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Mucosal modulation of contractility in bladder strips from normal and overactive rat models and the effect of botulinum toxin A on overactive bladder strips

Patrick C Campbell*, Bronagh McDonnell*, Kevin P Monaghan, Lauren Baysting, Oonagh Little and Karen D McCloskey

Centre for Cancer Research and Cell Biology, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, 97 Lisburn Road, Belfast, BT9 7AE, Northern Ireland, UK

* Patrick C Campbell and Bronagh McDonnell contributed equally to this study

Author for correspondence:

Karen D McCloskey

Centre for Cancer Research and Cell Biology,

School of Medicine, Dentistry and Biomedical Sciences,

Queen's University Belfast,

97 Lisburn Road,

Belfast, BT9 7AE, Northern Ireland, UK

k.mccloskey@qub.ac.uk

0044 2890 972386

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Short title: Mucosa and botox on bladder contraction

Abstract

Aims To investigate the local, regulatory role of the mucosa on bladder strip contractility from normal and overactive bladders and to examine the effect of botulinum toxin A (BoNT-A).

Methods Bladder strips from Spontaneously Hyperactive Rat (SHR) or normal rats (Sprague Dawley, SD) were dissected for myography as intact or mucosa-free preparations. Spontaneous, neurogenic and agonist-evoked contractions were investigated. SHR strips were incubated in BoNT-A (3 hours) to assess effects on contractility.

Results Spontaneous contraction amplitude, force-integral or frequency were not significantly different in SHR mucosa-free strips compared with intacts. In contrast, spontaneous contraction amplitude and force-integral were smaller in SD mucosa-free strips than in intacts; frequency was not affected by the mucosa. Frequency of spontaneous contractions in SHR strips was significantly greater than in SD strips.

Neurogenic contractions in mucosa-free SHR and SD strips at higher frequencies were smaller than in intact strips. The mucosa did not affect carbachol-evoked contractions in intact vs mucosa-free strips from SHR or SD bladders.

BoNT-A reduced spontaneous contractions in SHR intact strips; this trend was also observed in mucosa-free strips but was not significant. Neurogenic and carbachol-evoked contractions were reduced by BoNT-A in mucosa-free but not intact strips. Depolarization-induced contractions were smaller in BoNT-A treated mucosa-free strips.

Conclusions The mucosal layer positively modulates spontaneous contractions in strips from normal SD but not overactive SHR bladder strips. The novel finding of BoNT-A reduction of contractions in SHR mucosa-free strips indicates actions on the detrusor, independent of its classical action on neuronal SNARE complexes.

Introduction

Local control of bladder contractility by cells in the mucosal layer which include urothelium, lamina propria interstitial cells (IC-LP), nerves, smooth muscle and microvessels is increasingly recognised (1). *In vitro* myography of bladder strips has been widely used to decipher the contribution of the tissue layers in the bladder wall to contractility of the strip. The influence of urothelial and lamina propria components which collectively comprise the mucosa, on spontaneous, neurogenic and agonist-evoked contractions is dependent on complex signalling processes.

Intrinsic, non-voiding or spontaneous contractions occur in normal, healthy bladders during filling and contribute to the maintenance of optimal bladder tone (2). Experimentally, spontaneous contractions in *ex vivo* bladder strips from human or rodent bladders are regarded to be representative of non-voiding contractions, which are pronounced in pathophysiologies. Intact strips exhibit robust spontaneous contractions; interestingly mucosal and detrusor layers separated by sharp dissection also have spontaneous contractions (4, 5). Smooth muscle in the mucosa known as muscularis mucosae has physiological and pharmacological properties that are distinct from detrusor smooth muscle and have been reported for some species but not rat (3, 4). Muscularis mucosae contractions may act to periodically move the urothelium layer, preventing prolonged contact with elements in the urine; moreover, it is reported that muscularis mucosal contraction directly contributes to the contractility of guinea-pig intact strips (3).

Cells within the mucosal layer release signalling molecules which modulate bladder strip contraction. Purinergic receptor-mediated (5) and muscarinic receptor-mediated release of ATP (6) from urothelial cells is reportedly associated with mucosal layer contraction. The urothelium also exerts an inhibitory influence on bladder strip contractility and several groups have noted the existence of an urothelium derived inhibitory factor, UDIF. Hawthorn et al. (7) reported smaller carbachol-evoked contractions in intact strips of pig bladder compared with mucosa-free strips; moreover, mucosa-free strip contractions were inhibited by the presence of a second intact strip indicating release of a modulatory signal. The exact identity of this diffusible UDIF is not yet known although several candidates have been proposed and excluded (8).

Botulinum toxin type A (BoNT-A) is used to treat anti-muscarinic refractory detrusor overactivity (DO) where involuntary detrusor contractions can be observed and overactive bladder syndrome (OAB) where DO may or may not be demonstrable (9). The established mechanism of action of BoNT-A on neurones involves cleavage of synaptosome-associated protein-25 (SNAP-25), a component of the neuronal SNARE complex, preventing neuronal transmitter release. It is likely that there are other targets of BoNT-A in the bladder which contribute to its effect on OAB symptoms including an effect on sensory bladder function. In spinal cord injured (SCI) rats, BoNT-A significantly reduced ATP release into the bladder lumen, presumably from the urothelium (10) and in patients with neurogenic and idiopathic detrusor overactivity, BoNT-A decreased expression of sensory receptors $P2X_3$ and TRPV1 (11). SNAP-25 expression has been identified in human and mouse urothelium and BoNT-A was found to inhibit ATP release from urothelial cells (12). In contrast, in another study, SNAP-25 was not detected in human urothelial or muscle cells but in nerve fibres that permeated the urothelium and muscle layers (13).

Increased expression of Cx26 and Cx43 gap junction proteins on urothelial cells and IC-LP respectively in SCI rat overactive bladder was consistent with the ability of gap junction uncouplers to inhibit spontaneous activity (14). Reduced Cx43 expression in patients with DO treated with BoNT-A may indicate that IC-LP are affected by BoNT-A (15). A study on suburothelial vs intradetrusor BoNT-A injection in patients found that both modes were similarly effective (16).

We hypothesize that BoNT-A may have multiple, cellular targets of action in the bladder wall. The aims of the present study were to: (1) investigate the impact of the mucosa on spontaneous, neurogenic and carbachol-evoked contractions in normal and overactive bladder using the spontaneously hypertensive rat (SHR, 17) and (2) determine whether BoNT-A has a direct effect on these contractile parameters in intact and mucosa-free strips from overactive bladder.

Methods

Tissue preparations

Male SHRs (18-20 weeks, N=18) were purchased from Charles River Laboratories International Inc. and male Sprague Dawley rats (18 weeks, N=12) were purchased from Harlan UK and were sacrificed by cervical dislocation or CO_2 inhalation in accordance with Schedule 1 Animal (Scientific Procedures) Act (UK Home Office), European Directive 2010/63/EU and local ethical committee approval. Bladders were removed, opened longitudinally and pinned to a Sylgard dissecting dish. The trigone was removed and the bladder body was divided longitudinally. In one half, the mucosal layer was removed by sharp dissection under a dissecting microscope and the underlying detrusor layer cut into four strips (10mm x 2mm x 2mm); four full-thickness strips were prepared from the remaining half.

In vitro myography

Bladder strips were mounted vertically in organ bath chambers, attached by thread at one end to tension transducers and by thread at the other end to a fixed point. Tissues were continually perfused (2 $ml.min^{-1}$) with oxygenated (95% O_2/5% CO_2, 37^oC) Krebs' solution (mM: 120 NaCl, 5.9 KCl, 11 glucose, 25 $NaHCO_3$, 1.2 Na_2HPO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$). An initial tension of 1g was applied and the strips left to equilibrate for 1 hour during which time spontaneous contractions typically developed.

Bladder strips were stimulated with carbachol (1 µM, 5 min), ATP (5mM, 3 min) or 60mM K^+ in addition to stimulation of intramural nerves via electrical field stimulation (EFS, 70V, 0.3ms pulse width, 10s duration over the frequency range 0.5, 1, 2, 4, 8, 16Hz). Spontaneous activity and stimulation responses were recorded using Intracept Chart v4.9 software and analysed as contraction amplitude and force-integral Area Under Curve (AUC) using Clampfit v10.3 and Prism v5.0 (GraphPad) software. A 5 minute portion of the recording where spontaneous activity was established, prior to the perfusion of any agonists, was used for analysis. For amplitude and frequency measurement, a threshold was set which was 10% of the maximal contraction amplitude above the baseline; mean amplitude and frequency over the 5 minutes was calculated for each strip. Contractions in response to carbachol, ATP or 60mM K^+ were measured as AUC over 3 minutes from contraction onset. Data are presented as scatter plots with median indicated for spontaneous activity figures or as box and whisker plots showing median, interquartile range and minimum/maximum values. N and n denote number of animals and strips respectively. Data sets from strips were compared with Mann Whitney tests or for EFS protocols, two-way ANOVA with Bonferroni post-hoc test using Prism with $P<0.05$ considered as significant ($P<0.05$, $P<0.01$ and $P<0.001$ are denoted as *, ** and *** respectively on graphs).

Incubation with botulinum toxin A

Tissue strips from SHR bladders were assigned to control or treatment groups. In the treatment group, tissues were incubated in BoNT-A (30nM, Botox®, Allergan) reconstituted with HEPES-Krebs' solution (mM: 125 NaCl, 5.36 KCl, 11 glucose, 10 HEPES, 0.44 KH_2PO_4, 0.33 NaH_2PO_4, 1 $MgCl_2$, 1.8 $CaCl_2$) for 3 hours in a tissue culture incubator (37 ^oC, humidified 20% O_2). Control group tissues were incubated in HEPES-Krebs' solution in the same conditions.

Results

Spontaneous activity and mucosal modulation

Tissue strips from SHR overactive (17) and Sprague Dawley (SD) normal bladders typically developed spontaneous activity during the equilibration period. This activity was insensitive to tetrodotoxin (1μM) confirming its non-neurogenic nature. Representative examples are shown in **Figure 1a** for activity in intact and mucosa-free strips from each animal model. The summary data demonstrates that in SHR strips, absence of the mucosal layer did not affect AUC, average amplitude or frequency ($P>0.05$; N=15, intact n=23, mucosa-free n=20 (**Figure 1b,** $P>0.05$). In SD bladders, spontaneous activity AUC and amplitude were smaller in mucosa-free strips (**Figure 1c,** $P=0.02$ and $P=0.003$ respectively; N=8, n=15 intact, n=15 mucosa-free) whereas frequency was not affected ($P>0.05$).

Comparison of the data between strains demonstrated that the frequency of spontaneous contractions was smaller in both intact and mucosa-free strips from SD vs SHR bladders ($P=0.005$ and $P=0.004$ respectively). Contraction amplitude was not different in intact or mucosa-free strips and AUC was smaller in mucosa-free SD strips ($P=0.01$).

Contractions evoked by carbachol

Application of carbachol (1 μM) evoked similar contractions in SHR intact (N=9, n=14) and mucosa-free (N=9, n=14) tissues (**Figure 2a)** ($P>0.05$). Similarly, in SD bladder, carbachol-evoked contractions were not different whether or not the mucosal layer was present (N=8, n=16 intact and mucosa-free, $P>0.05$). Summary data is presented in **Figure 2b.** Responses to carbachol were not significantly different between strains either for intact or mucosa-free strips ($P>0.05$).

Neurogenic contractions

Electrical field stimulation was used to evoke neurogenic contractions across a frequency range (0.5, 1, 2, 4, 8, 16Hz). These responses were sensitive to tetrodotoxin (1μM) confirming their neurogenic origin. Neurogenic contraction amplitude in SHR mucosa-free strips (N=11, n=18) at the higher frequencies (8 and 16 Hz) was significantly smaller than in intact strips (N=11, n=18) ($P<0.01$) indicating that the mucosal layer contributed positively to contraction amplitude (**Figure 3a, b**). In the normal rat SD bladder, neurogenic contractions were also significantly smaller in intact strips (N=4, n=7) compared with mucosa-free strips (N=4, n=7) at 8Hz ($P<0.01$) and 16Hz ($P<0.001$, **Figure 3c, d**).

The effect of botulinum toxin A on contractions in SHR bladder strips

Intact and mucosa-free SHR strips were incubated in 30nM BoNT-A for 3 hours as described in methods (18). Smith et al. (10) and van Uhm *et al.* (19) used similar protocols to treat normal bladder tissues with BoNT-A; it has been suggested that BoNT-A may reach cells within the bladder wall via endocytosis and transcytosis (12). In this series of experiments, control tissues were treated in the same fashion but with the omission of BoNT-A. Contractions were generally smaller after the 3 hour incubation although they were within the range of values reported for experiments in figures 1-3. Spontaneous activity was reduced by BoNT-A (**Figure 4a, b**) in intact strips as measured by AUC (N=4, n=4 control; N=6, n=6 treated; $P=0.026$). In mucosa-free strips, AUC was reduced by BoNT-A however this was

not significant. Contraction frequency was not affected by BoNT-A in intact or mucosa-free strips ($P>0.05$).

Carbachol-evoked contractions (**Figure 4c, d**) were significantly reduced by BoNT-A in mucosa-free strips (n=7 (N=7) control, n=11 (N=9) treated; $P<0.05$) whereas these were not affected in intact strips (n=6, N=6 control; n=12, N=10 treated; $P>0.05$). The shape of carbachol responses was similar in BoNT-A treated and untreated strips however, the magnitude was markedly smaller. ATP-evoked contractions in mucosa-free strips were not significantly affected by incubation in BoNT-A (n=7, N=7 control; n=11, N=9 treated). Depolarisation-induced contractions by 60mM external K^+ solution, were reduced by BoNT-A in mucosa-free strips ($P<0.05$) indicating that smooth muscle contraction was negatively impacted. Interestingly, depolarization-evoked contractions in intact strips were not significantly affected by BoNT-A ($P>0.05$), suggesting that mucosal signalling may have masked the effect on mucosa-free contraction.

Neurogenic-contractions were significantly reduced by BoNT-A (**Figure 5a, b**) in mucosa-free strips at 4, 8 and 16 Hz (N=6, n=7 control; N=9, n=11 treated; $P<0.05$) whereas those in intact strips were not affected (N=6, n=6 control; N=9, n=12 treated; $P>0.05$).

Discussion

Spontaneous activity

The present study has shown that the mucosal layer provides a positive contribution to myogenic spontaneous activity in normal SD rat bladder but not in the overactive model, SHR. Little is known of SHR bladder strip contractility; overactivity of the SHR bladder itself is considered to be due to both peripheral and spinal mechanisms with a defect in noradrenergic control of micturition reflexes (17). The smooth muscle content of the SHR bladder wall is reportedly similar to normal rat bladder with no difference in the size, orientation or spacing of smooth muscle bundles (20). Non-contractile elements of the SHR bladder mucosa were reported to exhibit hypertrophy including increased type I collagen, a higher number of inflammatory cells and larger suburothelial spaces. Our findings show that spontaneous activity in SHR is of significantly higher frequency than in SD strips. In normal bladder models, the effect of mucosal layer removal (lamina propria and urothelium) or removal of only the urothelium on bladder strip contractility has been reported. In a study of normal SD rat bladder strips, spontaneous contraction amplitude was apparently smaller in strips with urothelium removed compared with intacts (statistics not reported in 18). This phenomenon has also been reported for guinea-pig bladder where removal of the mucosa resulted in reduced mucosa-free strip spontaneous activity (3). There may be contractile elements within the mucosal layer which contribute to the larger activity in intact bladder strips; in guinea-pig bladder, this has been attributed to muscularis mucosae smooth muscle contraction (3); however, this is perhaps unlikely to be the case in the present study given that such a tissue layer has not been reported for rat bladder (4). An explanation for enhanced activity in normal bladder intact strips may involve release of excitatory factors such as ATP from the mucosal layer which would act to enhance contraction of the detrusor smooth muscle (5, 6).

Neurogenic contractions

Our observation that removal of the mucosal layer reduced neurogenic contraction amplitude of SHR and normal bladder strips at all frequencies, with these differences being significant at higher frequencies. The mechanisms underlying this observation are not understood but it may be related to the relative proportions neurotransmitters where purinergic mechanisms are thought to be dominant at the lower frequencies and a larger cholinergic component at higher frequencies. Our finding that that carbachol-contractions were similar in intact and mucosa-free strips in SHRs consistent with Schneider *et al.* (22), indicates that expression of cholinergic receptors across the SHR bladder wall does not account for the neurogenic contraction difference. The findings in the present study for normal bladder are consistent with (23) which reported that neurogenic contractions were smaller in urothelium-free strips. Aronsson *et al.* (24) reported that TTX-sensitive neurogenic contractions in normal SD bladders were similar in intact and urothelium-free strips where the urothelium had been removed by collagenase treatment and mechanical scraping. The mode of removal of the urothelial layer by rubbing or the entire mucosa (urothelium and lamina propria) by sharp dissection as per the present study may account for some of the differences in observations within the published studies.

Carbachol-evoked contractions

In both SHR and normal bladder, carbachol-evoked contractions were similar in intact and mucosa-free strips indicating that the mucosal layer did not make a significant contribution. This is consistent with the finding of similar relative contributions of muscarinic receptor subtypes and muscarinic receptor-mediated contractions in SHR bladders compared with controls (22). In keeping with the present study, Aronsson *et al.* (24) found carbachol contractions over the range 10 nM–0.1 mM to be similar in mucosa-free and intact SD strips. In contrast (21) reported that intact SD strips were less sensitive to carbachol and concluded the mucosa exerted an inhibitory influence. Again, the opposite was reported by Munoz et al (23) who found carbachol-contractions to be smaller in urothelium-free strips. There are likely to be several reasons for differences in findings by independent groups which may include mode of removal of the mucosa; whether the lamina propria remained and the concentration range of carbachol used.

The lack of effect of mucosal-removal in the present study on carbachol-evoked contractions may reflect a composite from concomitant removal of a mucosal-derived inhibitory factor and removal of mucosal contractile elements.

Impact of BoNT-A on SHR contractility

The benefits of BoNT-A treatment to anti-muscarinic-refractory DO and OAB syndrome are widely accepted. While the canonical mechanism of action of BoNT-A on neurons is well understood, it is likely that there are additional effects on other cells of the bladder wall that modulate bladder activity. We adopted similar methodology to test the effect of BoNT-A on bladder tissue to others where the mechanism of access to cells within the tissue is considered to be endocytosis and transcytosis during a 3 hour incubation (10, 12, 18, 19). *In vivo* injections of BoNT-A to guinea-pig bladders reportedly did not generate cSNAP-25^{+} fibres at 6 hours; between 12 and 24 hours was required for this mechanism (13). Our experimental design therefore enabled us to test BoNT-A on contractility independent of the canonical mechanism of action where the presence of cSNAP-25 is used as a marker of neurotoxin action. Our findings demonstrate that BoNT-A directly reduced spontaneous activity in intact and mucosa-free strips (although the latter did not reach significance) which may point to an action of BoNT-A on smooth muscle or interstitial cells to diminish spontaneous activity in the SHR model of overactive bladder. Furthermore, neurogenic contractions and carbachol-evoked contractions were decreased by BoNT-A in mucosa-free strips. A reduction of neurogenic contractions in mice treated with BoNT-A, 48 hours previously was also reported by Ikeda et al (25) and Takahashi et al (18) reported smaller contractions after BoNT-A incubation. Interestingly, the impact of BoNT-A on neurogenic and carbachol-evoked contractions was not found in intact strips indicating that BoNT-A was acting on cells in the detrusor and that its effect in intact strips may have been masked by inhibitory mucosal signalling. ATP-evoked contractions showed a trend towards reduction by BoNT-A, however, this was not significant. The finding that depolarization-induced contractions were reduced by BoNT-A in mucosa-free strips but not in intacts, was also consistent with the view that an action of BoNT-A on detrusor smooth muscle contraction was being masked by mucosal signalling.

In conclusion, our findings demonstrate that the mucosal layer positively modulates spontaneous contractions in normal rat bladder strips but not in the overactive SHR model. An overall mucosal effect on carbachol-evoked contractions was not found in normal bladder

or SHR strips. The mucosal layer positively modulates higher frequency neurogenic contraction in strips from both strains.

BoNT-A reduced spontaneous activity in SHR bladder strips and also reduced neurogenic, carbachol-evoked and depolarization-induced contractions in SHR mucosa-free strips indicating an action on the detrusor layer, independent of the mucosa. This work reveals effects of BoNT-A on bladder contractility in addition to its classical effect on the neuronal SNARE complex.

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Figure Legends

Figure 1. Impact of the mucosal layer on spontaneous activity in overactive and normal rat bladder strips.
a: Representative traces of spontaneous contractile activity in intact or mucosa-free strips from SHR (overactive) and SD (normal) rat bladder.
b: Scatter plots (median denoted by line) of area under curve (AUC), average contraction amplitude and frequency in SHR bladder; these were not different in mucosa-free strips (intact n=23, N=15; mucosa-free n=20, N=15).
c: AUC and contraction amplitude were smaller in mucosa-free SD strips; frequency was not affected by the presence/absence of the mucosa (intact n=15, N=8; mucosa-free n=15, N=8). Mann Whitney tests; * denotes P<0.05).

Figure 2. Carbachol-evoked contractions are not impacted by the mucosal layer in SHR or SD rat bladder strips.
a: Representative traces of cholinergic contractions evoked by carbachol (1μM) in intact or mucosa-free strips from SHR (overactive) and SD (normal) rat bladder.
b: Summary plots (median, interquartile range and min/max) of area under curve (AUC) in both animal models. Carbachol-contractions were similar in the presence and absence of the mucosal layer in SHR (intact and mucosa-free n=14, N=9) and normal SD (intact and mucosa-free n=16, N=8) strips (Mann Whitney tests; p>0.05).

Figure 3. Neurogenic contractions are enhanced by the mucosal layer in SHR bladder strips
a. Representative traces of electrical field stimulation (EFS) evoked contractions in intact and mucosa-free SHR bladder strips.
b. Summary plots (median, interquartile range and min/max) of mean contraction amplitude at frequencies tested (n=18, N=11). EFS-contractions in mucosa-free strips were smaller at each frequency, this was significant at 8Hz and 16Hz (** p<0.01, 2 way ANOVA repeated measures, Bonferroni post-hoc test).
c. Representative traces of EFS-evoked contractions in intact and mucosa-free SD bladder strips.
d. Summary plots (median, interquartile range and min/max) of mean contraction amplitude at frequencies tested (n=7, N=4). EFS-contractions in mucosa-free strips were smaller at each frequency, this was significant at 8Hz and 16Hz (** p<0.01, *** p<0.001, 2 way ANOVA repeated measures, Bonferroni post-hoc test).

Figure 4. Impact of BoNT-A incubation on spontaneous activity and agonist-evoked contractions in SHR strips
a. Representative traces of spontaneous activity in mucosa-free SHR bladder strips in the presence and absence of BoNT-A.
b. Summary plots (median, interquartile range and min/max) of mean spontaneous activity (AUC and amplitude) in intact and mucosa-free strips. Activity was smaller in the BoNT-A treated strips compared with controls (n=4, N=4 control; n=6, N=6 treated). Mann Whitney tests; * denotes P<0.05).
c. Representative traces of contractions evoked by carbachol (1μM), ATP (5mM) and 60mM K^+ in detrusor SHR strips, control and pre-incubated in BoNT-A.
d. Summary plot (median, interquartile range and min/max) of mean evoked contractions (AUC) in mucosa-free strips by carbachol (1μM), ATP (5mM) or 60mM K^+. Carbachol and K^+-evoked contractions were smaller in the BoNT-A treated strips compared with controls (n=7, N=7 control; n=11, N=9 treated). Mann Whitney tests; * denotes P<0.05.

Figure 5. Impact of BoNT-A incubation on neurogenic contractions in SHR mucosa-free strips

a. Representative traces of neurogenic-contractions in mucosa-free SHR bladder strips in the presence and absence of BoNT-A.

b. Summary plots (median, interquartile range and min/max) of mean contraction amplitude where BoNT-A reduced contractions at each frequency tested (N=6, n=7 control; N=9, n=11 treated; two-way ANOVA repeated measures, Bonferroni post-hoc test; * $p<0.05$, ** $p<0.01$).

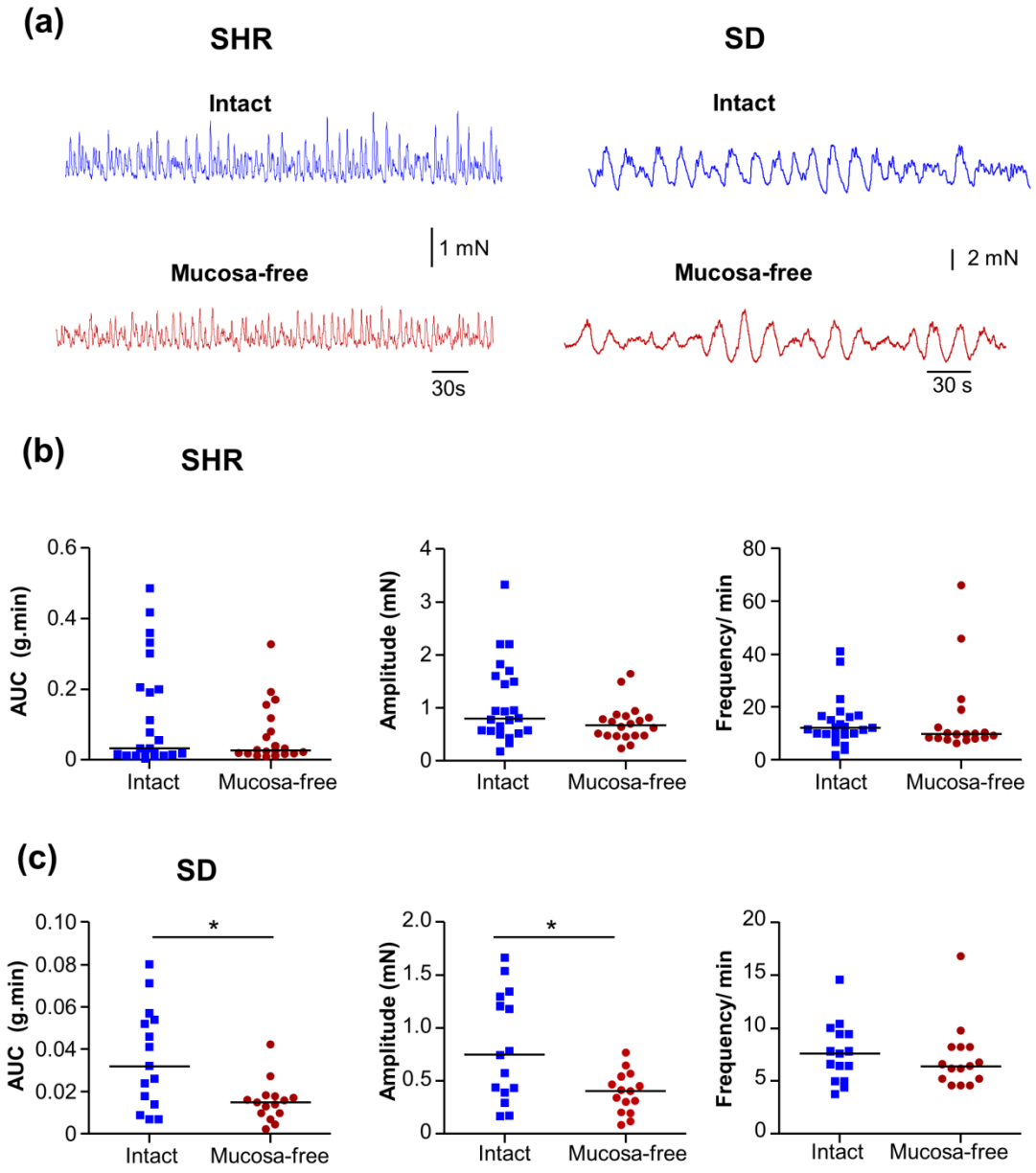


Figure 1. Impact of the mucosal layer on spontaneous activity in overactive and normal rat bladder strips.

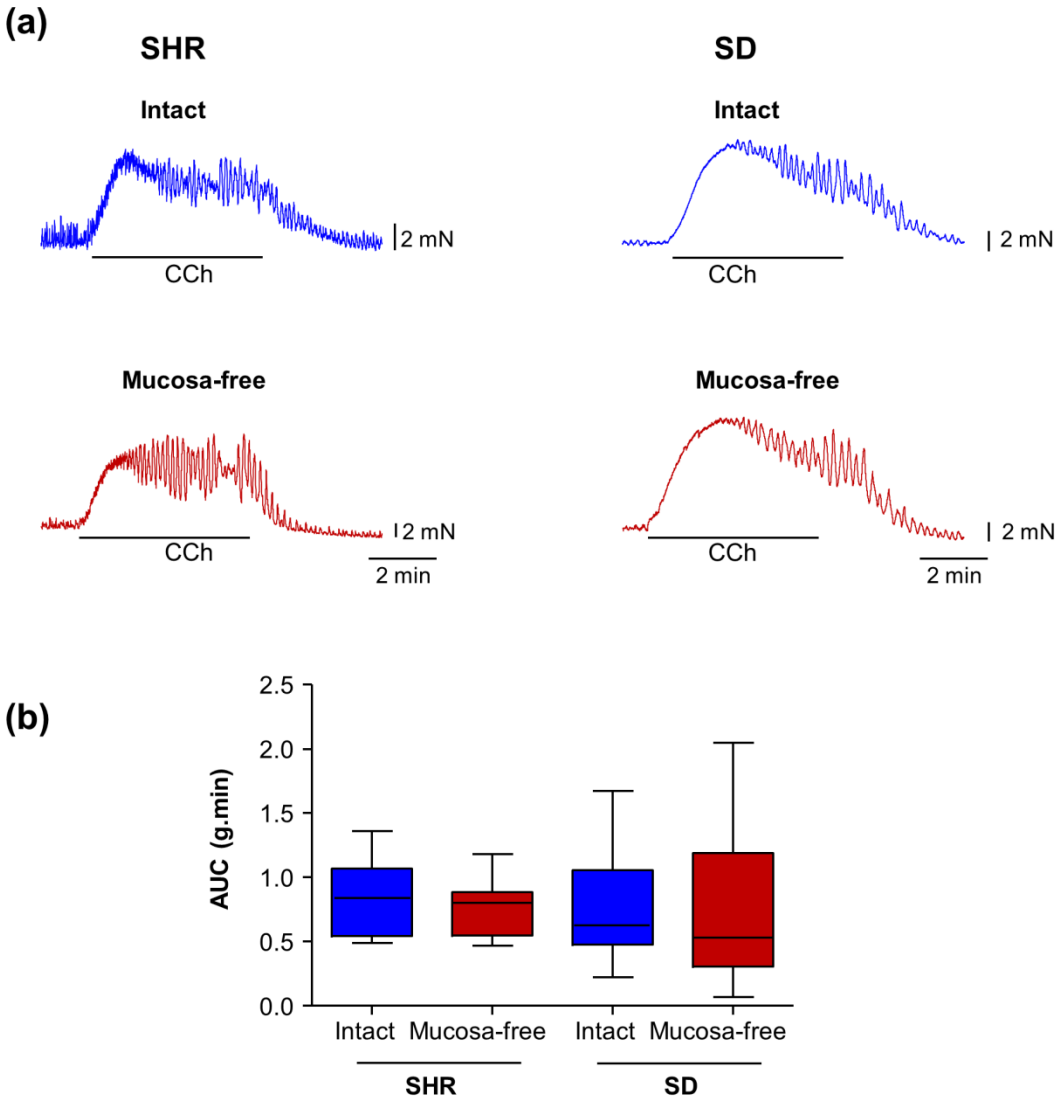


Figure 2. Carbachol-evoked contractions are not impacted by the mucosal layer in SHR or SD rat bladder strips.

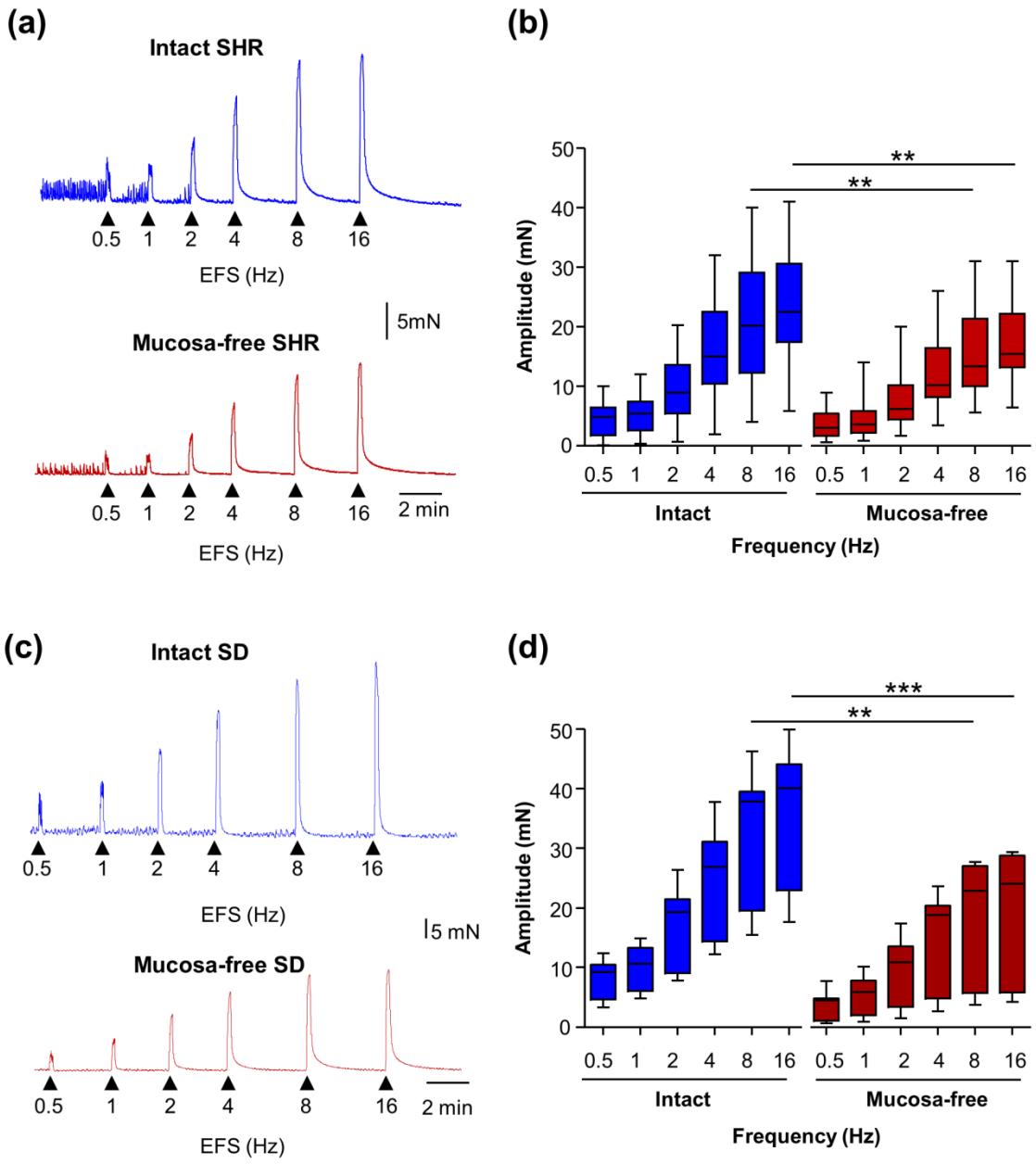


Figure 3. Higher frequency neurogenic contractions are enhanced by the mucosal layer in SHR and SD bladder strips

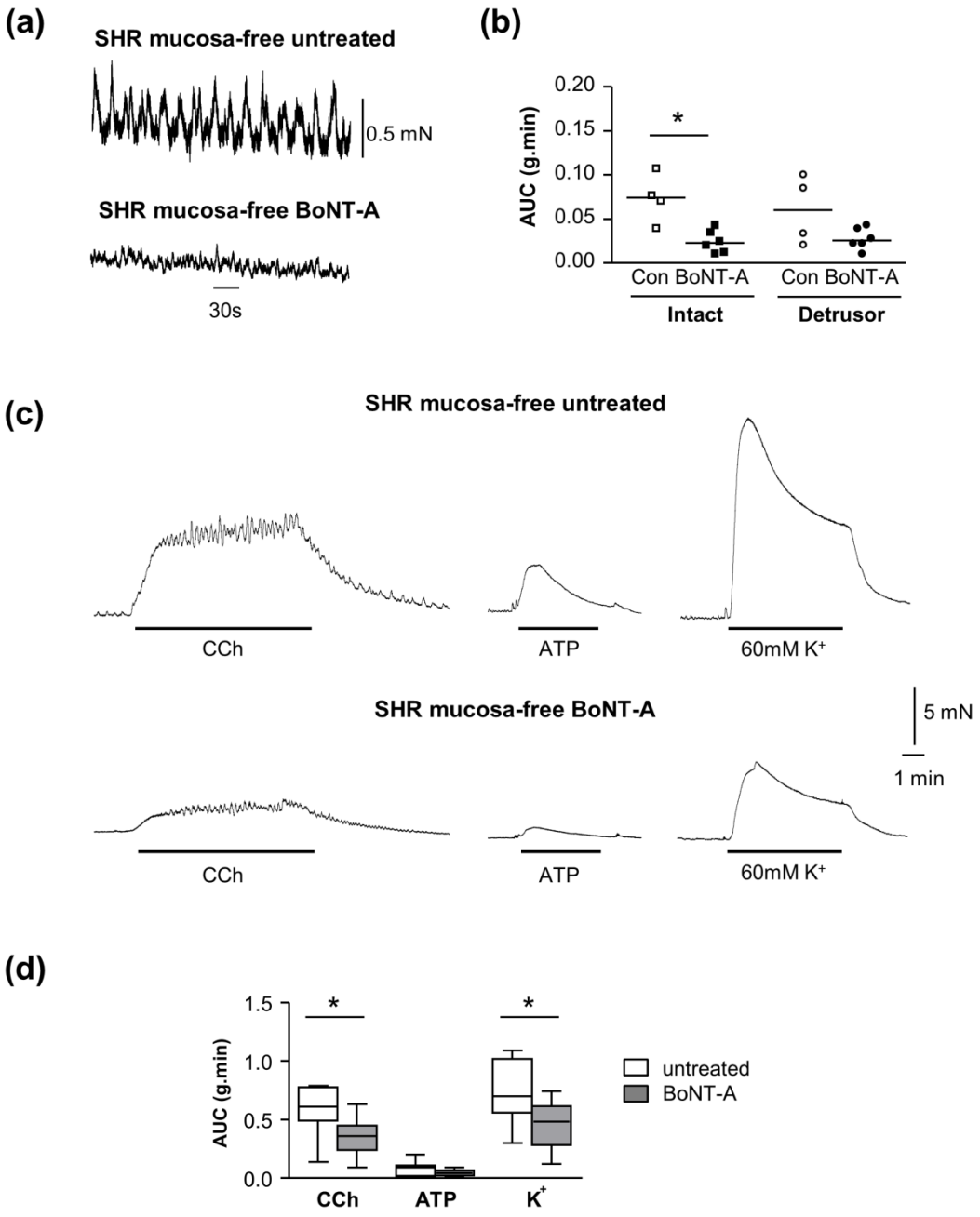
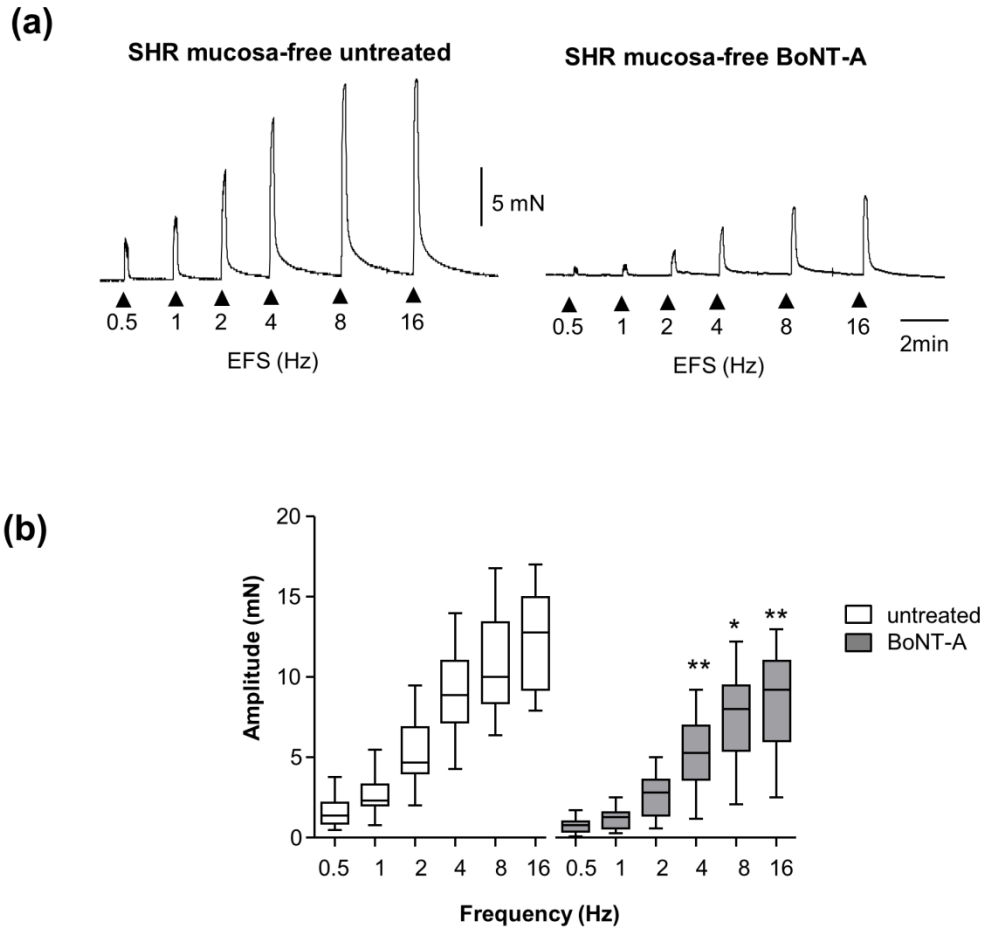


Figure 4. Impact of BoNT-A incubation on spontaneous activity and agonist-evoked contractions in SHR strips


(a)
SHR mucosa-free untreated
SHR mucosa-free BoNT-A
5 mN
0.5 1 2 4 8 16
EFS (Hz)
0.5 1 2 4 8 16
EFS (Hz)
2min
(b)
Amplitude (mN)
20
15
10
5
0
0.5 1 2 4 8 16 0.5 1 2 4 8 16
Frequency (Hz)
**
*
**
untreated
BoNT-A