Pain 130 (2007) 76–83

PAIN

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Botulinum Toxin type A reduces capsaicin-evoked pain and neurogenic vasodilatation in human skin

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Received 9 March 2006; received in revised form 19 October 2006; accepted 30 October 2006

Abstract

The effect of Botulinum Toxin type A (BoNT/A) on pain and neurogenic vasodilatation induced by application to the human skin of thermal stimuli and capsaicin was evaluated in a double blind study. A capsaicin cream (0.5 ml of a 0.075%) was applied to the skin of both forearms of eighteen subjects randomly pretreated with either BoNT/A (Botox®) or 0.9% saline (NS). Capsaicin was applied to a skin area either inside (protocol A) or adjacent to the BoNT/A treated area (protocol B). Pre-treatment with BoNT/A did not affect thermal-specific and thermal-pain thresholds (by quantitative sensory testing). However, capsaicin-induced pain sensation (by a visual analogue scale), flare area (by acetate sheet) and changes in cutaneous blood flow (CBF, by laser Doppler flowmetry) were reduced when capsaicin was administered inside (protocol A) the BoNT/A treated area. In Protocol B, capsaicin-induced pain was unchanged, and capsaicin-induced flare/increase in CBF were reduced only in the area treated with BoNT/A, but not in the BoNT/A untreated area. Results indicate that (i) BoNT/A reduces capsaicin-induced pain and neurogenic vasodilatation without affecting the transmission of thermal and thermal-pain modalities; (ii) reduction in capsaicin-induced pain occurs only if capsaicin is administered into the BoNT/A pretreated area; (iii) reduction in neurogenic vasodilatation by BoNT/A does not contribute to its analgesic action. BoNT/A could be tested for the treatment of conditions characterised by neurogenic inflammation and inflammatory pain.

Keywords: Botulinum toxin; Capsaicin; Neurogenic inflammation; Pain

1. Introduction

Botulinum Toxin type A (BoNT/A) is currently used for the treatment of focal muscle overactivity and spasticity (Tsui et al., 1986) and certain autonomic disorders, including hyperhidrosis and scialorrea (Bushara and Park, 1994; Erbguth and Naumann, 1999). The beneficial effect of BoNT/A is considered to result from the blockade of either neuromuscular or autonomic cholinergic junctions. In the last few years, attention has been paid to the use of BoNT/A for the treatment of different pain conditions. Pain diseases alleviated by BoNT/A encompass two main categories: those related to muscle disorders, including dystonia (Greene et al., 1990), spasticity (Wissel et al., 2000), myofascial pain (Cheshire et al., 1994; Porta, 2000), chronic pelvic pain (Brisinda et al., 2004), tension-type headache (Gobel et al., 2001), temporomandibular dysfunction (Freund et al., 2000), and those possibly related to neurovascular disorders, including migraine headache (Silberstein, 2001;

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0304-3959/$32.00
doi:10.1016/j.pain.2006.10.030

Evers et al., 2004; Troost, 2004; Dodick et al., 2005). Additional pain disorders treated with BoNT/A are facial chronic pain (Borodic et al., 2001; Borodic and Acquadro, 2002; Saper, 2002), trigeminal neuralgia (Allam et al., 2005) and pain related to spinal cord pathology (Jabbari et al., 2003).

The precise mechanism of the analgesic effect of BoNT/A is still poorly understood, and additional actions to the sole cholinergic blockade and muscular relaxation may contribute to this. Previous investigation apparently excluded a direct peripheral antinociceptive effect of BoNT/A in human skin (Blersch et al., 2002). In particular, results obtained from the measurement of thermal and electric pain thresholds, acute pain perception, hyperalgesia and flare in response to capsaicin application to the human skin seemed to rule out a direct analgesic effect of BoNT/A (Voller et al., 2003). However, findings obtained in facial pain (Borodic et al., 2001; Borodic and Acquadro, 2002) and in a rat model of inflammatory pain (Cui et al., 2002; Aoki, 2003; Cui et al., 2004) and in humans by transcutaneous electrical stimulation (Kramer et al., 2003) focused on the possibility that BoNT/A somehow limits the functioning of a subset of capsaicin-sensitive and neuropeptide-containing primary sensory neurons (Durham et al., 2004).

The aim of the present study was to investigate the effect of BoNT/A on pain and neurogenic vasodilatation induced by application of thermal stimuli and capsaicin to the human skin. We used capsaicin because this drug, by a unique molecular mechanism, the stimulation of the transient receptor potential vanilloid-1 (TRPV1) (Caterina et al., 1997), causes both pain and neurogenic inflammatory responses (Holzer, 1991; LaMotte et al., 1992; Geppetti and Holzer, 1996; Serra et al., 1998). The effect of subcutaneous administration of BoNT/A on capsaicin-induced neurogenic vasodilatation and pain was studied by the measurement of the flare area, the cutaneous blood flow (CBF) by laser Doppler flowmetry (LDF) (Baron et al., 1999; Schmelz et al., 2000) and by a subjective visual analogue pain scale (VAS), respectively. Thresholds for thermal and thermoalgesic modalities were measured by using the quantitative sensory testing (QST) (Verdugo and Ochoa, 1992; Yarnitsky et al., 1995). Results indicate that treatment with BoNT/A attenuates both pain and neurogenic vasodilatation evoked by capsaicin application, without affecting thermal-specific and thermal-pain thresholds.

2. Materials and methods

2.1. Subjects and design

Eighteen healthy, drug-free volunteers (10 males, 8 females), aged 30–47, were recruited from our department staff. All participants gave their informed consent, and the study was approved by the local Ethics Committee. Subjects were advised not to take any medication or analgesics 48 h before pain assessments. Forearms of each participant were randomly treated with BoNT/A or the equivalent volume of sterile saline solution 0.9% (NS) in a double-blind fashion: the subject and investigator were unaware of the side of the active treatment. A second investigator was in charge of preparing and administering the injections and of monitoring the effect of BoNT/A on sweat production in the follow up (Minor test). As application of capsaicin to the human skin produces two associated phenomena, burning pain and a flare reaction (e.g., Simone et al., 1989), two different protocols were performed with the aim to discriminate the possible effect of BoNT/A treatment on the flare response, pain or both. The sole difference between the two protocols was the area of capsaicin administration. In protocol A, capsaicin cream was applied inside the skin area pretreated with BoNT/A or NS, while in protocol B capsaicin cream was applied to a skin area adjacent, but not included in the area pretreated with BoNT/A or NS. Measurements of CBF and flare were carried out around the area of capsaicin administration that corresponded to the treated skin (BoNT/A or NS) for protocol A, and both the treated and untreated skin for protocol B (Fig. 1).

2.2. Procedures and measures

On day one the area to be treated with BoNT/A or NS was assessed by calculating 5 cm from elbow crease, tracing a line joining the ulnar and the radial forearm margins. A segment of 4 cm was delineated with a pen in the central part of the line. A line of 6 cm was drawn perpendicularly to the first one, thus describing a rectangular area of approximately 24 cm^2 . These landmarks facilitated the recognition of the treated zone during each experimental step. Because of the uneven distribution of nociceptive fields in forearm skin, the physiological property of summation to reach pain perception and the large shape of flare produced by capsaicin administration (Serra et al., 1998), a large cutaneous area was treated in order to involve a relevant number of capsaicin-sensitive fibres. The entire area was subdivided into smaller areas (1 cm^2) for a total of 24 sub-areas. Two units (0.02 ml) of BoNT/A (Botox®-Allergan, 100 U diluted in 1 ml of NS) or an equivalent volume of NS was injected subcutaneously into each 1 cm^2 sub-area for a total of 48 U (0.48 ml).

On day 28 (4 weeks later) the second investigator performed a Minor starch iodine test by painting the skin with iodine solution and then dusting it with starch powder, to detect the area of anhydrosis produced by BoNT/A pre-treatment. During the cycloergometer test used to produce sweating the subject was blindfolded and therefore not able to see the colour resulting from the Minor test. Invariably in all subjects a complete focal anhydrosis was present and, accordingly, successive experimental steps were carried out. All procedures, as listed below, were performed on both forearms.

2.3. Quantitative sensory testing

On day 29 quantitative sensory testing (QST) for thermal-specific and thermal-pain modalities was carried out in the rectangular areas treated with BoNT/A or NS of forearms in all the 18 subjects to verify the direct effect on thermal and thermal-pain sensory fibres. Heat (HS) and cold sensitive (CS)

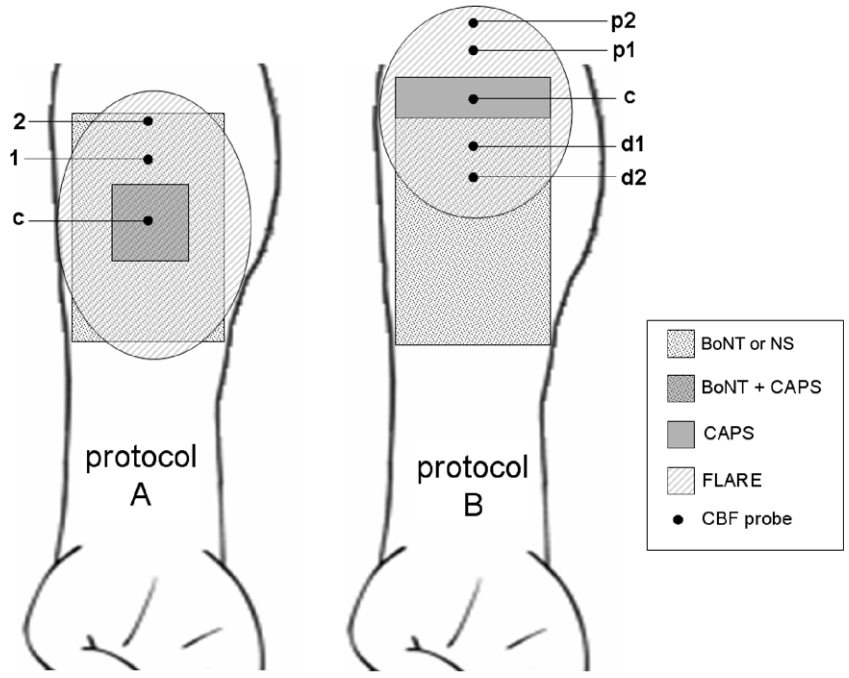

Fig. 1. Schematic representation of protocol A (left) and protocol B (right). Areas of BoNT/A or vehicle (NS) administration (rectangular areas), areas of capsaicin administration (dark grey), areas of flare (diagonally light grey) and sites of cutaneous blood flow (CBF) recording are indicated. CBF was recorded inside the area treated with capsaicin (CBFc) and at 1 cm (CBF1, CBFp1, CBFd1) and at 2 cm (CBF2, CBFp2, CBFd2) from the site of capsaicin application.

thresholds and heat (HP) and cold (CP) pain thresholds were detected by using a computer controlled thermode based on Peltier system and a thermal sensory analyzer (TSA 2001, Medoc Ltd., Ramat Yishai, Israel). The thermode contact area of 3×3 cm was applied to the middle of the forearm pretreated area. Temperatures ranged between 32–0 °C for CS and CP, and 32–50 °C for HS and HP. The rate of temperature changes was 0.5 °C/s, and interstimulus interval was 30 s. For each modality five successive stimuli were delivered and results were averaged. The interval between different modalities was 30 s. The method of limits was used to calculate each value following a standard procedure (Verdugo and Ochoa, 1992; Gruener and Dyck, 1994).

2.4. Capsaicin application and assessment of pain (VAS), flare and CBF

On day 30, capsaicin cream (0.075%) was applied to all subjects, on one arm at random. In protocol A (10 subjects), the cream (0.5 ml) was applied to a skin area of 2×2 cm in the middle of BoNT or NS treated rectangular area. In protocol B (8 subjects), the cream (0.5 ml) was applied to a skin of 1×4 cm external, but adjacent, to the upper side of the rectangular pretreated area (Fig. 1). On day 31 capsaicin cream was also applied to the second arm, following the same modalities of administration as the first ones according to the two protocols. Each subject reported the pain induced by capsaicin applying a vertical sign on a line of 10 cm, ranging from 0 (no pain sensation) to 10 (maximal pain sensation) at 5 (VAS_5), 10 (VAS_{10}), 20 (VAS_{20}), 30 (VAS_{30}) and 45 (VAS_{45}) min. After 45 min, when the maximum flare was achieved, capsaicin was gently removed to allow a correct CBF detection. The reddened area after capsaicin administration was outlined on an acetate sheet and measured with a digital planimeter. CBF was recorded by LDF (Perimed 5001, Sweden). In protocol A, the first probe was positioned in the middle of the capsaicin-treated area (CBFc) and the others at 1 cm (CBF1) and 2 cm (CBF2) from the external margins, along the major axis of the rectangular area, always above the area treated with capsaicin and inside the BoNT/A or NS treated skin. In protocol B, probes were positioned as indicated above with respect to capsaicin administration. However, probes were applied both below and above the capsaicin-treated area, e.g., inside or outside the BoNT/A or NS-treated skin (see Fig. 1, CBFd1, CBFd2, CBFp1, CBFp2, respectively). CBF was recorded for 2 min in order to obtain a regular flow in each site. The mean CBF was expressed in arbitrary units.

2.5. Statistical analysis

All data in the text and figures are means ± standard deviation (SD). Comparisons of values obtained in BoNT/A and NS pretreated area were performed by using the one-way repeated measures analysis of variance (ANOVA). Correlation between flare areas and VAS values was performed by the least squares method. A p value <0.05 was considered statistically significant.

3. Results

3.1. Thermal-specific and thermal-pain thresholds and spontaneous pain

Four weeks after the injections of either BoNT/A or NS, mean thermal and thermal-pain thresholds were

recorded. Values obtained in the BoNT/A pretreated arms were not statistically different from those obtained in the NS pretreated arms (Fig. 2). Capsaicin application caused a sensation of burning pain in both arms of all the subjects. In protocol A the magnitude of the capsaicin-evoked pain was significantly reduced in the skin pretreated with BoNT/A in comparison to the skin pretreated with NS (Fig. 3). In the BoNT/A treated arm the VAS value was significantly reduced by 45% as compared to the NS after 5 min (1.1 ± 0.3 vs. 2 ± 0.5; $p < 0.001$. Means $\pm$ SD), by 17.8% after 10 min (2.5 ± 0.4 vs. 3.1 ± 0.4; $p < 0.05$), by 40.3% after 20 min (4.1 ± 0.7 vs. 6.9 ± 0.8; $p < 0.001$), by 35.5% after 30 min (5.4 ± 0.7 vs. 8.4 ± 0.8; $p < 0.001$), and by 35.7% after 45 min (5.5 ± 0.7 vs. 8.5 ± 0.9; $p < 0.001$). In protocol B no significant difference was observed in pain perception following capsaicin application between the two sides pretreated with either BoNT/A or NS (Fig. 3).

3.2. Flare area on acetate sheet

Application of capsaicin produced a reddening of the skin exposed to the drug and the surrounding area. Consistently, in all subjects, the flare area, calculated 45 min after capsaicin administration, was significantly decreased in the BoNT/A treated arms as compared to the NS pretreated arm. The mean flare area was reduced by 18.8% in protocol A. In protocol B a significant reduction of 24.4% was seen in the partial area (PA) pretreated with BoNT/A (Table 1). A significant correlation was detected between the amplitude of the

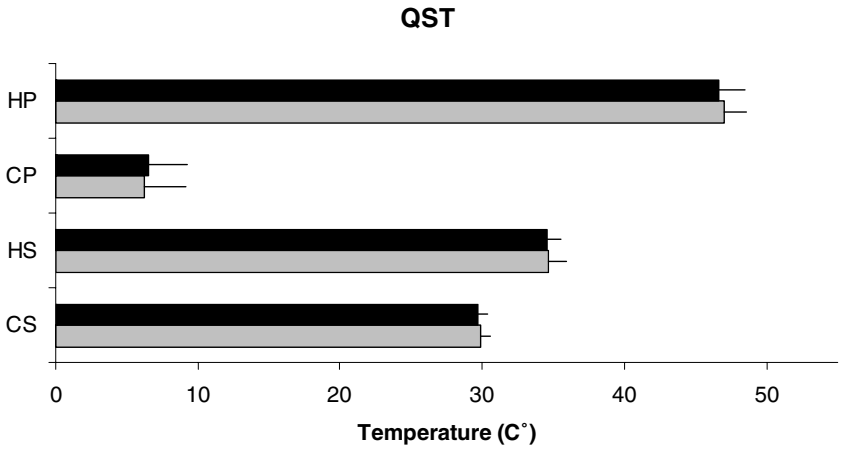

Fig. 2. Cold (CS) and heat (HS) sensitive thresholds and cold pain (CP) and heat pain (HP) thresholds values were detected in both BoNT/A pretreated (black columns) and vehicle pretreated (grey columns) forearms. All values are means $\pm$ SD of 18 subjects. No significant difference was detected by comparing BoNT/A and vehicle.

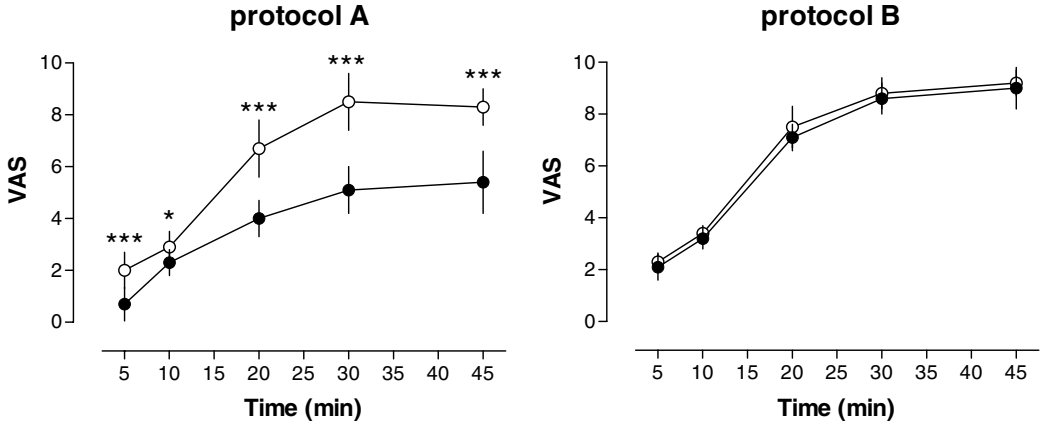

Fig. 3. Capsaicin-induced pain expressed by VAS values (means $\pm$ SD), for protocol A (10 subjects) on the left and protocol B (eight subjects) on the right. Black labels represent the results in BoNT/A pretreated skin, white labels the results in vehicle pretreated one. A significant reduction of VAS values was observed at each time point in the BoNT/A compared with vehicle pretreated forearms in protocol A ($^{*}p < 0.05$; $^{***}p < 0.001$). No significant difference was observed in protocol B.

Table 1
Effect of BoNT/A on the flare response produced by capsaicin application in the human skin

	Protocol A	Protocol B	
		TA	PA
Area of flare (cm^2)			
BoNT/A	$27.7 \pm 3.3^{**}$	$32.5 \pm 3.9^{**}$	$7.3 \pm 1.2^{*}$
NS	34.0 ± 3.8	38.9 ± 4.0	9.7 ± 1.7
Decrement (%) BoNT/A vs NS	−18.5%	−16.5%	−24.7%

Areas of reddened skin reported on acetate sheets show a significant decrement in BoNT/A pretreated area compared with vehicle ones for both protocol A and B. In protocol B two flare areas have been calculated: total area (TA) and partial area (PA) referred to the only spreading to pretreated skin. Values are means ± SD, $^{**}p < 0.001$, $^{*}p < 0.01$ (BoNT vs NS).

reddened areas and the pain VAS values in protocol A both for BoNT/A treated side ($r = 0.78$, $p < 0.01$) and NS treated side ($r = 0.70$, $p < 0.05$) (Fig. 4).

3.3. Cutaneous blood flow

Capsaicin application was associated with a marked increase in CBF as detected by the LDF technique. In protocol A capsaicin-evoked increase in CBFc was higher than the increase in CBF1, that, in turn, was higher than the increase in CBF2. CBFc was similar in both BoNT/A and NS treated arms. In contrast, CBF1 was significantly reduced by 40.9% ($p < 0.001$) in the side pretreated by BoNT/A as compared to the NS treated side. Similarly, CBF2 was reduced by 48.2% ($p < 0.001$) in the side pretreated with BoNT/A. In protocol B, capsaicin-evoked increase in CBFc was not statistically different in both BoNT/A and NS treated arms, and was similar to that observed in protocol A. When recordings were performed inside the treated areas, CBF was reduced (CBFd1 by 41.6%, $p < 0.01$, and CBFd2 by 40.9%, $p < 0.01$) in BoNT/A pretreated skin as compared to the NS pretreated side. In contrast, if CBF was recorded in the skin area not pretreated with BoNT/A or NS no significant difference in both CBFp1 and CBFp2 was observed (Fig. 5).

4. Discussion

The present study shows that treatment of the human skin with BoNT/A, while did not modify thermal and thermal-pain thresholds, reduced pain evoked by capsaicin application. Failure of BoNT/A to affect thermal or thermal-pain thresholds is consistent with previous studies, where, thresholds to heat and cold pain, electrical stimulation (Blersch et al., 2002), heat pain and current pain threshold/tolerance (Voller et al., 2003) were not changed in the skin pretreated with BoNT/A. Present results are at variance with a previous study (Voller et al., 2003) showing that capsaicin-induced pain and flare were not affected by BoNT/A. One possible explanation for the discrepancy is that in the study by Voller et al. (2003) the skin area, where capsaicin, injected intradermally, might have diffused, exceeding the small skin area (2.25 cm^2) treated with BoNT/A. Studies with TRPV1 knockout mice can shed some light into the apparent contradiction that BoNT/A reduced capsaicin-induced pain, but did not affect thermal and thermal-pain thresholds. Genetic deletion of TRPV1 did not affect responses to acute noxious thermal stimuli, but completely impaired the mouse ability to develop thermal hyperalgesia (Caterina et al., 2000; Davis et al., 2000), thus, suggesting that acute thermal-pain is independent from direct TRPV1 stimulation.

In the present study the inhibitory effect of BoNT/A on capsaicin-induced pain was observed solely if capsaicin was administrated inside the skin area pretreated with BoNT/A (protocol A), but not if it was applied

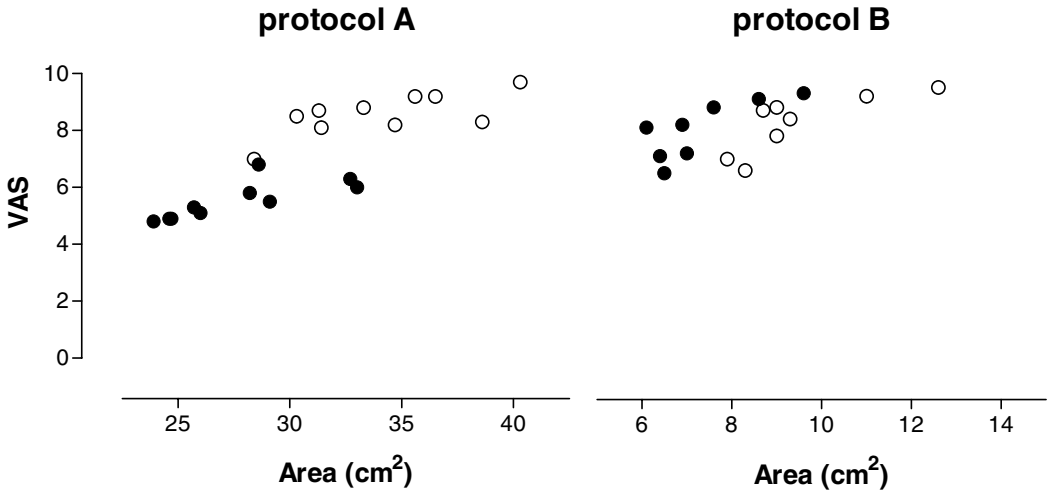

Fig. 4. Correlation between flare area and pain (VAS) in BoNT (black) and NS (white) arms in protocol A ($n = 10$) and protocol B ($n = 8$).

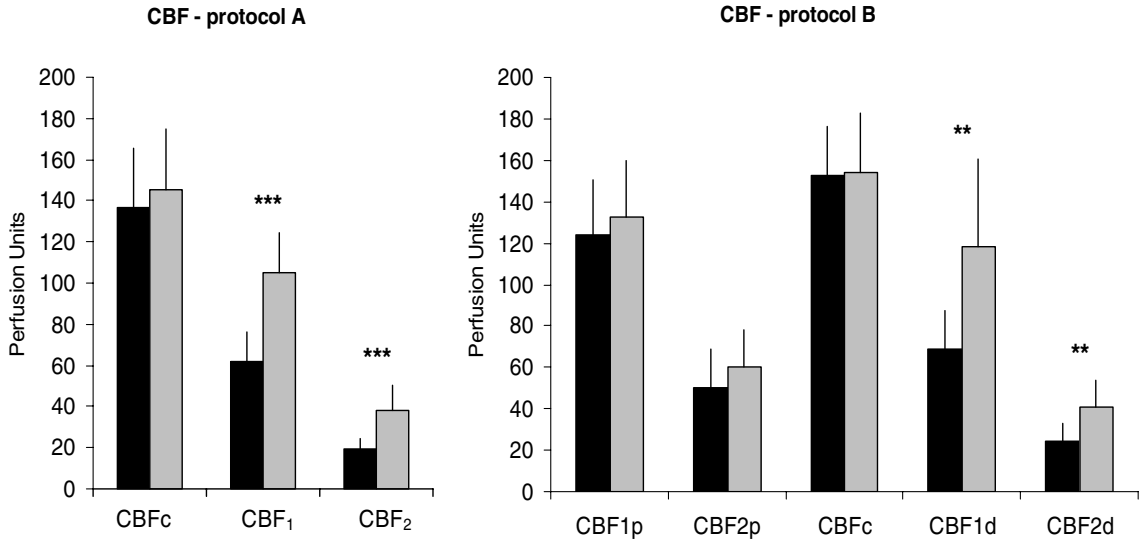

Fig. 5. Cutaneous blood flow (CBF) detected by laser Doppler flowmeter (LDF) expressed in perfusion units (arbitrary units) in BoNT/A pretreated (black columns) and vehicle pretreated (grey columns) forearms in protocol A (left) and protocol B (right). No significant difference was observed at the site of capsaicin administration (CBFc) for both protocols and for CBF1p and CBF2p for protocol B. A significant reduction in CBF was observed both for CBF1 and CBF2 (protocol A) and for CBF1d and CBF2d for protocol B in BoNT/A pretreated forearms. ($^{**}p < 0.01$; $^{***}p < 0.001$).

to a skin area adjacent to that pretreated with BoNT/A (protocol B). In contrast, the flare response/increase in CBF produced by capsaicin was inhibited by BoNT/A either if capsaicin was applied to the same area or in an area adjacent to that treated with BoNT/A. Present data confirm, in part, a previous study which shows that pretreatment with BoNT/A reduced the flare response by painful transcutaneous electrical stimulation (Kramer et al., 2003). However, the reduction in vasodilatation observed in BoNT/A treated side does not seem to contribute significantly to capsaicin-induced pain.

Stimulation of sensory C and A-delta fibres by capsaicin is due to its unique ability to activate the TRPV1 (Caterina et al., 1997; Szallasi, 2002). This effect produces the typical sensation of burning pain and a series of neuropeptide-mediated, inflammatory responses that includes arterial vasodilatation (Geppetti and Holzer, 1996). The present findings, showing that to limit capsaicin-induced pain BoNT/A must be applied to the same skin area, suggest that BoNT/A interferes either directly on capsaicin-induced TRPV1-activation or on the neurophysiological events that follow this activation in the nerve axon and its arborisation. However, a specific mechanism through which BoNT/A affects TRPV1-mediated nerve excitation has not been demonstrated so far.

The hypothesis that BoNT/A alters central transmission has been proposed because the toxin reduced the inflammatory pain phase in the formalin pain model in rats by decreasing glutamate release (Aoki, 2002, 2003; Cui et al., 2004). Furthermore, diminished activity of nociceptive neurons after BoNT/A administration was confirmed by the reduction in *c-fos* gene and Fos protein expression (Welch et al., 2000; Cui et al., 2002). However, attempts to demonstrate a direct action of BoNT/A in nociceptive neurons have not been consistently reproduced (Blersch et al., 2002). Recent evidence has shown that BoNT/A blocks SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors)-dependent TRPV1 exocytosis to the neuron surface (Morenilla-Palao et al., 2004). Thus, it may be proposed that reduction in TRPV1 translocation to the plasma membrane is responsible for reduced pain and neurogenic vasodilatation by BoNT/A.

Capsaicin releases vasodilatatory neuropeptides by a local efferent function (mediated by direct TRPV1 activation) or via an axon reflex arrangement (Maggi and Meli, 1988; Amann and Maggi, 1991). In the present study, CBF recorded at the site of capsaicin application (CBFc) was not affected by BoNT/A treatment that, however, inhibited CBF at sites distant from the area of application of capsaicin (CBF1 and CBF2). Thus, BoNT/A does not seem to affect neurogenic vasodilatation produced by the direct effect of capsaicin on TRPV1 (CBFc), but, rather, inhibits the increase in CBF presumably mediated by axon reflexes (CBF1 and CBF2). It should be, however, noted that failure of BoNT/A to inhibit the increase in blood flow at CBFc could have been due to the remarkable and probably saturated response at this site, compared to the moderate or mild responses seen at distance from the area of drug application. It is possible that all sensory nerve fibres are recruited by capsaicin at the site of drug application (CBFc), whereas at distance from its application (CBF1 and CBF2) only those fibres invaded by axon reflexes contribute to the vasodilatation. The dose of

BoNT/A may therefore be sufficient to block the vasodilatation outside the treatment area, presumably produced by activation of a lower number of fibres, but not the maximum vasodilatation produced inside the treatment area.

BoNT/A blocks Ca^{2+}-dependent release of neurotransmitter in cholinergic synapses, by cleaving SNARE proteins essential for exocytosis (Simpson, 1981). The high specificity of BoNT/A for cholinergic nerves is due to the high affinity of BoNT H-chain for membrane receptors located on presynaptic cholinergic terminals (Montecucco and Schiavo, 1994). Recently, indirect evidence has suggested that BoNT/A inhibits transmitter release from neuropeptide containing nerve terminals (Welch et al., 2000; Ishikawa et al., 2001; Caputi, 2004; Durham et al., 2004). The ability of BoNT/A to reduce norepinephrine release from sympathetic nerves has also been reported (Morris et al., 2002). The sympathetic system and in particular norepinephrine/α_1-adrenoceptors seem to play an important role in capsaicin-induced pain and local inflammatory responses in the skin in animal models and in man (Drummond, 1995; Drummond, 1998; Lin et al., 2003; Ren et al., 2005), while the sympathetic sudomotor activity seems to have no effect on capsaicin ongoing pain and hyperalgesia (Wasner et al., 2000). Thus, it is possible that BoNT/A treatment reduces neurogenic vasodilatation and pain by inhibiting the release of norepinephrine. However, the contribution, if any, of BoNT/A in the regulation of transmitter release from adrenergic or neuropeptide containing nerve fibres has not been clarified yet. In conclusion, although the mechanism(s) underlying the inhibitory effects remains unknown, the proved ability of BoNT/A to inhibit neurotransmitter exocytosis and the different responses of capsaicin-induced pain and flare in relation to BoNT/A treated area could suggest that, in our study, BoNT/A could act at two different levels of capsaicin model, and more precisely at the direct nervous afferent branch that is related to capsaicin-induced pain, for example by blocking SNARE-dependent TRPV1 exocytosis, and at the efferent branch of the axonal reflexes by inhibiting neuropeptide release and the neurogenic vasodilatation. The present findings reinforce the hypothesis that BoNT/A represents an interesting opportunity to treat pain conditions characterised by neurogenic inflammation and inflammatory pain.

Note: During the completion of the revised version of the present study, Gazerani et al. (2006) reported that intramuscular injection of BoNT/A into the forehead of healthy volunteers reduced both pain and flare induced by capsaicin. These data are in agreement with our present findings. However, the original and novel finding of the present study, that application outside the BoNT/A-treated area reduced capsaicin-evoked flare, but not pain, does not support the hypothesis (Gazerani et al., 2006) that inhibition of sensory neuropeptide release is the exclusive and main underlying mechanism of the reduction by BoNT/A of capsaicin-evoked pain.

Acknowledgement

The study was in part supported by MIUR.

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