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α_2-Adrenoceptor-mediated modulation of the nitrergic innervation of the canine isolated ileocolonic junction

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1 The effects of specific α-adrenoceptor agonists and antagonists on electrically-evoked non-adrenergic non-cholinergic (NANC) relaxations, previously demonstrated as nitrergic, were investigated in isolated circular muscle strips of the canine ileocolonic junction.

2 During a substance P-induced contraction and in the presence of atropine and guanethidine, the specific α_1-adrenoceptor agonist, phenylephrine and antagonist, prazosin, as well as the specific α_2-adrenoceptor antagonist, yohimbine, had no effect on the NANC relaxations evoked by electrical field stimulation. In contrast, clonidine and the more specific α_2-adrenoceptor agonist, UK-14,304, significantly reduced the electrically-induced relaxations, preferentially those in response to low frequency stimulation. The inhibitory effect of UK-14,304 on these relaxations was antagonized by yohimbine.

3 During a noradrenaline-induced contraction, clonidine, but not UK-14,304 significantly augmented the relaxations to electrical stimulation.

4 The adrenoceptor agonists and antagonists used had no effect on concentration-response curves to NO or on the relaxation induced by nitroglycerin.

5 These results indicate that stimulation of prejunctional α_2-adrenoceptors inhibits the nitrergic NANC relaxations induced by field stimulation and thus suggest prejunctional regulation of nitric oxide release via α_2-adrenoceptors in the canine ileocolonic junction.

Keywords: Canine ileocolonic junction; NANC neurotransmission; nitric oxide; prejunctional α_2-adrenoceptors

Introduction

Over the last decade, a great deal of attention has been focused on the concept that neurotransmitter release can be modulated via prejunctional receptors located on, in or near the axon terminals or varicosities. Activation of these receptors can be achieved by specific molecules released from adjacent neurones or cells (heteroregulation) or from the same neurone (autoregulation), resulting in a change in the amount of neurotransmitter that is released. Hence, this process is of key importance in the determination of the synaptic efficiency and the coordination of neuronal communication. Although most of the present knowledge concerning prejunctional modulation is derived from noradrenergic neurones, prejunctional receptors have also been demonstrated on cholinergic neurones as well as on central 5-hydroxytryptamine (5-HT), dopamine and γ-aminobutyric acid (GABA) neurones (Westfall & Martin, 1991). However, evidence suggesting this concept in the peripheral non-adrenergic non-cholinergic (NANC) nervous system, and especially the nitrergic innervation, is rather scarce. In the gastrointestinal tract, prejunctional inhibition of NANC neurotransmission has only been demonstrated in the rat gastric fundus (MacDonald *et al.*, 1990; Lefebvre & Smits, 1992), the guinea-pig proximal colon (Kojima *et al.*, 1988), the rat anococcygeus muscle (Li & Rand, 1989), and the canine lower oesophageal sphincter (Barnette *et al.*, 1990).

Recently, we provided evidence that nitric oxide (NO) or a NO-releasing substance mediates the NANC nerve-induced relaxations in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990a) and, using a bioassay, we actually demonstrated the release of a substance which pharmacologically behaves like NO in response to NANC nerve stimulation (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991b). Based on these results, we suggested NO as an inhibitory NANC neurotransmitter in this tissue. Interestingly, the NANC relaxations in the canine ileocolonic junction evoked by field stimulation up to 16 Hz, 1 ms are entirely mediated by NO (Boeckxstaens *et al.*, 1990a) and thus nitrergic. Furthermore, the involvement of other putative NANC neurotransmitters such as adenosine 5'-triphosphate, vasoactive intestinal polypeptide, GABA and 5-HT had been excluded earlier (Boeckxstaens *et al.*, 1990b–e). Recently, electrophysiological evidence was also provided confirming the role of NO in the ileocolonic junction (Ward *et al.*, 1992). This knowledge, together with morphological evidence demonstrating the presence of NO synthase in its myenteric neurones (unpublished observations), make the ileocolonic junction an interesting model to study possible prejunctional regulation of NO release. Since the ileocolonic junction receives a prominent adrenergic innervation and since prejunctional modulation of NANC neurotransmission via adrenoceptors has been described (Kojima *et al.*, 1988; MacDonald *et al.*, 1990; Lefebvre & Smits, 1992), we suggest a possible prejunctional adrenergic regulation of the NO release at the canine ileocolonic junction.

Methods

Tissue preparation

Mongrel dogs of either sex (body weight 10–30 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and a laparotomy was performed. The ileum and colon were resected 5 cm above and 3 cm below the ileocolonic junction respectively. After the resected specimen was cleaned and rinsed, the mucosa was removed from the ileum and ileocolonic junction by means of sharp dissection. Circular muscle strips of the ileocolonic junction were cut, approximately 1.5 cm long and 0.3 cm wide and mounted in organ

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baths (25 ml) (Pelckmans et al., 1989) filled with a modified Krebs-Ringer solution (mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, CaEDTA 0.026 and glucose 11.1). The solution was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2.

Isometric tension recording

One end of each muscle strip was connected to a metal rod while the other end was attached to a strain gauge transducer (Statham UC2) for continuous recording of isometric tension. After the muscle strips were brought to the optimal point of their length-tension relationship (Pelckmans et al., 1989), they were washed three times and then allowed to equilibrate for at least 45 min before experimentation.

Experimental protocols

All experiments were performed in the presence of atropine (0.3 μM) and guanethidine (3 μM) and during a contraction induced by substance P (0.1 μM) or noradrenaline (30 μM). After each contraction, the muscle strips were washed at least 3 times with an interval of 5 min resulting in a cycle time of at least 20 min.

In a first series of experiments, the muscle strips were contracted with substance P (0.1 μM). Substance P was chosen as contractile agent to avoid any interference with the adrenoceptor agents used. In a first protocol, the effects of the specific adrenoceptor agonists and antagonists clonidine (1 μM), UK-14,304 (0.1–3 μM), prazosin (1–3 μM), phenylephrine (3–10 μM) and yohimbine (0.1–1 μM) were investigated on relaxations induced by electrical impulses (2–16 Hz, 0.5–2 ms, pulse trains lasting 10 s), NO (1 μM) and nitroglycerin (1 μM).

In another protocol, the effects of these agents were also studied on concentration-response curves to NO (0.3–30 μM). Finally, the effect of UK-14,304 (0.1 μM) on the electrically-induced relaxations and on those to NO (1 μM) and nitroglycerin (1 μM) was reinvestigated in the presence of yohimbine (0.1 μM).

In a second series of experiments, the effects of clonidine (1 μM) and UK-14,304 (1 μM) on the relaxations to electrical stimulation (2–16 Hz, 1–2 ms), NO (0.3–30 μM) and nitroglycerin (1 μM) were re-evaluated during a noradrenaline (30 μM)-induced contraction.

Electrical impulses (rectangular waves, 100 mA, 9 V) were delivered by a GRASS stimulator and a direct current (d.c.) amplifier in trains of stimuli of 10 s. Pharmacological agents were added at least 10 min before stimulation of the tissue.

Drugs used

The following drugs were used: atropine sulphate (Federa, Brussels, Belgium), clonidine (Boehringer Ingelheim, Germany), guanethidine (Ciba-Geigy, Switzerland), noradrenaline hydrogentartrate (Fluka AG, Buchs SG, Switzerland), phenylephrine (Sigma Chemical Co, St. Louis, MO, U.S.A.), prazosin (Pfizer, Sandwich, England), substance P, yohimbine hydrochloride (Sigma Chemical Co, St. Louis, MO, U.S.A.). UK-14,304 tartrate (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) was a gift of Pfizer Central Research, Sandwich, England.

All drugs were administered as aqueous solutions. Ascorbic acid (57 mM) was added to the stock solution of noradrenaline and phenylephrine. The solutions were prepared on the day of experimentation and administered in volumes not exceeding 0.5% of the bath volume. NO solutions were prepared as described by Kelm et al. (1988).

Presentation of results and statistical analysis

Results are expressed as g contraction or as percentage decrease of the noradrenaline or substance P-induced contraction and are shown as mean ± s.e.mean for the number of dogs indicated.

For statistical analysis, a two tailed Student's t test for paired observations was used. P values of less than 0.05 were considered to be significant.

Results

In the presence of atropine (0.3 μM) and guanethidine (3 μM), and during a substance P (0.1 μM)-induced contraction, the specific α_1-adrenoceptor antagonist prazosin (1–3 μM), and the specific α_2-adrenoceptor antagonist yohimbine (0.1–1 μM) had no effect on the spontaneous activity of the canine ileocolonic junction (n = at least 6). In contrast, the specific α_1-adrenoceptor agonist, phenylephrine (3 μM: 0.4 ± 0.2 g; 10 μM: 0.4 ± 0.2 g, n = 8), and the specific α_2-adrenoceptor agonists, clonidine (1 μM; 0.4 ± 0.2 g, n = at least 6) and UK-14,304 (0.1 μM; 0.7 ± 0.5 g; 1 μM: 1.1 ± 0.2 g; 3 μM: 0.9 ± 0.1 g, n = at least 6), induced a tonic contraction

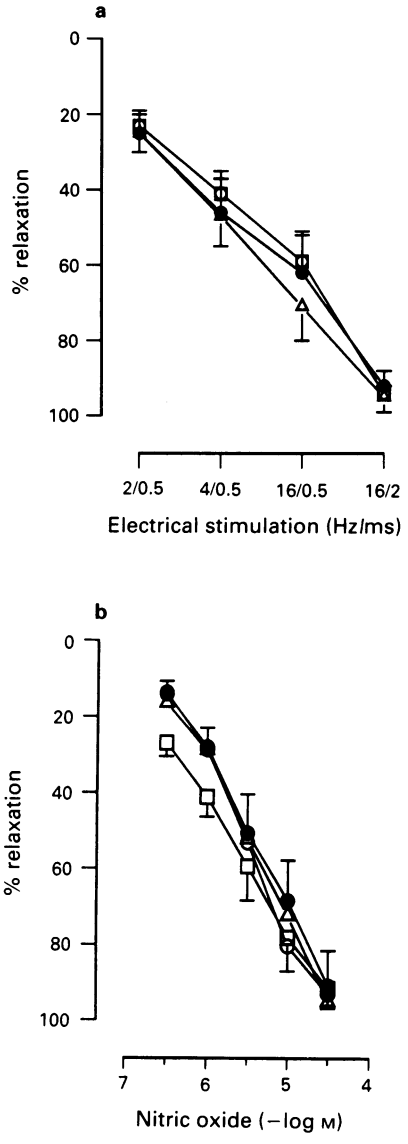

Figure 1 Effect of yohimbine (△, 1 μM), phenylephrine (□, 10 μM) and prazosin (●, 3 μM) on (a) electrically-evoked NANC relaxations (□, 2–16 Hz, 0.5–2 ms) and (b) the concentration-response curve to NO (○, 0.3–3 μM) in the canine ileocolonic junction during a substance P (0.1 μM)-induced contraction. Results are shown as mean ± s.e.mean for 5–8 experiments and are expressed as percentage decrease of the substance P-induced contraction.

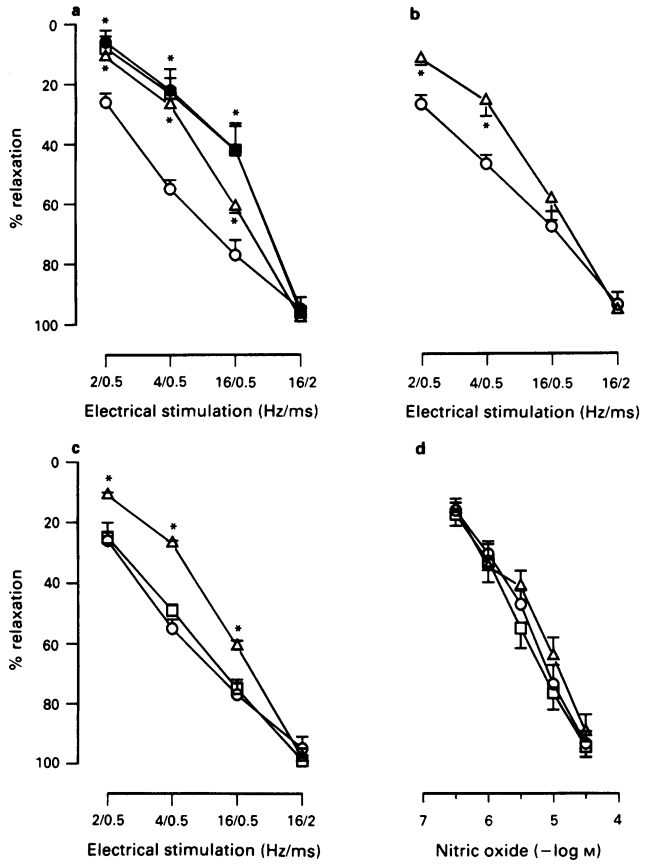

Figure 2 Effect on electrically-evoked NANC relaxations (2–16 Hz, 0.5–2 ms) (○) of (a) UK-14,304 (△, 0.1 μM; □, 1 μM; ●, 3 μM), of (b) clonidine (△, 1 μM) and of (c) UK-14,304 (0.1 μM) in the absence (△) and in the presence of (□) of yohimbine (0.1 μM). Effect of UK-14,304 (△, 3 μM) and clonidine (□, 1 μM) on the concentration-response curve to NO (○, 0.3–3 μM) in the canine ileocolonic junction (d). All experiments were performed during a substance P (0.1 μM)-induced contraction. Results are shown as mean ± s.e.mean for 4–6 experiments and are expressed as percentage decrease of the substance P-induced contraction. $^*P<0.05$, significantly different from control, Student's t test for paired observations.

confirming our previous results (Pelckmans *et al.*, 1990). None of the adrenoceptor agents affected the substance P-induced contraction (3.2 ± 0.5 g, $n = 7$).

Phenylephrine, prazosin, and yohimbine had no effect on the nitrergic relaxations evoked by electrical impulses (2–16 Hz, 0.5–2 ms), on the concentration-response curve to NO (0.3–30 μM) (Figure 1) or on the relaxations to nitroglycerin (1 μM) (results not shown). However, the α_2-adrenoceptor partial agonist clonidine (1 μM) and the more specific α_2-adrenoceptor agonist UK-14,304 (0.1–3 μM) significantly reduced the electrically-evoked NANC relaxations, preferentially those in response to low frequency stimulation (2 and 4 Hz, 0.5 ms) (Figures 2a, b and 3). The inhibitory effect of UK-14,304 was concentration-dependent (Figure 2a) and was antagonized by the specific α_2-adrenoceptor antagonist, yohimbine (0.1 μM) (Figures 2c and 3). Yohimbine also inhibited the contraction to UK-14,304 (0.1 μM) (Figure 3). Neither clonidine nor UK-14,304 had an effect on the concentration-response curve to NO (Figure 2d) or on the relaxation to nitroglycerin (1 μM, Figure 3).

Since in previous studies all our experiments were performed during a noradrenaline (30 μM)-induced contraction, a second series of experiments was designed in which we re-evaluated the effect of UK-14,304 (1 μM) and clonidine (1 μM) under such experimental conditions. To obtain relaxations of similar amplitude as during a substance P-induced contraction, the pulse duration of electrical impulses had to

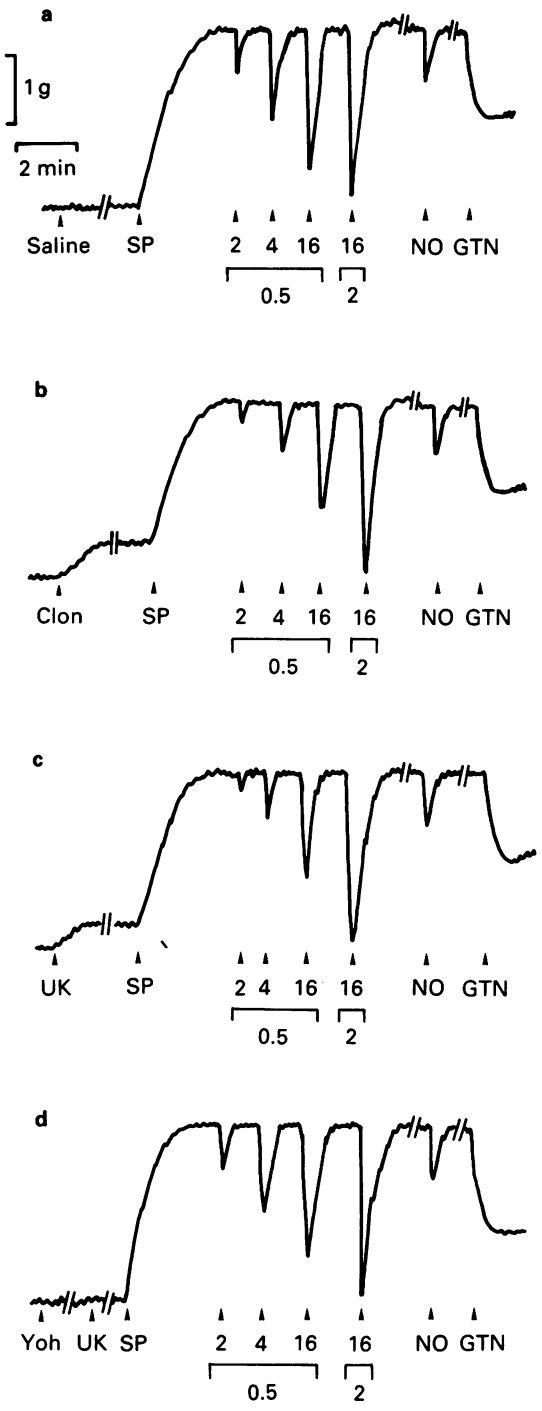

Figure 3 Isometric tension recordings showing (a) the control and the effect of (b) clonidine (Clon, 1 μM) and UK-14,304 (UK, 0.1 μM) in (c) in the absence and (d) the presence of yohimbine (Yoh, 0.1 μM) on NANC relaxations induced by electrical stimulation (2–16 Hz, 0.5–2 ms), NO (1 μM) and nitroglycerin (GTN, 1 μM) in a circular muscle strip of the ileocolonic junction. All experiments were performed during a substance P (SP, 0.1 μM)-induced contraction. Breaks in the tracings represent periods of tissue equilibration. Similar results were obtained in tissues from 3–5 other dogs.

be raised from 0.5 ms to 1 ms. In contrast to the results described above, UK-14,304 had no effect on the electrically-evoked (2–16 Hz, 1–2 ms) relaxations. On the other hand, clonidine significantly enhanced these NANC relaxations (Figures 4 and 5), preferentially those in response to low frequency stimulation (2–4 Hz, 1 ms).

Neither UK-14,304 nor clonidine had an effect on the concentration-response curve to NO, on the relaxation to

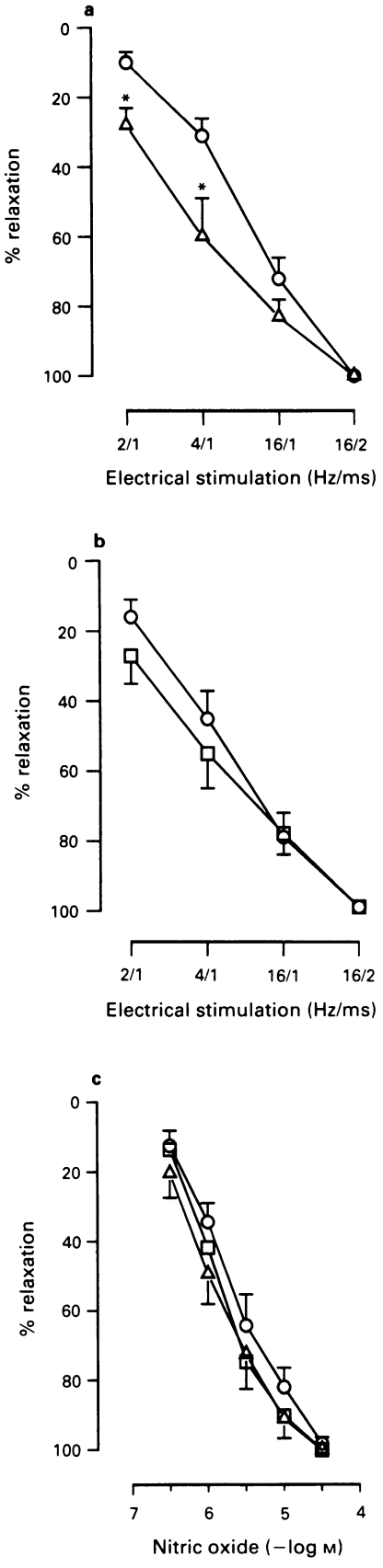

Figure 4 Effect of (a) clonidine (Δ, 1 μM) and (b) UK-14,304 (□, 1 μM) on electrically (2–16 Hz, 1–2 ms) evoked NANC relaxations (○) and (c) the concentration-response curve to NO (○, 0.3–30 μM) during a noradrenaline (30 μM)-induced contraction in the canine ileocolonic junction. Results are shown as mean ± s.e.mean for 6–7 experiments and expressed as percentage decrease of the noradrenaline contraction. $^*P<0.05$, significantly different from control, Student's t test for paired observations.

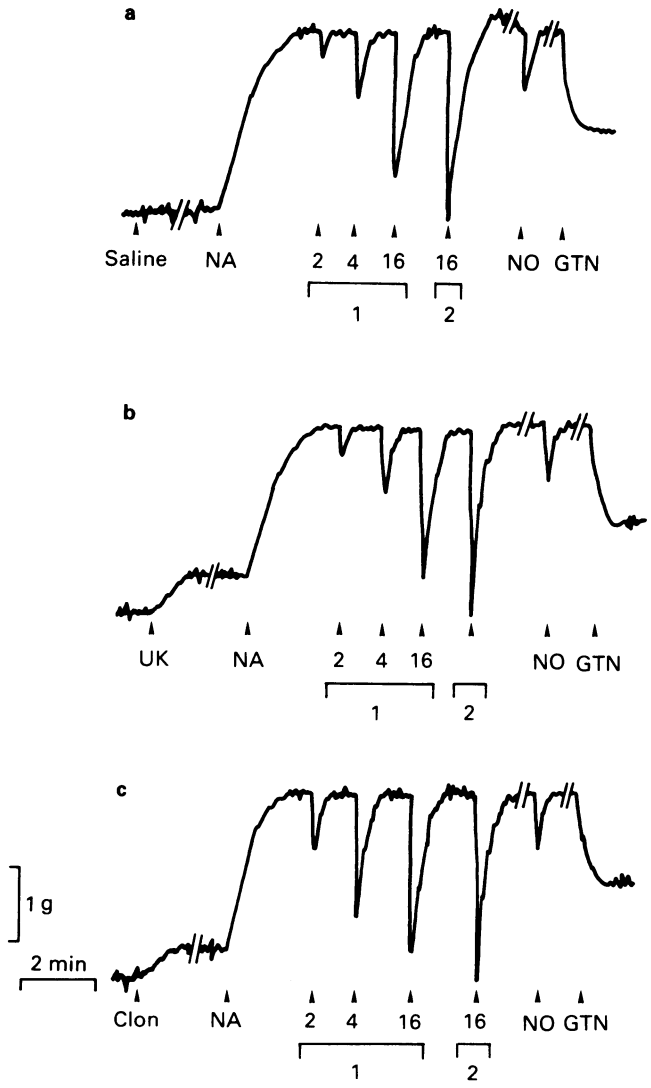

Figure 5 Isometric tension recordings showing (a) the control and the effect of (b) UK-14,304 (UK, 1 μM) and (c) clonidine (Clon, 1 μM) on NANC relaxations induced by electrical stimulation (2–16 Hz, 1–2 ms), NO (1 μM) and nitroglycerin (GTN, 1 μM) in a circular muscle strip of the canine ileocolonic junction. All experiments were performed during a noradrenaline (NA, 30 μM)-induced contraction. Breaks in the tracings represent periods of tissue equilibration. Similar results were obtained in tissues from 5–6 other dogs.

nitroglycerin (Figures 4 and 5) or on the contraction to noradrenaline.

Discussion

It is now well established that adrenergic neurones possess α_2-autoreceptors localized on the axon terminals. These prejunctional α_2-adrenoceptors are involved in the modulation of the calcium-dependent release of noradrenaline through a negative feedback mechanism mediated by the neurotransmitter itself (Langer, 1981). α_2-Adrenoceptors however subserve many more functions than autoinhibition of noradrenaline release and have also been found localized on non-adrenergic nerve terminals. Activation of such prejunctional α_2-adrenoceptors inhibits the calcium-dependent release of neurotransmitters such as acetylcholine, 5-HT and dopamine (Vizi, 1979; Gothert & Hunt, 1980; Dubocovich, 1984). The present study provides evidence indicating that these receptors are also involved in the prejunctional modulation of nitric oxide

release from NANC nerves in the canine ileocolonic junction.

In previous studies (see Introduction), we demonstrated that the inhibitory NANC innervation of the canine ileocolonic junction is mainly nitrergic; the NANC nerve-mediated relaxations induced by electrical impulses up to 16 Hz, 1 ms are completely blocked by inhibitors of the L-arginine:NO pathway, and in addition, the involvement of other putative NANC neurotransmitters, such as adenosine 5'-triphosphate (ATP) and vasoactive intestinal polypeptide (VIP), in the electrically-evoked NANC relaxations has been excluded. As such, the canine ileocolonic junction is a suitable model to study the nitrergic neurotransmission. Here, we demonstrate that in the absence of noradrenaline (muscle strips contracted with substance P), only the α_2-adrenoceptor agonists, clonidine and UK-14,304, significantly reduced the electrically-evoked NANC relaxations, especially those to low frequency stimulation. This effect was antagonized by the specific α_2-adrenoceptor antagonist, yohimbine, indicating that this effect was mediated by α_2-adrenoceptors. As previously reported (Pelckmans *et al.*, 1990), the canine ileocolonic junction possesses postjunctional excitatory α_2-adrenoceptors, explaining the contractile response to clonidine and UK-14,304. It seems rather unlikely however that these postjunctional receptors are involved in the inhibitory effect of clonidine and UK-14,304 on the electrically-evoked NANC relaxations, especially because these adrenoceptor agonists had no effect on the responses to exogenous NO. Furthermore, although clonidine did not affect the tone of the tissue in 3 out of 6 experiments, it did however inhibit the NANC relaxations in response to electrical stimulation in these experiments. Therefore, our results suggest the presence of prejunctional α_2-adrenoceptors which upon activation inhibit NO release. This is further confirmed by the observation that, compared to data obtained with α_2-adrenoceptor agonists on adrenergic neurones, the prejunctional receptor on the nitrergic NANC neurones has many features in common with the adrenergic autoreceptor. As demonstrated in numerous peripheral preparations (for review, see Gillespie, 1980), the inhibitory effect of α_2-adrenoceptor agonists on noradrenaline release is inversely proportional to the frequency of stimulation. In our study, both UK-14,304 and clonidine were indeed most effective on the relaxations induced by low frequency stimulation, but ineffective on those in response to high frequency stimulation (16 Hz). Furthermore, when there is much noradrenaline in the vicinity of the prejunctional α_2-adrenoceptor, it is well established that α_2-adrenoceptor agonists are least effective in inhibiting neurotransmitter release. This explains why UK-14,304 failed to affect nitrergic relaxations when the muscle strips were precontracted with high concentrations of noradrenaline. In contrast, however, it has been reported that partial agonists such as clonidine might even increase neurotransmitter release under such conditions (Medgett *et al.*, 1978; Gillespie, 1980), as also indicated in our experiments (muscle strips contracted with noradrenaline). Indeed, if the maximal inhibitory effect is already being produced by noradrenaline itself, it will be impossible to produce further inhibition by another agonist. If the agonist however has a lower efficacy than noradrenaline, its effect may be manifested as an antagonism of the effect of noradrenaline. This mechanism explains the potentiating action of clonidine on the NANC nerve-mediated relaxations observed in the canine ileocolonic junction during a noradrenaline-induced contraction. Finally, the prejunctional α_2-adrenoceptor activation by exogenous noradrenaline may also be responsible for the finding that the duration of the electrical stimuli applied during a noradrenaline-induced contraction had to be raised from 0.5 to 1 ms to obtain relaxations of the same amplitude as during a substance P-induced contraction. Similarly, endogenous noradrenaline released upon electrical stimulation can act on these α_2-adrenoceptors as well to inhibit NO release, an effect which should be blocked by yohimbine, thereby enhancing the NANC relaxations. However, as all experiments were performed in the presence of guanethidine to block the adrenergic neurotransmission, yohimbine had no such effect. Finally, circulating catecholamines released *in vivo* during stress situations can also influence NO release via activation of these prejunctional α_2-adrenoceptors, possibly playing a role in the motility disturbances observed under such conditions. Whether the prejunctional α_2-adrenoceptor is localized on the nerve terminals or varicosities or on the somatodendritic region of the nitrergic NANC neurones cannot be determined from our experiments.

Prejunctional modulation of NANC neurotransmission in the gastrointestinal tract was previously decribed in only a few tissues (see Introduction); in the rat gastric fundus (MacDonald *et al.*, 1990) and the guinea-pig proximal colon (Kojima *et al.*, 1988), the prejunctional inhibition of the NANC relaxations was reported to be mediated by α_2-adrenoceptors. Recently Lefebvre & Smits (1992) showed in the rat gastric fundus that UK-14,304 blocked the NANC relaxations induced by short train stimulation, previously demonstrated to be mediated by NO (Li & Rand, 1990; Boeckxstaens *et al.*, 1991a), suggesting prejunctional inhibition of NO release via α_2-adrenoceptor activation. In contrast to our results, clonidine did not affect the NANC relaxations in the rat gastric fundus. It should also be emphasized that in contrast to the canine ileocolonic junction, VIP is released by the NANC nerves of the rat gastric fundus as well as NO (De Beurme & Lefebvre, 1988), complicating the study of prejunctional regulation of NO release.

In conclusion, our results provide evidence indicating the presence of prejunctional α_2-adrenoceptors localized on nitrergic neurones in the canine ileocolonic junction. Activation of these receptors inhibits the nitrergic relaxations evoked by NANC nerve stimulation, suggesting that nitric oxide release is modulated via prejunctional α_2-adrenoceptors. This finding might suggest an interaction between the adrenergic and nitrergic innervation in the canine ileocolonic junction. Stimulation of the splanchnic nerves *in vivo* results in contraction of the ileocolonic junction via stimulation of α-adrenoceptors on the smooth muscle (for review, see Ouang, 1992). As adrenergic stimulation is excitatory, one might speculate on the presence of prejunctional inhibition of the release of NO, the main inhibitory neurotransmitter in this tissue, by adrenergic fibres favouring the contractile response. As mentioned in the Introduction, such prejunctional mechanisms are of key importance for the coordination of neuronal communication. The exact physiological importance of the described α_2-adrenoceptors however still needs to be determined. Also whether autoregulatory mechanisms exist in nitrergic neurotranmission or prejunctional regulation of the adrenergic and/or cholinergic innervation by NO occurs in the canine ileocolonic junction certainly needs further study.

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