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Differential effects of ω-conotoxin GVIA on cholinergic and non-cholinergic secretomotor neurones in the guinea-pig small intestine

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1 Ussing chambers were used to study the effects of the specific N-type Ca^{2+} channel antagonist, ω-conotoxin GVIA, on neurally evoked secretion across isolated submucosa/mucosa preparations from the small intestine of the guinea-pig.

2 Cholinergic and non-cholinergic neurones were stimulated with 10 μM dimethylphenylpiperazinium (DMPP). Non-cholinergic secretomotor neurones were preferentially stimulated with 100 nM 5-hydroxytryptamine (5-HT), while cholinergic secretomotor neurones were preferentially stimulated with 3 μM 5-HT in the presence of the 5-HT_2 receptor antagonist ketanserin (30 nM).

3 ω-Conotoxin GVIA (1 nM – 1 μM) depressed the secretion evoked by DMPP in a concentration-dependent manner, but a substantial residual response was observed. Hyoscine (100 nM) significantly depressed secretion evoked by DMPP, but did not prevent further depression of secretion by ω-conotoxin GVIA.

4 The toxin was substantially more effective when the non-cholinergic secretomotor neurones were preferentially activated with 100 nM 5-HT, with a decrease in the response of more than 75% of the control value in the presence of 1 μM ω-conotoxin GVIA.

5 ω-Conotoxin GVIA (1 μM) was relatively ineffective against secretion evoked by preferential activation of cholinergic secretomotor neurones with 3 μM 5-HT in the presence of 30 nM ketanserin, inhibiting the response by less than 33%. However, this inhibition was significant. Both 100 nM hyoscine and 300 nM tetrodotoxin abolished this effect of ω-conotoxin GVIA.

6 It is concluded that N-type Ca^{2+} channels play a major role in transmitter release from non-cholinergic secretomotor neurones, but are not important for release from cholinergic secretomotor neurones in the guinea-pig small intestine.

Keywords: Secretomotor neurones; calcium; N-type channels; ω-conotoxin GVIA; cholinergic neurones; non-cholinergic neurones; submucous plexus; 5-hydroxytryptamine (5-HT)

Introduction

The movement of water and electrolytes across the mucosa is one of the many intestinal functions that is under the control of the enteric nervous system (Keast, 1987; Cooke, 1989). In the guinea-pig small intestine, the final neurones responsible for this control include both cholinergic and non-cholinergic secretomotor neurones, which have been deduced to be immunoreactive for neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP), respectively (Bornstein & Furness, 1988). These two populations of neurones are differentially excited by various agonists. For example, 5-hydroxytryptamine (5-HT) excites both populations of neurones, but the non-cholinergic secretomotor neurones typically are excited by lower concentrations via 5-HT_2 receptors, while significantly higher concentrations are needed to excite cholinergic neurones via 5-HT_3 receptors (Johnson *et al*., 1994). However, studies of the effects of various secretogogues on the mucosa have been hampered by the difficulty of ensuring that any effect is not secondary to an action either on the secretomotor neurones or their terminals. As Ca^{2+} ions are essential for normal secretion across the mucosa (Kaunitz *et al*., 1995), conventional blockade of synaptic transmission with inorganic Ca^{2+} antagonists is not useful in this context.

The relatively recent discovery that various organic toxins can specifically block the Ca^{2+} channels in nerve terminal membranes to block transmitter release suggests that such toxins may be useful in studies of intestinal secretion. However, the most widely used toxin, ω-conotoxin GVIA, which specifically blocks N-type Ca^{2+} channels (Olivera *et al*., 1985; Tsien *et al*., 1988), does not block transmission at some autonomic junctions (Wright & Angus, 1995; Rathner *et al*., 1995), while being highly effective at others (De Luca *et al*., 1990; Bridgewater *et al*., 1995; Wright & Angus, 1995; Hong *et al*., 1996). The present study was undertaken to determine whether blockade of N-type Ca^{2+} channels by ω-conotoxin GVIA modified transmission from secretomotor neurones in the submucous plexus to the mucosal epithelium of the guinea-pig small intestine.

Submucosal secretomotor neurones were stimulated with agonists which partially differentiate between cholinergic and non-cholinergic neurones and the response of the mucosal epithelium was monitored as the short circuit current across intact sheets of mucosa and submucosa (Keast *et al*., 1985; Johnson *et al*., 1994). The results indicate that ω-conotoxin GVIA blocks release from the non-cholinergic secretomotor neurones, but may have little effect on release from cholinergic secretomotor neurones.

Methods

Guinea-pigs (200 – 350 g) of either sex were killed by being stunned and then having their carotid arteries and spinal cords severed. Segments of small intestine were removed and placed in physiological saline (composition in mM: NaCl 118, KCl 4.8, NaH_2PO_4 1, $NaHCO_3$ 25, $MgSO_4$ 1.2, glucose 11, $CaCl_2$ 2.5, equilibrated with 95% O_2/5% CO_2) and sheets of mucosa and

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submucosa were prepared as described previously (Hendriks *et al*., 1989; Johnson *et al*., 1994). The intestinal segment was cut open along its mesenteric border and placed in a dissecting dish maintained at 4°C and containing the physiological saline. It was then flushed with saline to remove all intestinal contents and fresh saline was added to the dish. The opened sheet was pinned flat mucosa down and the longitudinal and circular muscle layers dissected away. The segment was then divided into sheets, each approximately 1 cm^2. The sheets of mucosa and submucosa were then mounted in Ussing chambers, which had a cross-sectional area of 0.38 cm^2. In the Ussing chamber, the submucosal surface of each segment was exposed to the normal physiological saline and the mucosal surface exposed to saline of similar composition but with mannose substituted for glucose. This prevents glucose absorption which, because it is coupled to sodium, might otherwise interfere with interpretation of the measurement of secretion.

Secretion was monitored as an increase in the short circuit current measured with a voltage-clamp circuit; earlier studies have shown that such increases are the result of electrogenic secretion of electrolytes, predominantly chloride ion, across the mucosal epithelium (for review see Keast (1987)). Thus, the short circuit current is a useful index of secretion.

In each experiment, 4 preparations from adjacent parts of the same intestinal segment were mounted in separate chambers: one acted as a control, while the others were used for the analysis of the actions of the toxin. Drugs were only added to the solution bathing the submucosal surfaces of the preparations. The 4 preparations were left to equilibrate for 1 h after being mounted in the Ussing chambers. 5-HT (1 μM) was then added to each of the submucosal half-chambers to obtain an index of the level of secretion the individual preparations could produce, this was washed out after 2.5 min. These responses were used to normalize the later responses. After another 20 min interval, the preparations were stimulated 3 times at 20 min intervals by a 2.5–3 min exposure to either 5-HT or dimethylphenylpiperazinium (DMPP), (see below). ω-Conotoxin GVIA was then added to the submucosal half-chambers of 3 of the 4 Ussing chambers and allowed to equilibrate for 30 min before the secretogogue was reapplied to all 4 Ussing chambers. In most experiments, after washout of the secretogogue, the toxin was again added to the 3 preparations previously exposed to ensure that the concentration of toxin remained constant and after another 30 min the secretogogue was readministered. The results of these two exposures were then pooled to provide an estimate of the response to the stimulant. When specific antagonists were used to block muscarinic receptors on the mucosa, 5-HT receptors on neurones or neuronal sodium channels, these were added 15 min before the addition of the toxin and replaced after each washout. In the experiments in which the effect of ketanserin was compared with that of tetrodotoxin (TTX), the TTX was added between the two additions of ω-conotoxin GVIA and only a single response of each preparation was used in the analysis. In each experiment, one preparation was not exposed to ω-conotoxin GVIA, but was exposed to all other agents used. This chamber provided a control to ensure that the changes in responses seen were not due to some time-dependent factor other than the toxin.

Responses were measured by taking the maximum amplitude of the change in short circuit current (see Figure 1). This value was then normalized by dividing the amplitude by that of the original response to 1 μM 5-HT from the same preparation to determine the relative change in short circuit current (expressed as a percentage). For quantitative analysis, the responses from preparations from a least 5 animals (in most cases, 6 animals) were measured, normalized and expressed as means ± s.e. The significance of changes in responses produced by ω-conotoxin GVIA, hyoscine and TTX was assessed by comparing responses in the presence of these drugs with those in the control chamber by either one way analysis of variance or two way analysis of variance as appropriate.

Drugs used included DMPP (Sigma), hyoscine (Sigma), 5-HT (Sigma), ketanserin tartrate (Research Biochemicals Incorporated), TTX (Sigma) and ω-conotoxin GVIA (synthesized by Mr James P. Flinn, Department of Pharmacology, University of Melbourne; (Flinn *et al*., 1995).

Results

As described elsewhere (Keast *et al*., 1985), DMPP produced a biphasic increase in short circuit current (Figure 1). This secretory response was markedly depressed by ω-conotoxin GVIA, an effect that was clearly concentration-dependent (Figures 1 and 2). However, even at concentrations as high as 1 μM, the toxin did not abolish the responses to DMPP nor did it appear to have any effect on basal secretion. As previous studies had indicated that DMPP acts exclusively on the secretomotor neurones in submucosa-mucosal preparations from guinea-pig small intestine (Keast *et al*., 1985), these observations suggested that the toxin might be specific for one class of secretomotor neurones. Subsequent experiments were designed to test this possibility.

If ω-conotoxin GVIA acts specifically on cholinergic secretomotor neurones, but has no effect on the non-cholinergic secretomotor neurones, then it would be predicted that blockade of muscarinic receptors on the mucosa would abolish the effects of the toxin on secretion evoked by DMPP. However, while hyoscine (100 nM) depressed the secretory response to DMPP (Figure 2), it did not prevent the inhibition of this response by ω-conotoxin GVIA (Figure 2). This suggests that at least part of the effect of the toxin is on the non-cholinergic secretomotor neurones.

Johnson *et al*. (1994) found that concentrations of 5-HT lower than 1 μM excite non-cholinergic secretomotor neurones via 5-HT_2 receptors, but have little or no effect on cholinergic secretomotor neurones. Accordingly, the effects of the toxin on secretory responses evoked by 100 nM 5-HT were examined (Figures 3 and 4). ω-Conotoxin GVIA profoundly depressed

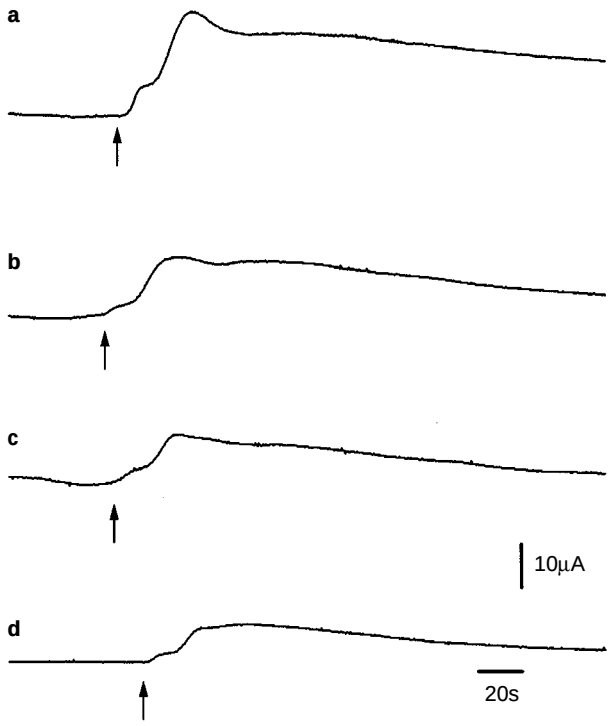

Figure 1 Changes in the short circuit current (I_{SC}) evoked by addition of 10 μM DMPP (arrows) to the serosal side of an Ussing chamber in (a) control solution, (b) 10 nM ω-conotoxin GVIA, (c) 100 nM ω-conotoxin GVIA and (d) 1 μM ω-conotoxin GVIA. The toxin depressed but did not abolish the increased secretion evoked by the nicotinic agonist.

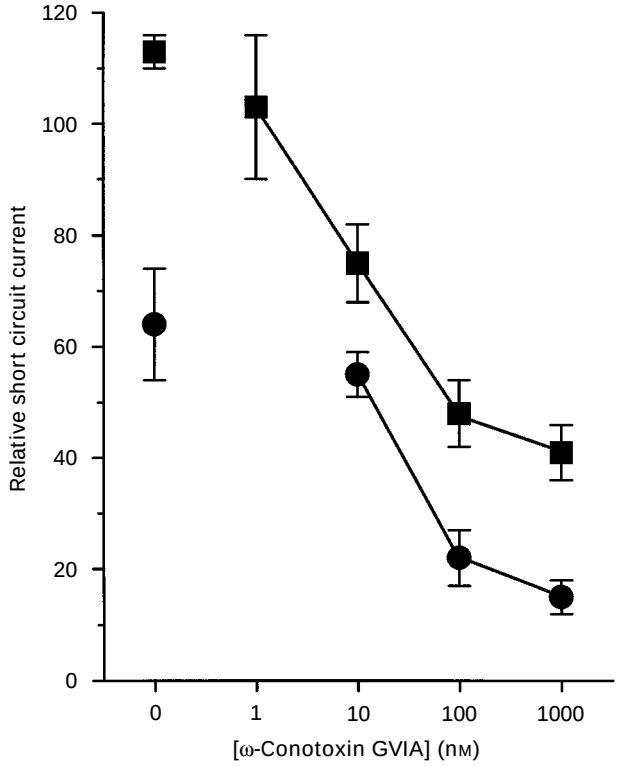

Figure 2 Concentration-response curves for the effects of ω-conotoxin GVIA on secretion evoked by DMPP in control solution (■) and in the presence of 100 nM hyoscine (●). The relative short circuit current values were calculated by measuring the maximum amplitude of each response and dividing this by the amplitude of control responses to 1 μM 5-HT measured in the same preparation before the addition of any other drugs or toxins. Each point represents the mean of results from 6 animals; vertical lines show s.e. ω-Conotoxin GVIA depressed, but did not abolish, secretory responses evoked by DMPP both in the presence and absence of hyoscine. In each case, this effect was concentration-dependent ($P<0.0025$, two way analysis of variance). Hyoscine significantly depressed the response to DMPP at all concentrations of the toxin ($P<0.0025$).

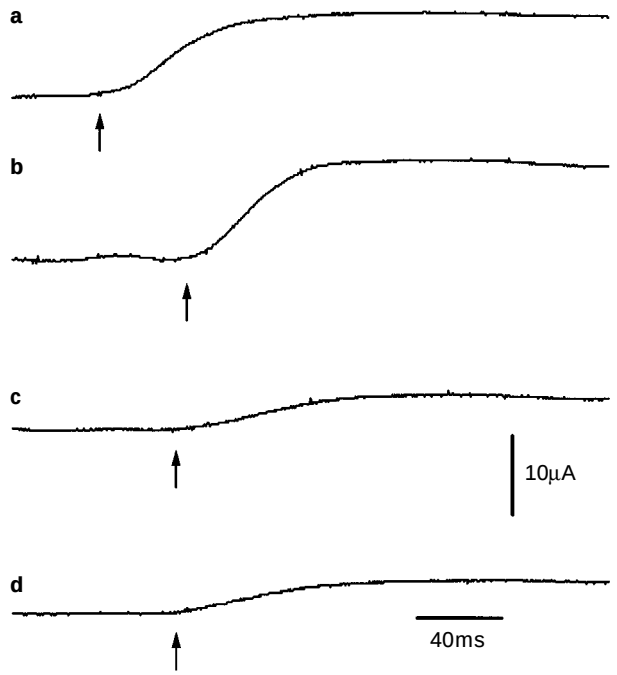

Figure 3 Increases in the short circuit current (I_{SC}) evoked by 100 nM 5-HT (arrows) in (a) control solution, (b) 10 nM ω-conotoxin GVIA, (c) 100 nM ω-conotoxin GVIA and (d) 1 μM ω-conotoxin GVIA. The toxin depressed, but did not abolish, the secretory response in a concentration-dependent manner.

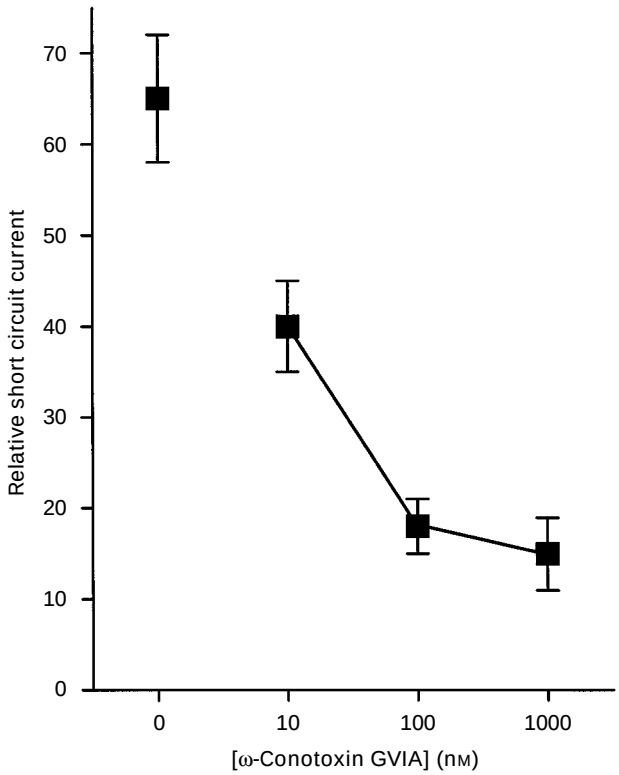

Figure 4 Concentration-effect curve for the effects of ω-conotoxin GVIA on the increased secretion evoked by 100 nM 5-HT. Each point represents the mean of results from 6 animals; vertical lines show s.e. The effect of the toxin was clearly concentration-dependent ($P<0.0025$, one way analysis of variance).

these responses, with the relative values of short circuit current falling from 65±7% in control ($n=5$) to 15±4% in 1 μM toxin ($n=6$) and this phenomenon was concentration-dependent ($P<0.001$, one way analysis of variance). This suggested that the toxin can act on neurotransmitter release from the non-cholinergic secretomotor neurones. However, a small residual response remained even with 1 μM toxin, this was probably due to a direct effect of 5-HT on the mucosal epithelium.

At concentrations greater than or equal to 1 μM, 5-HT excites both cholinergic and non-cholinergic secretomotor neurones, the former via 5-HT_3 receptors and the latter predominantly via 5-HT_2 receptors (Johnson *et al.*, 1994). Thus, in the presence of ketanserin (a 5-HT_2 receptor antagonist), high concentrations of 5-HT preferentially excite cholinergic secretomotor neurones. Figure 5 illustrates the effect of ω-conotoxin GVIA on secretion evoked by 3 μM 5-HT in the presence of ketanserin. As can be seen, the toxin produced only a small depression of the secretory response under these conditions (control 154±15% ($n=6$), 1 μM toxin 111±14% ($n=6$)). Furthermore, hyoscine (100 nM) substantially depressed responses to this high concentration of 5-HT (control 154±15% ($n=6$), in the presence of hyoscine but without toxin 55±9% ($n=6$); $P<<0.01$, two way analysis of variance) and in the presence of this muscarinic antagonist the toxin had no significant effect on secretion (without toxin 55±9% ($n=6$), with 1 μM toxin 47±8% ($n=6$), $P>0.6$). Similarly, ω-conotoxin GVIA had no significant effect in the presence of TTX (300 nM) (without toxin 35±4%, with 1 μM toxin 40±8%; $P>0.8$, one way analysis of variance, $n=6$). Responses to 5-HT in the presence of ketanserin and hyoscine

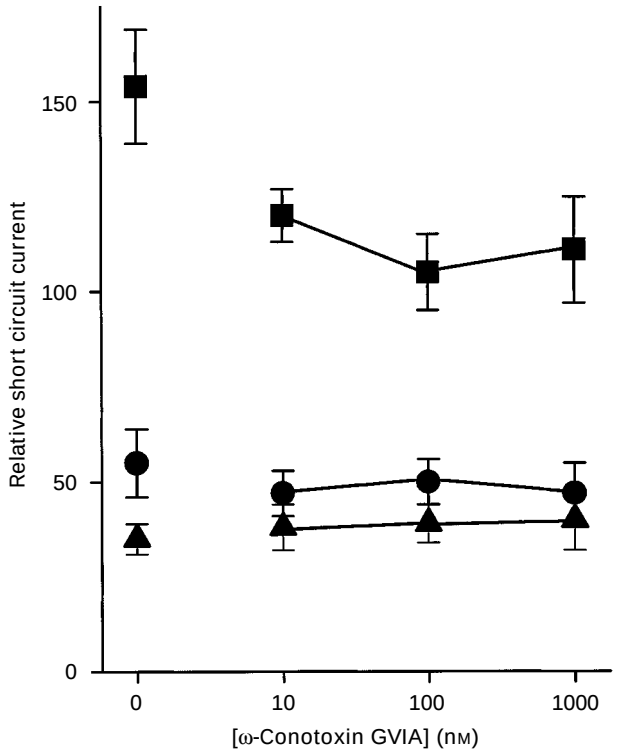

Figure 5 Concentration-effect curves showing the effects of ω-conotoxin GVIA on secretion evoked by 3 μM 5-HT in the presence of 30 nM ketanserin (■), 30 nM ketanserin plus 100 nM hyoscine (●) or 300 nM TTX (▲). Each point represents the mean of results from 6 animals; vertical lines show s.e.mean. The toxin reduced responses in the presence of ketanserin alone by less than 50%, while it had no effect in the presence of hyoscine or TTX.

(in the absence of ω-conotoxin GVIA, 55±9% ($n=6$)) were somewhat larger than those seen in the presence of TTX (in the absence of ω-conotoxin GVIA, 35±4% ($n=6$); this difference was statistically significant ($P<0.02$, two way analysis of variance), but it is not clear whether this was biologically significant.

Discussion

These results indicate that blockade of N-type Ca^{2+} channels virtually abolishes secretion evoked by stimulation of non-cholinergic secretomotor neurones, but is much less effective on secretion evoked by stimulation of cholinergic secretomotor neurones. ω-Conotoxin GVIA does not act directly on enterocytes to prevent secretion, because the direct effect of 5-HT on the mucosal epithelium (i.e. the TTX-insensitive component) is unaffected by it. Thus, the toxin acts on one or more populations of secretomotor neurones. Although the highest concentration of toxin used here, 1 μM, was 100 times that needed to block release at many synaptic and neuroeffector junctions (Pruneau & Angus, 1990; Wright & Angus, 1996), higher concentrations are often used to study N-type calcium channels on neurones (Wang *et al*., 1996) and ω-conotoxin GVIA is selective for N-type calcium channels at concentrations up to 10 000 times greater than needed to block such channels (Whorlow *et al*., 1996).

The conclusion that ω-conotoxin GVIA acts primarily on the terminals of non-cholinergic secretomotor neurones is based on several observations. First, the toxin depresses responses to 100 nM 5-HT more than either responses to DMPP or responses to 3 μM 5-HT in the presence of ketanserin. At 100 nM, 5-HT preferentially excites non-cholinergic secretomotor neurones, while DMPP excites both cholinergic and non-cholinergic secretomotor neurones and 3 μM 5-HT preferentially excites cholinergic secretomotor neurones when 5-HT_2 receptors are blocked (Keast *et al*., 1985; Johnson *et al*., 1994). Thus, when cholinergic secretomotor neurones are excited blockade of N-type calcium channels is less effective than when the non-cholinergic secretomotor neurones are selectively activated.

When both cholinergic and non-cholinergic secretomotor neurones were excited, hyoscine depressed the response, but did not abolish the effect of the toxin (Figure 2). This confirms that the toxin acts upon the non-cholinergic secretomotor neurones. In contrast, when cholinergic secretomotor neurones were preferentially excited by 3 μM 5-HT in the presence of ketanserin, hyoscine abolished the effect of the toxin (Figure 5), indicating that there was no residual toxin-sensitive non-cholinergic component.

Furthermore, the toxin-resistant components of the response to 5-HT and/or DMPP are substantially depressed by hyoscine, the former to levels very similar to those seen in TTX. Thus, the neurogenic secretion that is resistant to ω-conotoxin GVIA is largely, or wholly, mediated by ACh.

The observation that the response to 3 μM 5-HT in the presence of hyoscine and ketanserin was slightly greater than that seen in TTX differs from the results of an earlier study (Johnson *et al*., 1994), in which no significant difference was observed. However, the statistical power of the present study was substantially greater than that in the previous study, suggesting that the difference may be real. This would imply that 3 μM 5-HT excites non-cholinergic neurones that are insensitive to ω-conotoxin GVIA or that cholinergic secretomotor neurones release a transmitter other than ACh (see below).

There are three possible explanations for the observation that ω-conotoxin GVIA reduces the neurogenic secretory response evoked by 3 μM 5-HT in ketanserin (Figure 5). The toxin sensitive component may be due to activation of non-cholinergic secretomotor neurones via 5-HT_3 receptors (Johnson *et al*., 1994). In this case, the remaining neurogenic response to 5-HT would be due to activation of cholinergic secretomotor neurones. However, hyoscine depressed control responses in ketanserin to a level only marginally greater than those seen in TTX suggesting that this is not a complete explanation. Further, the toxin did not depress the hyoscine-insensitive response indicating that very few ω-conotoxin GVIA sensitive non-cholinergic secretomotor neurones were excited in ketanserin.

It is also possible that cholinergic neurones express two or more distinct Ca^{2+} channels and that ω-conotoxin GVIA blocks only one of these. There is ample precedent for the idea that a single population of neurones can express more than one Ca^{2+} channel. In the hippocampus, about 50% of transmitter released from CA3 and CA1 neurones is prevented by blockade of N-type channels, the remainder is mediated via P- or Q-type channels (Wheeler *et al*., 1994a, b). Furthermore, the movements of fluorescently labelled vesicles in the terminals of hippocampal neurones indicate that these terminals express N-type Ca^{2+} channels plus at least one other type of Ca^{2+} channel (Reuter, 1995). In the gut, N-channel blockade abolishes cholinergic transmission to the longitudinal muscle evoked by single electrical stimuli, but a component of cholinergic transmission during high frequency trains of stimuli resists blockade of these Ca^{2+} channels (Hong *et al*., 1996).

Finally, there may be two distinct populations of cholinergic secretomotor neurones, one of which expresses N-type Ca^{2+} channels, while the other does not. Cholinergic neurones elsewhere in the autonomic nervous system are affected differently by N-channel blockade. For example, vagal efferents supplying the rabbit heart are relatively unaffected by ω-conotoxin GVIA (Wright & Angus, 1995), while cholinergic transmission in the guinea-pig taenia caeci is substantially depressed by this toxin (De Luca *et al*., 1990) as is transmission to the longitudinal muscle of the guinea-pig ileum (Hong *et al*., 1996). There are 3 populations of cholinergic neurones pro-

jecting to the intestinal mucosa in the guinea-pig small intestine (Furness *et al*., 1984; Brookes *et al*., 1991). These include neurones immunoreactive for neuropeptide Y (NPY), cholesystokinin, somatostatin and calcitonin gene-related peptide, all of which may alter secretion; neurones immunoreactive for substance P (SP) and neurones immunoreactive for the calcium-binding protein, calretinin. While the NPY-im munoreactive neurones are clearly secretomotor neurones and the SP-immunoreactive neurones are almost certainly sensory neurones (Bornstein & Furness, 1988; Kirchgessner *et al*., 1992), the functions of the calretinin-immunoreactive neurones have not been identified and no secretogogues, other than ACh, have been identified with them. Nevertheless, the calretinin-immunoreactive neurones probably receive fast cholinergic synaptic input (see Bornstein & Furness, 1988), an observation consistent with them being motor neurones. This also indicates that they would be excited by DMPP similar to other cholinergic secretomotor neurones. In contrast, the SP-immunoreactive cholinergic neurones rarely receive cholinergic inputs (Bornstein *et al*., 1989) and may not be responsive to DMPP.

The results of this study show that, while ω-conotoxin GVIA blocks transmitter release from some enteric secretomotor neurones, some cholinergic secretomotor neurones are relatively unaffected by this toxin. Thus, care must be taken in interpreting experiments in which ω-conotoxin GVIA is used to block synaptic, or junctional, transmission in the gastrointestinal tract. Failure of the toxin to block a particular phenomenon does not necessarily indicate that the response is not mediated via neuronal calcium channels.

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