Excitatory Cholinergic and Purinergic Signaling in Bladder Are Equally Susceptible to Botulinum Neurotoxin A Consistent with Co-Release of Transmitters from Efferent Fibers

Gary W. Lawrence, K. Roger Aoki, and J. Oliver Dolly

International Centre for Neurotherapeutics, Dublin City University, Glasnevin, Dublin, Ireland (G.W.L., J.O.D.); and Allergan Inc., Irvine, California (K.R.A.)

Received April 16, 2010; accepted June 23, 2010

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 BoNT/A in alleviating smooth muscle spasms associated with overactive bladder. BoNT/A, BoNT/C1, or BoNT/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 cleaved vesicle-associated membrane protein proved to be much less effective. Acetylcholine (ACh) 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 desensitizing agonist of P2X1 and P2X3 or a nonselective ATP receptor antagonist. Both transmitters were released in the innervated muscle layer and, thus, persisted after removal of urothelium. Atropine or a desensitizer of the P2X1 or P2X3 receptors did not alter the rate at which muscle contractions were weakened by BoNT/A. Moreover, although cholinergic and purinergic signaling could be partially delineated by using high-frequency EFS (which intensified a transient, largely atropine-resistant spike in muscle contractions that was reduced after P2X receptor desensitization), they proved equally susceptible to BoNT/A. Thus, equi-potent blockade of ATP co-released with ACh from muscle efferents probably contributes to the effectiveness of BoNT/A in treating bladder overactivity, including nonresponders to anticholinergic drugs. Because purinergic receptors are known mediators of sensory afferent excitation, inhibition of effenter 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 overactive bladder (OAB) (Schurch and Dykstra, 2009), involving either neurogenic detrusor overactivity (NDO) or idiopathic detrusor overactivity, can be achieved by injecting botulinum toxin (BoNT) type A (BoNT/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 $M_r = 25k$ (SNAP-25), which is essential for synaptic vesicle fusion (Blasi et al., 1993). It is noteworthy that BOTOX often proves effective in nonresponders 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 the bladder (Burnstock et al., 1978). A purine-gated ion channel, $P_{2X_3}$, is found in detrusor muscle (Vial and Evans, 2000). In addition, another subtype, $P_{2X_2}$, is enriched on sensory afferents, and its role in voiding control has been established by the pronounced bladder hyporeflexia seen in a $P_{2X_2}$ knockout 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 signaling were determined and evidence was obtained that ACh and ATP (or metabolites) are co-released from the same efferent nerve fibers by showing that they are equally susceptible to inhibition by BoNT/A. Possible mechanisms whereby such blockade could contribute to attenuation of sensory and motor signaling in bladder are discussed.

Materials and Methods

Materials. PPADS was purchased from Tocris Bioscience (Eching, Germany). BoNTs were obtained from Metabiologics Inc. (Madison, WI) as dichains, except BoNT/E, which was activated with trypsin before use. Specific neurotoxicities of BoNTs after (Madison, WI) as dichains, except BoNT/E, which was activated, Germany). BoNTs were obtained from Metabiologics Inc.

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, Inc., Stevenage, UK) in Krebs/Ringer solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 23.8 mM NaHCO3, 1.2 mM KH2PO4, 2.5 mM CaCl2, and 11.7 mM glucose) and gassed with 5% CO2/95% O2; in some experiments, intraperitoneal injection into mice (after nicking, where required) with trypsin before use. Specific neurotoxicities of BoNTs after injection into mice (after nicking, where required) were determined and evidence was obtained that ACh and ATP (or metabolites) are co-released from the same efferent nerve fibers by showing that they are equally susceptible to inhibition by BoNT/A. Possible mechanisms whereby such blockade could contribute to attenuation of sensory and motor signaling in bladder are discussed.

Equi-Potent Inhibition of Ach and ATP Release by BoNT/A

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-Inactivating Serotypes Proved to Be Less Potent. With the aim of dissecting neurotransmission into distinct components and delineating their sensitivity to BoNTs, rat bladder strips were stimulated by 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. 1A, a and b). Raising the pulse frequency to 8 Hz 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. 1A, a and 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 caused by nerve stimulation because 1 μM TTX dramatically reduced the size of contractions (compare trace d with b in Fig. 1A), indicating a requirement for TTX-sensitive Na+ channels that are expressed in neurons but not muscle, as shown previously under 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). It is noteworthy that 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 more than 4 h (Fig. 1, Ba and 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. It is noteworthy that the rate of neuroparalysis by BoNT/A is concentration-dependent (Fig. 1D). Similar relationships were observed for BoNT/E and BoNT/C1, with the latter being slightly faster (Fig. 1E). BoNT/D, BoNT/F, and BoNT/G were very slow acting, and no dose–response relationship was evident. It is noteworthy that the relationships between concentration and paralysis time for BoNT/E and BoNT/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). Although BoNT/A showed more variation, the data could also be fitted with a power function (Table 1). Such concentration dependencies are in accordance 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 fea-
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. 4, A and C) or αβMeATP (Fig. 4, B and 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. 4, A and B) or 1.5-ms pulses at 32 Hz (Fig. 4, C and 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 internalize and are inactivated by BoNT/A at exactly the same rate, even when purinergic signaling 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 that seems to be caused largely by purinergic signaling, but this remains susceptible to BoNT/A. Unlike the vesicle-associated membrane protein-cleaving toxins, others acting on SNAP-25 (BoNT/C1 and BoNT/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 overactivity and is 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 the possible effects of BoNT/A on the complex array of cholinergic and additional signaling molecules acting in healthy and diseased bladder (Apostolidis et al., 2006; Chancellor et al., 2008).

Electrically induced atropine-resistant bladder contractions have been known to be blocked by BoNT/A (MacKenzie et al., 1982; Smith et al., 2003), although less effectively than by BoNT/A, BoNT/C1, and BoNT/E herein, probably because of the lower BoNT concentrations used.
previously (Smith et al., 2003). It is noteworthy that neither natural (Fig. 1) or recombinant BoNT/D (unpublished observation) proved to be as effective as BoNT/A (BoNT/C1 or BoNT/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 because of differences between the stimulation paradigms used. Purinergic excitation was confirmed herein by using two strategies: 1) P2X desensitization with αβMeATP (Kasakov and Burnstock, 1982) and 2) 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 signaling equally, consistent with co-release of ATP and ACh from nerve fibers, 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 signaling molecules from uroepithelial cells because 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 fibers innervating detrusor muscle (Schulte-Baukloh et al., 2007) and SV2 occurs in neuro-varicosities apposing P2X1 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 signaling 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 fiber conductance without affecting smooth muscle excitability (Burnstock, 2007) or afferent fiber excitation (Yoshimura et al., 2001) and has minimal effect on the 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 (Plea et al., 1993) or idiopathic detrusor instability, possibly because of 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 signaling may underlie symptoms of bladder instability that are resistant to anticholinergics; hence, P2X1 receptors are being considered as a drug target for the relief of detrusor overactivity (Ruggieri, 2006). Most likely, the effectiveness of BoNT/A in the management of OAB, even in nonresponders to antimuscarinics, is caused by 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 that seems 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 transient receptor
potential vanilloid type 1- and calcitonin gene related peptidase-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 signaling bladder filling (Burnstock, 2007). However, P2X₃ expression becomes raised during inflammation (Dang et al., 2008) or OAB (idiopathic detrusor overactivity 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 are reduced in the P2X₃ knockout 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 radio-iodinated BoNT/A showed preferential binding to cholinergic (rather than peptidergic or noradrenergic) terminals innervating mouse ileum (Black and Dolly, 1987). Thus, although direct evidence is lacking for afferent excitation via ATP released from bladder efferents (indeed, several other possible mechanisms to alleviate sensory signaling have also been forwarded (Apostolidis et al., 2006; Chancellor et al., 2008, 2009)), blockade of efferent release of nociceptor-activating ATP offers a cogent explanation for relief by BoNT/A of pain/urgency; this would be in accordance with its established selectivity for cholinergic neurons and antinociceptive outcomes in the urology clinic.

It is noteworthy that this model does not exclude a sensory signaling 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 fibers 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 fibers, a concept summarized below but developed and reviewed in detail by others (Apodaca et al., 2007; Khandelwal et al., 2009; Birder, 2010). Both efferent and afferent fibers are localized in close proximity to (and some within) the urothelium, and the epithelial cells express a variety of muscarinic and purergic receptors; thus, both nerve types can transmit chemical signals to the epithelium. In response, uroepithelial cells can themselves release transmitters, including ATP, nitric oxide, prostaglandin growth factors, and (via a nonvesicular mechanism) ACh. Such transmitters can excite neighboring epithelial cells in a paracrine manner, amplify excitatory signaling via autocrine positive feedback mechanisms (e.g., activation of uroepithelial 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 fibers. In addition, both ATP and ACh are released from urothelium upon stretch; thus, mechanical stimuli can supplement and amplify chemical signaling.

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 uroepithelial cells is unlikely because of 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 fibers 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, because this would mitigate mechanical stimulation of the urothelium. A third possibility is that BoNT/A blocks the release from afferent fibers 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 signaling in the absence of any detectable effect on bladder contractility (Smith et al., 2008); however, because of 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/A may inhibit both direct activation of nociceptors (via exocytosed ATP) and indirect activation (possibly with amplification) of the sensory fibers through the urothelium sensory web.

Acknowledgments

We thank Dr. Mark Hollywood for assistance in establishing the smooth muscle assay.

References


Volkanudt H 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.

Address correspondence to: J. O. Dolly, International Centre for Neurotherapeutics, Dublin City University, Glasnevin, Dublin 9, Ireland. E-mail: oliver.dolly@dcu.ie