British Journal of Pharmacology (2002) 135, 1841–1843
www.nature.com/bjp

COMMENTARY

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

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A Special Report in this issue of the *British Journal of Pharmacology*, by Jackson and Cunnane, suggests that pharmacological agents can be employed to help distinguish different types of autonomic nerves, in this case varicose nerve terminals, present in peripheral tissues. Their study makes the important point that, while fluorimetric studies of individual cells or parts of cells can elucidate functional mechanisms in great detail, it also brings us up against the problem that similar looking structures can possess different properties. Specifically, varicose nerve terminals found in peripheral tissues, whose properties may differ radically in terms of neurotransmitters and associated structures and proteins, appear to be much the same size and look similar. In the tissue studied by Jackson and Cunnane, the vas deferens, the diversity of neurotransmitters involved in mediating contraction has long intrigued and entertained pharmacologists – so controversy cannot be far away.

Jackson and Cunnane demonstrate, through a process of elimination, that 'adrenergic neurone blockers' can help them distinguish non-sympathetic nerve terminals. This was cleverly done using two drugs – 6-hydroxydopamine (6-OHDA) and bretylium – which owe their specificity of action to their uptake by the neuronal monamine transporter into nerve terminals. Thus, their high concentration allows selective toxicity against sympathetic nerves, albeit by different mechanisms, with 6-OHDA destroying the terminals in which it is concentrated whereas, in adult neurones, bretylium blocks the ability of the action potential to depolarise the varicosity and prevents exocytosis of neurotransmitter(s).

After treatment with 6-OHDA, Jackson and Cunnane studied vas deferens preparations that should have been devoid of nerves fibres containing functional neuronal and vesicular monoamine transporters. However, they still found a population of varicose nerve terminals that, when stimulated electrically, increased their intracellular calcium levels. In an untreated vas deferens, this population of non-sympathetic varicose nerve fibres would be visually indistinguishable from those in which the monoamine transporters are functionally active. Jackson and Cunnane suggest that these could be identified pharmacologically. Subsequent to other analysis, and provided that varicose nerve terminals remain functionally intact, sympathetic and non-sympathetic nerve terminals can be distinguished by their susceptibility or resistance to breylium. Indeed, the authors find that the majority of the terminals studied were resistant to bretylium and, therefore, these nerve terminals in vas deferens should be considered sympathetic (but lacking uptake1 sites and presumably non secretory) or non-sympathetic.

The unmasking of functionally different varicose nerve terminals re-ignites the controversy over the identity, number and relative localization of neurotransmitters capable of contracting the vas deferens. In this respect, following the initial analysis of contractions of guinea-pig vas deferens stimulated *via* the hypogastric nerve (Hukovic, 1961) and throughout the ensuing decade, it was universally believed that noradrenaline was the sole neurotransmitter responsible. This belief persisted in spite of the failure of almost every drug interfering with sympathetic responses – including reserpine, α-adrenoceptor antagonists and even adrenergic neurone blockers such as bretylium and guanethidine (except at very high concentrations) – to block nerve-mediated contractions. Indeed, the excitatory junction potential (EJP) in the vas deferens was once considered a classic example of the electrical effects of noradrenaline, persistently 'decorating' textbooks even after the EJP was shown to be purinergic.

The above dogma was broken by Ambache & Zar (1971), who argued that the balance of pharmacological evidence was heavily against a single, noradrenergic transmission process. Instead, it seemed more likely that contraction of the vas deferens was caused by another, as yet unknown, neurotransmitter. Noradrenaline undoubtedly is present in nerves in the vas deferens and released by electrical stimulation, but proposed to perform an inhibitory function over the other neurotransmitter process (Ambache *et al*., 1972). This proposal was double heresy, because prejunctional receptors had not yet been conceived and the identity of the second neurotransmitter was unknown. Over the next decade, evidence accrued for two neurotransmitters in the vas deferens, one clearly noradrenergic and the other clearly not (then called non-adrenergic, non-cholinergic or NANC, McGrath, 1978; Blakeley *et al*., 1981; French & Scott, 1981). There was no hint at this time of the identity of the non-adrenergic neurotransmitter – even Geoff Burnstock, surprisingly, had not yet suggested ATP (Burnstock *et al*., 1980). Further, it was equally surprising that the failure of adrenergic neurone blockers (such as guanethidine) and sympatholytics (such as 6-OHDA) to abolish the contraction of the vas deferens, did not dent the perception that only noradrenergic nerves control this tissue.

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Here, your present commentator has to own up to a lack of conviction. I had made several observations which convinced me (but not yet others) that there should be two sets of nerves responsible for contractions of the rat vas deferens. First, contractions were relatively resistant to guanethidine (McGrath, 1978). Secondly, 6-OHDA could eliminate most of the histochemical trace of adrenergic nerves or chemically-measurable noradrenaline; yet, these non-adrenergic, non-cholinergic contractions were larger than after reserpine (or as large as controls, since the NANC responses were increased after escaping from α_2-adrenoceptor-mediated prejunctional inhibition; Brown *et al*., 1983). Finally, sympathetic and NANC-evoked contractions are dominant in different parts of the vas deferens (Pennefather *et al*., 1975; McGrath, 1978). There had to be another set of nerves additional to those releasing noradrenaline, but my observations failed to resist or blunt the prevailing dogma.

This issue was swept away when purinergic receptor antagonists emerged in the mid 1980s and helped to show ATP mediated the NANC response (Sneddon & Westfall, 1984). Also, vigorous treatment with 6-OHDA abolished the EJP in the vas (as reported by Jackson and Cunnane) suggesting that ATP, like noradrenaline, might come from 6-OHDA sensitive nerves. The known co-existence, in varicosities, of vesicles containing both noradrenaline and ATP then made sense. The co-transmission concept took hold and held sway for two decades, but without understanding which neurotransmitter was physiologically important in the vas deferens. Eventually, Mulryan *et al*. (2000) showed that $P2X_1$ receptor knockout in mice impaired the movement of sperm and their numbers in the ejaculate, whereas blockade of adrenergic responses did not, making the purinergic component the more relevant. This functional property reminds us of one caveat to viewing the vas deferens as an exemplar of peripheral co-transmission: the noradrenergic component is inoperative before sexual maturity in the rat (MacDonald & McGrath, 1984) and, thereafter, dependent on testosterone (MacDonald & McGrath, 1980).

So what is the explanation for my earlier observations of resistance of contractile responses in rat vas deferens to guanethidine and 6-OHDA? I had assumed a very small proportion of the original population of nerves remained functional after drug treatment, and these few residual nerves could produce a large response. This explanation seemed reasonable because the postjunctional action potential initiated by ATP would propagate through the well-coupled smooth muscle cells of vas deferens and, in the absence of restraint from prejunctional α_2-adrenoceptors, large responses would still ensue. However, the observations of Jackson and Cunnane now refute these assumptions, because they report that a proportion of varicose nerve terminals survive after 6-OHDA pretreatment and a large population of nerve terminals still respond with Ca^{2+} transients in the presence of bretylium. Jackson and Cunnane further show that bretylium or 6-OHDA pretreatment prevents purinergic contractile and postjunctional electrical responses in mouse vas deferens (see also Allcorn *et al*., 1986). So, the enigma remains.

The authors' observations remind us that the link between individual nerves and their post-junctional response is quite tenuous. There is no direct evidence that a single varicosity produces a postjunctional response to both ATP and noradrenaline. Extension of the single cell visualization approach should allow us to understand such issues. There is also histochemical evidence for the presence of diverse putative neurotransmitters, with different distribution through the wall (mostly on the luminal side (Ventura *et al*., 1998)) and the noradrenaline-containing nerves are very differently distributed along the length of the vas deferens, being far less dense and more inclined towards the lumen in the epididymal end, which exhibits the greater adrenergic contraction (Anton *et al*., 1977). Thus, the dense plexus of noradrenaline-containing nerves in the prostatic part of the vas deferens contributes little to the adrenergic contractile response. Indeed, Jackson and Cunnane report, on the basis of the blocking actions of atropine, that the vas deferens is innervated by cholinergic nerves. These observations confirm previous reports of an atropine-sensitive component of contraction in mouse vas deferens (Kaschube & Zetler, 1989). Jackson and Cunnane show that the cholinergic neurogenic contraction is enhanced by bretylium suggesting that sympathetic nerves may modulate ACh release, in which case drugs would affect both sympathetic and parasympathetic nerves in complex ways. Thus, functional cholinergic nerves in the mouse vas deferens could equally well be labelled during Ca^{2+} imaging studies. The most likely explanation for conflicting data on the effects of drugs on strings of different varicosities in the Ca^{2+} imaging studies of Brain & Bennett (1997) and O'Connor *et al*. (1999) as stated in the present Special Report is that different regions of sympathetic nerves or indeed non-sympathetic nerves may have been unknowingly investigated by these investigators. An important message in the Special Report of Jackson and Cunnane is that experimental scientists must make strenuous efforts to identify the type and region of nerve studied in Ca^{2+} imaging experiments else spurious and unequivocal conclusions will be drawn. Clearly, one can begin to address these difficulties by using bretylium or 6-OHDA.

The structures visualized by Jackson and Cunnane were near the external surface of the vas deferens. A limitation of current technology is that looking inside 3-dimensional structures, even with confocal microscopes, is limited to tens of microns and places only relatively superficial structures within grasp. The current study certainly suggests that most of the terminals visualized near the surface of mouse vas deferens do not possess transporters capable of concentrating bretylium sufficiently to block their calcium response to electrical stimulation. Thus, presenting different regions of this tissue to the microscope after appropriate dissection should be an important extension of this work. However, only a methodology that can show the nature of the post-junctional response, as well as prejunctional properties, at deeper sites will resolve these issues.

The multidimensional data collected by the confocal microscope has been reduced to two dimensions to suit the current limitations of the published print medium. This reduces the excitement inherent in this type of work. It is worth visiting the authors' website Cunnane (2002) to view some excellent movies of their data.

Overall, the paper by Jackson and Cunnane, while cautionary in nature, points to an exciting new chapter in unravelling the diversity of autonomic neuroeffector mechanisms. We look forward to the combination of pharmacological nous with vital stains, image analysis, and, since the

species of choice is mouse, functional genomics. The *British Journal of Pharmacology* encourages submission of papers applying such creative combinations of technologies to the study of the action of drugs.

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