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SPECIAL REPORT

Bretylium or 6-OHDA-resistant, action potential-evoked Ca^{2+} transients in varicosities of the mouse vas deferens

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Action potential-evoked calcium transients in varicosities in mouse vas deferens were monitored using laser scanning confocal microscopy. Their significance was examined by comparison with excitatory junction potentials (EJPs) and neurogenic contractions, both indirect measurements of transmitter release. Bretylium abolished EJPs, as well as the ATP and NA-mediated phases of contraction. However, bretylium revealed a prominent late component of contraction that was atropine-sensitive. Bretylium abolished calcium transients in 21%, enhanced in 16% and had no effect in 63% of varicosities examined. Pre-treatment with 6-OHDA reduced NA levels to below detectable levels but many strings of varicosities still responded to nerve impulses with 'normal' calcium transients. Varicosities in which calcium transients were abolished by these agents were sympathetic. The identity of those varicosities in which calcium transients were resistant to bretylium (sympathetic but no uptake-1 sites, parasympathetic, sensory) remains to be established.
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Abbreviation: EJP, excitatory junction potential

Introduction Electrophysiological techniques have shown unequivocally that the action potential invades all sympathetic varicosities but releases the transmitters ATP (Brock & Cunnane, 1988a) and NA (Msghina *et al*., 1993) in a highly intermittent manner. One explanation for intermittence is that the action potential may often fail to elicit Ca^{2+} entry into sympathetic varicosities. This aspect may now be examined directly by using laser scanning confocal microscopy, a method shown to resolve the action potential-evoked entry of Ca^{2+} into individual varicosities. In vas deferens, a preparation which is densely innervated by sympathetic nerves, Brain & Bennett (1997) showed that the nerve action potential elicited a Ca^{2+} transient in every varicosity examined. Thus, action potential-evoked Ca^{2+} entry in individual varicosities is non-intermittent, while Ca^{2+}-dependent transmitter release is highly intermittent. It was also demonstrated that Ca^{2+} entry could be modulated in some, but not all, varicosities by α_2- and P2X-receptor agonists and antagonists (O'Connor *et al*., 1999). However, the interpretation of these findings crucially depends on whether the varicose nerve terminals studied were sympathetic. Undoubtedly some were sympathetic but the possibility that others were parasympathetic/sensory was not considered. One approach is to use bretylium to identify sympathetic nerve terminals. Bretylium enters sympathetic varicosities through uptake-1 sites where it acts as a local anaesthetic and blocks action potential propagation in the secretory terminals and hence transmitter release (Boura & Green, 1960; Brock & Cunnane, 1988b). Bretylium should therefore abolish action potential-evoked Ca^{2+} transients in all varicosities containing uptake-1 sites. Similar results would be expected following destruction of sympathetic nerve terminals with 6-OHDA. Here we report on the effects of bretylium and 6-OHDA on action potential-evoked Ca^{2+} transients in short strings of varicosities in the mouse isolated vas deferens.

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Methods *Ca^{2+} imaging* Aganglionic vasa deferentia were removed from 8–12 week-old Balb/C mice and placed in Krebs solution containing (mM): NaCl 118.8, $NaHCO_3$ 25, NaH_2PO_4 1.13, KCl 4.7, $CaCl_2$ 1.8, $MgCl_2$ 1.2, glucose 11.1 and gassed with 95% O_2/5% CO_2 to pH 7.4. The vas deferens was sucked into a pipette containing a solution of Oregon Green 488 BAPTA1, dextran 10 kDa (Molecular Probes) and left to load for either 5 or 8 h to allow the indicator to travel variable distances along the nerve, and then subsequently washed for 3 h. Dynamic movies can be viewed on the Web at: http://users.ox.ac.uk/~cunnane/Sympathetic.htm.

Stimuli and recording The prostatic end of a vas deferens was mounted in a 2 ml organ bath and placed on the stage of a Leica TCS NT inverted laser scanning confocal microscope. The preparation was secured with a pair of parallel platinum electrodes and stimulated using an optically isolated stimulator (Digitimer DS2). The pulse width was set between 0.06–0.4 ms and the applied voltage was adjusted to give a reliable change in fluorescence following a single stimulus. The stimulus voltage was then increased by 20% to ensure that it was suprathreshold. The stimuli were electronically synchronized with the confocal microscope scans. The 488 nm wavelength of an argon ion laser was used for exciting fluorescence together with a 515 nm long pass emission filter. When detecting Ca^{2+} transients, sets of images were captured for 56 s every 3 min at 33–34°C in the presence of nifedipine and prazosin (1 μM). This protocol prevented excessive photobleaching and phototoxicity. In a typical preparation several terminal branches, each

containing about 6–8 varicosities, were examined. It is important to note that all varicosities on individual strings were equally affected (inhibited, enhanced or unchanged) by bretylium.

Intracellular recording Conventional intracellular recording techniques were used to record EJPs in smooth muscle cells as a measure of ATP release (Cunnane & Manchanda, 1988). In control experiments nifedipine and prazosin (1 μM) had no significant effects on EJPs.

Mechanical responses Neurogenic contractions were recorded as previously described (Allcorn *et al*., 1986). Nifedipine and prazosin were not present in the Krebs solution.

6-OHDA pre-treatment 6-OHDA was dissolved in sterile saline 0.9% w v^{-1} solution and mice were injected intraperitoneally (100 mg kg^{-1}). Tissue was removed after allowing 2 days for sympathetic nerve terminals to degenerate.

Data analysis As Oregon Green is a non-ratiometric dye, changes in intracellular Ca^{2+} concentration are reported as changes in fluorescence divided by basal fluorescence ($\Delta F/F$). Data are expressed as mean$\pm$s.e.mean and analysed statistically using a one tailed paired *t*-test and statistical significance taken as $P<0.05$. Imaging data has been normalized with control responses being expressed as 100%.

Drugs Stock solutions of D-amphetamine sulphate, atropine sulphate and bretylium tosylate, were dissolved in distilled water, prazosin in dimethyl sulphoxide and nifedipine in ethanol. Vehicle controls had no detectable effect on Ca^{2+} transients. All compounds were obtained from Sigma (Dorset, U.K.).

Results *Effects of bretylium on EJPs* EJPs were recorded to ensure that bretylium reliably blocked ATP release. EJPs evoked by trains of stimuli at 0.33 Hz (Figure 1) were abolished after 1–2 h exposure to bretylium (20 μM; Figure 1a). Amphetamine (20 μM) reversed the inhibitory effect of bretylium on EJP amplitude (Figure 1a). The mean data summarizing the degree of inhibition of EJPs produced by bretylium and reversal by amphetamine are respectively, control, 31.6$\pm$1.8 mV; bretylium 0.6$\pm$0.3 mV, $n=19$ animals, $P<0.005$; amphetamine 22$\pm$4.4 mV, $n=3$ animals.

Effects of bretylium on neurogenic contractions To test that NA release was abolished, long trains of stimuli were used to elicit a noradrenergic contraction. Both the purinergic and noradrenergic contractions were abolished but even after 3–5 h exposure to bretylium, a significant TTX-sensitive contraction remained in response to trains of 100 stimuli at 10 Hz in six preparations. The bretylium-insensitive contraction varied in amplitude from preparation to preparation (18–121% of the control noradrenergic component) but was abolished when atropine (2 μM) was applied (Figure 1).

Effects of bretylium on action potential-evoked Ca^{2+} transients Bretylium was used to determine whether the varicosities were sympathetic in origin. Surprisingly, bretylium (20 μM) had no effect on action potential-evoked Ca^{2+} transients in most preparations. Bretylium abolished the Ca^{2+} transient in every varicosity on the same string in only four out of 19 preparations (21%) loaded for 5 h, but always to the same extent in all varicosities on the same nerve terminal branch (Figure 2, -100.2 ± 2.7% of control, $n=43$ varicosities, $n=8$ strings, $n=4$ animals; $P<0.005$). However, when Ca^{2+} transients were abolished by bretylium, the inhibitory effect was partially reversed by amphetamine (20 μM; Figure 2d, -17.8 ± 6.1% of control). In 12 out of 19 preparations,

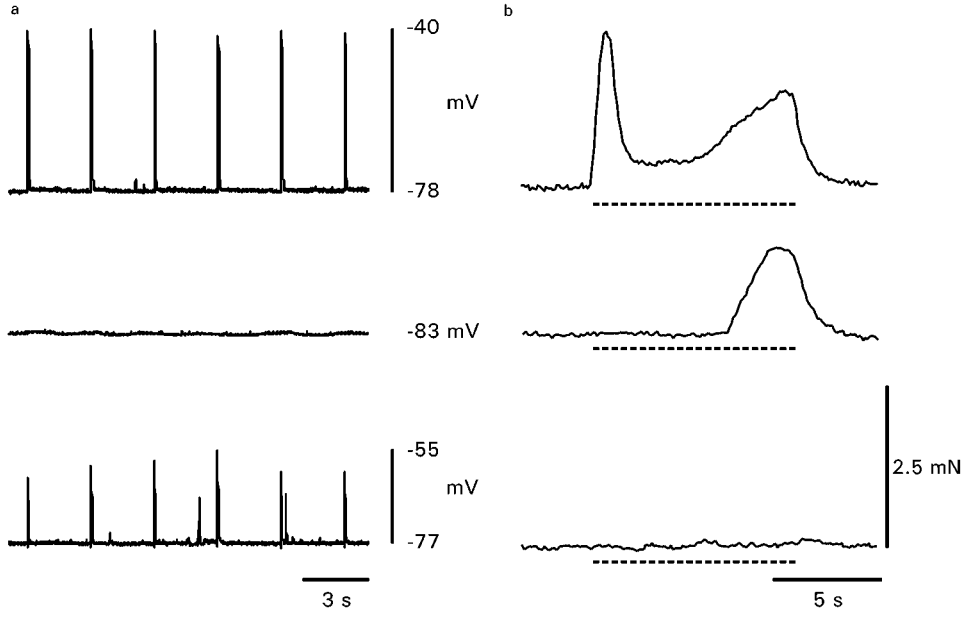

Figure 1 Effects of bretylium on EJPs in mouse vas deferens. (a) Control EJPs evoked at 0.33 Hz, 1 h after bretylium (20 μM) and reversal by amphetamine (20 μM). (b) Upper panel: control biphasic contraction evoked by a train of 100 stimuli at 10 Hz; Middle panel: monophasic contraction evoked after 5 h exposure to bretylium (20 μM); Lower panel: bretylium-resistant contraction abolished by atropine (2 μM).

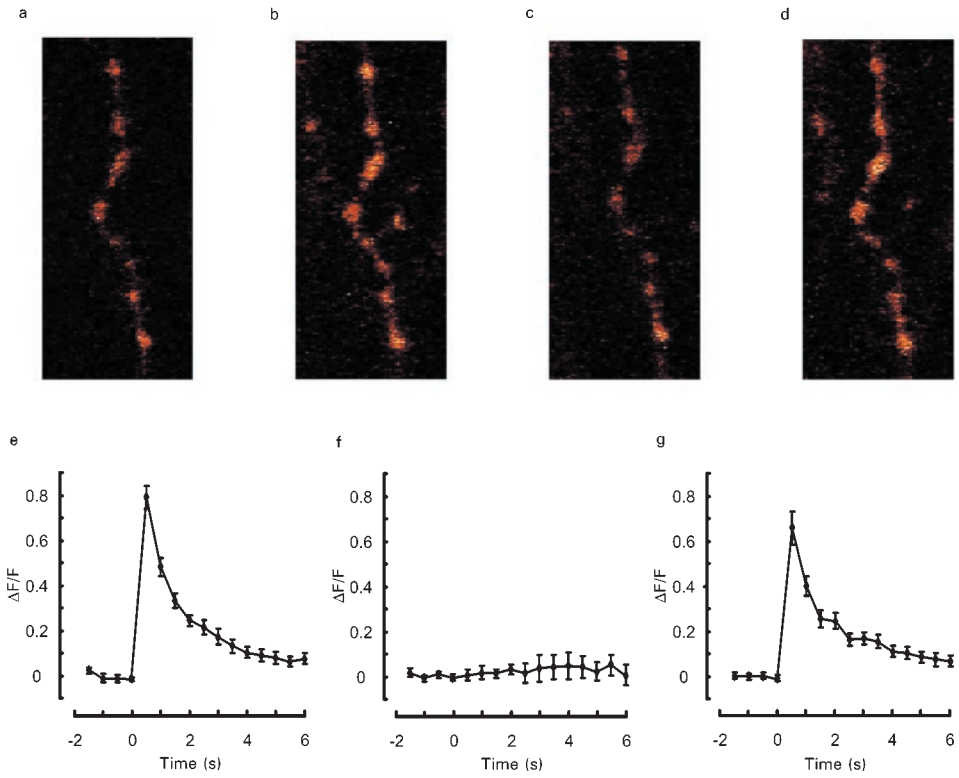

Figure 2 Action potential-evoked Ca^{2+} transients in a string of sympathetic varicosities before and after bretylium. (a) Basal. (b) Response to a single action potential. (c) Block of action potential-evoked Ca^{2+} transient by bretylium (20 μM, 1 h). (d) Reversal of bretylium by amphetamine. Graphs showing the mean data. (e) Control. (f) Bretylium and (g) Amphetamine reversal. Data from 43 varicosities in eight strings, four preparations.

bretylium did not inhibit the action potential-evoked Ca^{2+} transient. A graph expressing $\Delta F/F$ against time for 94 varicosities in response to a single action potential before, and 2 h after the bath application of bretylium (20 μM, $104 \pm 4\%$, $n=94$ varicosities, $n=24$ strings, $n=12$ animals; $P>0.05$) is shown in Figure 3a. Interestingly, within the same microscopic field (40 μm^2), separate strings of varicosities responded differently to bretylium. For example, the branching string on the right (see Figure 3b) was bretylium-sensitive while the remaining varicose strings were bretylium-resistant. In three out of 19 preparations, bretylium (20 μM) actually increased the amplitude of the action potential-evoked Ca^{2+} transient ($151.1 \pm 11.7\%$ of control, $n=23$ varicosities, $n=7$ strings, $n=3$ animals; $P<0.005$). When neurones were loaded for 8 h as opposed to 5 h, the probability of finding bretylium-sensitive varicose strings was similar (sensitive, $-106.6 \pm 4.0\%$, $n=48$ varicosities, $n=6$ animals, $P<0.005$; resistant, $-3.1 \pm 3.3\%$, $n=58$ varicosities, $n=8$ animals, $P>0.05$). Small shifts in the basal fluorescence of some dye-loaded nerves accounts for the 'apparent' degree of inhibition being $>100\%$.

Effects of 6-OHDA It was not possible to quantify the effects of 6-OHDA on varicosity degeneration because of the variable number of varicose strings labelled within a preparation. However, two criteria were used to establish the effectiveness of 6-OHDA pre-treatment, namely that EJPs were abolished and the NA content of the vas deferens fell below detectable levels. When these criteria were met, pre-terminal axon bundles still responded to nerve stimulation with 'normal' Ca^{2+} transients (Figure 4a). In the terminal region, numerous microscopic fields still 'contained Ca^{2+}' in what looked like 'remnants' of varicosities; these did not respond to nerve stimulation (Figure 4b). However, some varicosities looked 'normal' and responded (Figure 4c) with Ca^{2+} transients indistinguishable from controls (Figure 4d).

Discussion On first impressions, there seems no good reason to doubt that the varicose nerve terminals studied in vas deferens using Ca^{2+} imaging techniques would not be sympathetic. However, the effects of bretylium on the action potential-evoked Ca^{2+} transient varied from string to string. In some (21%) of the preparations Ca^{2+} transients were abolished, in some (16%) enhanced and others (63%) unaffected. Quite clearly, in the majority of varicose strings, bretylium failed to block the action potential-evoked Ca^{2+} transient. In some preparations, bretylium-sensitive and bretylium-insensitive strings of varicosities were observed in the same field of view (see Figure 3; although it is uncertain that these strings belong to the same neurone). In all cases, varicosities in individual strings responded similarly to bretylium i.e. Ca^{2+} transients were either abolished, potentiated or unaffected in an entire string. The bretylium-induced block was reversed by amphetamine, showing that these varicosities were sympathetic, and that the block was real and not due to photobleaching of the Ca^{2+} indicator, or due to the stimulus failing to initiate the action potential. These

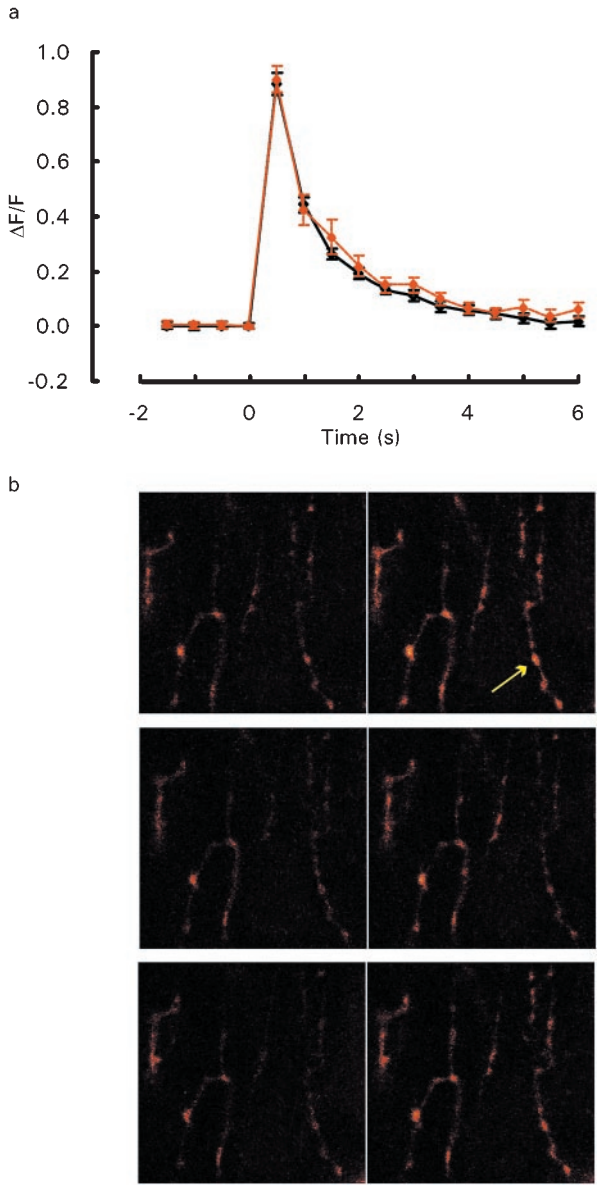

Figure 3 Action potential-evoked Ca^{2+} transients in a string of varicosities before and after bretylium. (a) Lack of effect of bretylium on the action-potential evoked Ca^{2+} transient–mean of 94 varicosities, 24 strings, 12 preparations. (b) Field of varicosities showing both bretylium-sensitive (the yellow arrow points to one varicosity on a bretylium-sensitive string) and bretylium-resistant varicosities. Top panel, left basal, right response to a single action potential. Middle panel, left basal, right response to a single action potential after bretylium (20 μM, 1 h). Bottom panel, reversal of bretylium-sensitive string by amphetamine (20 μM).

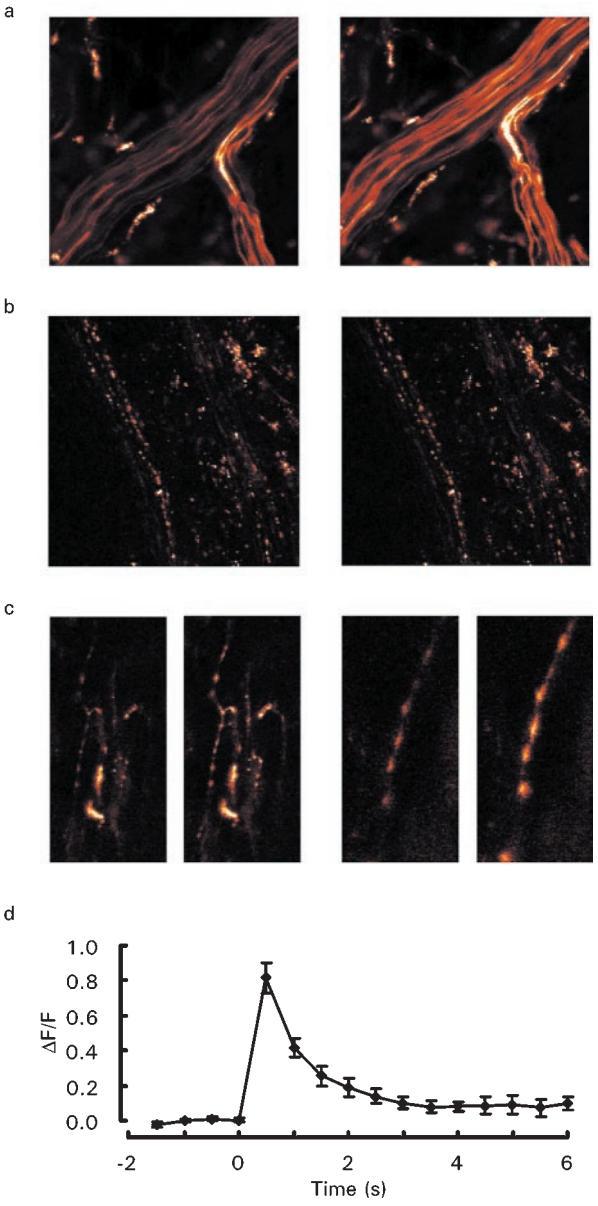

Figure 4 The effects of 6-OHDA on axon bundles and varicosities in mouse vas deferens. (a) 6-OHDA pre-treatment had no effect on the action potential-evoked Ca^{2+} transient in preterminal axon bundles. (b) Remnants of varicosities which failed to respond after 6-OHDA. (c) Left two panels showing low resolution images of fields of 6-OHDA-resistant varicosities; right two panels magnified view showing basal Ca^{2+} and action potential-evoked Ca^{2+} transient in 6-OHDA pretreated vas deferens. (d) The action potential-evoked Ca^{2+} transient in varicosities resistant to 6-OHDA has a similar configuration to the control (see Figure 2e). In the same preparation, EJPs were abolished and NA levels fell below detectable levels using HPLC.

findings raise fundamental questions regarding the anatomical origin of the varicosities studied.

First, some of the varicosities in which bretylium failed to affect the Ca^{2+} transient may be sympathetic but located in regions in which the varicosities do not possess enough functional uptake-1 sites for bretylium to block action potential propagation. NA transporters are found, at variable density, in all regions of sympathetic nerves (Gillespie & Kirpekar, 1966; Stjärne *et al*., 1970; Hökfelt & Ljungdahl, 1972). Recently, the co-localization of an uptake-1 marker with dopamine β-hydroxylase in, but not between, all varicosities has been reported (Schroeter *et al*., 2000). Thus all sympathetic varicosities possess uptake-1 transporters. If so, then in our experiments bretylium was probably taken up in all regions of sympathetic nerves, distributed according to the density of uptake-1 sites present. However, these data do

not reveal if these transporters were located in the cytosol or in the membrane and whether they are functional, nor exclude the possible occurrence of non-clustered uptake-1 sites. Indeed, Allcorn *et al*. (1986) have shown that in vasa deferentia taken from 6-OHDA pre-treated rodents, catecholamine containing varicosities in the longitudinal muscle layer were almost totally eliminated while a considerable number persisted in the inner circular muscle layer. Presumably these sympathetic neurones did not possess sufficient uptake-1 sites for 6-OHDA to act. To determine whether uptake-1 sites were functional in all regions, mice were pre-treated with 6-OHDA. Bretylium or 6-OHDA pre-treatment did not affect the nerve action potential-evoked Ca^{2+} transients previously characterized in preterminal axon bundles (Jackson *et al*., 2001). These data suggest that uptake-1 sites are not present in sufficient numbers to block action potential propagation in the parent axons.

Second, some of the varicosities in which bretylium failed to affect the transients may be non-sympathetic. Although the vas deferens is densely innervated by sympathetic nerves, morphological studies have shown that cholinergic and sensory nerves are also present (see Ventura *et al*., 1998). Indeed, the parasympathetic innervation of the vas deferens is derived from the visceral sacral outflow, *via* the pelvic nerves. A single nerve bundle carrying both the sympathetic and parasympathetic postganglionic fibres, as well as the accompanying sensory fibres, enters the vas deferens at the prostatic end (Ventura *et al*., 1998).

To identify whether the Ca^{2+} transients are evoked in non-sympathetic nerves during confocal studies is technically demanding at present. To determine whether the varicosities are sensory, capsaicin could be used, as it is known to increase intracellular Ca^{2+} in sensory neurones (Wächtler *et al*., 1998). However, not all sensory neurones are capsaicin-sensitive (Marsh *et al*., 1987; Wächtler *et al*., 1998). Similarly, it is not yet possible to identify cholinergic varicosities. However, we find that the longitudinal muscle appears to be innervated by cholinergic nerves (Figure 1b) since bretylium-resistant neurogenic contractions were abolished by atropine. These observations confirm previous studies (Allcorn *et al*., 1986; Kaschube & Zetler, 1989). Interestingly, the cholinergic contraction is enhanced by bretylium suggesting that sympathetic nerves may modulate ACh release, in which case the use of drugs such as α_2-adrenoceptor agonists and antagonists would affect both sympathetic and parasympathetic nerves. Thus, functional cholinergic nerves in the mouse vas deferens could equally well be labelled during Ca^{2+} imaging studies.

Why do we need to address these questions immediately? Increasing numbers of studies of nerves using Ca^{2+} imaging techniques are being performed. The results presented here show the importance of using specific pharmacological tools to identify the type of nerve terminal imaged. This addition to the experimental protocol (although time consuming) will help to clarify inconsistency of responses in the same preparation. For example, Brain & Bennett (1997) reported that the α_2-adrenoceptor agonist clonidine decreased the nerve-evoked Ca^{2+} transient in all varicosities by $\sim$55% while the same laboratory later reported that clonidine reduced the Ca^{2+} transient in only 50% of varicosities with some showing no change at all (O'Connor *et al*., 1999). A plausible explanation for the conflicting data may be because different regions of sympathetic nerves were being studied unknowingly or indeed populations of non-sympathetic nerves. It is interesting that sometimes, whole strings of varicosities were resistant to clonidine as indeed they were to other α_2-adrenoceptor and P2 receptor agonists. At present, one can begin to address these difficulties by using bretylium or 6-OHDA.

In conclusion, the results strongly caution against assuming that all varicosities in the mouse vas deferens in which the nerve impulse evokes a Ca^{2+} transient are sympathetic in origin. Bretylium can be used at the end of an experiment to determine if the Ca^{2+} transients were indeed elicited in a sympathetic nerve.

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