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Pharmacology, Biochemistry and Behavior 81 (2005) 100 – 113
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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Cholinergic modulation of the urethro genital reflex in spinal cord-transected rats

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Received 12 February 2004; received in revised form 12 February 2004; accepted 24 February 2005

Abstract

The aim of this study was to determine which of the muscarinic receptor subtypes are involved in the modulation of the urethrogenital reflex (UGR) in male, spinal cord-transected rats. The electromyographic (EMG) responses of the bulbospongiosus muscle (BS) to the topical spinal application of muscarine and the combination of muscarine and the selective muscarine receptor antagonists methoctramine (M2), AFDX (M2), 4DAMP (M3) and tropicamide (M4) were determined before and after the elicitation of UGR by way of the mechanical stimulation of the urethra.

When 50- and 100-μg doses of muscarine were applied without urethral stimulation, a rhythmic activity of the BS was observed, similar to the one found when UGR was evoked. The M3 and M4 – but not the M2 – antagonists prevented BS response to muscarine when urethral stimulation was not performed. When UGR was elicited following urethral stimulation muscarine produced an increase in burst duration and a decrease in burst frequency. The M2 antagonist reverted the effects of muscarine on the UGR, while the M3 and M4 antagonists produced a significant increase in the frequency and in the bursts number, when compared to the control muscarine response.

The differences observed in BS responses to muscarine and muscarine antagonists before and after UGR elicitation were probably linked to the intrinsic effects of the endogenous acethylcholine (Ach) released after urethral stimulation.

The present results suggest a cholinergic modulation of UGR in spinal cord-transected rats mediated by the M2, M3 and M4 muscarinic receptor subtypes.
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Keywords: Sexual behavior; Ejaculation; Sexual climax; Spinal cord; Acetylcholine; Muscarinic Receptors; Urethrogenital reflex

1. Introduction

The urethrogenital reflex (UGR) is a spinal reflex that has been considered as an experimental model of sexual climax in both male and female rats (Chung et al., 1988; McKenna et al., 1991). In male spinal cord-transected rats, the UGR can be evoked by the mechanical stimulation of the urethra. It is characterized by penis glans erections, rhythmic discharges on the bulbospongiosus muscle (BS), and the expulsion of the urethral contents (Chung et al., 1988).

The UGR has been used to identify neural structures associated with normal copula and to determine the effects of drugs on these structures. It was reported that serotonergic neurons on the paragigantocellularis nucleus control the inhibition of the UGR (Marson and McKenna, 1992, 1994), and also inhibit male sexual behavior and other ex-copula sexual reflexes (Yells et al., 1992). The intrathecal injection of serotonine (5-hydroxytryptamine) in spinalized rats inhibits UGR (Yells et al., 1992; Marson and McKenna, 1992, 1994), while the intrathecal

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doi:10.1016/j.pbb.2005.02.011

injection of muscarine facilitates the reflex (Durán et al., 2000).

The local application of muscarine on the spinal cord produces rhythmic BS bursts in anesthetized, spinal cord-transected rats, similar to those found in the UGR (Gil et al., 2000).

It has also been reported that ejaculation latency, intromission frequency and intercopulatory interval are all decreased by intrathecally-applied muscarine, and that trained animals are unable to copulate when the receptor antagonist homatropine is administered (Durán et al., 2000).

Pharmacological and molecular biological evidence suggests the presence of M1, M2, M3 and M4 muscarinic receptor subtypes in the spinal cord (Höglund and Baghdoyan, 1997; Lograsso et al., 2002; Radhakrishnan and Sluka, 2003), and several physiological processes are mediated by muscarinic cholinergic neurotransmission in the spinal cord. For example, spinal cord cholinergic systems are involved in the regulation of hemodynamic responses in vivo (Bhargava et al., 1982; Magri and Buccafusco, 1988; Sundaram et al., 1989; Calaresu et al., 1990; Takahashi and Buccafusco, 1991; Feldman and Buccafusco, 1993) and in nociceptive transmission (Detweiler et al., 1993; Iwamoto and Marion, 1993; Naguib and Yaksh, 1994; Abram and O'Connor, 1995; Abram and Wine, 1995).

Other studies suggest that M2 receptor subtypes are involved in spinal cord-mediated analgesia (Takahashi and Buccafusco, 1991; Iwamoto and Marion, 1993), and other researchers have reported that the M3 receptor subtype is involved in cholinergic mediated analgesia (Honda et al., 2000). The M1 and M3 muscarinic receptor subtypes are also involved in the transcutaneous electric nerve stimulation (TENS) analgesia (Radhakrishnan and Sluka, 2003), and the M4 receptor has also recently been linked to spinal cord analgesic responses (Duttaroy et al., 2002; Mulugeta et al., 2003).

Furthermore, previous evidence points to the important role of cholinergic spinal cord neurotransmission in the modulation of sexual behavior (Gil et al., 2000; Vargas et al., 2004). However, the exact nature of this neuromodulation, and the specific muscarinic receptor subtypes involved, are not yet known.

In considering the UGR as a valuable experimental model of spinal-level sexual behavior, we were interested in determining which muscarinic receptor subtypes are involved in that type of modulation. Consequently, the goal of this study was to identify the muscarinic receptor subtypes responsible for the rhythmic response of the BS to muscarine topical administration on the spinal cord (Gil et al., 2000), both before and after UGR elicitation. For each of the responses, the effects of specific muscarinic agonists and selective muscarinic antagonist receptor subtypes were tested on male, spinal cord-transected rats.

2. Materials and methods

2.1. Animals

118 male Wistar rats (300–450 g) from our facilities were used. All the animals were sexually naïve. Three animals were housed per cage, and they were exposed to a reverse light cycle (lights on: 10:30 p.m., lights off: 10:30 a.m.), with room temperature at 21 °C. Food and water were supplied ad libitum. The principles of laboratory animal care, as well as the local requirements of our institutional ethics committee, were followed in the research.

2.2. Surgical procedures

The subjects were anesthetized with urethane (1.6 g/kg in 20% solution, ip). The trachea was cannulated and a spinal cord transection was performed at the T6 anatomical level. Gelfoam was inserted between the transected spinal segments in order to prevent bleeding. The pelvic urethra was catheterized with PE-50 polyethylene tubing (0.965 mm id) through an incision made in the bladder. A small lumbar laminectomy was performed at L4-S2 in order to expose the lumbar enlargement. In previous work, we had found that this is the most appropriate segment in which to elicit the UGR (Gil et al., 2000). The dura was cut, and the spinal cord was covered with cotton saturated in saline solution. The BS muscle was exposed, and a pair of platinum needles (Grass S2) was inserted into the distal region of the muscle (Holmes et al., 1991) for recording purposes.

2.3. Drugs and application

Muscarine chloride (tetrahydro-4β-hydroxy-N,N,N,5α-tetramethyl-2α-furanmetanaminium); methoctramine tetrahydrochloride (N,N' bis [6-[[(2-methoxyphenyl)methyl]amino]hexyl]-1,8-octanediamine)(M2 antagonist); 4-DAMP (methiodide 4-D-diphenylacetoxy-*N*-methylpiperidine) (M3 antagonist) and tropicamide (*N*-ethyl-2-phenyl-*N*-[4-pyridilmethylacrilamide) (M4 antagonist) were acquired from Sigma (St. Louis, Mo.). Arecaidine (1,2,5,6-tetrahydro-1-methyl-3-pyridine-carboxylic acid but-2-ynyl-ester tosylate) (M2 agonist) and AFDX-116 (11-[[2-[(diethylamino)-methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-b][1,4]benzodiazepin-6-one) (M2 antagonist) were acquired from Tocris (Ballwin, Mo.). Six treatment groups were randomly selected: muscarine ($n=22$); methoctramine ($n=24$); 4-DAMP ($n=24$); tropicamide ($n=24$); arecaidine ($n=15$); AFDX ($n=9$). All the drugs were dissolved in 200 μL of normal saline solution just before the topical application on the spinal cord. A piece of cotton soaked in the drug solution was left on for 20 min as an equilibration period. Extent of the spread of the drugs was measured at the end of the experiment in 18 subjects by placing a piece of

organic dye-soaked cotton on the exposed spinal segments (L5-S1) for 20 min. The dye spread from the L4 to S2 spinal segments. The final pH for all of the drug solutions was 6.5, except for muscarine which has pH value of 6. Since subsequent to the local application, the responses are related with the total amount of the drug in the application site, so we refer to the dose as the total quantity applied in micrograms (μg).

2.4. Recording

The electromyographic (EMG) activity of the BS muscle was used to record responses to drugs following the application of muscarine without urethral stimulation (control responses) and after elicitation of UGR by way of urethral stimulation. Urethral pressure was recorded by means of a pressure transducer connected in parallel to the pump through a three-valve stopcock. Both signals were recorded by a polygraph (Grass M7) using conventional techniques. Paper speed for recording BS was 50 mm/min in all experiments without urethral stimulation. For UGR, 50 mm/min was used in 76, and 100 mm/min in 18 observations.

2.5. BS responses to muscarine and selective muscarine receptor antagonists

I. BS response curves to 1 μg ($n=6$); 10 μg ($n=10$) and 100 μg ($n=6$) of muscarine.
II. BS responses to 25, 50 and 100 μg ($n=8$ for each concentration) of the three selective muscarine antagonist subtypes (methoctramine (M2); 4 DAMP (M3); tropicamide (M4)).

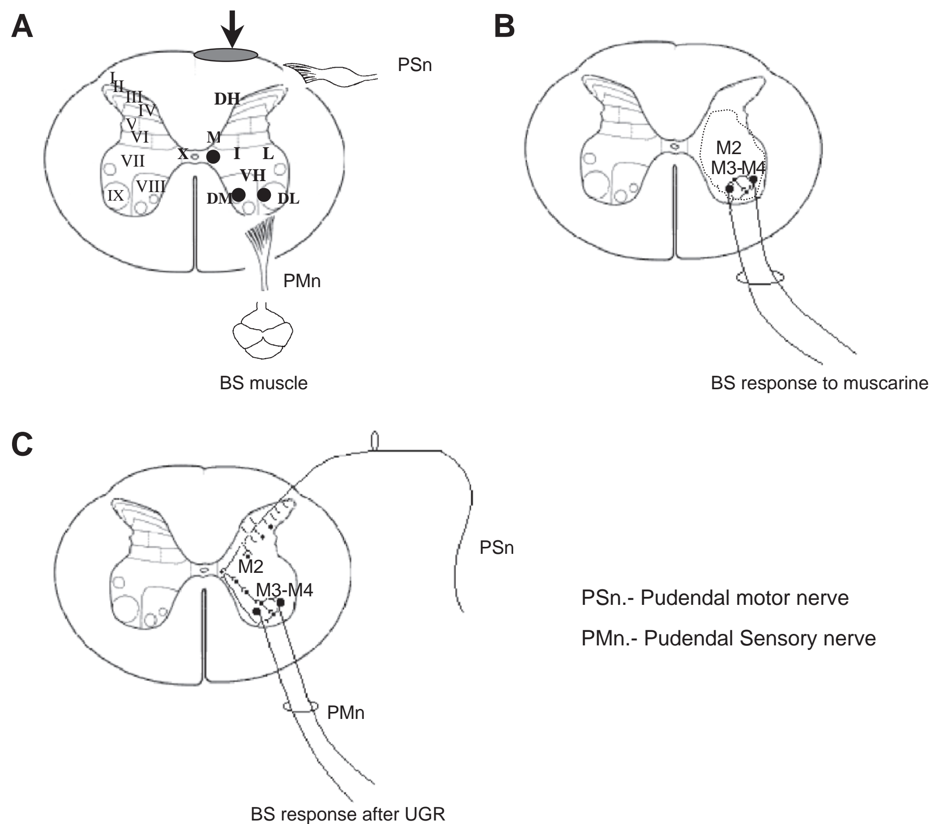

Fig. 1. Schematic diagram of the experimental design. L5-S1 spinal cord segments were exposed, and the drugs were applied topically. The spinal cord areas where the muscarinic receptor subtypes M2, M3 and M4 are probably located during UGR elicitation are shown in panel (A). The left side shows the location of the laminae. The right side shows the subdivisions mentioned in the text: DH—dorsal horn; M—medial gray; I—intermediate gray; L—lateral gray; VH—ventral horn; DM—dorsomedial, and DL—dorsolateral nucleus. The dots correspond to the probable sites where interneurons and motoneurons involved in the local circuit activated during UGR are located. The arrow indicates the site of drug application. PSn indicates the sensorial branch of the pudendal nerve. PMn indicates the pudendal motor branch. BS represents the bulbospongiosus muscle, where the responses were recorded. Panel (B) is a diagram illustrating the probable sites activated by local application of muscarine without urethral stimulation. The effects observed are probably mediated by drug diffusion on interneurons and motoneurons possessing M2, M3 and M4 receptors. In panel (C), pudendal sensory nerve afferents were activated following urethral stimulation. These afferents relay information to interneurons in the medial and intermediate gray, which then project onto motoneurons of the pudendal nerve, located mainly at DL and DM. According to previous studies (Marson et al., 2003; Stewart and Maxwell, 2003), these interneurons mainly possess inhibitory M2 receptors. The pudendal motoneurons probably mainly possess M3 and M4 receptors which displayed excitatory responses. Given the network connections between neurons, excitatory-inhibitory synchronous activity is probably established in a local spinal circuit during UGR.

III. BS responses to 10 µg muscarine plus 25 µg, 50 µg and 100 µg of M2, M3 and M4 muscarinic antagonists (n=6 for each concentration).
IV. BS response curves to 25, 50 and 100 µg of arecaidine (M2 agonist).

2.6. UGR responses

UGR was produced by the injection of normal saline solution into the urethra (200 µl/min, Harvard Syringe Pump) while occluding the penis glans.

In order to determine the drugs' effects on UGR, the reflex was evoked twice at threshold intensity (5–7 mm Hg). Following the control responses, the drugs were applied and allowed to equilibrate for 20 min. UGR was then elicited every 3 min with the aforementioned stimulation intensity until the reflex was inhibited (mean of successful trials ±SD: 6±4; range 4–10 for 2.6.I procedure and 7±4, range 3–14 for procedures 2.6.II and 2.6.III). UGR was considered to be inhibited when there was no response to urethral stimulation in three consecutive trials (Durán et al., 1997). Afterward, the drug-soaked cotton was removed and replaced with a fresh piece of cotton soaked in normal saline solution.

I. UGR responses to 1 µg, 10 µg, and 100 µg of muscarine (n=6 for each concentration).
II. UGR responses to 25, 50 and 100 µg of M2, M3 and M4 selective antagonist subtypes (n=8 for each concentration).
III. UGR responses to 10 µg of muscarine plus 25 µg, 50 µg and 100 µg of each selective muscarinic antagonist (M2, M3 and M4; n=8 for each concentration).
IV. In order to corroborate the role of the M2 receptor subtype on UGR modulation, a second selective M2 antagonist, AFDX, was administered to a separate group of male rats. A UGR dose response curve to 25, 50 and 100 µg of AFDX (n=3 for each concentration) was performed under the aforementioned conditions for methoctramine.
V. In order to determine whether or not arecaidine (M2 agonist) induces recovery of the UGR, the following procedures were carried out:
   i. Following the elicitation and subsequent inhibition of UGR under control conditions, three of the subjects rested for 3 h were exposed to a new saline application and the urethral stimulation was repeated. After we determined that UGR had not been elicited (three unsuccessful trials), the procedure was repeated after administering 100 µg of arecaidine.
   ii. Following UGR inhibition under control conditions, arecaidine (25, 50 and 100 µg; n=3 for each concentration) was applied and the UGR was induced using the previous threshold stimulation intensity.
   iii. In three other subjects, four different stimulation intensities were applied successively (5, 10, 60 and 120 mm Hg) in order to evoke UGR. After inhibition of the reflex with the previous intensity, the next one was applied, in order to determine whether or not recovery is dependent on stimulus intensity. After UGR inhibition with maximum intensity (120 mm Hg), 100 µg of arecaidine was applied for 20 min, and stimulus with 60 mm Hg of pressure was repeated.

2.7. Data analysis

The following parameters were evaluated: (i) presence of rhythmical BS bursts following administering of muscarine, and antagonist plus muscarine; (ii) UGR latency; (iii) UGR burst frequency; (iv) UGR burst

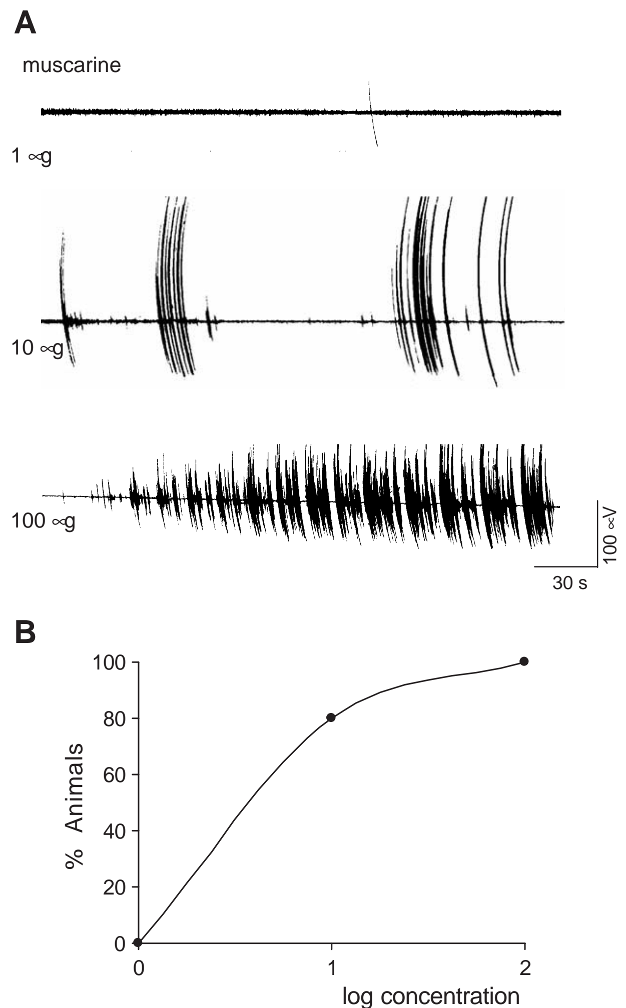

Fig. 2. EMG activity of the BS following local spinal muscarine application. (A) Examples of EMG activity of BS in response to 1 (upper record), 10 (middle record) and 100 µg (bottom record) of the drug. (B) Dose–response curve to muscarine. As shown, no rat whatsoever responded to 1 µg, but 80% and 100% of the rats responded to 10 and 100 µg of the agonist, respectively.

duration, and (v) number of UGR bursts. The data of subjects studied at each dose for the muscarine, methoctramine, 4DAMP and tropicamide groups were pooled. For the muscarine group, a comparison was made between UGR responses after 10 (UGR $n=60$) and 100 μg (UGR $n=50$) and the control responses (UGR $n=20$ and UGR $n=12$, respectively). For the methoctramine, 4DAMP and tropicamide groups, a comparison was made between UGR responses after 10 μg of muscarine (UGR $n=60$) and after muscarine plus each antagonist (UGR $n=48$ on average for the three concentrations of antagonists). Statistical analysis was carried out using a one-way ANOVA test, followed by a Student–Newman–Keuls test. A value of $p<0.05$ was considered to be statistically significant. For the arecaidine and AFDX groups, a comparison was made between the control rats (UGR $n=6$) and the treated rats (UGR $n=18$ on average). A paired Student t-test was used for statistical analysis. Differences were considered significant if $p<0.05$.

3. Results

In Fig. 1 a schematic diagram of the experimental design is shown.

3.1. BS responses to the application of muscarine

As shown in Fig. 2A, muscarine elicited BS response only at 10- and 100-μg doses. BS response to muscarine occurred in clusters separated by several seconds, or even minutes (Fig. 2A, middle and bottom records). Five to seven such episodes were elicited in 8 out of 10 subjects treated with 10 μg. As reported previously (Gil et al., 2000), burst frequency within each episode was similar to the one found in UGR ($F(15,65)=0.656$, $p>0.1$, one-way ANOVA). In four subjects, BS muscle activity in response to muscarine continued for the remainder of the experiment, but the bursts showed smaller amplitude and shorter duration than those shown in the middle record of Fig. 2A. At 100 μg of muscarine (Fig.

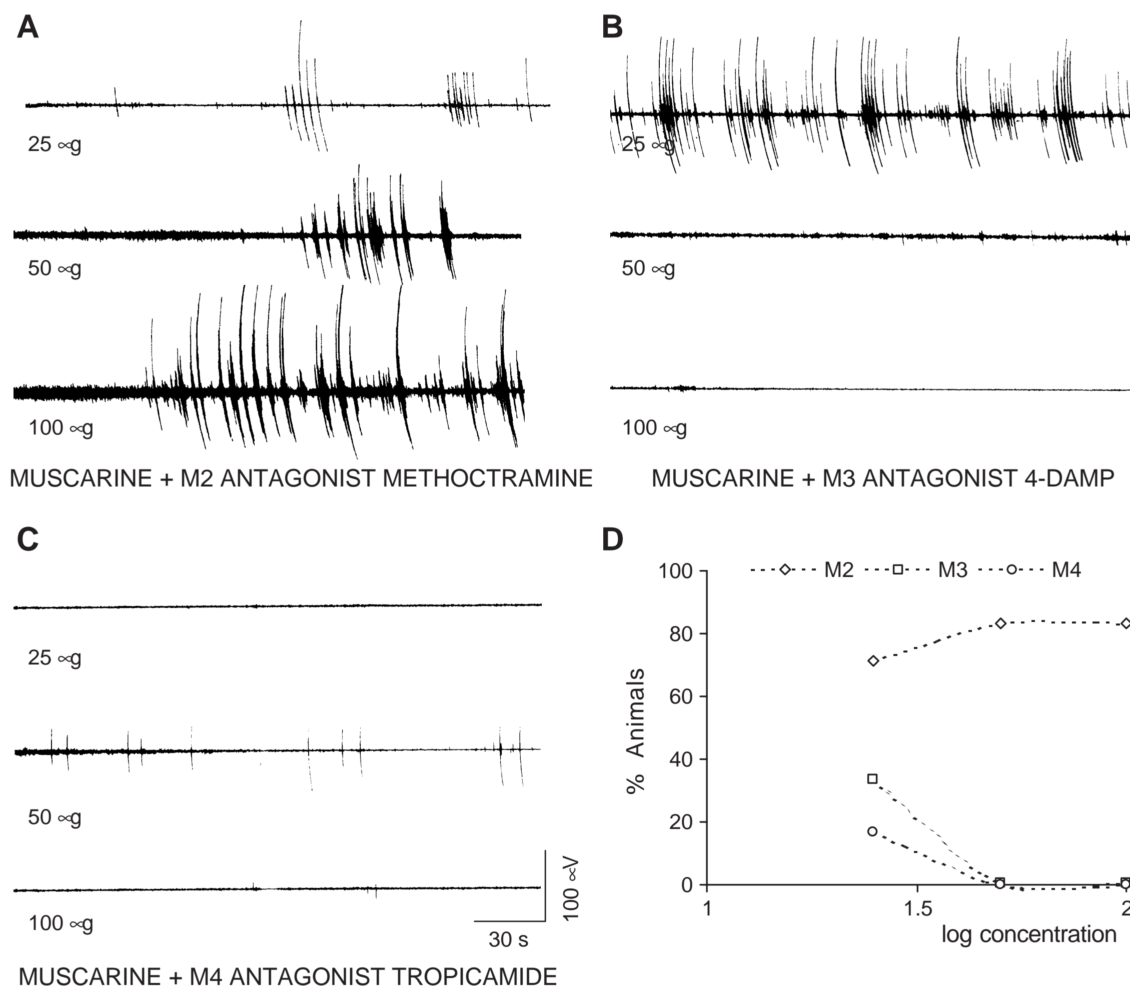

Fig. 3. Effects of M2, M3, and M4 muscarinic antagonist receptor subtypes on BS response to muscarine. Effects of 10 μg of muscarine in the presence of 25, 50 and 100 μg of the three different selective receptor antagonist subtypes: (A) M2 receptor antagonist methoctramine; (B) M3 receptor antagonist 4-DAMP, and (C) M4 receptor antagonist tropicamide. In panel (D), the quantal dose–response curve is shown for muscarine in the presence of methoctramine (◇), 4-DAMP (□) and tropicamide (○). $n=6$ for all antagonist doses.

2A, bottom record), BS episodes of activity were observed in all six subjects tested. These appeared at shorter intervals than those produced by the middle concentration of the drug ($F(15,65)=3.356$, $p<0.05$, one-way ANOVA), and burst duration and frequency were different at the two drug concentrations ($F(15,65)=4.26$ and 4.27 respectively, $p<0.05$, one-way ANOVA). In all six subjects, BS activity continued for the entire observation period (1 h on average). The dose–response curve to the three different concentrations of the drug is shown in Fig. 2B.

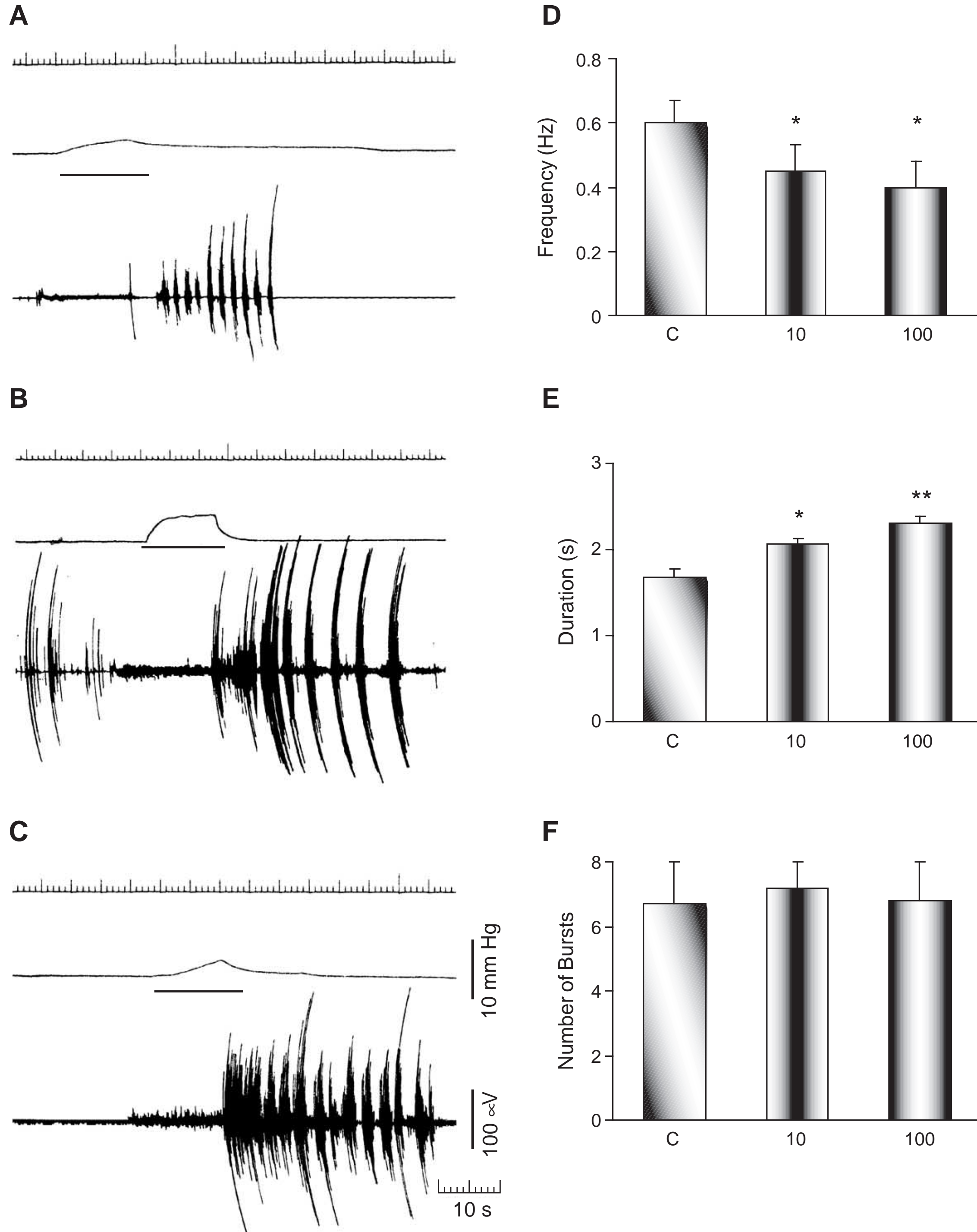

Fig. 4. UGR changes in response to muscarine. (A) Control UGR before muscarine application. (B) UGR after 10 µg of muscarine. (C) UGR after 100 µg of muscarine. The records are from different subjects. As shown, muscarine produces an increase in burst duration and a decrease in burst frequency. In panels (D–F), quantitative changes in burst frequency (D), burst duration (E) and number of bursts (F) are shown. Data are expressed as the mean±SEM. *$p<0.01$; **$p<0.001$, Student–Newman–Keuls test. In panels (A–C), upper record—time recording; middle record—urethral pressure. The line below the pressure record shows the stimulus duration; bottom record: EMG activity of BS muscles. The same calibrations apply to all figures.

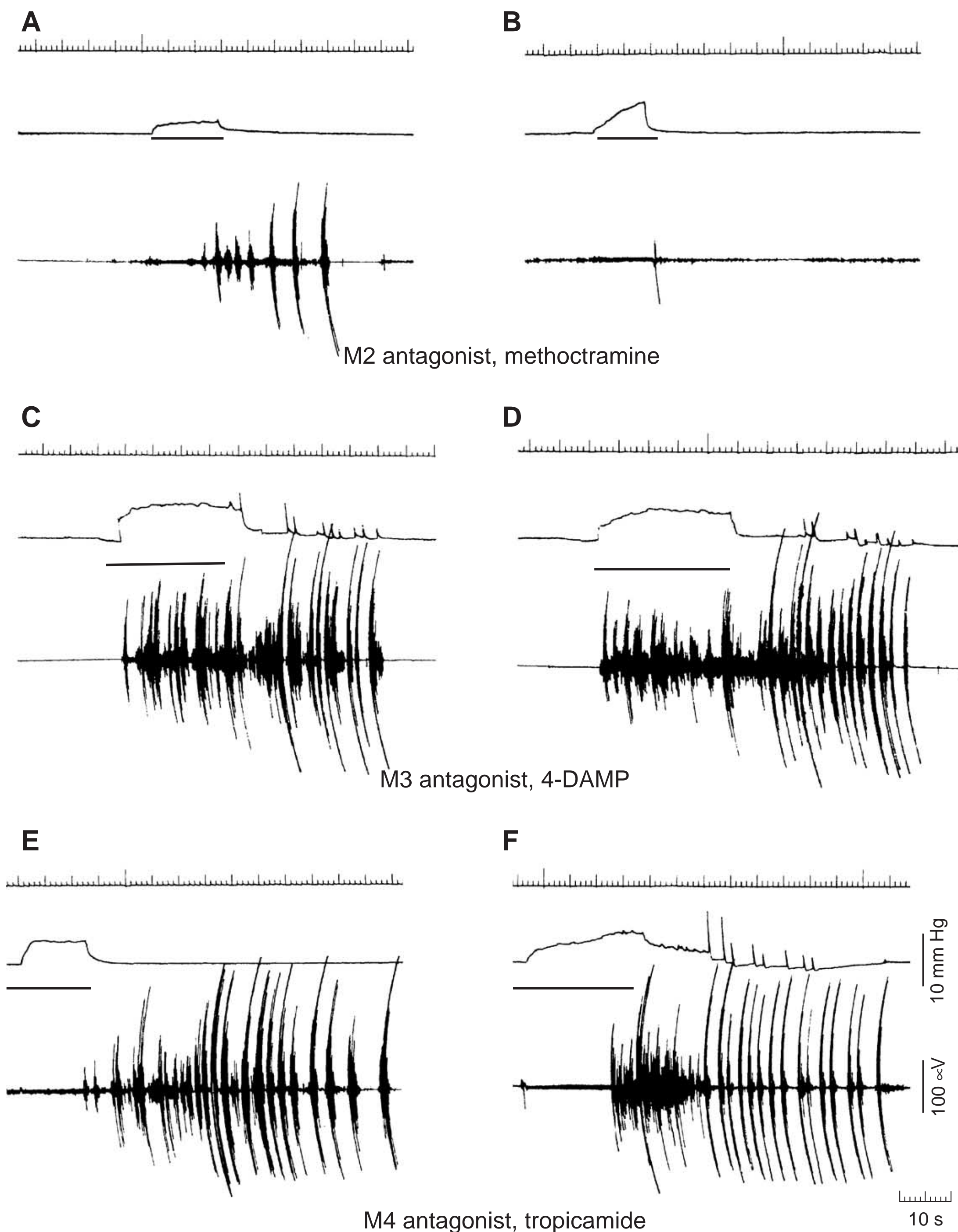

Fig. 5. UGR changes to muscarine response in the presence of M2, M3 and M4 receptor antagonist subtypes. (A–B) UGR after application of 10 μg muscarine, plus 50 μg and 100 μg of methoctramine, respectively. Decreases in burst duration, frequency and number were observed. After 100 μg of methoctramine (B), a lack of response was observed. (C–D) UGR after 10 μg muscarine, plus 50 μg and 100 μg of 4-DAMP, respectively. The M3 antagonist did not reverse the effects of muscarine. (E–F) UGR after 10 μg of muscarine, plus 25 and 100 μg of tropicamide, respectively. An increase in the number and frequency of bursts, accompanied by a decrease in burst duration, may be seen, along with rhythmical BS bursting near the end of the stimulus. All records are from different animals. Upper record—time recording; middle record—urethral pressure. The line below the pressure record shows the stimulus duration; bottom record—EMG activity of BS. The same calibrations apply to all figures.

3.2. BS responses to the application of selective muscarine receptor antagonists methoctramine (M2), 4DAMP (M3) and tropicamide (M4)

When the selective antagonists methoctramine (M2), 4DAMP (M3) and tropicamide (M4) were applied alone on the spinal cord, no changes in BS background activity were observed at any of the test doses (recordings not shown).

3.3. BS response to muscarine plus selective muscarine antagonists

Examples of BS recordings in the presence of muscarine plus antagonists are shown in Fig. 3A–C. The dose–response curve to muscarine in the presence of the three antagonists is shown in Fig. 3D. BS response was observed in the presence of methoctramine (M2) in four out of six subjects at 25 μg and in five out of six subjects at 50 or 100 μg (Fig. 3A). On the other hand, BS response was decreased in a dose-dependent manner in the presence of 4DAMP (M3) (Fig. 3B). The response was absent in presence of all doses of tropicamide (M4) (Fig. 3C). BS response was observed in only three subjects at the lowest dose of 4DAMP (M3), but there was no response in any of the subjects when the dose of both antagonists (M3 and M4) was increased to 50 μg and 100 μg. As may be seen, the maximum inhibition effect was observed at 50 μg of the antagonists.

3.4. BS response to arecaidine (M2 agonist)

There was no BS response to arecaidine, even at 100 μg.

3.5. Effects of muscarine on the UGR

The control response of the BS muscle after UGR is shown in Fig. 4A. In Fig. 4B and C, changes in UGR

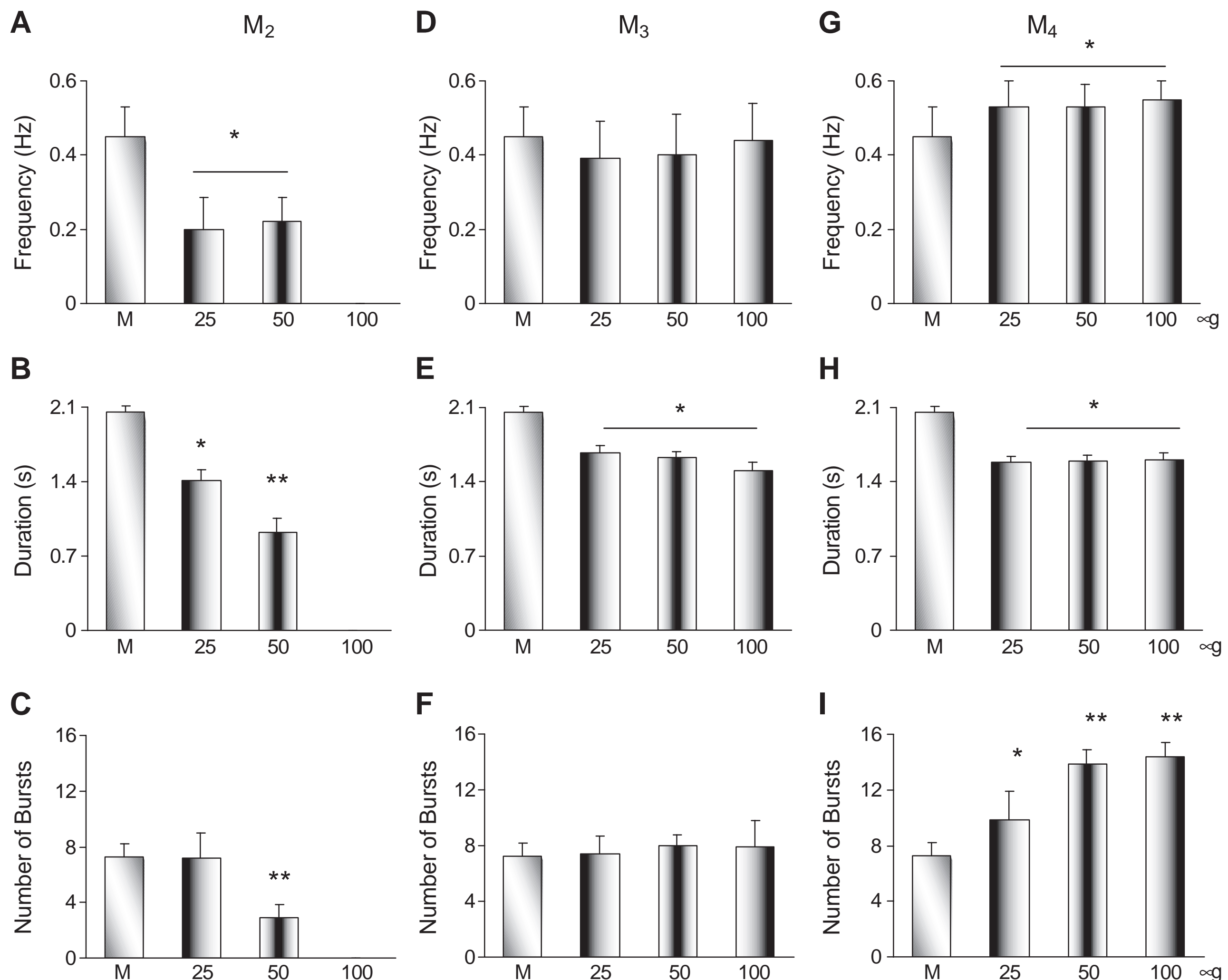

Fig. 6. Changes in UGR parameters following the application of muscarine plus the different muscarinic antagonist receptor subtypes (M2, M3 and M4). Changes in the UGR response to muscarine (burst frequency, duration and number) in the presence of the M2 (A–C), M3 (D–F) and M4 antagonists (G–I) are shown. Data are expressed as mean±SEM; *p<0.01; **p<0.001, Student–Newman–Keuls test (10 μg of muscarine plus 25, 50, and 100 μg of the antagonists).

Table 1
UGR changes produced by AFDX

Parameters	Control	AFDX 25	Control	AFDX 50	Control	AFDX 100
Latency (s)	1.9±0.2	2.8±0.7	1.96±0.5	8.8±2.3*	2.2±0.7	No UGR
Frequency (Hz)	0.7±0.02	0.6±0.1	0.7±0.1	0.5±0.03**	0.7±0.2	No UGR
Duration (s)	1.8±0.07	1.76±0.03	1.8±0.06	1.6±0.08	1.8±0.04	No UGR
No. of bursts	9.8±2	7±1.8	5.4±1.3	7.1±1.5	6±1.4	No UGR

Data are expressed as the mean±SEM (UGR number: control=6; 25 μg=18; 50 μg=14; 100 μg=9). Control columns refer to responses before drug application. The concentration of antagonist is indicated in the upper row. *$p<0.01$; **$p<0.03$, *t*-paired Student test.

after muscarine applications of 10 and 100 μg, respectively, are displayed. Muscarine produced a significant increase in burst duration and a significant decrease in burst frequency (Fig. 4E, D), which are dose-dependent. As demonstrated in Fig. 4C, the tonic response to the mechanical stimulus increased at 100 μg, and the long-lasting BS activity elicited by muscarine was inhibited during the urethral stimulation. No significant changes in UGR latency or burst number (Fig. 4F) were found after muscarine application, when compared to latency and burst number in the control responses. Similar responses to those shown in Fig. 4B–C were found in all subjects tested with these agonist doses.

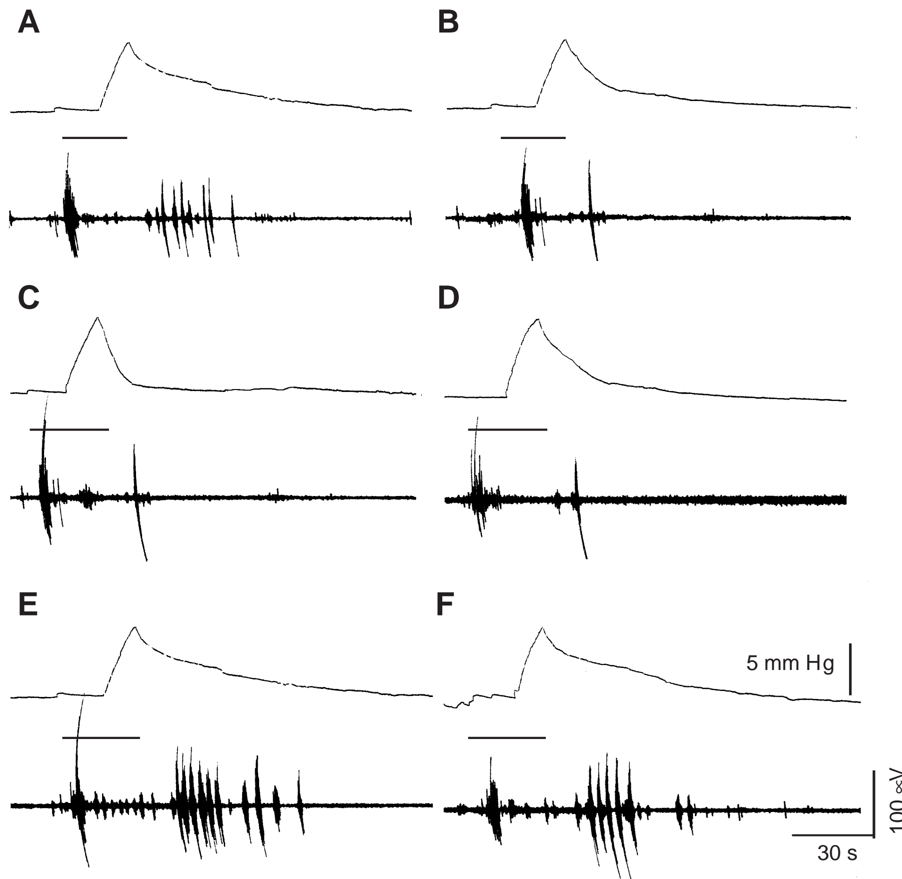

Fig. 7. UGR recovery following arecaidine application. (A) Control UGR for the second stimulation. The stimulus elicited a tonic BS response followed by characteristic UGR activity at the end of stimulation. (B) An example of UGR decline after repetitive stimulation: Response after the eighth consecutive stimulation. Only a tonic response and a single burst at the end of stimulation were observed. (C–D) Responses to the first and third stimuli after saline application. As may be seen, UGR was not elicited. The response to this stimulation was similar to that shown in panel (B). (E–F) Responses to the first (E) and the second (F) stimulation following the application of 100 μg of arecaidine are shown. As may be observed, UGR was recovered after the arecaidine application. All records are from the same subject. The upper line shows pressure changes in the urethra; the line below the pressure record shows the stimulus duration. Bottom line shows EMG activity. The same calibrations apply to all figures.

3.6. Effects of muscarine plus selective muscarinic antagonist receptor subtypes on the UGR

A dose-dependent decrease in UGR muscarine response was observed in the presence of the M2 antagonist methoctramine, as shown in Fig. 5A–B. A light response was observed at 50 μg, but no response whatsoever was observed at 100 μg. As evidenced in Fig. 6A–C, methoctramine reduced burst frequency, duration and number in a dose-dependent manner.

The application of muscarine plus 50 and 100 μg of the M3 antagonist 4DAMP are shown in Fig. 5C–D. Rhythmical BS bursting during the stimulus application was observed with this antagonist, similar to the effect observed with tropicamide (M4) (Fig. 5E–F). In terms of burst duration, a significant decrease was observed after 4DAMP application, but there were no changes in the frequency and number of bursts when compared to responses to the muscarine control (Fig. 6D–F).

The main effect of tropicamide (M4 antagonist) was to increase the number of bursts (Figs. 5E–F and 6I). Furthermore, there was a significant increase in burst frequency and a significant decrease in burst duration compared to the muscarine control (Fig. 6G–H).

3.7. UGR response to application of AFDX (M2)

The changes in UGR after AFDX application to the spinal cord are summarized in Table 1. As may be seen, the application of AFDX, an M2 selective antagonist, produced a response similar to the one obtained with methoctramine. A gradual, dose-dependent decline in UGR was observed. After the lower drug application (25 μg), no significant changes in UGR were observed. However, at 50 μg latency was longer and burst frequency was reduced. At 100 μg, UGR could not be elicited in any subject. UGR decline was also found in the other four stimulation intensities applied in the present study.

3.8. UGR recovery following application of the M2 agonist arecaidine

When 25 μg of arecaidine was applied, UGR could not be re-elicited in any of the subjects, even after three consecutive attempts. However, after 50 μg UGR was re-elicited, and the same results were obtained with 100 μg. There were no significant differences in the results observed in all the subjects tested. As in previous reports (Durán et al., 1997), repetitive stimulation induced exhaustion of the UGR, which continued for at least 3 h after the last stimulus (Durán et al., 1997). This behavior is shown in Fig. 7B.

In this study, the decline in UGR occurred at all the stimulus intensities applied; however, after application of arecaidine (M2 agonist), UGR recovery was observed. Thinking the observed recovery might have been related to the time elapsed without stimulation, we tried to re-elicit the reflex 20 min after the saline application in three subjects in which the UGR had been exhausted. As shown in Fig. 7C–D, the UGR was not elicited with this procedure. However, when 100 μg of arecaidine was applied in the same subjects (Fig. 7E–F), UGR was evoked successfully eight times. At the ninth stimulus, and twice more afterward, the BS response was a single burst, similar to the one observed in Fig. 7B. Similar results were found in the three subjects. Subsequent to the arecaidine application, there was clearly longer latency and reduced frequency of UGR in comparison with the control responses. All the results obtained after the application of 100 μg of arecaidine was pooled, and they are summarized in Table 2.

Table 2
Parameters of UGR before and after arecaidine (A)

Parameters:	Control	A. 50 μg ($n=3$)	Control	A. 100 μg ($n=9$)
Latency (s)	6.2±1.5	10.3±3.1*	5.7±1.2	10.7±2*
Number of bursts	5.9±0.6	6.4±0.8	6.4±0.7	6.8±1.3
Frequency (Hz)	0.7±0.09	0.5±0.08**	0.7±0.08	0.5±0.06**
Suc/Total trials	20/30	13/29	22/34	20/52

Data are expressed as the mean±SEM.
Control columns are the responses before drug application. *$p<0.05$; **$p<0.03$, Student t test. Suc/Total trials: number of successful trials/total stimulation trials.

4. Discussion

In this study, the electromyographic responses of the bulbospongiosus muscle to muscarine– and muscarine plus selective antagonist receptor subtypes M2, M3 and M4, without and following mechanical urethral stimulation to elicit UGR, were registered.

4.1. BS muscarinic responses

As previously reported (Gil et al., 2000), in this study BS response to muscarine was also elicited. It took place in clusters, and the intervals within clusters were related to the muscarine dose. In addition, the number of bursts per cluster and the burst frequency were very similar to those previously found (Gil et al., 2000) in the elicitation of UGR. It is important to point out that when no urethral stimulation was performed, no autonomic response or locomotive-like activity was observed, only BS muscle discharges, often accompanied by penis erections. These last two responses were also present during UGR.

The BS response to 10 μg of muscarine was not reverted by the M2 antagonist methoctramine, and the M2 agonist arecaidine was unable to reproduce muscarine response at any concentration. Quite the opposite, both M3 and M4 receptor antagonists inhibited the bulboespongiosus response to muscarine with similar potency.

These results could indicate that M2 receptors are not involved in muscarine response. However, they also suggest that the M2 receptor probably could activate inhibitory mechanisms in interneurons, which are abolished in our model by spinal cord transection at the T6 level. It was demonstrated that a descending inhibitory serotonergic pathway in the brainstem controls spinal sexual reflexes (Marson and McKenna, 1990). Thus, in the absence of this inhibitory mechanism, only the excitatory facilitated response was observed in our experimental model. With regards to the effects observed with the M3 and M4 antagonists, these could indicate that M3 and M4 receptors could mediate excitatory responses.

Several studies have shown that the M2 subtype is the main muscarinic receptor in the spinal dorsal horn, where it makes up approximately 90% of the spinal cord muscarinic receptors (Duttaroy et al., 2002). Other authors have found M2 receptors in both the dorsal horn and in motoneurons (Villiger and Faull, 1985; Kurihara et al., 1993; Höglund and Baghdoyan, 1997; Yung and Lo, 1997), and recent reports indicate that the M2 receptor is not only concentrated in synaptic regions, but is also present on the entire surface of the plasma membrane of cell bodies in the dorsal horn neurons. It was thus hypothesized that the effect of muscarine on neurons with M2 receptors could be carried out by way of a diffuse, non-synaptic method of transmission (Vizi, 2000). It was also suggested that M2 receptors could not be involved in fast synaptic responses (Stewart and Maxwell, 2003). Several other studies have shown that cholinergic agonists mediate inhibitory responses in the spinal cord (Chen and Pan, 2004), and that the M2 receptor subtype is mainly involved in those inhibitory responses (Haberberger et al., 2000; Chen and Pan, 2004).

There is also important evidence concerning the co-localization of M2 receptors with inhibitory neurotransmitters as GABA and NOS, mainly in Lamina III of the dorsal horn (Stewart and Maxwell, 2003). Moreover, a large population of M2 receptors has been located around Lamina X (Stewart and Maxwell, 2003), where descending serotonergic fibers associated with the paragigantocellularis nucleus have been identified (Marson et al., 2003). Other studies have also demonstrated important interactions between cholinergic receptors and inhibitory neurotransmitters in the spinal cord (Li et al., 1994). On the other hand, in lumbar dorsal root ganglia (DRG), the expression of M3 receptors has been associated with the neuronal increase in Ca^{2+}, which could be explained by the release of calcium from internal stores via inositoltrisphosphate (Haberberger et al., 2000). Other researchers studying the neonatal rat spinal cord have found that the excitatory responses associated with nociceptive transmissions are linked to the depolarization of motoneurons that possess the M3 receptor (Kurihara et al., 1993). Furthermore, the relationship between the M3 receptor and the excitatory and contractile responses to muscarine agonists has been demonstrated by various authors (Caulfield, 1993; Eglen et al., 1996; Longhurst et al., 1995).

The existence of M4 receptors on the spinal cord was suggested by Höglund and Baghdoyan (1997), for whom they represent only a small fraction of the total muscarinic receptor population, and recently these receptor subtypes have been located on the dorsal horn (Mulugeta et al., 2003).

As for the role of M4 receptors in the spinal cord, there exist a limited amount of information. However recent studies have shown that they are associated with other receptor subtypes in muscarine-mediated analgesia (Duttaroy et al., 2002; Mulugeta et al., 2003, Honda et al., 2004; Kang and Eisenach, 2003).

Traditionally the M2 and M4 receptors have been associated with inhibitory responses mediated by the inhibition of adenylate cylase activity, however recent studies carried out on M2 and M4 knockout mice have found significant differences in both their functions and protein components. Hence, there is a possibility that M2 and M4 activate different signaling pathways (McClatchy et al., 2002). In support, other authors have found a significant increase in the baseline locomotor activity of M4 knockout mice, as well as increased sensitivity to locomotor stimulant dopamine D1 agonists (Gomeza et al., 1999) when compared to wild mice.

As may be seen in Fig. 3A, BS response to 10 μg of muscarine plus the M2 antagonist methoctramine is dependent on the antagonist dose. This could indicate that inhibitory mechanisms are gradually blocked after the increase of the M2 antagonist concentration, which facilitates the excitatory responses.

Since the M3 receptor has been found in motoneurons, where it mediates depolarizing responses (Kurihara et al., 1993), and M4 has also been found in the spinal cord (Höglund and Baghdoyan, 1997; Mulugeta et al., 2003), it could be possible that the BS response to muscarine observed in this study could be mediated by the stimulation of both receptors. The presence of M3 and M4 receptors was confirmed, because the blockage of these receptors also inhibited muscarine-induced bulbospongiosus responses (Fig. 3B–C). Since the blockage observed after the application of M3 and M4 antagonists showed similar – but not identical – patterns (Fig. 3B–C), we can speculate that their responses could be mediated by different mechanisms. The limited selectivity of 4-DAMP (M3, pKb 8.9–9.3) and tropicamide (M4, pKb 9.4) (Caulfield, 1993) could also point to the role of both M3 and M4 in the responses of the bulbospongiosus muscle to muscarine.

4.2. BS responses following the elicitation of UGR by way of urethral stimulation

The main UGR responses observed after muscarine application were an increase in burst duration and a decrease in burst frequency. However, the most significant observation was the inhibition of UGR after M2 antagonist application

and the positive response to M3 and M4 antagonists, since these responses were diametrically opposed to those observed when no urethral stimulation was applied. This apparent contradiction could be explained if one considers that after UGR elicitation, the urethral stimulation could activate terminal afferents in the pudendal and pelvic nerves, which could then release greater amounts of acetylcholine. On the other hand, when urethral stimulation was not applied, we only observed a response to 10 μg of muscarine. Therefore, after UGR the activation of a great number of receptors is possible, and larger populations of interneurons and motoneurons may thus be recruited.

It is important to point out that M2 receptors have already been located on interneurons and motoneurons, in sites where this cholinergic receptor was probably involved in the integration or modulation of both excitatory and inhibitory mechanisms (Stewart and Maxwell, 2003). It is also noteworthy that after the endogenous release of Ach during UGR, several other excitatory mechanisms could be modulated after the activation of M2 receptors, for example, muscarinic excitatory responses, probably mediated by M3 receptors, nicotinic responses (Haberberger et al., 2004; Cordero-Euraskin et al., 2004), or even excitatory responses mediated by other neurotransmitters (e.g., glutamate). M2 could also modulate inhibitory responses mediated by other cholinergic receptors (e.g., M4) and by serotonergic mechanisms. A descending inhibitory serotonergic pathway for the UGR was previously demonstrated (Marson and McKenna, 1990; Marson et al., 2003), which was inhibited in our model by the spinal cord transection. This modulation, mediated by M2 receptors most likely located on interneurons in the medial dorsal horn and the dorsal gray commissure (DGC), may drive the activity of other motoneurons and interneurons, which could then give rise to synchronous patterns of rhythmic motor activity such as the UGR, in which alternate excitatory and inhibitory mechanisms are involved. This hypothesis is supported by recent studies in which large populations of M2 receptors have been found in spinal cord areas (Stewart and Maxwell, 2003; Duttaroy et al., 2002), where strong neuronal activity has been observed during the elicitation of UGR (Marson et al., 2003). It was also observed that the M2 – and probably M4 – receptors may bring about greater activation of G proteins when compared with M3 receptors, which may explain the predominance of M2 responses in some cholinergic-stimulated tissues (Baumgold and Drobnick, 1989). Thus, if the likely integrative function of M2 receptors in the synchronization of excitatory and inhibitory mechanisms during UGR were blocked with the M2 antagonists methoctramine or AFDX, we could explain the gradual, inhibitory, dose-dependent response observed in our results (Fig. 5A–B). The opposite effect was observed when the M2 receptors were stimulated by the agonist arecaidine (Fig. 7E–F), which induces UGR recovery and may confirm our suggestion.

A potential explanation for the results observed after M3 and M4 antagonist application could be related to the increase in acethylcholine release after urethral stimulation from the pudendal and pelvic afferent neurons. Given the more limited populations of M3 and M4 receptors in the spinal cord (Höglund and Baghdoyan, 1997), the high concentration of Ach released during UGR could overcome the blockage of the 4DAMP (M3) and tropicamide (M4) antagonists. Only the facilitation produced by Ach was observed in our data (Fig. 5C–F). Again, it is important to remember that in this model, excitatory responses are facilitated, because the serotonergic descending inhibition from the paragigantocellularis nucleus was abolished by way of the spinal cord transection at T6.

Another potential explanation for the results obtained with M3 and M4 antagonists could be the potentiation of excitatory responses by the activation of other excitatory neurotransmitters, but it is also possible that M4 receptors may function as auto-receptors in interneurons or motoneurons, since the response observed with tropicamide (M4 antagonist) was stronger than that observed with 4DAMP (M3 antagonist). The presence of M4 auto-receptors has been demonstrated in other sites in the central nervous system (McKinney et al., 1993).

Marson et al. (2003), using Fos immunohistochemistry techniques, demonstrated that the spinal circuits associated with the UGR involve afferent neurons from the pudendal sensory nerves, which enter the spinal cord at segments L6-S1 and relay on neurons in the medial dorsal horn and dorsal gray commissure (DGC). These authors also found that efferent output involves preganglionic neurons in the lateral gray of L5-S1 and lateral and medial gray of T13-L2. Spinal interneurons were also found in the dorsal horn, and in the intermediate and medial gray of T12-S1. These studies demonstrated a multi-segmental spinal circuit, activated during the UGR, which is inhibited by a descending serotonergic pathway from the paragigantocellularis nucleus. Another recent study (Stewart and Maxwell, 2003), using immunoreactivity methods, has shown that the muscarinic M2 receptor was present in the grey matter of the lumbar spinal cord, with a high density of labeling found in the superficial dorsal horn motor nuclei and in lamina X. In that study, a significant amount of presumed inhibitory interneurons showed immunoreactivity for the M2 receptor, while excitatory neurons did not display immunoreactivity.

Although our work did not show any direct evidence regarding the exact location of the muscarinic receptors involved in UGR, based on in our results and the recent, aforementioned published data, allow us to suggest that in the elicitation of UGR, M2 receptors, probably colocated with other inhibitory and excitatory receptors on interneurons located in the medial dorsal horn, mainly in Lamina X, are involved in modulatory functions. Those interneurons receive synaptic input from afferents of the pelvic and hypogastric nerves, and project synaptically to other interneurons and motoneurons within a network of recurrently connected neurons. The excitatory activity of M3, and

probably M4, receptors located on motoneurons situated on the dorsomedial (DM) and dorsolateral (DL) nucleus of the L5-S1 spinal segment could explain the contractile responses observed on the bulbospongiosus muscles. It is possible, therefore, that a cholinergic spinal cord system mediated by M2, M3 and M4 receptors could modulate synchronous patterns of motor activity such as UGR, other sexual reflexes and motor behaviors, after the recruitment of cholinergic neurons which could produce oscillatory activity by means of interconnection with other interneurons and motoneurons in a local spinal circuit.

In support of this hypothesis, the regulation of the intrinsic response properties of motoneurons by muscarine and other metabotropic synaptic receptors has been demonstrated in the spinal cord of the turtle (Delgado-Lezama et al., 1997; Svirkis and Hounsgaard, 1998; Alaburda et al., 2002), and both excitatory (via M3-type receptors) and inhibitory (via M2-type receptors) have been identified on motoneurons of the spinal cord of neonatal rats (Jiang and Dun, 1986). It is known that in addition to UGR and sexual behavior facilitation, the stimulation of spinal cholinergic neurons elicits locomotor-like activity in the neonatal rat's spinal cord (Cowley and Smith, 1994, 1997) and in the spinal cord of decerebrate cats during fictive-locomotion (Huang et al., 2000).

Further support for our suggestion comes by the finding of a cholinergic propriospinal system, which has been located in the spinal cord by some researchers (Sherriff and Henderson, 1994), and which is made up of cholinergic neurons mainly located around the central canal (Lamina X). This system may be active during programmed motor activities such as UGR, sexual behavior, and other motor activities (Carr et al., 1995; Huang et al., 2000). Since these cholinergic neurons are interconnected on the spinal cord, forming longitudinal and transverse bundles (Houser et al., 1983; Barber et al., 1984; Borges and Iverson, 1986; Woolf, 1991), it is possible that cholinergic modulation of sexual reflexes is carried out by interneurons and motoneurons bunched in this specific area of the spinal cord (Segment L5-S1), activated during muscarine application, and that M2, M3 and M4 receptors located on these neurons are involved in UGR modulation.

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