Prejunctional modulatory action of neuropeptide Y on responses due to antidromic activation of peripheral terminals of capsaicin-sensitive sensory nerves in the isolated guinea-pig ileum

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- 1 The effect of neuropeptide Y (NPY) on motor responses produced by activation of capsaicin-sensitive primary afferents in the guinea-pig isolated ileum was determined by use of capsaicin itself and electrical mesenteric nerve stimulation as stimuli.
- 2 NPY inhibited or suppressed the cholinergic contractile response produced by electrical mesenteric nerve stimulation while leaving the contractile response to a threshold concentration of capsaicin.
- 3 NPY had no effect on motor responses produced by a submaximal concentration of substance P, the putative endogenous mediator of the 'efferent' function of sensory fibres in this preparation.
- 4 It is concluded that NPY exerted a prejunctional inhibitory action on transmitter release from peripheral endings of capsaicin-sensitive nerves at interneuronal synapses.

Keywords: Neuropeptide Y; capsaicin; sensory nerves; antidromic activation; ileal motility

Introduction

Capsaicin-sensitive primary sensory neurones synthesize several neuropeptides, e.g. tachykinins and calcitonin gene-related peptide (CGRP). The latter are transported to both central and peripheral nerve endings at which level the peptides are released to produce their sensory and 'efferent' function, respectively (Szolcsanyi, 1984; Maggi & Meli, 1988; Holzer, 1988). Pharmacological evidence indicates that these sensory nerve endings are endowed with various receptors for transmitters and autacoids (e.g. opioids, γ -aminobutyric acid (GABA), and prostaglandins), the activation of which produces changes in excitability, thereby regulating the amount of released transmitter (Szolcsanyi & Bartho, 1978; Yaksh *et al.*, 1980; Grbovic & Radmanovic, 1983; Takaki *et al.*, 1986; Bartho *et al.*, 1987; Go & Yaksh, 1987; Saria *et al.*, 1988a,b; Maggi *et al.*, 1988a,b; 1989a; Giuliani *et al.*, 1989a,b). These modulatory influences can also be observed in peripheral tissues, indicating that the 'efferent' function of these primary sensory neurones can be regulated by other components of the autonomic nervous system. Neuropeptide Y (NPY) is a 36 amino acid peptide, widely distributed in the mammalian peripheral nervous system; it has been shown to be present in sympathetic nerves where it is co-localized with noradrenaline and released by electrical nerve stimulation (Lundberg *et al.*, 1982; 1983; 1984; Fried *et al.*, 1985; Stjarne *et al.*, 1986). A dense plexus of nerve fibres containing NPY-like immunoreactivity is present in the heart, respiratory and digestive tracts of various species (Furness *et al.*, 1983; Lundberg *et al.*, 1983; Sundler *et al.*, 1983; Uddman *et al.*, 1984; Sternini & Brecha, 1985; Potter, 1988). In addition to sympathetic NPY fibres of extramural origin, the gastrointestinal tract also contains an abundance of nerve cells and fibres containing immunoreactive NPY-like material that are devoid of noradrenaline and originate in intramural ganglia (Furness *et al.*, 1983; Sundler *et al.*, 1983). Among other effects, NPY has been shown to exert a prejunctional inhibitory influence on transmitter release from both cholinergic and noradrenergic nerves in the guinea-pig heart (Lundberg *et al.*, 1984; Potter, 1987). Recently, a prejunctional modulatory action of NPY on peripheral terminals of capsaicin-sensitive sensory nerves in the guinea-pig isolated left atria, bronchi and trachea has been reported (Giuliani *et al.*, 1989b; Grundemar *et al.*, 1990).

Here we present data indicating that NPY exerts a potent inhibitory action on motor responses produced in the guinea-pig ileum when the sensory fibres of the mesenteric nerve are activated by electrical antidromic stimulation but not by capsaicin. The present data suggest that the intrinsic neurones of the intestine could influence the excitability of extrinsic sensory nerves, at least with regard to their 'efferent' function.

Methods

Adult guinea-pigs of either sex were stunned and exsanguinated. The ileum, except the terminal 20 cm, was used for experiments. A 2.5 cm length of ileum with (or without) the attached mesenteric vessels and nerves was excised and suspended under a tension of 0.5 g in an organ bath containing 15 ml of Krebs solution at 36°C, and aerated with 5% CO₂ in O₂. The composition of the modified Krebs solution was (mM): NaCl 120.9, KCl 5.9, NaHCO₃ 14.4, NaH₂PO₄ 1.1, CaCl₂ 1.9, MgCl₂ 1.2 and glucose 11.5. The preparations were left to settle in the organ bath for 1.5–2 h. The tissues were treated with guanethidine (2 μ M) and hexamethonium (50–200 μ M) before and during the experiments. The mesenteric vessels and nerves were placed between a pair of annular platinum electrodes for mesenteric nerve (MN) stimulation. Field stimulation was effected via two platinum ring-electrodes placed around the segment. The MN stimulation parameters were: 20 Hz, 0.5 ms, supramaximal currents; stimulation was given for 30 s at 5.5 min intervals. The continuous field stimulation parameters through the experiment were: 0.1 Hz, 0.5 ms, supramaximal currents. The mechanical activity of the longitudinal muscle was recorded through an isotonic transducer.

The drugs used were: atropine sulphate (Dainippon Seiyaku), capsaicin (Sigma), guanethidine sulphate (Tokyo-Kasei), hexamethonium bromide (Sigma), neuropeptide Y (Human) (NPY; Peptide Institute), substance P (Peptide Institute). Capsaicin (10 mM) was dissolved in 100% dimethylsulphoxide (DMSO); when used, this stock solution was diluted one-hundred fold with distilled water. DMSO at 0.1% had no effect of its own. An available vial of neuropeptide Y contains 0.02 μ mol of NPY. Thus, 0.1 μ M NPY was the maximal concentration available for the present experiments.

All values are presented as the means $\pm$ s.e.mean. The statistical significance of differences between means was estimated with Student's *t* test for unpaired data. A *P* value of <0.05 was considered statistically significant.

Results

NPY ($0.1 \mu\text{M}$) produced either a small, transient relaxation or had no effect on spontaneous ileal motility (Figures 1a and 2a). In the presence of guanethidine ($2 \mu\text{M}$) and hexamethonium ($50\text{--}200 \mu\text{M}$), supramaximal mesenteric nerve (MN) stimulation (20 Hz , 0.5 ms) consistently caused a contraction, i.e., a capsaicin-sensitive contractile response, which was irreversibly abolished by capsaicin ($1 \mu\text{M}$) as shown in Figures 1b and 2b. This MN-evoked response has been characterized previously as being dependent upon activation of capsaicin-sensitive sensory nerves which release a tachykinin-like transmitter responsible for the cholinergic contractile effect (Szolcsanyi & Bartho, 1978; Takaki *et al.*, 1987). In the present study, it was reconfirmed that the contractions evoked by MN stimulation were abolished by atropine ($1 \mu\text{M}$). Contractions evoked by

Figure 1 A typical tracing showing the inhibitory effect of neuropeptide Y (NPY, $0.1 \mu\text{M}$; at the arrow) on the cholinergic contractile response to mesenteric nerve (MN) stimulation (at the dots) in the guinea-pig isolated ileum (guanethidine pretreated, hexamethonium $50 \mu\text{M}$ in the bath) in (a). In (b), neuropeptide Y (NPY $0.1 \mu\text{M}$; at the first arrow) did not reduce the contractile response induced by capsaicin *per se* (Cap, $1 \mu\text{M}$; at the second arrow) in (ii) to compare with a control response in (i) (guanethidine pretreated, hexamethonium $200 \mu\text{M}$ in the bath); (i) and (ii) were from different preparations of the same animal. Capsaicin abolished the response to MN stimulation (at the dots) as described previously (Szolcsanyi & Bartho, 1978; Takaki *et al.*, 1987).

Figure 2 A typical tracing showing no inhibitory effect of neuropeptide Y (NPY, $0.1 \mu\text{M}$, at the second arrow) on the contractile response induced by exogenous substance P (SP; 1 nM , at the first, third and fourth arrow) in (a). In the middle trace, after confirming the inhibitory effect of NPY on the contractile response to mesenteric nerve (MN) stimulation (at the dots), SP was applied. In (b), capsaicin (Cap, $1 \mu\text{M}$) abolished the contractile response to MN stimulation; (a) and (b) were from the same preparation. W, washing for 30 min.

MN stimulation were abolished in the presence of high magnesium (12.5 mM):low calcium (1.25 mM), as shown in Figure 3a ($n = 7$), or high magnesium (12.5 mM): very low calcium (0.25 mM) ($n = 5$) Krebs solution. In normal medium, as shown in Figure 3b, the first application of capsaicin ($1 \mu\text{M}$) caused a strong contraction, which is known to be due to activation of capsaicin-sensitive sensory nerves that release a tachykinin-like transmitter, such as substance P (Holzer, 1988), and irreversibly abolished the contractile response to MN stimulation. No contraction was elicited by a second application of capsaicin ($1 \mu\text{M}$) ($n = 7$); this event is thought to be a specific marker for the action of capsaicin on sensory nerves (Maggi & Meli, 1988). Capsaicin caused only a small contraction in the presence of high magnesium (12.5 mM):low calcium (1.25 mM) medium (Figure 3a) and did not cause any contraction in the presence of high magnesium (12.5 mM): very low calcium (0.25 mM) medium for 60 min, respectively. However, in both cases, after replacement with the normal medium, a second application of capsaicin had no effect. An example is shown in Figure 3a. In contrast, after treatment of the high magnesium (12.5 mM):low (1.25 mM) or very low calcium (0.25 mM) medium (without capsaicin) for 60 min, a first application of capsaicin ($1 \mu\text{M}$) caused a strong contraction after replacement with the normal medium ($n = 4$). Thus, a first application of capsaicin was still effective in these high magnesium:low or very low calcium medium.

NPY ($0.01 \mu\text{M}$) did not affect the capsaicin-sensitive contractile response to MN stimulation. The response was inhibited to $69.6 \pm 5.9\%$ ($n = 8$) at $0.05 \mu\text{M}$ and at $0.1 \mu\text{M}$ was inhibited to $59.9 \pm 3.6\%$ ($n = 7$) of controls (Figure 4), 3–18 min after application of NPY. Sufficient NPY was not available to use concentrations higher than $0.1 \mu\text{M}$ in the present experiments. However, no significant difference between effects of $0.05 \mu\text{M}$ and $0.1 \mu\text{M}$ was observed. Thus, $0.1 \mu\text{M}$ NPY seems to be close to the maximal effective dose. A typical example is shown in Figure 1a. During the presence of NPY for 30 min, the inhibition of the response to MN stimulation persisted; a decline in the effects of NPY to 90% and 82.5% of the maximal effect was observed at least 11 and 14 min after an application in 2 out of 7 cases. The inhibitory effect of NPY was fully reversible 30 min later, after thorough washing of the preparation (Figure 2a).

On the other hand, NPY ($0.1 \mu\text{M}$) caused a transient slight but statistically significant inhibition ($13.3 \pm 4.8\%$, $n = 4$, after 1 min) on the twitch responses induced by continuous field stimulation (0.1 Hz , 0.5 ms), but after 5 min the responses recovered completely (Figure 5). These twitch responses were

Figure 3 Effect of high magnesium (12.5 mM):low calcium (1.25 mM) Krebs solution on the capsaicin-sensitive contractile response to MN stimulation (at the dots) and the contractile effects of capsaicin *per se*. In (a), the left trace showing the control response to MN stimulation, the middle trace showing that the responses to MN stimulation were abolished in the presence of high magnesium:low calcium medium for 60 min, but a small contraction was produced by a first application of capsaicin (Cap, $1 \mu\text{M}$; at the arrow), and the right trace showing that the responses to MN stimulation did not recover after 1 h washing with normal Krebs solution and a second application of capsaicin (Cap, $1 \mu\text{M}$; at the arrow) did not cause any effect. In (b), in normal Krebs solution, a first application of capsaicin (Cap, $1 \mu\text{M}$; at the arrow) caused a strong contraction and irreversibly abolished the responses to MN stimulation and a second application of capsaicin (Cap, $1 \mu\text{M}$; at the arrow) did not cause any effect after 1 h washing. W, washing.

Figure 4 Comparison of the capsaicin-sensitive contractile response to mesenteric nerve stimulation (MNS, 20 Hz, 0.5 ms, supramaximal currents for 30 s), capsaicin (Cap) or exogenously administered substance P (SP) in the guinea-pig isolated ileum (guanethidine pre-treated, hexamethonium 50–200 µM throughout). Open columns, control; stippled columns, 3–18 min after addition of neuropeptide Y (0.1 µM); striped column, after thorough washing for 30 min. Significantly different from controls, * $P < 0.001$.

Figure 5 Effect of neuropeptide Y (NPY) on the twitch responses to continuous electrical field stimulation at 0.1 Hz. NPY (0.1 µM) was applied at 0 min. Points indicate mean values of 4 determinations; vertical line indicates s.e.mean. Significantly different from the pre-drug responses, * $P < 0.05$.

unaffected by hexamethonium (50–200 µM) but abolished by atropine (1 µM).

In contrast, NPY (0.05 µM and 0.1 µM) had no inhibitory effect on contractile responses due to activation of capsaicin-sensitive sensory nerves by application of capsaicin (1 µM, $n = 6$) as shown in Figure 1b, or contractile responses to exogenous substance P (1 nM, $n = 7$) as shown in Figure 2, at concentrations matched to produce a contractile response similar to that produced by MN stimulation (Figure 4). The concentration of capsaicin was just above threshold and at concentrations higher than 3 µM, capsaicin exerted a non-specific action on the smooth muscle. The concentration of substance P, which caused about 45% of the amplitude of the maximal contraction, was submaximal (1% of the maximal effective concentration). At lower concentrations than 1 nM, substance P did not produce contractions of constant amplitude. Under the present experimental conditions, maximal contractile effects were obtained with 10 µM capsaicin, which included a non-specific action on the intestinal muscles, and 0.1 µM substance P.

Discussion

Under appropriate experimental conditions, it is possible to elicit cholinergic motor responses produced by antidromic activation of capsaicin-sensitive afferents in the guinea-pig isolated ileum (Szolcsanyi & Bartho, 1978; Takaki *et al.*, 1987). These responses are highly reproducible and appear particu-

larly suitable for studying the modulatory influence of substances on the excitability of the sensory nerves. The present findings indicate (i) that NPY exerted a potent inhibitory effect on motor responses produced when the capsaicin-sensitive sensory nerves were antidromically activated by electrical MN stimulation but not when they were activated by capsaicin and (ii) that NPY inhibited just transiently the twitch contractions mediated via intrinsic cholinergic motor neurones which were activated by electrical field stimulation.

As NPY did not affect responses to substance P, the putative endogenous mediator of the contractile responses produced by activation of capsaicin-sensitive nerves, present data strongly suggest a prejunctional action of NPY. This conclusion is consistent with the well known neuromodulatory action of NPY on autonomic nerves (Lundberg *et al.*, 1984; Wahlestedt *et al.*, 1986; Potter, 1988) and capsaicin-sensitive sensory nerves in the guinea-pig isolated left atria, bronchi and trachea (Giuliani *et al.*, 1989b; Grundemar *et al.*, 1990). It has been previously shown by Maggi *et al.* (1988b,c; 1989a) that the activation of peripheral endings of capsaicin-sensitive sensory nerves by electrical field stimulation (antidromic stimulation) and capsaicin differ in their sensitivity to a potent blocker of N- and L-type voltage-sensitive calcium channels, ω -conotoxin fraction GVIA. In particular, ω -conotoxin suppressed the responses produced by electrical field stimulation while leaving the responses to capsaicin unaffected. In the present study, high magnesium (12.5 mM):low (1.25 mM) or very low calcium (0.25 mM) medium, which is known to block voltage-sensitive calcium channels and inhibit transmitter release, abolished the contractile response (capsaicin-sensitive) to MN stimulation but did not block the specific effect of capsaicin on sensory nerves. These findings support the proposal by Giuliani *et al.* (1989a,b) that the responses produced by these two stimuli are dependent on electrical antidromic stimulation and independent of (capsaicin) activation of ω -conotoxin-sensitive voltage-dependent calcium channels. A selective inhibition of sensory nerve excitability when they are activated by electrical field stimulation but not when they are activated by capsaicin has been reported previously for opioids (Bartho *et al.*, 1987) and for the neuropeptide, galanin (Giuliani *et al.*, 1989a), and for NPY (Giuliani *et al.*, 1989b; Grundemar *et al.*, 1990) in the guinea-pig bronchi, trachea, ear and left atria. This may reflect a peculiar mode of action of capsaicin which is actually capable of concentrating extracellular calcium within sensory neurones (Wood *et al.*, 1988) in such a way that even micromolar calcium concentrations in the medium are sufficient to sustain a full release of sensory neuropeptides from peripheral endings of capsaicin-sensitive nerves (Maggi *et al.*, 1988d; 1989b).

NPY has been shown to inhibit calcium currents in rat dorsal root ganglia and abolish the depolarization-coupled (high K medium) release of substance P (Ewald *et al.*, 1988; Walker *et al.*, 1988). As NPY selectively inhibits the responses produced via the ω -conotoxin-sensitive mode of activation of sensory fibres (Maggi *et al.*, 1988b), the present findings agree with those of Walker *et al.* (1988) suggesting inhibition of voltage-sensitive calcium channels as a likely mechanism for the prejunctional inhibitory action of NPY on excitability of primary sensory neurones.

The NPY containing nerve terminals found elsewhere in the gut wall are those of intrinsic non-noradrenergic neurones (Furness *et al.*, 1983; Sundler *et al.*, 1983). In addition to capsaicin-sensitive sensory nerves, vagal parasympathetic fibres were found to be contained in the mesenteric nerves (Hirst & McKirdy, 1974) which when stimulated, can release NPY from intramural non-noradrenergic neurones. The released NPY could act in turn on sensory nerve endings at interneuronal synapses within the myenteric plexus.

The present findings suggest a possible mode of interaction between enteric and sensory nerves at interneuronal synapses, that is, a form of prejunctional inhibition of sensory nerve excitability by NPY.

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