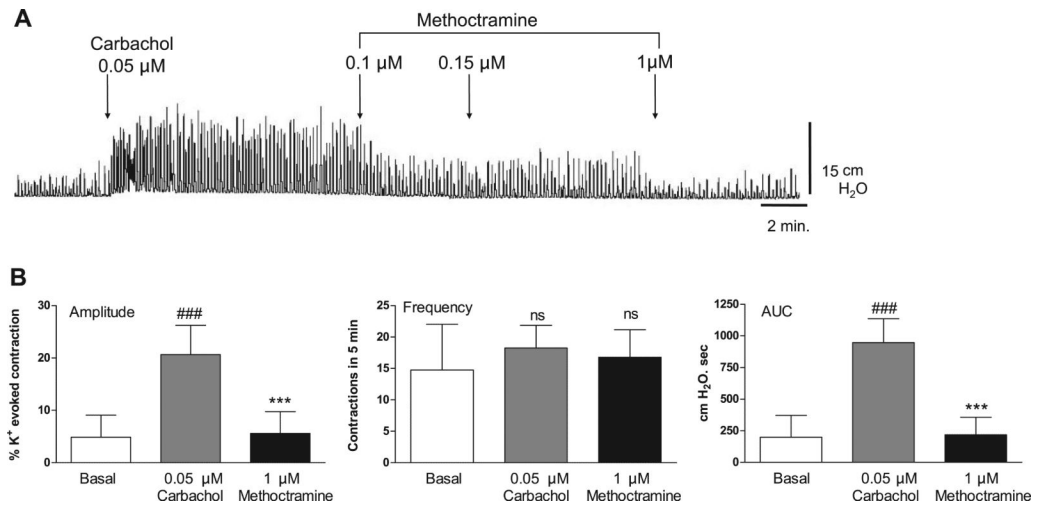
*A:* concentration-response study of effect of L-type  $\text{Ca}^{2+}$  channel opener *S*-(-)-BAY K 8644 on basal spontaneous contractions. Values are means (SD)  $n = 5$ . *B:* BAY K 8644 enhances spontaneous contractions after AF-DX 116 has blocked carbachol facilitation of spontaneous contractions. Values are means (SD);  $n = 4$ . *C:* IBTX enhances spontaneous contractions after AF-DX 116 has blocked carbachol facilitation of spontaneous contractions. Values are means (SD);  $n = 6$ . \* $P < 0.05$ ; \*\* $P < 0.01$  vs. basal. \*\*\* $P < 0.001$  vs. AF-DX 116.



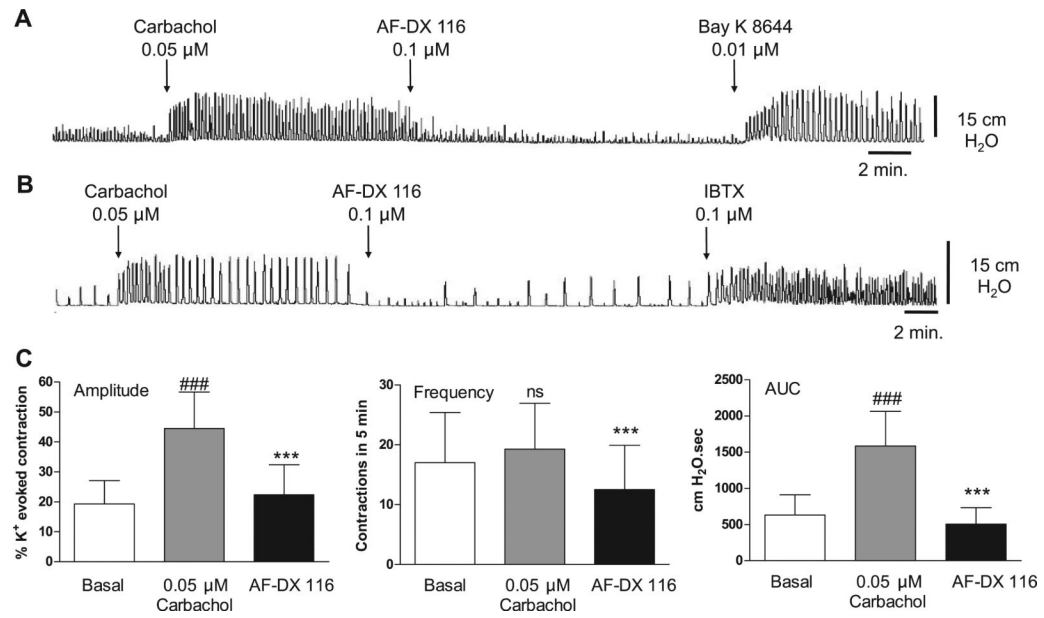


**Fig. 5.**

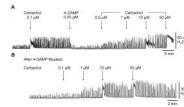
$M_2$  and  $M_3$  antagonism does not affect amplitude or AUC of basal spontaneous contractions. *A*: effect of 0.1  $\mu\text{M}$  AF-DX 116 on basal spontaneous contractions. Values are means (SD);  $n = 4$ . *B*: effect of 0.05  $\mu\text{M}$  4-diphenylacetoxy-*N*-(2-chloroethyl)piperidine hydrochloride (4-DAMP mustard) on basal spontaneous contractions. Values are means (SD);  $n = 12$ . \* $P < 0.05$  vs. basal. M, muscarinic.



**Fig. 6.** Suppression of carbachol-enhanced spontaneous contractions by methoctramine. Values are means (SD);  $n = 4$ . \*\*\* $P < 0.001$  vs. carbachol. ### $P < 0.001$  vs. basal.

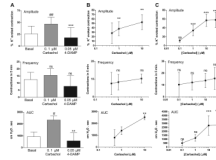
**Fig. 7.**

Inhibition of carbachol-enhanced spontaneous contractions by the  $M_2$  antagonist AF-DX 116. *S*-(–)-BAY K 8644 ( $n = 4$ ) or IBTX ( $n = 6$ ) still increases spontaneous contractions in the presence of AF-DX 116. Values are means (SD). \*\*\* $P < 0.001$  vs. carbachol. ### $P < 0.001$  vs. basal.



**Fig. 8.**

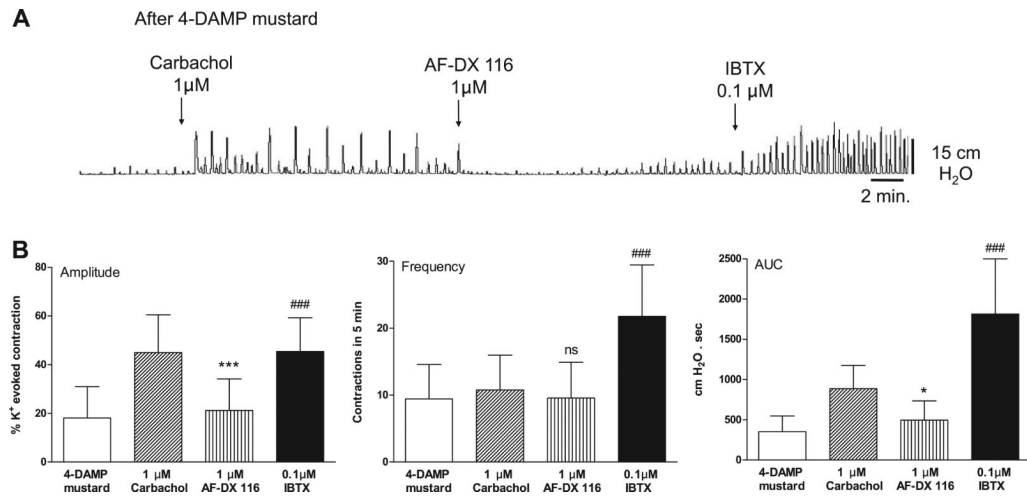
*A:* blocking effect of 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) on carbachol-enhanced spontaneous contractions can be overcome by increasing carbachol concentration. *B:* concentration response of carbachol after 4-DAMP mustard.



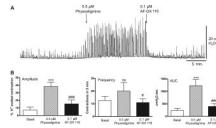
**Fig. 9.**

**A:** effect of 4-DAMP on carbachol-enhanced spontaneous contractions. Values are means (SD);  $n = 4$ .  $**P < 0.01$ ;  $***P < 0.001$  vs. carbachol.  $\#P < 0.05$ ;  $\#\#P < 0.01$  vs. basal. **B:** concentration response of carbachol in the presence of 4-DAMP. Values are means (SD);  $n = 4$ .  $**P < 0.01$  vs.  $0.5 \mu\text{M}$  carbachol. **C:** concentration response of carbachol after 4-DAMP mustard. Values are means (SD);  $n = 6$ .  $***P < 0.001$  vs.  $0.1 \mu\text{M}$  carbachol.

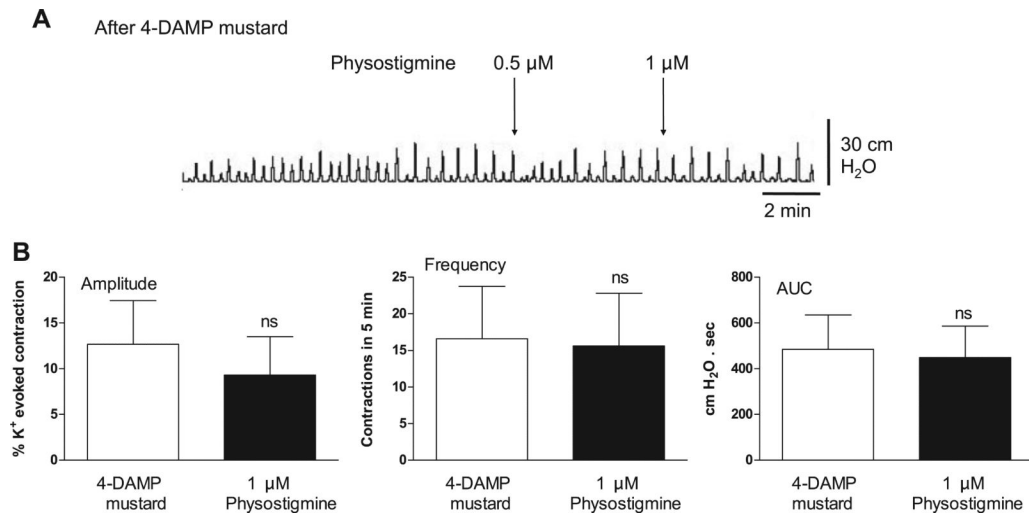




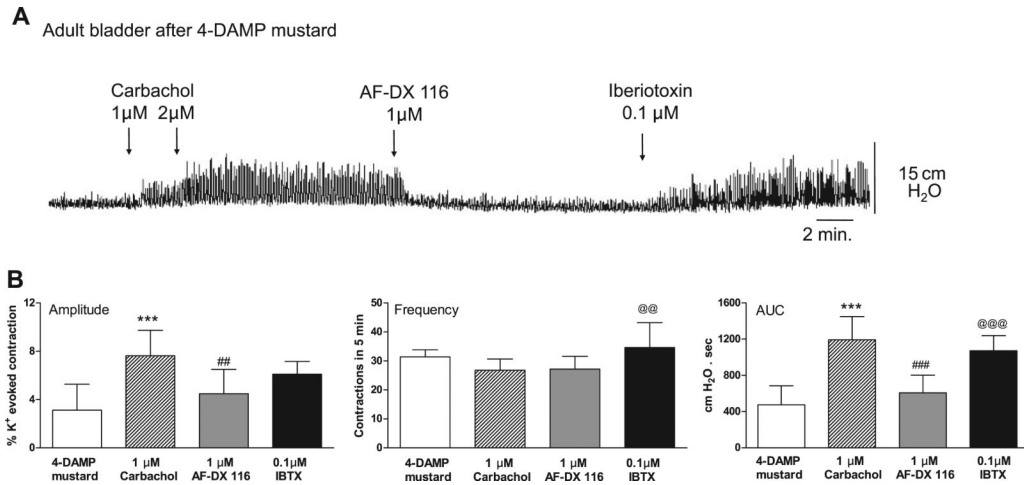
**Fig. 10.** Effect of AF-DX 116 on carbachol-enhanced spontaneous contractions. *A*: after combined M<sub>2</sub>-M<sub>3</sub> receptor blockade, IBTX is still effective. *B*: effect of AF-DX 116 and IBTX on carbachol-enhanced spontaneous contractions after 4-DAMP mustard. Values are means (SD); *n* = 9. \**P* < 0.05; \*\*\**P* < 0.001 vs. carbachol. ###*P* < 0.001 vs. AF-DX 116.



**Fig. 11.** Effect of physostigmine on basal spontaneous contractions. Facilitatory effect of physostigmine on spontaneous bladder contractions is reversed by AF-DX 116. Values are means (SD);  $n = 5$ . \*\*\* $P < 0.001$  vs. basal. # $P < 0.05$ ; ### $P < 0.001$  vs. physostigmine.



**Fig. 12.** Physostigmine does not affect basal spontaneous contractions after irreversible M<sub>3</sub> receptor antagonism with 4-DAMP mustard. Values are means (SD); *n* = 5.



**Fig. 13.**

Effect of carbachol, AF-DX 116, and IBTX on basal spontaneous contractions of adult bladders pretreated with 4-DAMP mustard. After  $M_3$  receptors have been inactivated, spontaneous contractions of adult bladders can be enhanced by  $\geq 1 \mu\text{M}$  carbachol. Effects of AF-DX 116 and IBTX are similar to those in neonatal bladders. Values are means (SD);  $n = 5$ . \*\*\* $P < 0.001$  vs. basal. ## $P < 0.01$ ; ### $P < 0.001$  vs. carbachol. @@ $P < 0.01$ ; @@@ $P < 0.001$  vs. AF-DX 116.