Excitatory cholinergic and purinergic signalling in bladder are equally susceptible to botulinum neurotoxin A consistent with co-release of transmitters from efferent fibres

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Running Title Page

Running Title: Equi-potent inhibition of ACh and ATP release by BoNT/A

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ABBREVIATIONS: αβMeATP, α,β-methylene-adenosine-5'-triphosphate; ACh, acetylcholine; BoNT, botulinum neurotoxin; IDO, idiopathic detrusor over-activity; NDO, neurogenic detrusor over-activity; OAB, over-active bladder; P2, purinergic receptor; PPADS, pyridoxal-5’-phosphate-6-azophenyl-2’, 4’-disulphonic acid; SNAP-25, synaptosomal-associated protein of Mr = 25k; SV2, synaptic vesicle protein 2; TTX, tetrodotoxin; VAMP, vesicle-associated membrane protein

Section: Neuropharmacology
Abstract

Mediators of neuromuscular transmission in rat bladder strips were dissected pharmacologically to examine their susceptibilities to inhibition by botulinum neurotoxins (BoNTs) and elucidate a basis for the clinical effectiveness of type A in alleviating smooth muscle spasms associated with over-active bladder. BoNT/A, /C1 or /E reduced peak and average force of muscle contractions induced by electric field stimulation (EFS) in dose-dependent manners by acting only on neurogenic, tetrodotoxin-sensitive responses. BoNTs that cleave vesicle-associated membrane protein (VAMP) proved much less effective. Acetylcholine and ATP were found to provide virtually all excitatory input because EFS-evoked contractions were abolished by the muscarinic receptor antagonist, atropine, combined with either a desensitising agonist of P2X₁ and P2X₃ or a non-selective ATP-receptor antagonist. Both transmitters were released in the innervated muscle layer and, thus, persisted after removal of urothelium. Neither atropine nor a desensitiser of the P2X₁ or P2X₃ receptors altered the rate at which muscle contractions were weakened by BoNT/A. Moreover, though cholinergic and purinergic signalling could be partially delineated using high frequency EFS (which intensified a transient, largely atropine-resistant, spike in muscle contractions that was reduced following P2X-receptor desensitisation), they proved equally susceptible to BoNT/A. Thus, equi-potent blockade of ATP co-released with acetylcholine from muscle efferents likely contributes to the effectiveness of BoNT/A in treating bladder over-activity, including non-responders to anti-cholinergic drugs. As purinergic receptors are known mediators of sensory afferent excitation, inhibition of efferent ATP release by BoNT/A could also help to ameliorate acute pain and urgency sensation reported by some recipients.
Introduction

Safe and effective management of over-active bladder (OAB) (Schurch and Dykstra, 2009), involving either neurogenic detrusor over-activity (NDO) or idiopathic detrusor over-activity (IDO), can be achieved by injecting botulinum toxin (BoNT) type A (A, the active ingredient in BOTOX®) — an effect attributed to blockade of acetylcholine (ACh) release from smooth muscle efferents. Its specificity arises from binding to gangliosides plus synaptic vesicle protein 2 (SV2) (Dong et al., 2006) and efficacy from proteolysis of synaptosomal-associated protein of Mr = 25k (SNAP-25), which is essential for synaptic vesicle fusion (Blasi et al., 1993). Notably, BOTOX® often proves effective in non-responders to antimuscarinic drugs (Schurch and Dykstra, 2009) and many recipients report sensory benefits that suggest decreased release of other neurotransmitters/modulators (Apostolidis et al., 2006). In fact, BoNT/A blocks atropine-resistant contractions in guinea-pig bladder (MacKenzie et al., 1982). Purines are implicated because ATP elicits muscle contractions and electric field stimulation (EFS) induces its release in bladder (Burnstock et al., 1978). A purine-gated ion channel, P2X1, is found in detrusor muscle (Vial and Evans, 2000). Also, another subtype P2X3 is enriched on sensory afferents and its role in voiding control has been established by the pronounced bladder hypo-reflexia seen in a P2X3 knock-out mouse (Cockayne et al., 2000). Hence, inhibition of ATP release could attenuate sensory symptoms (Apostolidis et al., 2006). Although ACh and ATP can be co-released from nerves (Zimmermann, 2008), clinical evidence that BOTOX® often attenuates urgency and pain before urodynamic improvement led to a suggestion that inhibition of ATP release from distended urothelium precedes any cholinergic block (Khera et al., 2004). A direct action of BoNT/A on sensory afferents has also been proposed (Apostolidis et al., 2006) because it blocks the release of pain and inflammatory mediators from cultured sensory neurones (Apostolidis et al., 2006; Meng et al., 2009) and brain slices (Meng et al., 2009).
Herein, electrically-induced contractions of rat bladder strips were investigated to determine their susceptibility to BoNT serotypes that cleave different proteins essential for synaptic vesicle fusion. Moreover, contributions of cholinergic and purinergic inputs to motor signalling were determined and evidence was obtained that ACh and ATP (or metabolites) are co-released from the same efferent nerve fibres by showing that they are equally susceptible to inhibition by BoNT/A. Possible mechanisms whereby such blockade could contribute to attenuation of sensory, as well as motor, signalling in bladder are discussed.

**Methods**

**Materials.** PPADS was purchased from Tocris Biosciences (Eching, Germany). BoNTs were obtained from Metabiologics Inc. (Madison, WI) as dichains, except /E which was activated with trypsin before use. Specific neurotoxicities of BoNTs following intraperitoneal injection into mice (after nicking, where required) were determined by the manufacturer (x 10^8 median lethal doses per mg): /A (2.5); /B (0.9), /C1 (0.2), /D (0.6), /E (0.6), /F (0.2) and /G (0.1). Atropine, α,β-methylene-adenosine-5'-triphosphate (αβMeATP) and buffer salts were supplied by Sigma Aldrich (Dublin, Ireland).

**Tissue preparation.** Bladders were excised from adult rats (2-4 months), cut into longitudinal strips and mounted in 10 ml tissue baths (Myobath: World Precision Instruments, Stevenage, U.K.) in Krebs Ringer solution (in mM: NaCl 118, KCl 4.7, MgSO4 1.2, NaHCO3 23.8, KH2PO4 1.2, CaCl2 2.5, and glucose 11.7) and gassed with 5% CO2/95% O2; in some experiments, the urothelial layer was removed before sectioning and mounting the muscle.

**Functional studies.** Due to limitations in the amounts of BoNTs available, a large static bath was employed, as used previously in this laboratory (Lawrence et al., 2007) and by others (Simpson, 1980) to study the action of these proteins on innervated skeletal muscle. This recording format gives a lower efficiency of EFS for nerve (and muscle) due to reduced
shunt resistance; hence, the pulse frequencies and widths required to elicit muscle contractions (see Figure legends for details) are larger than those reported in studies utilising smaller baths (e.g. MacKenzie et al., 1982). Notably, others have successfully employed conditions almost identical to those described here (Dveksler et al., 1985). Moreover, to minimise the possibility of direct muscle stimulation, care was taken to ensure that the current amplitude employed was no more than five-fold greater than the threshold for triggering nerve-mediated contractions. The strips were tensioned (0.4g) and equilibrated (1h) before EFS, via Ag/AgCl electrodes introduced into the top and bottom of the bath, respectively. Constant current EFS (250 mA) was supplied from a Multistim DE300 stimulator (Digitimer, Welwyn Garden City, U.K.) in square pulses (see Figure legends for details); polarity was switched between consecutive pulses. Muscle tension was recorded using isometric force transducers (FORT25) linked to a LabTrax 4/16 data acquisition system. Values for peak changes in muscle tension (i.e. the difference between the maximum force generated during EFS and the basal level immediately prior to EFS) and contractile area (i.e. the area under the force-time trace) during EFS were measured using DataTrax 2 software (World Precision Instruments).

**Results**

BoNT/A inhibits EFS-evoked TTX-sensitive contractions of rat bladder strips: other SNAP-25-cleaving BoNTs are equally effective whereas the vesicle-associated membrane protein (VAMP)-inactivating serotypes proved less potent. With the aim of dissecting neurotransmission into distinct components and delineating their sensitivity to BoNTs, rat bladder strips were stimulated using a variety of pulse protocols before and upon exposure to these inhibitors of synaptic vesicle fusion. During 1 min excitation with 50 ms pulses at 4 Hz, muscle contractions were most intense during the first seconds and then receded (Fig. 1Aa,b). Raising the pulse frequency to 8Hz increased contractile force over the whole 1 min.
stimulation period, particularly at the start. No contractions were observed if strips were stimulated with much narrower pulses, 1.5 ms (not shown), unless the pulse frequency was increased, e.g. to 32 hz, which induced a transient peak of tension followed by a more slowly declining signal (Fig. 1Aa,b, and see later). A second train produced contractions profiles similar to the preceding signals (Fig. 1Ac) but, in general, of lower tension. Accordingly, contraction force declined steadily during a repetitive series of EFS (Fig. 1Ba). Elicited responses were due to nerve stimulation because 1 μM TTX dramatically reduced the size of contractions (c.f. traces in Fig. 1Ad with b) indicating a requirement for TTX-sensitive Na+ channels that are expressed in neurons but not muscle, as shown previously using similar EFS conditions (Dveksler et al., 1985). Application of 3 nM BoNT/A greatly accelerated the decline in evoked muscle tension, which was virtually abolished within 2 h (Fig. 1Bb). Notably, subsequent inclusion of 1 μM TTX exerted minimal effect on the residual response in BoNT/A-treated strips (Fig. 1Cb), unlike control muscle that retained a large TTX-sensitive signal for over 4 h (Fig. 1 Ba, Ca). Thus, in rat bladder BoNT/A selectively inhibits release of excitatory neurotransmitters from a source excited by the opening of TTX-sensitive voltage-activated Na+ channels. Importantly, the rate of neuroparalysis by BoNT/A is concentration-dependent (Fig. 1D). Similar relationships were observed for BoNT/E and /C1, with the latter being slightly faster (Fig. 1E). BoNT/D, /F and /G were very slow-acting and no dose-response relationship was evident. Notably, the relationships between concentration and paralysis time for /E and /C were fit well by a power function (Fig. 1E, Table 1), a property well established for the action of BoNTs at the neuromuscular junction (Simpson, 1980; Lawrence et al., 2007). Though BoNT/A showed more variation, the data could also be fitted with a power function (Table 1). Such concentration-dependencies accord with BoNTs being directly responsible for a loss of muscle tension after their application to bladder strips.
Partial delineation of cholinergic and purinergic contractile components. Shortening of the pulse width induced an initial transient spike in tension preceding a sustained phase; increasing frequency intensified both features (Fig. 2A). As some commentators have suggested that high-frequency stimulation may reflect aberrant neural excitation in NDO (Smith et al., 2003; Apostolidis et al., 2006), contractile responses to 32 Hz EFS were scrutinised. A proposed involvement of ACh and ATP in the spike and sustained phases was probed using, respectively, atropine to antagonise muscarinic receptors and αβMeATP to activate and desensitise P2X-receptors. Notably, only the sustained phase was reduced significantly by atropine (Fig. 2B); indeed, atropine often had little effect when EFS trains were reduced in duration from 1 min to 10s, thereby, eliciting only the spike (e.g. Fig. 2B). In contrast, αβMeATP selectively removed the spike (Fig. 2C). Sequential application of αβMeATP and then atropine virtually abolished evoked contractions, indicating that ACh plus ATP accounts for virtually all excitatory signalling in rat bladder (Fig. 2D). Whereas atropine reproducibly inhibited the sustained phase more effectively than the initial spike elicited by stimulation with 1.5 ms pulses at 32 Hz (Fig. 2E; p < 0.001, n = 8), upon stimulation with 50 ms pulses at 8Hz the difference in reduction was not statistically significant (Fig. 2F, n = 8). On the other hand, αβMeATP was reliably more effective in reducing the initial spike than the sustained response after excitation with narrow pulses at high frequency (Fig. 2G; p < 0.05, n = 9) but, again, no significant difference in the reduction of each phase was noted for stimulation with 50 ms pulses at 8 Hz (n = 9). Thus, reducing the width and increasing the frequency of electrical pulses elicits a partial temporal separation of the purinergic and cholinergic components of the excitatory input.

The innervated muscle layer can produce excitatory ATP. Further evidence for excitatory purinergic signals was obtained by sequential application of atropine then a broad range P2 antagonist, PPADS. Atropine induced a rapid reduction in EFS-evoked muscle
tension to a level that was then sustained for several hours (Fig. 3A), but subsequent addition of PPADS after the atropine-resistant responses had stabilized caused a progressive further decrease in muscle tension (Fig. 3B). Importantly, the atropine-sensitive and -resistant signals were preserved in the muscle layer after urothelium removal (Fig. 3C) and the latter were inhibited by PPADS (Fig. 3D). This confirms that both excitatory ATP and ACh can be released from a source within the innervated muscle layer, though it does not exclude urothelium serving as an additional source of these transmitters.

**Cholinergic and purinergic signals are blocked by BoNT/A with equal efficacy.** The relative susceptibilities to BoNT/A of cholinergic and purinergic signals were determined by recording paralysis in the absence or presence of either atropine (Fig. 4A, C) or αβMeATP (Fig. 4B, D). Neither changed significantly the rate of paralysis by BoNT/A, with respect to untreated control strips, irrespective of stimulation with 50 ms pulses at 8 Hz (Fig. 4A, B) or 1.5 ms pulses at 32 Hz (Fig. 4C, D). These data are consistent with ACh and ATP being released from the same source. Although not inconceivable, it seems unlikely that these two neurotransmitters could be released from two independent cell populations that both internalise and are inactivated by BoNT/A at exactly the same rate, even when purinergic signalling is preferentially activated by high frequency narrow pulse stimulation.

In summary, EFS causes co-release of ACh and ATP from the same TTX-sensitive source in the rat bladder muscle layer, in the absence or presence of urothelium, and each is inhibited with equal efficacy by BoNT/A. High frequency stimulation intensifies an initial spike in muscle tension which appears to be largely due to purinergic signalling, but this remains susceptible to BoNT/A. Unlike the VAMP-cleaving toxins, others acting on SNAP-25 (C1 and C2) and SNAP-25 (E) are also effective inhibitors of excitatory transmission in the bladder and, thus, could potentially be used as therapeutics for OAB.

**Discussion**
The ability of BoNT/A to inhibit transmitter release has been exploited for treating detrusor over-activity, as well established for its management of skeletal muscle spasms and spasticity (Jankovic et al., 2008). It has proved very effective in reducing voiding frequency and incontinence episodes attributable to lowered bladder pressure during filling and voiding, together with improvements in bladder capacity and compliance (Apostolidis et al., 2006; Schurch and Dykstra, 2009). Patients also report major benefits in reduced urgency sensation. Consequently, much effort has been expended on investigations into possible effects of BoNT/A on the complex array of cholinergic and additional signalling molecules acting in healthy and diseased bladder (Apostolidis et al., 2006; Chancellor et al., 2008).

Electrically-induced atropine-resistant bladder contractions were known to be blocked by BoNT/A (MacKenzie et al., 1982; Smith et al., 2003), though less effectively than by /A, /C1 and /E herein, probably due to the lower BoNT concentrations used previously (Smith et al., 2003). Notably, neither natural (Fig. 1) or recombinant /D (unpublished observation) proved as effective as /A (/C1 or /E), in contrast to a previous report (Smith et al., 2003). The reasons for such a discrepancy are not clear, and direct comparison of the data herein with those in the earlier report is not possible due to differences between the stimulation paradigms employed.

Purinergic excitation was confirmed herein using two strategies: (i) P2X desensitisation with αβMeATP (Kasakov and Burnstock, 1982) and (ii) P2 antagonism with PPADS (North, 2002). Both virtually abolished atropine-insensitive contractions, as expected from an absence of atropine-resistant contractility in mice lacking P2X1 (Vial and Evans, 2000). Moreover, BoNT/A blocked the cholinergic and purinergic signalling equally, consistent with co-release of ATP and ACh from nerve fibres, possibly the same vesicles [as in other tissues (Volknandt and Zimmermann, 1986; Richardson and Brown, 1987; Zimmermann, 2008)]. Urothelium also releases ATP, which is stimulated by bladder distension (Ferguson et al., 1997), and it has been reported that BoNT/A inhibits urothelial ATP release in a rat spinal cord injury.
model (Khera et al., 2004). However, it remains unclear how BoNT/A could block release of any signalling molecules from uroepithelial cells as they do not express either SV2 or SNAP-25 [see below and Chancellor, et al. (2008) for review]. In fact, immuno-histochemistry has indicated that SNAP-25 is restricted to fibres innervating detrusor muscle (Schulte-Baukloh et al., 2007) and SV2 occurs in neuro-varicosities apposing P2X$_1$-receptor clusters (Hansen et al., 1998). Herein, several pieces of evidence indicate that rat bladder efferents are the source of ATP that is blocked by BoNT/A. Atropine-resistant purinergic signalling was preserved in bladder muscle stripped of urothelium, albeit at a lower level that may reflect some influence of the latter on neuromuscular transmission (see below). Nevertheless, contractile responses were virtually abolished by TTX that blocks nerve fibre conductance without affecting smooth muscle excitability (Burnstock, 2007) or afferent fibres excitation (Yoshimura et al., 2001) and has minimal effect on urothelial release of ATP (Ferguson et al., 1997; Sadananda et al., 2009). BoNT/A specifically inhibited TTX-sensitive contractions, consistent with the minor TTX-resistant component elicited by direct muscle stimulation (Burnstock et al., 1978).

Although neuromuscular transmission in healthy human bladder is > 95% atropine-sensitive, the purinergic component can increase to 40% in cases of interstitial cystitis (Palea et al., 1993) or idopathic detrusor instability, possibly due to altered P2X-receptor expression (O’Reilly et al., 2002) or reduced expression of ectopic nucleotidases in the detrusor (Harvey et al., 2002). Consequently, abnormally-elevated purinergic excitatory signalling may underlie symptoms of bladder instability that are resistant to anti-cholinergics and, hence, P2X$_1$ receptors are being considered as a drug target for the relief of detrusor over-activity (Ruggieri, 2006). Most likely, the effectiveness of BoNT/A in the management of OAB, even in non-responders to anti-muscarinics, is due to its inhibition of the co-release of ATP with ACh.
An intriguing aspect of BoNT/A therapy is the reported alleviation of sensory urgency and pain which appears to be independent of muscle relaxation. A mechanism proposed in a recent review (Chancellor et al., 2009) is that inhibition of efferent ATP release by BoNT/A leads to reduced excitation of sensory afferents. P2X₃, enriched on TRPV1- and CGRP-expressing bladder sensory afferents (Cockayne et al., 2000; Apostolidis et al., 2006), is thought to be important for sensing ATP released from distended urothelium and signalling bladder filling (Burnstock, 2007). However, P2X₃ expression becomes raised during inflammation (Dang et al., 2008) or OAB (IDO and, especially, NDO) and, thus, may also be implicated in increased sensory urgency and pain associated with these conditions. Indeed, bladder capacity before reflex voiding is raised and inflammatory pain responses reduced in the P2X₃ knock-out mouse (Cockayne et al., 2000). Although a direct action of BoNT/A on afferents to block the release of pain and inflammatory mediators (Lucioni et al., 2008; Meng et al., 2009) could also contribute to its amelioration of sensory symptoms, studies with radiiodinated BoNT/A showed preferential binding to cholinergic (rather than peptidergic or noradrenergic) terminals innervating mouse ileum (Black and Dolly, 1987). Thus, though direct evidence is lacking for afferent excitation via ATP released from bladder efferents [indeed, several other possible mechanisms to alleviate sensory signalling have also been forwarded (Apostolidis et al., 2006; Chancellor et al., 2008; Chancellor et al., 2009)], blockade of efferent release of nociceptor-activating ATP offers a cogent explanation for relief by BoNT/A of pain/urgency; this would accord with its established selectivity for cholinergic neurons and anti-nociceptive outcomes in the Urology clinic.

Importantly, this model does not exclude a sensory signalling role for ATP released from urothelium but, rather, provides an explanation for the apparent non-cholinergic actions of BoNT/A by its inhibition of purine release from a neural source. Indeed, it is possible that relief of sensory symptoms by BoNT/A arises wholly, or in part, from its inhibition of...
communication from nerve fibres to the urothelium. In fact, the latter has been termed a “sensory web” because it integrates chemical and mechanical stimuli into responses that can excite sensory nerve fibres, a concept summarised below but developed and reviewed in detail by others (Apodaca et al., 2007; Khandelwal et al., 2009; Birder, 2010). Both efferent and afferent fibres are localised in close proximity to (and some within) the urothelium and the epithelial cells express a variety of muscarinic and purinergic receptors; thus, both nerve types can transmit chemical signals to the epithelium. In response, uro-epithelial cells can themselves release transmitters, including ATP, nitric oxide, prostaglandins growth factors and (via a non-vesicular mechanism) ACh. Such transmitters can excite neighbouring epithelial cells in a paracrine manner, amplify excitatory signalling via autocrine positive-feedback mechanisms (e.g. activation of uro-epithelial muscarinic receptors can elicit increased urothelial release of ATP) and communicate back to nerves, for example, via elevated ATP release to activate P2X receptors on sensory fibres. Additionally, both ATP and ACh are released from urothelium upon stretch; thus, mechanical stimuli can supplement and amplify chemical signalling.

As noted above, injection of BoNT/A into rat bladders modulates the secretion of neurotransmitters from the epithelium (Khera et al., 2004; Smith et al., 2008), but a direct action on secretion from uro-epithelial cells is unlikely due to their lack of its substrate, SNAP-25 (Chancellor et al., 2008). Indeed, evoked release of nitric oxide is altered after BoNT/A injection even though this transmitter is not released by vesicular exocytosis (Smith et al., 2008). Another explanation is that inhibition by BoNT/A of ACh and ATP release from nerve fibres blocks excitation by these transmitters of the urothelium sensory web. Alternatively (or additionally), a similar outcome may accrue indirectly from a reduction in the frequency and force of detrusor contraction, as this would mitigate mechanical stimulation of the urothelium. A third possibility is that BoNT/A blocks the release from afferent fibres of
ATP and other transmitters that stimulate the urothelium (Apostolidis et al., 2006; Smith et al., 2008). Some investigators have even suggested that BoNT/A might block afferent signalling in the absence of any detectable effect on bladder contractility (Smith et al., 2008); however, due to the observed large variability in contraction force for strips obtained from different subjects, such an interpretation cannot be made conclusively with the small sample group used in that study. Moreover, it is difficult to reconcile this notion with the preferential binding by BoNTs to cholinergic nerves in the peripheral nervous system (Black and Dolly, 1987), as noted earlier. Rather, by inhibiting efferent release of ACh and ATP, BoNT may inhibit both direct activation of nociceptors (via exocytosed ATP) and indirect activation (possibly with amplification) of the sensory fibres through the urothelium sensory web.

**Acknowledgement**

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References


Volknandt W and Zimmermann H (1986) Acetylcholine, ATP, and proteoglycan are common to synaptic vesicles isolated from the electric organs of electric eel and electric catfish as well as from rat diaphragm. *J Neurochem* **47**:1449-1462.


Footnotes

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Legends for Figures

**Fig. 1** BoNT/A inhibits TTX-sensitive neuromuscular transmission in rat bladder strips: the rate of paralysis is concentration-dependent, as is the blockade by /C1 or /E. (A) Typical traces of muscle tension for two bladder strips (a, b) stimulated for 1 min with 50 ms wide pulses at 4 Hz or 8 Hz, or 1.5 ms pulses at 32 Hz. (Ac, d) shows responses to second trains, applied 12 min after the first, without (Ac) or with (Ad) the addition of 1 μM TTX between trains. (B, C) muscle strips were stimulated by EFS (1.5 ms, 32Hz) in 1 min trains repeated every 5 mins whilst recording tension profiles in the absence (Ba) or presence (Bb) of 3 nM BoNT/A; arrow indicates time of addition. In (C) profiles are magnified on the time axis; [Ca(i), Cb(i)] were recorded immediately before addition of 3 nM BoNT/A to (Cb), [Ca(ii), Cb(ii)] 2 h later, and [Ca(iii), Cb(iii)] following subsequent addition of 1 μM TTX. (D, E) strips were stimulated by 1 min trains of EFS (50 ms, 8Hz) repeated every 5 mins. The contractile area (see Materials and Methods) was normalised as a % of the signal immediately before addition of BoNT/A (nM): 0.3 (□, n=3), 0.5 (△, n=3), 1 (▼, n=3), 2 (▲, n=3) 3 (■, n=5) or 10 (●, n=4). Control recordings (◇) were quantified after the addition of bovine serum albumin (10 mg/ml in bath). (E) Elapsed time (± s.e.m.; n ≥ 3) for contractile area to be reduced by 50% after addition of BoNT/A (◇) /C1 (△), D (■), E (□), F (●) or G (★). In (D) and (E), strips were obtained from at least two different bladders for each point.

**Fig. 2** Excitatory neuromuscular transmission in rat bladder consists of cholinergic and purinergic components, with the latter being selectively intensified by increasing EFS frequency. (A) A typical tension profile for a bladder strip during sequential 1 min. trains of EFS, using various pulse widths and frequencies. (B-D) were all obtained from bladder strips subjected to EFS trains of pulses 1.5 ms wide at 32 Hz. In (B) trains were alternated between 60 and 10 seconds in length and atropine added (1μM final concentration) at the time
indicated. (C) EFS was applied for 1 min then αβMeATP was added at the indicated times (bringing the bath concentration up to 100 μM) before applying further trains of EFS. In (D) αβMeATP addition was followed by two trains of EFS before adding 1 μM atropine and a further EFS train thereafter. To obtain the data plotted in panels (E-H), peak heights and contractile areas were measured for sequential responses to EFS [1.5ms pulses at 32 Hz (E,G) or 50 ms pulses at 8Hz (F, H)] and the subsequent signals expressed as a % of their antecedents. Where indicated, 1 μM atropine (E, F) or two sequential, additive doses of 50 μM αβMeATP (G, H) were included between the sequential bouts of EFS. For each bar, n ≥ 7; p-values were calculated using Student’s t-test. A minimum of 4 bladders were used for each panel in (E-H). An equal number of control and agonist/antagonist-treated strips were obtained from each bladder.

**Fig. 3** The non-cholinergic component of neuromuscular transmission arises from the muscle layer and is blocked completely by PPADS, a broad range antagonist of P2 receptors. Tension profiles are shown for bladder strips excited by EFS (1.5 ms pulses at 32Hz; A-D). The urothelium layer was removed before recording responses from only the smooth muscle (in C, D). Atropine (1μM) was added where indicated by ‘1’ and PPADS (A, C) by ‘2’.

**Fig. 4** BoNT/A inhibits cholinergic and purinergic components of neuromuscular transmission at equivalent rates. Bladder strips were stimulated by 1 min trains of EFS with 50 ms pulses at 8 Hz (A,B) or 1.5 ms pulses at 32 Hz (C,D), separated by 5 min recovery intervals, in the absence (○) or presence of (A,C) 1 μM atropine (□) or (B,D) 100 μM αβMeATP (▽; added in two steps as in Figure 2C) for 30 mins before adding 10 nM (A) or 3 nM BoNT/A (B-D); note that inclusion of atropine or αβMeATP reduced contractile area (c.f. Fig 2). Signals recorded after BoNT/A addition (at t = 0 mins.) were normalised as a % of the
response immediately before exposure to the toxin. Mean values (± s.e.m.) are plotted for data obtained using 4 or more bladder strips from at least two different animals; control and atropine/αβMeATP-treated bladder strips were always obtained from the same rats.
Table 1  Power functions<sup>a</sup> fit to the relationship between concentration and time to paralysis of rat bladder strips by various BoNTs

<table>
<thead>
<tr>
<th>BoNT/</th>
<th>A</th>
<th>B</th>
<th>R²</th>
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<tbody>
<tr>
<td>A</td>
<td>97.3±3.8</td>
<td>-0.28±0.04</td>
<td>0.747</td>
</tr>
<tr>
<td>C1</td>
<td>73.5±2.0</td>
<td>-0.27±0.02</td>
<td>0.920</td>
</tr>
<tr>
<td>E</td>
<td>89.2±2.7</td>
<td>-0.24±0.03</td>
<td>0.955</td>
</tr>
</tbody>
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<sup>a</sup> Data sets were fit to the equation \( y = A(x^B) \) and \( R^2 \) calculated using GraphPad Prism software.
Figure 2

A: EFS: 1.5ms @ 32Hz

B: 50ms @ 8Hz

C: 1.5ms @ 32Hz

D: 50ms @ 8Hz

E: % Preceding response (±s.e.m.)

F: % Preceding response (±s.e.m.)

G: % Preceding response (±s.e.m.)

H: % Preceding response (±s.e.m.)

EFS: 1.5ms @ 32Hz

50ms @ 8Hz

1.5ms @ 32Hz

50ms @ 8Hz

Atropine

Peak

Area

αβMeATP

Peak

Area
Figure 4

Graph A: EFS: 50ms @ 8Hz
- Control
- 1 μM atropine

Graph B: EFS: 50ms @ 8Hz
- Control
- 100 μM αβMeATP

Graph C: EFS: 1.5ms @ 32Hz
- Control
- 1 μM atropine

Graph D: EFS: 1.5ms @ 32Hz
- Control
- 100 μM αβMeATP

Tension (% value immediately before adding BoNT/A ± s.e.m.)

Mins. after BoNT/A addition