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RESEARCH PAPER

Bretylium abolishes neurotransmitter release without necessarily abolishing the nerve terminal action potential in sympathetic terminals

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Background and purpose: The antidysrhythmic bretylium is useful experimentally because it selectively abolishes neurotransmitter release from sympathetic peripheral nerve terminals. Its mechanism of action seemed settled, but recent results from optical monitoring of single terminals now suggests a new interpretation.
Experimental approach: Orthograde transport of a dextran-conjugated Ca^{2+} indicator to monitor Ca^{2+} in nerve terminals of mouse isolated vas deferens with a confocal microscope. In some experiments, local neurotransmitter release was detected by monitoring neuroeffector Ca^{2+} transients (NCTs) in adjacent smooth muscles, a local measure of purinergic transmission. Sympathetic terminals were identified with catecholamine fluorescence (UV excitation) or post-experiment immunohistochemistry.
Key results: Bretylium (10 μM) abolished NCTs at 60/61 junctions over the course of 2 h, indicating effective abolition of neurotransmitter release. However, bretylium did not abolish the field stimulus-induced Ca^{2+} transient in most nerve terminals, but did increase both action potential delay (by 2 ± 0.4 ms) and absolute refractory period (by 4 ± 2 ms). Immunohistochemistry demonstrated that 85–96% of terminals orthogradely filled with a dextran-conjugated fluorescent probe contained Neuropeptide Y (NPY). A formaldehyde–glutaraldehyde-induced catecholamine fluorescence (FAGLU) technique was modified to allow sympathetic terminals to be identified with a Ca^{2+} indicator present. Most terminals contained catecholamines (based on FAGLU) or secrete ATP (as NCTs in adjacent smooth muscle cells are abolished).
Conclusions and implications: Bretylium can inhibit neurotransmitter release downstream of Ca^{2+} influx without abolishing the nerve terminal action potential. Bretylium-induced increases in the absolute refractory period permit living sympathetic terminals to be identified.
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Keywords: sympathetic; confocal; calcium; bretylium; vas deferens; mouse

Abbreviations: FAGLU, formaldehyde–glutaraldehyde-induced catecholamine fluorescence; NCT, neuroeffector Ca^{2+} transient; Oregon-BAPTA-dex, Oregon Green 488 BAPTA-1 10 kDa dextran

Introduction

The mouse vas deferens is a useful model for studying sympathetic transmission as it has a dense, but not exclusive, noradrenergic innervation (Sjöstrand, 1965; Taxi, 1965; Yamauchi and Burnstock, 1969; Jones and Spriggs, 1975). With the increasing use of optical techniques to study living autonomic terminals (Brain and Bennett, 1997; Jackson *et al*., 2001; Lamont *et al*., 2003), it is now necessary to identify nerve terminal types in living tissue or at least to be able to identify the nerve terminal type on the stage of the microscope. The Falck technique (Falck *et al*., 1962) allowed catecholamines to be detected using light microscopes and was used to show that there is a dense noradrenergic innervation in rodent vasa deferentia (Sjöstrand, 1965), particularly in mice (Taxi, 1965; Jones and Spriggs, 1975). Sympathetic terminals can also be identified by the detection of intraterminal granular vesicles in electron micrographs (Merrillees *et al*., 1963; Richardson, 1966) or with immunohistochemistry. However, the extensive tissue processing required complicates all of these experimental approaches.

It has been shown, in mouse vas deferens (Brain *et al*., 2002, 2003), rat mesenteric vessels (Lamont and Wier, 2002) and rat urinary bladder (Heppner *et al*., 2005), that the focal release of ATP acting on postjunctional $P2X_1$ receptors can be detected by optically monitoring the Ca^{2+} concentration in smooth muscle cells. In the mouse vas deferens, the focal

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Ca^{2+} transients evoked by local ATP release are referred to as neuroeffector Ca^{2+} transients (NCTs). Although it has been assumed that such ATP release arises from sympathetic terminals, the bretylium sensitivity of these events has not yet been established.

It has been argued that bretylium (Boura and Green, 1959) prevents neurotransmitter release from sympathetic nerve terminals by abolishing the nerve terminal action potential (Haeusler *et al.*, 1969; Mitchell and Oates, 1970; Maxwell and Wastila, 1977; Brock and Cunnane, 1988); selectivity for sympathetic terminals occurs because bretylium is taken up into sympathetic terminals by a norepinephrine (NA) transporter-1 (or uptake-1). Hence, a potentially useful approach to identify noradrenergic terminals is to detect strings of varicosities in which the nerve action potential is abolished by bretylium (Jackson and Cunnane, 2002). However, Jackson and Cunnane (2002) showed that bretylium (20 μM) does not abolish the action potential-induced Ca^{2+} transient in the majority of nerve terminal varicosities orthogradely loaded with the dextran conjugate of a Ca^{2+} indicator. This causes a dilemma because the innervation of the mouse vas deferens is predominantly sympathetic (Taxi, 1965; Jones and Spriggs, 1975). Potential explanations include that

(a) the outer longitudinal layer was predominantly innervated by non-sympathetic nerves;
(b) the dextran-conjugated Ca^{2+} indicator selectively loads non-sympathetic terminals;
(c) the dextran-conjugated Ca^{2+} indicator selectively loads non-secretory regions of sympathetic terminals located proximally within the nerve terminal arborization;
(d) bretylium changes the shape of the nerve terminal action potential (and hence the configuration of the nerve terminal impulse) so that, although the total Ca^{2+} influx is not changed, the spatiotemporal Ca^{2+} pattern close to the membrane changes sufficiently to abolish transmitter release;
(e) bretylium inhibits transmitter release downstream of Ca^{2+} influx.

In the present study, hypotheses (a) and (b) are explored using immunohistochemistry, secretory terminals are identified on the basis of the action of bretylium on NCTs (c), whereas (d) and (e) are addressed using line scanning confocal Ca^{2+} imaging in nerve terminals. In addition, 3 methods for identifying sympathetic terminals in intact organs will be compared, namely the formaldehyde–glutaraldehyde-induced catecholamine fluorescence (FAGLU) technique, bretylium-induced action potential delay and the suppression of NCTs by bretylium.

Methods

Orthograde loading of nerve terminals

Vasa deferentia were removed from 8- to 12-week-old Balb/c mice (Harlan, UK), which had been killed by cervical fracture. Experiments were in accordance with the European Communities Council Directives (86/609/EEC of 24 November 1986). Each vas deferens was transferred to an organ bath; the cut prostatic end of each vas deferens was secured in a glass micropipette containing the 10 kDa dextran conjugate of either Alexa 568 or the Ca^{2+} indicator Oregon Green 488 BAPTA-1 (Oregon-BAPTA (1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetracetic acid)-dex; Invitrogen, Paisley, UK), using a protocol similar to that previously described (Brain and Bennett, 1997). The bathing physiological salt solution was re-circulated and contained (mM) NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.13, $CaCl_2$ 1.8, KCl 4.7, $MgCl_2$ 1.3 and glucose 11.1. pH and $[O_2]$ were regulated by continuously bubbling the solution reservoir with 95% O_2/5% CO_2. The preparations were left in contact with the dye for 5 h, removed from the micropipette and then left for a further 5 h, all at room temperature.

Immunohistochemistry

After orthogradely loading nerve terminals with Alex 568 dextran, vasa deferentia were fixed in 3% paraformaldehyde for 2 h and then cut using a vibratome to a thickness of 30–50 μm. Primary antibodies (raised in rabbit) were applied at a dilution of 1:1000 in 0.1% Triton X-100, overnight at 4 °C. The sections were then washed three times (10 min for each wash), and then incubated with Alexa 488-conjugated goat anti-rabbit secondary antibody (1:200) for 6–8 h at room temperature. The sections were washed a further three times, before being mounted on a slide in Vectashield (Vector Laboratories, Peterborough, UK). The primary antibodies used were rabbit polyclonal antibodies for Neuropeptide Y (NPY) (catalogue number NA1233; Affiniti, Devon, UK), Calcitonin gene related peptide (CGRP; CA1134), TH (TZ1010) and the vesicular ACh transporter (H-120; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). This NPY antibody has not been confirmed to work in mice. However, the amino-acid sequence of NPY is the same in mouse (Swiss Prot accession number P57774; positions 29–64), human (accession number P01303; 29–64) and rat (accession number P07808; 30–65). In preliminary experiments using a primary rabbit polyclonal IgG against the vesicular ACh transporter (H-160; Autogen Bioclear UK (Calne, Wiltshire, UK)), there was no positive labelling in either the vas deferens or the bladder (which contains a dense cholinergic innervation and hence acts as a positive control). Given the lack of labelling in the bladder vesicular, ACh transporter labelling was not used to investigate the cholinergic innervation of the vas deferens. In mouse bladder, dense AChE labelling could be detected (results not shown). To reduce the impact of poor antibody penetration into these sections, confocal images were acquired only within 5 μm of the cut surfaces.

FAGLU

The FAGLU technique (Furness *et al.*, 1978) allows the detection of catecholamines in nerve terminals and is a modification of the histochemical technique of Hillarp and Falck (Falck *et al.*, 1962). The only naturally occurring substances in peripheral nerves that generate a fluorescent product when reacted with aldehydes are noradrenaline, adrenaline, dopamine and 5-HT; these are only found in

sympathetic nerves and their terminals (Furness *et al*., 1978), although 5-HT may be found in other cell types such as mast cells. In the present study, a modification of the original FAGLU technique was used (Furness *et al*., 1987). At the end of the experiments in living organs, a solution containing 4% formaldehyde and 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer (adjusted to pH 7.0) was cooled at 0 °C and applied to the vas deferens while in an organ bath mounted on the stage of a confocal microscope. The solution was changed every 20 min to keep the temperature low. The solution was then changed to one containing FAGLU (as above) with 20% w/v sucrose (Nakamura, 1979). The chemistry of this reaction is described elsewhere (Björklund *et al*., 1973). The ultraviolet (UV) laser on the confocal microscope was used to excite fluorescence. In tissues loaded with Oregon-BAPTA-dex, UV illumination excited a fluorescent signal from dextran-loaded terminals when a 515 nm long pass emission filter was used. Hence, all experiments involving UV illumination and Oregon-BAPTA-dex were carried out using a 440 nm band pass emission filter. The emission spectrum of Oregon-BAPTA-dex when illuminated with UV light (355 nm wavelength) was assessed in a spectrophotometer (LS50B; Perkin Elmer, Seer Green, UK). There was a strong, narrow fluorescent peak at 395 ± 20 nm and a weak, broad peak with a maximum at 520 nm (commencing at 485 nm); there was negligible fluorescent emission at 440 ± 20 nm. When the FAGLU solution was added to the cuvette containing Oregon-BAPTA-dex, there was a 25% increase in the fluorescent signal at 520 nm (after 30 min), but still there was no fluorescent signal at 440 ± 20 nm. This implies that Oregon-BAPTA-dex in FAGLU-treated tissue is unlikely to fluoresce at 440 ± 20 nm when excited with the UV laser.

Nerve terminal Ca^{2+} imaging

In preparations orthogradely loaded with Oregon-BAPTA-dex, images were acquired using a Leica NT inverted confocal microscope. Nerve terminals filled with the Ca^{2+} indicator and a small number of smooth muscle cells close to the cut end of the tissue could be seen (Brain *et al*., 2002). In some cases, labelled smooth muscle cells were found where the adjacent nerve terminal varicosities were also filled with the indicator. All field stimuli (pulse width 0.1 ms; amplitude 50 V) were synchronized with the start of image acquisition, so that the interval between each stimulus and recording was fixed. During line scanning (0.54 ms per line for 256 lines, acquired and stimulated at 0.33 Hz for 60 s), stimuli were triggered 40 ms after the commencement of each frame.

Smooth muscle Ca^{2+} imaging

In these experiments, each vas deferens was exposed to the Ca^{2+} indicator Oregon Green 488 BAPTA-1 acetoxymethyl ester (Oregon-BAPTA-AM; Molecular Probes; 10 μM in 1% dimethylsulphoxide) for 2 h at 36 °C and then washed for at least 30 min in physiological salt solution. This filled several cell types within the preparation (although almost never nerve terminals); smooth muscle cells were identified by their characteristic morphology. During field stimulation, highly intermittent, focal smooth muscle Ca^{2+} transients (NCTs) were recorded as described previously (Brain *et al*., 2002).

Image analysis

Image analysis was performed with NIH Image 1.63 (http://rsb.info.nih.gov/nih-image/) or Image SXM (http://www.liv.ac.uk/~sdb/ImageSXM/) using custom-written macros. Nerve terminal and smooth muscle Ca^{2+} measurements were quantified as described previously (Brain and Bennett, 1997; Brain *et al*., 2002).

Drug preparation

Bretylium tosylate and amphetamine sulphate stock solutions (10 mM in water; Sigma-Aldrich (Poole, Dorset, UK)) were stored in aliquots at –20 °C. Formaldehyde (F8775; 37%) and glutaraldehyde solutions (G5882; 25%; Sigma) were diluted to the appropriate final concentration when required.

Statistics

In the immunohistochemistry and FAGLU experiments, 95% confidence intervals (95% CIs) were calculated by assuming that terminals were randomly selected from the population (and hence that counting followed binomial statistics).

Results

Immunohistochemistry

To identify the types of nerve terminal in the outer longitudinal layer of the mouse vas deferens accessible to orthograde labelling, terminals were loaded with Alexa 568 dextran, fixed and longitudinal sections were taken for immunohistochemistry. TH labelling was detected in at least two varicosities per terminal in 39 of the 81 dextran-labelled terminals (95% CI 39–59%). In many terminals, only a small proportion of the varicosities were positively labelled with TH (Figure 1a). In sections labelled with NPY, which also marks sympathetic terminals, 82 of the 90 terminals were NPY positive (95% CI 85–96%; Figure 1b).

From a sample of 91 terminals, five were CGRP positive (95% CI 3–12%; Figure 1c). Although the CGRP labelling was punctate, the terminals themselves, filled with an indicator that distributes throughout the cytoplasm, had a smoother profile than that seen with TH- or NPY-positive terminals.

In other tissues, 19 of the 312 terminals were AChE positive (95% CI 4–9%; Figure 1d). These AChE-positive terminals were often located in tight loops reminiscent of small blood vessels supplying the vas deferens.

FAGLU labelling

The FAGLU labelling technique was used to identify catecholamine-containing nerve terminals. Each vas deferens was exposed to FAGLU for 30–60 min. During this time, no specific fluorescence could be observed when excited with

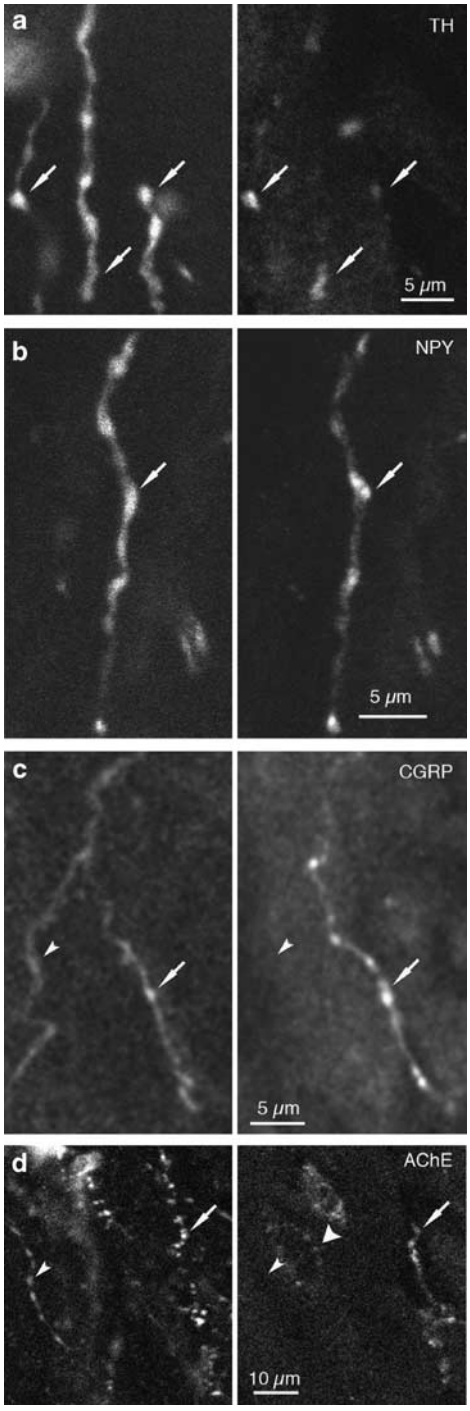

Figure 1 Immunohistochemistry of orthogradely loaded terminals. The left panels show a selection of terminals orthogradely loaded with Alexa 568 dextran from fixed tissue cut in longitudinal sections. The right panels show the corresponding regions in experiments in which the sections were labelled with TH (**a**), NPY (**b**), CGRP (**c**) or AChE (**d**). CGRP, Calcitonin gene related peptide; NPY, Neuropeptide Y.

488 nm light (Figure 2a), but with UV excitation structures resembling nerve terminal varicosities could be identified (Figure 2b).

To determine whether dextran-labelled terminals contained catecholamines, vasa deferentia were orthogradely loaded with Oregon-BAPTA-dex. Terminals (Figure 2c) were identified in which field stimulation caused an increase in fluorescent signal, indicating an increase in $[Ca^{2+}]_t$. When these live vasa deferentia were viewed with UV illumination (using a 440 nm band pass emission filter), there was significant tissue autofluorescence (Figure 2d). No nerve terminals could be identified. When FAGLU was added to the bath, there was some local tissue deformation and rotation, but this was not sufficient to either lose the field of view or prevent the identification of each nerve terminal. The location of each nerve terminal was identified with 488 nm wavelength light (for example, see Figure 2e). When exciting fluorescence with UV light, a fluorescent signal developed in 113 of the 117 nerve terminals (93–99%; number of preparations, $n_p = 7$; for example, see Figure 2f). Many terminals were fluorescent under UV illumination, but not when excited at 488 nm, implying that not all terminals in the preparation were filled with the Ca^{2+} indicator (or were poorly filled). It was possible to identify terminals that were filled with the dextran, but showed no UV fluorescence after treatment with FAGLU (Figures 2g and h).

Bretylium and FALGU

Nerve terminals orthogradely loaded with OG-BAPTA-dex and responding to field stimulation were identified. When exposed to bretylium (10–20 μM) for 2 h, all terminals recorded in this series maintained their nerve terminal $[Ca^{2+}]_t$ transient elicited by field stimulation (19 terminals; $n_p = 5$). Upon exposure to FAGLU, all of these terminals developed a UV fluorescent signal.

Bretylium and NCTs

In preparations loaded with Oregon-BAPTA-AM, to fill smooth muscle cells with the Ca^{2+} indicator, field stimulation at 1 Hz was used to elicit NCTs. Bretylium (10 μM) abolished almost all evoked NCTs (number of neuroeffector junctions, $n_j = 60/61$; $n_p = 7$; for example, see Figure 3). This effect was partially reversed by 10 μM amphetamine (to at least 10% of the control frequency), a drug that inhibits intraterminal bretylium accumulation (Ross and Gosztonyi, 1975). At the one junction where bretylium did not abolish NCTs, their frequency was reduced by more than 90%, and amphetamine (10 μM) caused complete recovery of NCTs (to within 10% of the control frequency).

In experiments where both nerve terminal varicosities and the underlying smooth muscle cells were loaded (using Oregon-BAPTA-dex), it was possible to record the nerve terminal Ca^{2+} transient and directly adjacent NCTs (Figure 3; $n_p = 3$). In such cases, NCTs were abolished without abolishing the nerve terminal action potential.

Bretylium and the nerve terminal Ca^{2+} transient

Nerve terminal varicosities were loaded with Oregon-BAPTA-dex to measure the delay between the stimulus and a rapid rise in $[Ca^{2+}]_t$ during line scanning confocal microscopy (which acts a proxy for the arrival of the action potential). After 60–90 min in bretylium (10 μM), the delay was in-

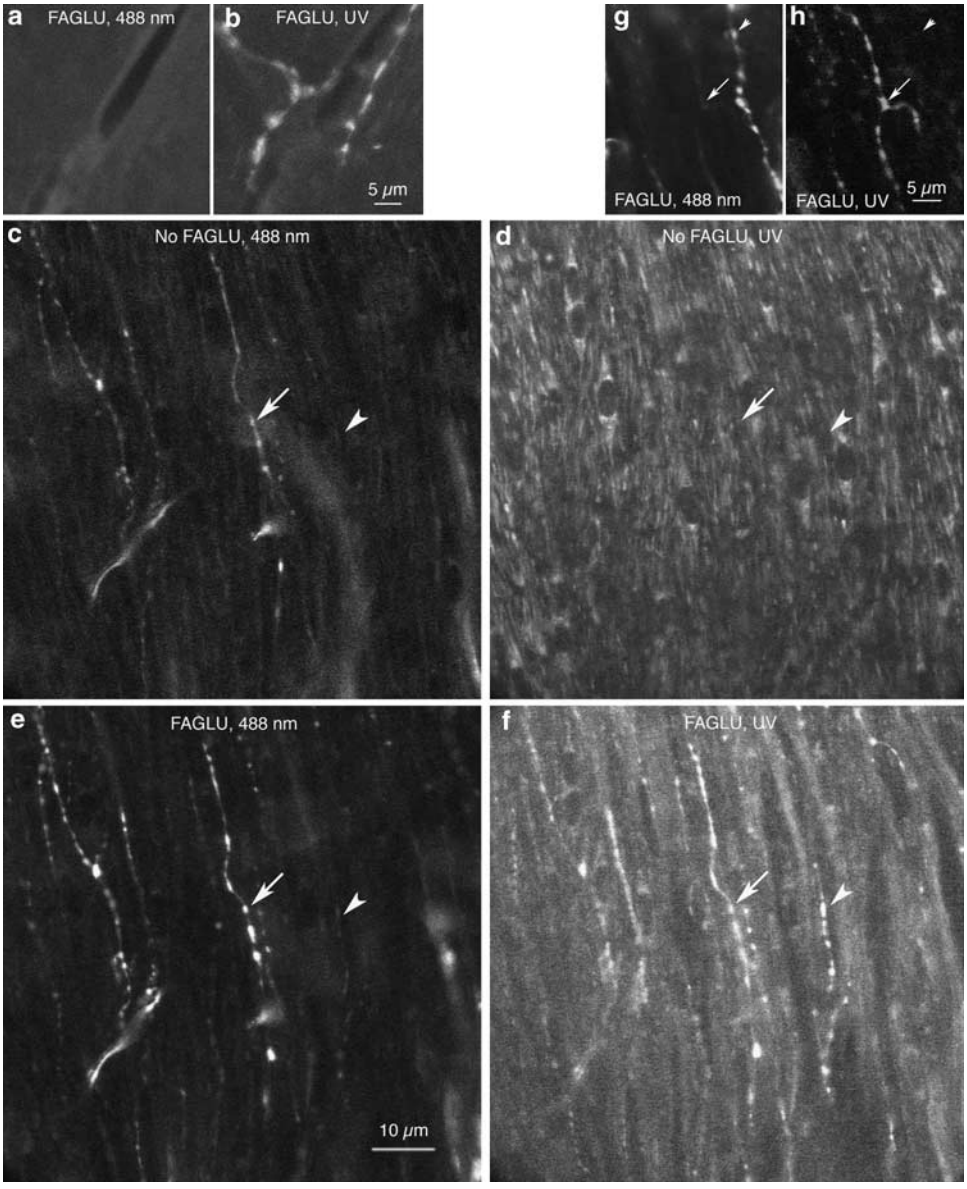

Figure 2 FAGLU fluorescence in the outer longitudinal layer. In the absence of nerve terminal Oregon-BAPTA-dex labelling and after the FAGLU protocol, 488 nm light excitation gives little fluorescence. (**a**) When excited with a UV laser (**b**), nerve terminal varicosities fluoresce. (**c**) A wide field image of Oregon-BAPTA-dex-filled terminals (for example, at the arrow) illuminated at 488 nm prior to the FAGLU protocol. When the same region is illuminated with UV light (**d**), there is significant autofluorescence from an unidentified cell types or structures. There is no UV fluorescent signal from terminals. After treatment with FAGLU, there is some degree of tissue deformation and an increase in the fluorescent signal in terminals excited at 488 nm. (**e**) When illuminated with UV light (**f**), many nerve terminal fluoresce, including some that are not (or that are poorly) filled with the Ca^{2+} indicator (arrowhead). (**g** and **h**) Another field, where there are Oregon-BAPTA-dex-filled terminals that are both FAGLU positive (arrow) and FAGLU negative (arrowhead). BAPTA , 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*,′*N*-tetracetic acid; FAGLU, formaldehyde–glutaraldehyde-induced catecholamine fluorescence; Oregon-BAPTA-dex, Oregon Green 488 BAPTA-1 10 kDa dextran.

creased by 2 ± 0.4 ms ($n_p = 7$; Figure 4a); an increased delay was observed in all terminals. No significant change in the rise time or the recovery of the $[Ca^{2+}]_t$ transient was detected. The onset of this increased delay commenced 60 min after the first exposure to bretylium (Figure 4b).

The refractory period in single nerve terminals was measured using paired-pulse supramaximal field stimuli and monitoring $[Ca^{2+}]_t$. Under control conditions, the absolute refractory period was 3 ms in all terminals tested (Figure 4c); that is paired pulse stimuli could not be elicited with a 2 ms delay, but could be elicited with a 3 ms delay between the pulses. After treatment with bretylium (10 μM) for 90 min, the refractory period was 7 ± 2 ms (in 7 terminals; $n_p = 3$; Figure 4d). The refractory period increased by at least 1 ms in all terminals. It was noted that even under control conditions, the time between the increase in $[Ca^{2+}]_t$ from consecutive field stimuli was slower than the interpulse interval (Figure 4c). This is consistent with a slower propagation speed of the second action potential in the pair.

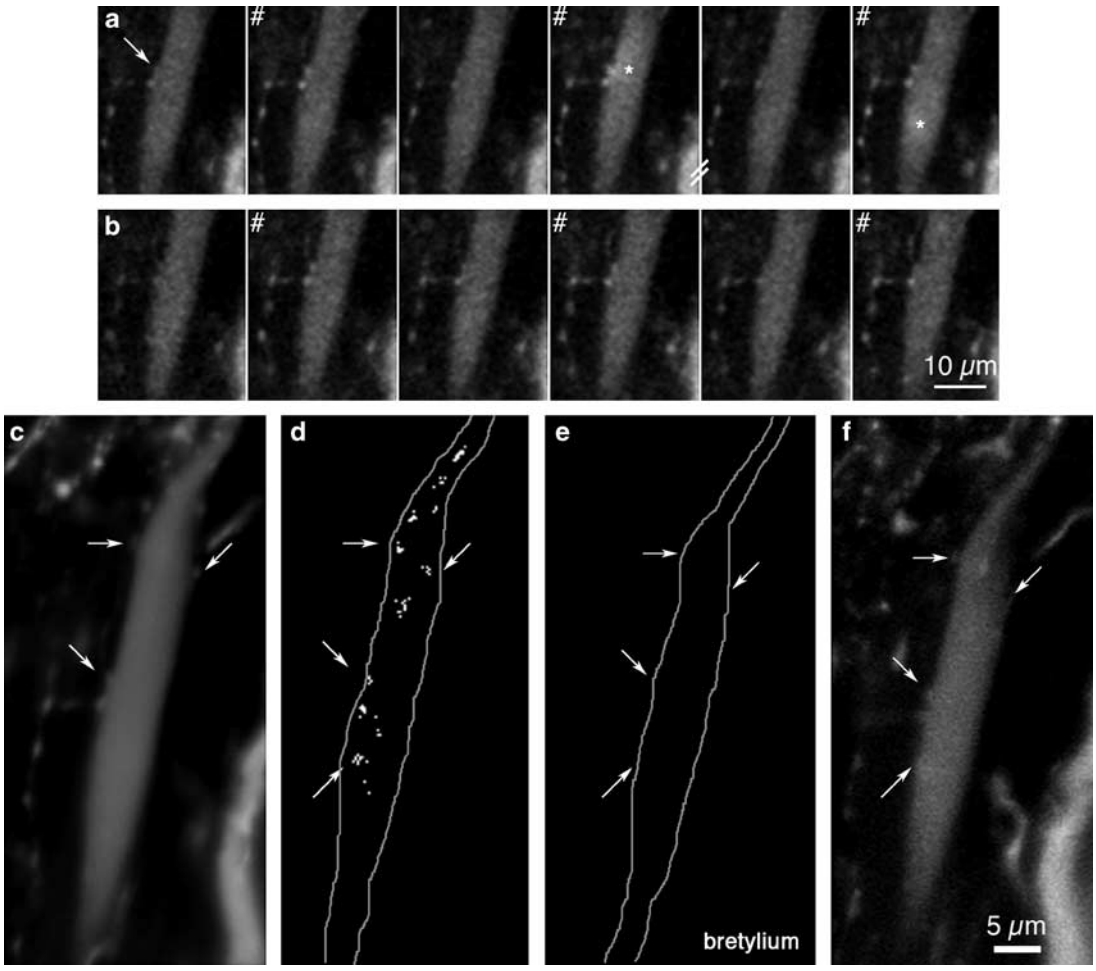

Figure 3 Nerve terminal $[Ca^{2+}]$ regulation, neuroeffector Ca^{2+} transients (NCTs) and bretylium. (**a**) A set of images, acquired every 500 ms, showing an Oregon-BAPTA-dex-labelled smooth muscle cell and nerve terminal varicosities (for example, at the arrows). Every field stimulus (#) resulted in an increase in $[Ca^{2+}]_t$, but only intermittent evoked local increases in $[Ca^{2+}]$ in the smooth muscle cell (NCTs; for example on the 4th and 6th frames, marked with an asterisk). After 90 min in bretylium, the $[Ca^{2+}]_t$ increased upon every field stimulus; however, no NCTs were detected. (**b**) A wider field view of this region (average of 400 frames; frames were aligned with Image SXM prior to averaging). Maps of the location of NCTs, occurring at some time over 30 min of recording, were constructed under both control conditions (**d**) and after exposure to bretylium (**e**). (**d** and **e**) A manually drawn cell outline (from **c** and **f**, respectively) as an aid for aligning corresponding areas. (**f**) An average of 10 frames, acquired at a slightly different plane of focus to demonstrate some nerve terminal varicosities that are not in the plane of focus in (**c**). BAPTA , 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*,′*N*-tetracetic acid; Oregon-BAPTA-dex, Oregon Green 488 BAPTA-1 10 kDa dextran.

Discussion and conclusions

In the guinea pig vas deferens, bretylium abolishes excitatory junction currents and markedly alters the shape of the nerve terminal impulse (Brock and Cunnane, 1988), an effect that has been interpreted as indicating an abolition of the nerve terminal action potential (for review see Boura, 1997). In the mouse vas deferens, bretylium abolishes excitatory junction potentials, but does not affect the nerve terminal Ca^{2+} transients in the majority of terminals in this tissue (Jackson and Cunnane, 2002); this apparent contradiction is explored in the present work.

The finding that bretylium abolishes NCTs (in the majority of cases) implies that NCTs arise from the release of ATP from sympathetic terminals. As such, the occurrence of NCTs adjacent to nerve terminal varicosities can be used to identify a given varicosity as sympathetic. The main problem with this approach is that in many sympathetically innervated tissues, neurotransmitter release occurs from within small bundles of terminals. In such cases, if different nerve terminal types are closely packed together, it may be impossible to use this optical approach to identify the source of the neurotransmitter. In the mouse vas deferens, sympathetic nerve terminals commonly run singly (Cottee *et al.*, 1996).

Bretylium had no detectable effect on either the time course of the action potential-induced $[Ca^{2+}]_t$ rise or the rate of $[Ca^{2+}]_t$ recovery. This supports the evidence of Jackson and Cunnane (2002) that bretylium does not affect Ca^{2+} handling in nerve terminals. However, the increase in delay between the stimulus and arrival of the action potential in the nerve terminal (as reported by an increase in $[Ca^{2+}]_t$) suggests that either the site of action potential initiation moves further away from the terminal or, more likely, that the speed of action potential propagation has slowed. The increase in absolute refractory period fits well with the classical observation that the effects of bretylium are more marked on high-frequency stimulation (Boura and Green, 1962) and represents the local anaesthetic-like action of

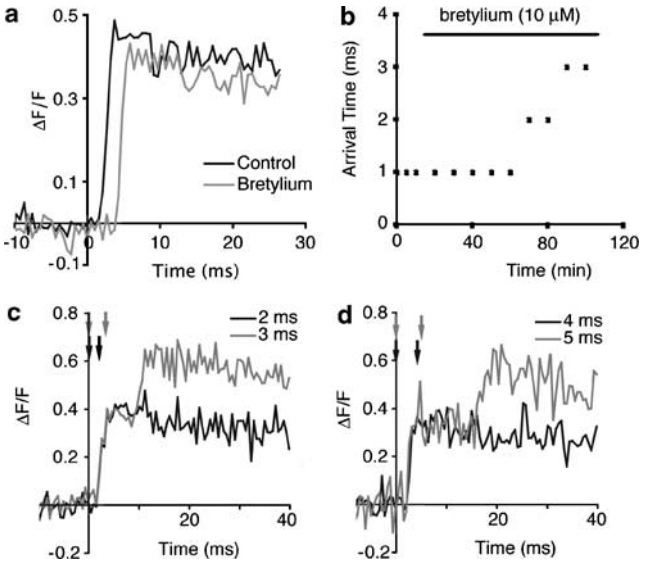

Figure 4 Line scanning Ca^{2+} imaging in nerve terminals with and without bretylium. (**a**) The average of four confocal line scans through a single terminal, with and without bretylium, showing the change in the fluorescent signal ($\Delta F/F$) of the Ca^{2+} indicator Oregon-BAPTA-dex. A single field stimulus was applied at 0 ms, and after a brief delay the nerve terminal action potential arrives in the terminal, causing an increase in $[Ca^{2+}]_t$. In the presence of bretylium, the $[Ca^{2+}]_t$ rises later, suggesting that the action potential arrives in the terminal later. The time taken for the onset of this delay is illustrated in (**b**), which represents the delay between the stimulus and first detectable increase in $[Ca^{2+}]_t$ in a single terminal. In a different preparation (**c** and **d**), supramaximal paired-pulse stimuli (arrows) were applied to measure the absolute refractory period. Each trace is the average of four line scans acquired before (**c**) and in the presence (**d**) of bretylium. Note in (**c**) the second pulse at a 2 ms interval fails to initiate an increase in $[Ca^{2+}]_t$ (suggesting that it fails to initiate an action potential or that any action potential fails to reach the terminal), but does transmit at a 3 ms interval. In the presence of bretylium, the second impulse will not transmit at a 4 ms (or briefer) interval, but will do so at a 5 ms (or longer) interval. BAPTA , 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*,′*N*-tetracetic acid; Oregon-BAPTA-dex, Oregon Green 488 BAPTA-1 10 kDa dextran.

bretylium that is specific to sympathetic nerves (Haeusler *et al*., 1969; Brock and Cunnane, 1988). The nerve terminal impulse, the second derivative of the action potential, is expected to change greatly with action potential slowing; therefore, it is possible that the nerve terminal impulse amplitude change described by Brock and Cunnane (1988) might be due purely to slowing rather than to a collapse of the nerve terminal action potential. An increase in absolute refractory period induced by bretylium could be used to optically identify sympathetic nerve terminals during nerve terminal $[Ca^{2+}]$ imaging.

The abolition of NCTs without abolishing the nerve terminal action potential in the adjacent terminal and the high proportion of catecholamine-containing terminals in which bretylium fails to abolish the nerve terminal Ca^{2+} transient, together imply that bretylium can inhibit neurotransmitter release without abolishing the nerve terminal action potential, at least in the mouse vas deferens. Whether this is by changing the shape of the nerve terminal action potential (in a manner that does not significantly affect Ca^{2+} influx), by locally modifying the spatiotemporal Ca^{2+} signal at the trigger site in a manner that does not affect the residual Ca^{2+} transient (hypothesis d) or by targeting exocytosis at a site downstream of Ca^{2+} entry (hypothesis e) has not been clearly established. Further evidence for a direct action of bretylium on transmitter release comes from chick sympathetic neurons, where bretylium (3 μM) can abolish norepinephrine overflow while having no effect on depolarization-induced Ca^{2+} current (under voltage clamp) or intracellular $[Ca^{2+}]$ transient during action potentials (evoked by 120 mA current pulses for 1 ms applied at 1 Hz for 10 s) (Przywara *et al*., 1991). Also, cooling-induced release of noradrenaline from cutaneous blood vessels (and hence vasoconstriction) is present in tetrodotoxin (TTX), but blocked by the addition of bretylium (Koganezawa *et al*., 2006). This suggests that bretylium, in addition to its effects on nerve terminal conduction, blocks noradrenaline release downstream of the action potential.

It seems odd that in the present experiments, the TH labelling was not consistent within a given terminal. The most parsimonious explanation is that of a methodological problem of poor permeability of the primary TH antibody into the tissue. Such non-uniformity was not present in the labelling with other primary antibodies such as that for NPY. In addition to nerve terminals, NPY immunoreactivity has been detected in Schwann cells (Ubink and Hokfelt, 2000). However, in the present immunohistochemistry experiments, varicosities were identified by othogradely loading with the dextran conjugate of Alexa 568 so that terminals could be morphologically identified.

Although NPY labelling has been used to identify sympathetic nerves (Stjärne *et al*., 1986; Ventura *et al*., 1998), there is a very low density of cholinergic terminals that contain both vasoactive intestinal peptide (VIP) and NPY in the guinea pig vas deferens (Song *et al*., 1994). If it is assumed that AChE marks cholinergic terminals and CGRP locates sensory terminals, the distribution of terminal types in the outer longitudinal layer was broadly in line with that reported for whole mouse vas deferens (Sjöstrand, 1965; Taxi, 1965; Yamauchi and Burnstock, 1969; Jones and Spriggs, 1975). These observations suggest that dextran-conjugated fluorescent probes do not selectively load any particular nerve terminal type. In the mouse vas deferens, only about 10% of axons are cholinergic (Yamauchi and Burnstock, 1969; Jones and Spriggs, 1975). Although the distribution of cholinergic fibres has not been clearly described in the mouse, in other rodents the cholinergic fibres are concentrated in the inner circular layer close to the epithelium (Risley and Skrepetos, 1964; Al-Zuhair *et al*., 1975; Ventura *et al*., 1998).

The observation that 93–99% of terminals developed UV-induced fluorescence in response to FAGLU is consistent with the observation that most 3,3-diethyloxardicarbocyanide ($DiOC_2(5)$) fluorescent varicosities in the mouse vas deferens also display such FAGLU fluorescence (Lavidis and Bennett, 1993). It is worth noting that the UV laser is capable of eliciting some fluorescent signal from the Ca^{2+} indicator Oregon-BAPTA-dex at long wavelengths. However, by sampling the fluorescent emission signal with a band pass 440 nm filter, it is possible to detect only the catecholamine-induced fluorescent signal. The density of varicosities in the

vas deferens of $2 \times 10^6\,mm^{-3}$ (56×10^6 per 10 mm length in the rat; Dahlström *et al.*, 1966; cross-sectional muscle area of $2.6\,mm^2$; Carvalho *et al.*, 1993) implies that about four varicosities per $30\,\mu m \times 30\,\mu m \times 2\,\mu m$ optical section should be observed. This is comparable to or lower than the density of FAGLU-positive varicosities observed in the present study (see Figure 2).

A limitation of the present study is that the high proportion of sympathetic terminals makes it difficult to find non-sympathetic terminals. Hence, it is not surprising that no terminals were found in which bretylium failed to affect the absolute refractory period.

Although FAGLU, juxtaposition of terminals and NCTs, and bretylium-induced refractory period prolongation have been shown to be viable methods for identifying catecholaminergic terminals in the mouse vas deferens, FAGLU seems the simplest approach in the absence of nerve terminal Ca^{2+} imaging, whereas bretylium-induced refractory period prolongation has the advantage of allowing living terminals to be identified when nerve terminal Ca^{2+} imaging is available. The direct mode of action of bretylium to inhibit neurotransmitter release, independent of effects on the nerve terminal action potential, should be considered when it is used as an investigational tool.

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Conflict of interest

The authors state no conflict of interest.

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