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Modulation of cholinergic neurotransmission in guinea-pig trachea by neuropeptide Y

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1 Neuropeptide Y (NPY) is localized to adrenergic nerves in guinea-pig airways but its function is not known.

2 NPY (1×10^{-10}–3×10^{-7} M) had no direct effect on guinea-pig tracheal smooth muscle *in vitro*.

3 NPY produced a concentration- and frequency-dependent inhibition of the cholinergic component of responses elicited by electrical field stimulation (EFS) whilst having no effect on the contractile response to exogenously applied acetylcholine (ACh).

4 Yohimbine was able to reverse significantly the inhibitory effect of noradrenaline on the cholinergic component to EFS without having any significant effect on the inhibition produced by NPY.

5 Neither blockade of β-adrenoceptors by propranolol, nor depletion of adrenergic nerves by incubation with 6-hydroxydopamine caused any significant alteration in the response to EFS in the presence of 3×10^{-7} M NPY. Bretylium tosylate incubation to prevent noradrenaline release produced a small but significant enhancement of the inhibitory effect of NPY on EFS at high frequencies.

6 NPY appears to reduce the cholinergic component to EFS via a prejunctional mechanism, acting directly on receptors on cholinergic nerve terminals, rather than affecting adrenergic mechanisms. NPY released by adrenergic nerves may modulate cholinergic neurotransmission in guinea-pig airways.

Introduction

Neuropeptide tyrosine (NPY) is a 36 amino acid peptide which is localized to blood vessels and smooth muscle within the guinea-pig trachea (Sheppard *et al.*, 1984; Uddman *et al.*, 1984; Sundler *et al.*, 1986). The distribution of NPY-like immunoreactivity appears to be similar to that of dopamine β-hydroxylase, suggesting a localization to adrenergic nerve fibres. An interaction of NPY with adrenergic mechanisms in various tissues has been widely documented (Lundberg *et al.*, 1982; Hokfelt *et al.*, 1983; Edvinsson *et al.*, 1984; Lundberg & Stjarne, 1984; Garzon *et al.*, 1986). NPY appears to interact with adrenergic mechanisms in several ways. NPY inhibits electrically-induced contraction of the rat (Lundberg *et al.*, 1982) and mouse (Stjarne *et al.*, 1986) vas deferens, suggesting that it may modulate neurotransmitter release. NPY reduces the outflow of noradrenaline (NA) via a prejunctional action (Dahlof *et al.*, 1985; Lundberg *et al.*, 1985), and in vascular smooth muscle NPY enhances the contractile effects of NA via a postjunctional effect (Ekblad *et al.*, 1984; Lundberg & Stjarne, 1984; Wahlestedt *et al.*, 1985). In rat brain preparations, NPY has been demonstrated to increase preferentially the number of α_2-, but not α_1-adrenoceptors (Agnati *et al.*, 1983), and NPY may enhance the activity of an α_2-adrenoceptor agonist at the prejunctional α_2-adrenoceptor (Martire *et al.*, 1986). Since adrenergic nerves and catecholamines may modulate cholinergic nerves in guinea-pig trachea (Grundstrom *et al.*, 1981), we have investigated whether NPY has an effect on cholinergic nerve-mediated contraction.

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Methods

Tissue preparation

Male Dunkin-Hartley guinea-pigs (250–500 g) were killed by cervical dislocation and the tracheae rapidly removed. After being stripped of connective tissue, the trachea was opened longitudinally by

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cutting through the cartilage. Each trachea was cut into eight transverse pieces, each containing 3–4 cartilaginous strips.

Care was taken not to damage the epithelial layer of the internal surface of the trachea and each segment was suspended in a 10 ml organ bath containing Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_2$ 25.5 and glucose 5.05. Indomethacin was present throughout at a concentration of 10^{-5} M. The solution was maintained at 37°C and gassed continuously with a mixture of 95% O_2 and 5% CO_2, pH 7.4. The tissues were allowed to equilibrate for 1 h, with frequent washing, under a resting tension of 1 g, which was found to be optimal for determining changes in tension. Isometric contractile responses were measured using Grass FT.03 force-displacement transducers and recorded on a polygraph (Grass Model 7D, Grass Instruments Co., Quincy, Mass., U.S.A.).

Electrical field stimulation

The effect of NPY on responses of the tissue to electrical field stimulation (EFS) were studied by suspending the tissues between parallel platinum wire electrodes (approximately 1.5 cm apart). Biphasic square wave pulses were delivered for 15 s periods from a Grass S88 stimulator, using a supramaximal voltage of 40 V at source and a pulse duration of 0.5 ms, for 15 s.

Frequency-response relationships were constructed using a frequency range of 0.5–64.0 Hz. Stimuli were delivered every 4 min, allowing sufficient time between stimulation for each tissue to return to its resting tension. The effect of NPY (1×10^{-10}–3×10^{-7} M) on such frequency-response relationships was studied.

The effect of α- and β-adrenoceptor blockade on frequency-response relationships obtained in the absence and in the presence of NPY were examined using yohimbine (2×10^{-6} M) and (±)-propranolol (2×10^{-6} M), respectively. Pre-incubation with 6-hydroxydopamine (1×10^{-3} M) for 1 h or bretylium tosylate (5×10^{-5} M) for 40 min, before beginning the experiments, was used to establish the effects of adrenergic nerve depletion and the suppression of noradrenaline release, respectively, on the corresponding frequency-response relationships established in both the absence and the presence of NPY (3×10^{-7} M).

In another set of experiments, the effects of yohimbine (2×10^{-6} M) on responses to EFS (40 V, 0.5 ms, 4 Hz) in the presence of noradrenaline (4×10^{-7} M) were studied.

The contractile responses induced by EFS could be abolished by either atropine (10^{-6} M) or tetrodotoxin (TTX, 10^{-6} M), confirming that they were due entirely to cholinergic nerve stimulation.

Acetylcholine concentration-response relationship

The effects of NPY (1×10^{-10}–3×10^{-7} M) on cumulative concentration-response relationships to acetylcholine (ACh) were studied using a concentration range of ACh of 10^{-8}–10^{-1} M.

Drugs and solutions

Drugs were obtained from the following sources: neuropeptide tyrosine (Bachem); acetylcholine chloride, indomethacin, (−)-noradrenaline bitartrate, yohimbine hydrochloride, (±)-propranolol hydrochloride, 6-hydroxydopamine hydrochloride and bovine serum albumin (BSA) (Sigma); bretylium tosylate (Wellcome). NPY stock solutions were made up in distilled water in the presence of bovine serum albumin (BSA; 2×10^{-4} g mg^{-1} peptide). Aliquots of NPY were freeze-dried and stored at −20°C. Indomethacin was made up in alkaline phosphate buffer (pH 7.8) of the following composition (mM): KH_2PO_4 20, Na_2HPO_4 120. Fresh drug solutions were made up daily. Drug additions did not exceed 1% of the bath volume. All concentrations referred to are final bath concentrations.

Analysis of results

Contractile responses were expressed as absolute changes in tension, and then transformed to a percentage of the maximal response for each tissue obtained due to either EFS (40 V, 0.5 ms, 64 Hz for 15 s) or ACh (10^{-2} M). The effects of exogenous drug additions on frequency-response curves were assessed by use of Student's t test for paired data. Comparisons between independent groups were assessed by use of Student's t test for unpaired data. Probability values of <0.05 were considered significant.

Results

Effect of neuropeptide Y

NPY (1×10^{-10}–3×10^{-7} M) had no effect on resting tone in guinea-pig trachea. Figure 1a shows the effect of NPY on the contractile response to EFS (40 V, 0.5 ms, for 15 s) at increasing frequencies from 0.5–64 Hz. Figure 1b shows the inhibition of repetitive responses to EFS (40 V, 0.5 ms, 4 Hz, for 15 s) in the presence of NPY (3×10^{-7} M). The inhibitory effect of NPY was removed with washing, and all responses obtained to EFS were abolished in the presence of TTX (10^{-6} M). NPY produced both a

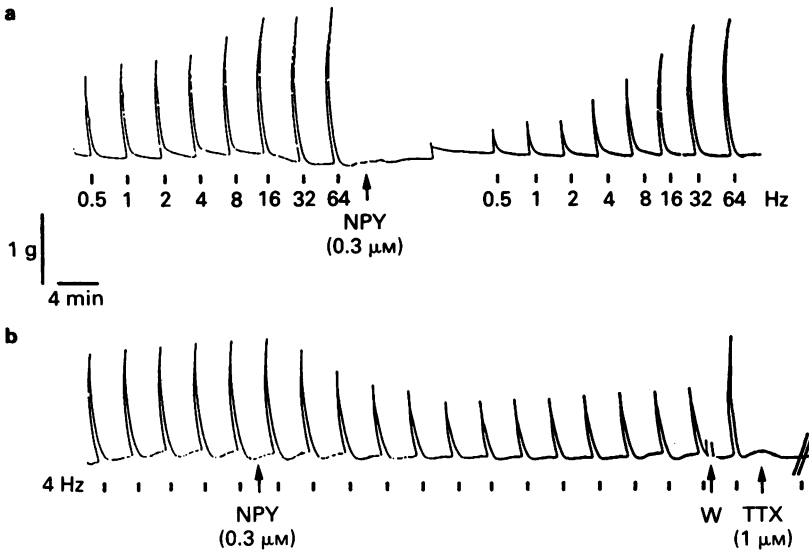

Figure 1 Effect of neuropeptide Y (NPY) on electrical field stimulation (EFS) in guinea-pig trachea *in vitro*. (a) A typical frequency-response tracing (0.5–64.0 Hz) in the absence and then in the presence of NPY (3×10^{-7} M). Stimulation parameters: 40 V, 0.5 ms, 15 s, every 4 min. (b) The inhibitory effect of NPY (3×10^{-7} M) on responses produced to EFS (40 V, 0.5 ms, 4 Hz, every 4 min). The inhibition of the response obtained is reversed by washing (W) and all responses are abolished after the introduction of tetrodotoxin (TTX, 10^{-6} M) for 10 min before resuming stimulation.

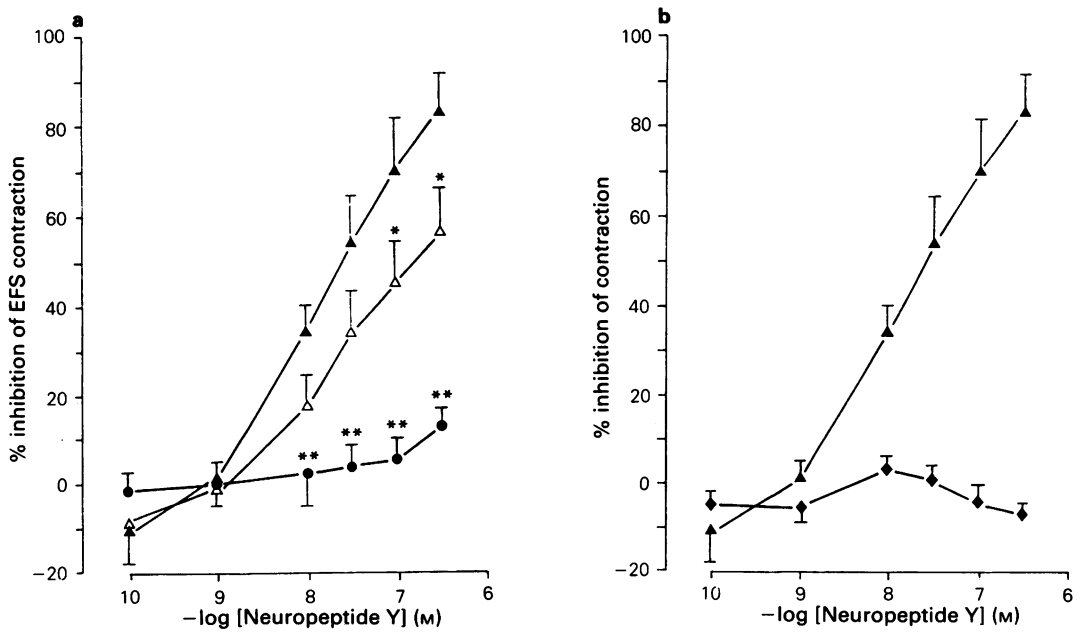

Figure 2 (a) Concentration- and frequency-dependent inhibition of responses to electrical field stimulation (EFS) (●) 64.0 Hz, (△) 8.0 Hz and (▲) 0.5 Hz, by neuropeptide Y (NPY). Points represent means, $n = 7$; vertical lines indicate s.e. mean. $IC_{50} = 1.03 \pm 0.11 \times 10^{-8}$ M at 0.5 Hz. * $P < 0.05$ and ** $P < 0.01$ as compared with responses obtained at 0.5 Hz, by means of Student's t test for paired data. (b) Concentration-dependent inhibition of responses to EFS (40 V, 0.5 ms, 0.5 Hz) (▲), and the degree of inhibition of matched contractile responses produced to exogenously-applied acetylcholine (3×10^{-6} M) (◆) in the presence of NPY (1×10^{-10}–3×10^{-7} M), $n = 7$.

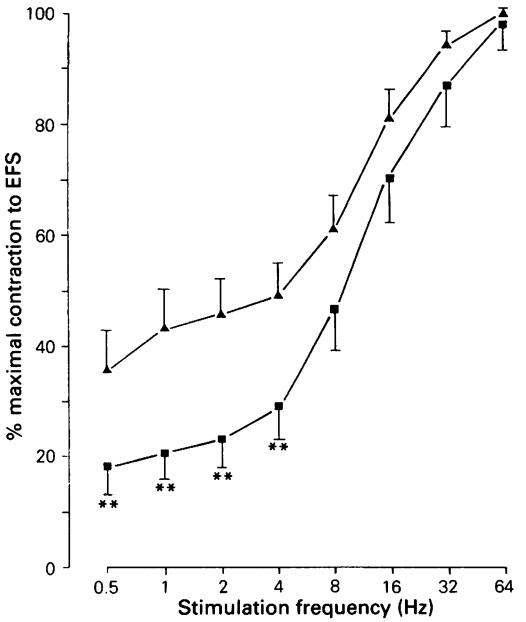

Figure 3 Frequency-response relationship in the absence (▲) and in the presence (■) of 3×10^{-7} M neuropeptide Y (NPY). All points represent the mean, $n = 7$; vertical lines indicate s.e. mean. ** $P < 0.01$ as compared with control values, by use of Student's t-test for paired data.

concentration- and frequency-dependent inhibition of the cholinergic component to EFS (Figure 2a and 3). The concentration of NPY causing 50% inhibition of the cholinergic responses to 0.5 Hz was $1.03 \pm 0.11 \times 10^{-8}$ M. NPY had no significant effect on responses to exogenously applied acetylcholine (ACh) (10^{-8}–10^{-1} M) (Figure 2b); the EC_{50} before NPY being $5.08 \pm 0.17 \times 10^{-6}$ M and after NPY $4.98 \pm 0.15 \times 10^{-6}$ M ($n = 7$). The suppression of the responses to EFS produced by NPY was reversible and there was no apparent development of tachyphylaxis to the peptide. Indomethacin was present throughout at the concentration of 10^{-5} M.

Effect of adrenoceptor blockade on responses to neuropeptide Y

The presence of yohimbine and/or propranolol (2×10^{-6} M), to block α- and β-adrenoceptors, respectively, did not significantly alter the inhibitory effect of NPY on responses produced to EFS. In contrast, however, repetitive stimulation using constant stimulation parameters (40 V, 0.5 ms, 4 Hz, every 4 min), yohimbine (2×10^{-6} M) was able to reverse significantly the inhibitory effect of noradrenaline (NA) (4×10^{-7} M) on responses to EFS, (Figure 4). Control experiments carried out in the presence of both yohimbine and propranolol and in the absence of NPY failed to reveal any significant alteration in responses obtained to EFS due to these two agents.

6-Hydroxydopamine (6-OHDA) pretreatment (10^{-3} M) for 1 h *in vitro*, which has previously been shown to deplete tissues of NA (Doggrell & Waldron, 1982), failed to alter the frequency-response relationship obtained in the presence of NPY (3×10^{-7} M). 6-OHDA alone did not produce any significant alteration in responses obtained to EFS when compared with control responses obtained in the absence of NPY.

Bretylium (5×10^{-5} M) pretreatment for 40 min *in vitro* to prevent NA release from adrenergic nerves (Danser *et al.*, 1987) also failed to reduce the inhibitory effect of NPY (3×10^{-7} M) on the frequency-response relationship, but significantly augmented the inhibitory effect of NPY produced at higher fre-

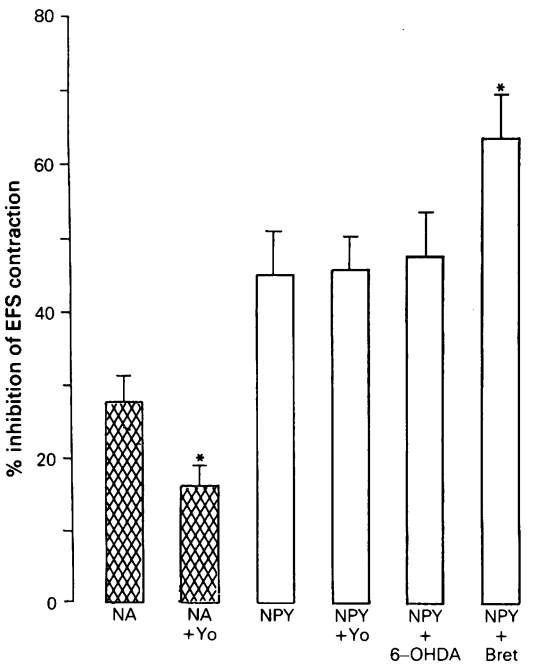

Figure 4 Histogram illustrating the degree of inhibition of responses produced to electrical field stimulation (EFS; 40 V, 0.5 ms, 4 Hz, for 15 s) by noradrenaline (NA, 4×10^{-7} M, $n = 6$) in the absence and in the presence of yohimbine (Yo, 2×10^{-6} M, $n = 6$), and also by neuropeptide Y (NPY, 3×10^{-7} M, $n = 7$) in the absence and presence of yohimbine (2×10^{-6} M, $n = 7$), 6-hydroxydopamine (6-OHDA, 10^{-3} M, $n = 8$) and bretylium tosylate (Bret, 5×10^{-5} M, $n = 9$). Columns represent the mean and vertical lines indicate s.e. mean. * $P < 0.05$ as compared with control responses.

quencies of stimulation. Bretylium alone did not significantly alter responses obtained to EFS when compared with those obtained in corresponding control experiments carried out in the absence of NPY. The effect of 6-OHDA and bretylium pretreatments on the inhibition of responses to EFS in the presence of NPY (3×10^{-7} M) is shown in Figures 4 and 5. Figure 5 also shows the effect of NPY alone, and in the presence of 6-OHDA and bretylium on EFS responses at different frequencies, together with a control curve constructed in the absence of any drug to demonstrate the stability of the response. Neither bretylium nor 6-OHDA reduced the contractile response to EFS.

Discussion

The neuronal control of guinea-pig airways is complex, with cholinergic excitatory, adrenergic inhibitory, non-adrenergic, non-cholinergic (NANC) inhibitory and NANC excitatory nerves (present within the bronchi but not the trachea) (Grundstrom *et al.*, 1981; Taylor *et al.*, 1984). In the guinea-pig, adrenergic innervation is closely associated with cholinergic nerves (Nadel & Barnes, 1984), and noradrenaline (NA) released from adrenergic nerves reduces the bronchoconstriction due to cholinergic nerve activation by prejunctional α_2-adrenoceptors on the cholinergic nerve terminals (Grundstrom *et al.*, 1981). NPY-like immunoreactivity has been shown to be localized to blood vessels and smooth muscle within the guinea-pig trachea, with a distribution similar to that of dopamine β-hydroxylase (Sheppard *et al.*, 1984; Sundler *et al.*, 1986), which suggests a possible role for NPY as a co-transmitter or neuromodulator within adrenergic neuronal mechanisms.

The co-localization of neuropeptide Y (NPY) with NA within sympathetic nerve fibres has been widely documented (Lundberg *et al.*, 1982; Sheppard *et al.*, 1984; Varndell *et al.*, 1984; Sundler *et al.*, 1986). Within blood vessels, NPY itself has a direct, Ca^{2+}-dependent vasoconstrictor action, but also enhances NA-induced contraction in blood vessels, although to a variable extent (Pernow *et al.*, 1986; Wahlestedt *et al.*, 1985). The effect of NPY on blood vessels may be exerted via a pre- or postjunctional effect, the prejunctional effect requiring only the C-terminal portion of the peptide, whereas the postjunctional effect requires the whole peptide sequence (Wahlestedt *et al.*, 1986). In other tissues such as the rat and mouse vas deferens, NPY has been shown to interact with adrenergic mechanisms ultimately to alter the responses to NA via both pre- and postjunctional mechanisms (Pernow *et al.*, 1986; Wahlestedt *et al.*, 1986).

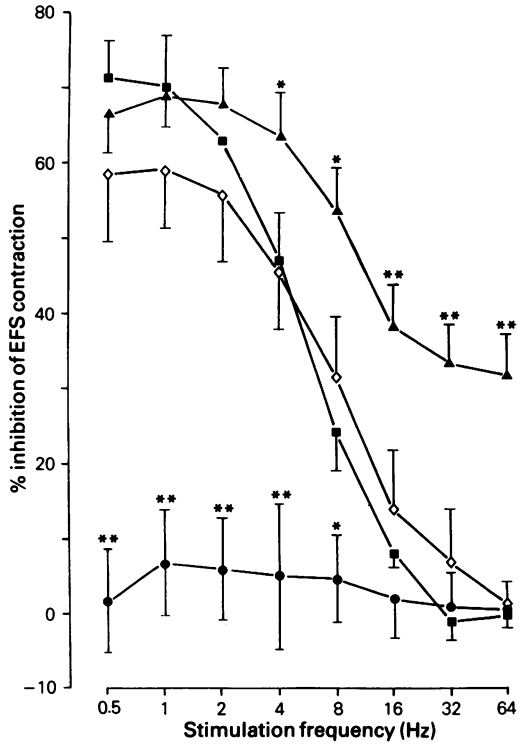

Figure 5 Inhibition of the cholinergic component to electrical field stimulation (EFS) in the presence of 3×10^{-7} M neuropeptide Y (NPY) (◇, $n = 7$), NPY and 6-hydroxydopamine (6-OHDA, 10^{-3} M) (■, $n = 8$), NPY and bretylium tosylate (5×10^{-5} M) (▲, $n = 9$), and in the absence of any drug (●, $n = 8$). All points represent a mean; vertical lines indicate s.e. mean. * $P < 0.05$ and ** $P < 0.01$ as compared with responses obtained in the presence of NPY alone (◇).

NPY had no direct effect on airway smooth muscle, in contrast to its potent effect in vascular smooth muscle in various species (Edvinsson *et al.*, 1984; Wahlestedt *et al.*, 1985; Pernow *et al.*, 1986; Rudehill *et al.*, 1986). In the guinea-pig trachea, however, NPY produced both a concentration- and a frequency-dependent inhibition of the cholinergic component due to electrical field stimulation (EFS). Since NPY failed to alter significantly matched responses produced to exogenously-applied ACh, this suggests that it may directly or indirectly reduce the release of ACh. The inhibitory effect of NPY might be mediated via adrenergic mechanisms, as has been documented in other smooth muscle preparations. The inhibitory effect of NA on cholinergic nerve contractile responses in guinea-pig trachea was partially blocked by yohimbine in agreement with previous observations (Grundstrom *et al.*, 1981), but

yohimbine in the same concentration had no effect on NPY-induced modulation of EFS. Similarly, propranolol had no effect on NPY-induced effects.

Pre-incubation with 6-hydroxydopamine (6-OHDA) for one hour has been shown to deplete completely the content of NA from adrenergic nerves (Doggrell & Waldron 1982; Matthews & McCafferty, 1982) and has also been shown to reduce the NPY content in several peripheral tissues (Lundberg *et al.*, 1982; 1985; Ekblad *et al.*, 1984; Grunditz *et al.*, 1984; Uddman *et al.*, 1984). In this preparation, 6-OHDA pretreatment had no significant effect on the inhibition of responses to EFS due to NPY, confirming that the effect of NPY was not due to release of NA.

Bretylium tosylate, an adrenergic neurone blocking drug with only a slight ability to block neuronal uptake into sympathetic nerves (Uptake 1), has been shown to block the output of NA due to EFS when pre-incubated for 40 min. Bretylium tosylate, however, failed to alter significantly the inhibitory effect of NPY on EFS. The presence of bretylium tosylate did, however, slightly, but significantly, enhance the inhibitory effect of NPY on EFS at high frequencies of stimulation. This could possibly be explained by the fact that bretylium tosylate has, although only to a minor extent, an ability to block the uptake of NA by acting as a competitive substrate for the neuronal uptake mechanism (Uptake 1). Bretylium also possesses a weak anti-cholinergic effect at very high concentrations, which may add to this enhanced inhibitory effect, although the fact that bretylium alone did not inhibit EFS would contradict this possibility.

There is a marked difference in innervation of airways in different species (Richardson, 1979; Barnes, 1986), and it is difficult to extrapolate from guinea-pig trachea to human airways. NPY has been localized to bronchial vessels and airway smooth muscle of human airways (Sheppard *et al.*, 1984), although this is less pronounced than in guinea-pig since the adrenergic nerve supply is scanty. Nevertheless, adrenergic nerve profiles have been observed in close association with cholinergic nerves in human airways (Daniel *et al.*, 1986), so it is possible that NPY may have a similar modulating effect on cholinergic tone of human airways.

In conclusion, it would appear that, in guinea-pig trachea, NPY acts via a pre- rather than a postjunctional mechanism to reduce the EFS-induced release of ACh from cholinergic nerve terminals. Neither blockade of α- and β-adrenoceptors, blockade of NA release, nor depletion of NA from adrenergic nerves (via 6-OHDA) reduced the inhibition by NPY of the cholinergic component to EFS, suggesting that NPY acts directly at a prejunctional receptor site located on the cholinergic nerve terminals, rather than interacting with adrenergic mechanisms. NPY released by adrenergic nerves may, therefore, modulate cholinergic neurotransmission in guinea-pig airways and thus influence airway calibre in this species.

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