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Inhibitory pathways in the circular muscle of rat jejunum

[1]Gwen Vanneste, [2]Patrick Robberecht & *,[1]Romain A. Lefebvre

[1]Heymans Institute of Pharmacology, Ghent University, Ghent, Belgium and [2]Department of Biological Chemistry and Nutrition, Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium

1 Conflicting data have been reported on the contribution of nitric oxide (NO) to inhibitory neurotransmission in rat jejunum. Therefore, the mechanism of relaxation and contribution to inhibitory neurotransmission of NO, adenosine 5′-triphosphate (ATP), vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) was examined in the circular muscle of Wistar–Han rat jejunum.

2 Mucosa-free circular muscle strips were precontracted with methacholine in the presence of guanethidine and exposed to electrical field stimulation (EFS) and exogenous NO, ATP, VIP and PACAP. All stimuli induced reduction of tone and inhibition of phasic motility. Only electrically induced responses were sensitive to tetrodotoxin (3×10^{-6} M).

3 NO (10^{-6}–10^{-4} M)-induced concentration-dependent relaxations that were inhibited by the soluble guanylyl cyclase inhibitor 1H-[1,2,4]-oxadiazolo-[4,3-*a*]-quinoxalin-1-one (ODQ; 10^{-5} M) and the small conductance Ca^{2+}-activated K^+-channel blocker apamin (APA; 3×10^{-8} M).

4 Relaxations elicited by exogenous ATP (10^{-4}–10^{-3} M) were inhibited by the P2Y purinoceptor antagonist reactive blue 2 (RB2; 3×10^{-4} M), but not by APA and ODQ.

5 The inhibitory responses evoked by 10^{-7} M VIP and 3×10^{-8} M PACAP were decreased by the selective PAC_1 receptor antagonist $PACAP_{6-38}$ (3×10^{-6} M) and APA. The $VPAC_2$ receptor antagonist PG99-465 (3×10^{-7} M) reduced relaxations caused by VIP, but not those by PACAP, while the $VPAC_1$ receptor antagonist PG97-269 (3×10^{-7} M) had no influence.

6 EFS-induced relaxations were inhibited by the NO-synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (3×10^{-4} M), ODQ and APA, but not by RB2, PG97-269, PG99-465 and $PACAP_{6-38}$.

7 These results suggest that NO is the main inhibitory neurotransmitter in the circular muscle of Wistar–Han rat jejunum acting through a rise in cyclic guanosine monophosphate levels and activation of small conductance Ca^{2+}-dependent K^+ channels.
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Keywords: Jejunal motility; NANC; rat (Wistar–Han); nitric oxide; ATP; VIP; PACAP; cGMP; SK channels

Abbreviations: ATP, adenosine 5′-triphosphate; AUC, area under the curve; cGMP, cyclic guanosine monophosphate; EFS, electrical field stimulation; ijp, inhibitory junction potential; iNOS, inducible nitric oxide synthase; L_o, optimal load; L-NAME, N^{ω}-nitro-L-arginine methyl ester; mRNA, messenger RNA; NANC, nonadrenergic, noncholinergic; NO, nitric oxide; ODQ, 1H[1,2,4,]oxadiazolo[4,3-*a*]quinoxalin-1-one; PACAP, pituitary adenylate cyclase-activating peptide; sGC, soluble guanylate cyclase; SK_{Ca} channel, small conductance Ca^{2+}-dependent K^+ channel; TTX, tetrodotoxin; VIP, vasoactive intestinal peptide

Introduction

The small intestine belongs to the affected area in human postoperative ileus (Holte & Kehlet, 2000) and rodent models are used to understand the pathogenesis of the disturbed motility in postoperative ileus. Circular muscle motility of the small intestine and, more specific, the jejunum is influenced in a pathological model of postoperative ileus in rats (Kalff *et al*., 1998). Furthermore, nitric oxide (NO), produced by inducible NO synthase (iNOS), was reported to play a significant role in rat postoperative jejunal smooth muscle dysfunction (Kalff *et al*., 2000). In order to study the influence of operative procedures on gastrointestinal motility, it is important to know its exact regulation in normal conditions.

Nonadrenergic, noncholinergic (NANC) neurones play an important role in gastrointestinal motility. NO is considered to be the major mediator of NANC relaxations in the gastrointestinal tract. However, adenosine 5′-triphosphate (ATP) and the neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) have also been reported to mediate NANC inhibitory responses. Each of these individual neurotransmitters exhibits variable effects not only between species and species strains but also between gut regions and muscle layers (Balsiger *et al*., 2000; Okishio *et al*., 2000a, b).

Conflicting results have been reported on the role of NO in inhibitory neurotransmission of rat jejunum. NO was reported to mediate 45.5, 25.5 and 3.5% of NANC inhibitory responses in longitudinal muscle of rat jejunum in Sprague–Dawley, Wistar ST and Wistar rat, respectively (Niioka *et al*., 1997; Okishio *et al*., 2000b). For the circular muscle layer of

*Author for Correspondence at: Heymans Institute of Pharmacology, De Pintelaan 185, Ghent 9000, Belgium;
E-mail: romain.lefebvre@UGent.be
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Lewis rat jejunum, it was reported that NO is not involved in NANC inhibitory responses (Balsiger *et al*., 2000). These discrepancies are also found when other species are considered. In the longitudinal muscle layer of the human jejunum, NO was reported to play a moderate role in NANC relaxations (Zyromski *et al*., 2001), while in the circular muscle of human jejunum NO was suggested to be the major mediator (Murr *et al*., 1999). In the circular muscle of mouse jejunum, NO was reported to play a moderate role in NANC neurotransmission (De Man *et al*., 2003).

Also with regard to the other putative NANC neurotransmitters, opposing results have been reported. In longitudinal muscle of Sprague–Dawley rat jejunum, exogenously applied ATP induced concentration-dependent relaxations (Fox *et al*., 1986), but further data on the involvement of ATP in rat jejunal NANC relaxations was not found. When considering other regions of the small intestine, ATP was reported to play a significant role in NANC inhibitory responses in longitudinal muscle of Wistar rat duodenum (Manzini *et al*., 1985), but this was contradicted by another study that found no involvement of ATP in NANC neurotransmission in the same tissue and species (Serio *et al*., 1990). ATP was also reported to play a role in NANC relaxations in longitudinal muscle of Wistar rat ileum (Kadowaki *et al*., 2003). In the circular muscle of human jejunum, ATP was suggested to participate in the creation of fast inhibitory junction potentials (ijp's) (Xue *et al*., 1999), but another report suggested that ATP has no role in NANC relaxations in the circular muscle of human jejunum (Murr *et al*., 1999). ATP was also reported to be the major mediator of relaxations in the circular muscle of mouse jejunum (De Man *et al*., 2003).

In longitudinal muscle of Sprague–Dawley and Wistar ST rat jejunum, PACAP and VIP do not contribute in the mediation of NANC inhibitory responses (Niioka *et al*., 1997; Okishio *et al*., 2000b). In the circular muscle layer of human jejunum, high doses of exogenous VIP were able to inhibit completely spontaneous contractile activity, but no role for VIP was found in NANC inhibitory neurotransmission (Murr *et al*., 1999). In contrast, in the circular muscle layer of hamster jejunum, VIP was suggested to participate in NANC relaxations (Matsuyama *et al*., 2002).

In view of our interest for the jejunum as a site struck in postoperative ileus, and in view of the conflicting results on the mediators of inhibitory NANC neurotransmission in this part of the gastrointestinal tract, the aim of our study was to investigate the effect and the contribution to inhibitory neurotransmission of NO, ATP, VIP and PACAP in the circular muscle of Wistar–Han rat jejunum. The mechanism of action of NO and ATP was investigated, concentrating on the role of cyclic guanosine monophosphate (cGMP) and small conductance Ca^{2+}-dependent K^+ channels (SK_{Ca} channels).

Methods

Tissue preparation

Male Wistar–Han rats (340–500 g) were purchased from Janvier, Le Genest St-Isle, France and had free access to water and commercially available rat chow. They were killed by decapitation and a $\pm$20 cm long segment of the gastrointestinal tract starting 5–10 cm distally from the ligament of Treitz was removed. The segment was flushed and put in aerated Krebs solution (composition in mM: NaCl 188.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1). The most proximal part of the segment was used for further preparation. The segment was opened along the mesenteric border and pinned mucosa side up in physiological salt solution. The mucosa was removed by sharp dissection under a microscope and eight to 12 full-thickness muscle strips (7×10 mm) were cut along the circular axis. All experimental procedures were approved by the Ethical Committee for laboratory animals from the Faculty of Medicine and Health Sciences at Ghent University.

Isometric tension recording

After a silk thread (USP 4/0) was attached to both ends, muscle strips were mounted in either 5 ml organ baths between two platinum electrodes (26×5 mm, 7 mm apart) or 3 ml organ baths. The organ baths contained aerated (5% CO_2 in O_2) Krebs solution, maintained at 37°C in the presence of 4×10^{-6} M guanethidine to block noradrenergic responses. When an experiment with the peptides VIP or PACAP was carried out, 50 mg l^{-1} bovine serum albumin and 30 mg l^{-1} bacitracin was added to the Krebs solution. Changes in isometric tension were measured using MLT 050/D force transducers (ADInstruments, U.K.) and recorded with Chart software and a PowerLab/8sp data recording system (ADInstruments, U.K.).

After an equilibration period of 60 min with flushing every 15 min at a load of 0.25 g, the length–tension relationship was determined. Muscle strips were stretched by load increments of 0.25 g and at each load level exposed to 10^{-5} M methacholine to determine the optimal load (L_o; the load at which maximal response to the contractile agent occurred).

Protocol

Strips were allowed to equilibrate for 30 min at L_o with flushing every 10 min and then precontracted with 10^{-5} M methacholine. Methacholine induced an initial fast increase in tone and phasic activity; tone then declined to a lower level with superimposed phasic activity (Figure 1). In the first part of the experiment, relaxations were induced by either application of exogenous NO (10^{-6}–10^{-4} M), ATP (10^{-4}–10^{-3} M), VIP (10^{-7} or 3×10^{-7} M) or by electrical field stimulation (EFS; 40 V, 0.1 ms, 1–8 Hz for 20 s or 4–8 Hz for 100 s) *via* the platinum plate electrodes by means of a Hugo Sachs Stimulator I type 215/I. The response consisted of a decrease in tone and a reduction of phasic activity. The different concentrations of NO or ATP, or the EFS trains at different frequencies, were given 5 min after the response to the previous stimulus has ended. Only one relaxant stimulus was studied per tissue, and the first concentration of a relaxant agent or the first EFS train was applied 10 min after adding methacholine. Strips were washed for 20 min with flushing every 5 min, and interfering drugs were then incubated for 20 min. Methacholine (10^{-5} M) was then again applied and the responses to NO, ATP, VIP or EFS were studied again in the presence of one of the following drugs: (1) *for NO- and EFS-induced relaxations*: the nerve blocker tetrodotoxin (TTX; 3×10^{-6} M), the NO-synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 3×10^{-4} M), the SK_{Ca} channel blocker

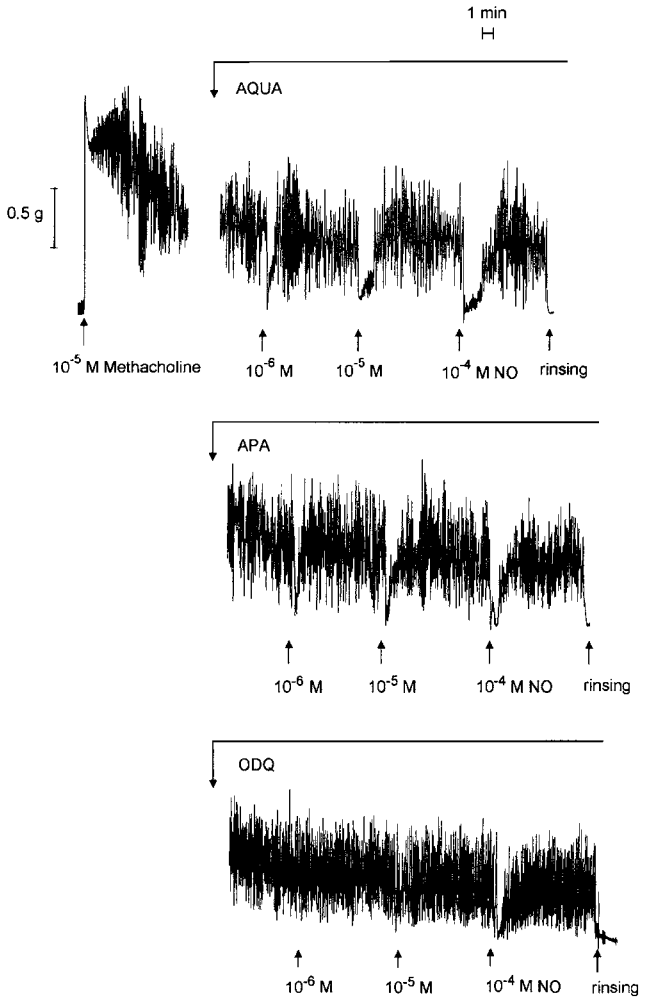

Figure 1 Traces showing the inhibitory responses of mucosa-free precontracted (methacholine;10^{-5} M) circular muscle strips of Wistar–Han rat jejunum to exogenously applied NO (10^{-6}–10^{-4} M) in control conditions (top), in the presence of apamin (APA; 3×10^{-8} M; middle) and ODQ (10^{-5} M; bottom). Arrows indicate the moment of administration. The upper trace was interrupted in the figure before the administration of NO started.

apamin (3×10^{-8} or 5×10^{-7} M), the soluble guanylyl cyclase (sGC) inhibitor 1H[1,2,4,]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ; 10^{-5} M), apamin (3×10^{-8} or 5×10^{-7} M) plus ODQ (10^{-5} M), the P2Y purinoceptor antagonist reactive blue 2 (RB2) (3×10^{-4} M), the $VPAC_1$ receptor antagonist PG 97-269 (3×10^{-7} M), the $VPAC_2$ receptor antagonist PG 99-465 (3×10^{-7} M) and the PAC_1 receptor antagonist $PACAP_{6-38}$ (3×10^{-6} M); (2) *for ATP-induced relaxations*: TTX, L-NAME, apamin, ODQ and RB2; and (3) *for VIP-induced relaxations*: TTX, L-NAME, apamin, RB2, PG 97-269, PG 99-465 and $PACAP_{6-38}$. The responses to EFS were also studied before and in the presence of the N-type Ca^{2+} channel blocker ω-conotoxin GVIA (10^{-6} M), and before and after exposing the tissues to capsaicin (10^{-6} M). As $PACAP_{1-38}$ (3×10^{-8} M)-induced relaxations could not be reproduced, this neuropeptide was studied in parallel strips in the presence or absence of TTX, apamin, RB2, PG 97-269, PG 99-465 and $PACAP_{6-38}$. The influence of RB2 (3×10^{-4} M) was also tested *versus* isoprenaline (3×10^{-7} M).

The reproducibility of the responses to NO, ATP, VIP or EFS was evaluated by running time-control strips in parallel, which did not receive the interfering drugs or received the solvent of these drugs when it was not water.

Data analysis

The duration of the inhibitory responses was determined as 20 or 100 s for EFS (i.e. the length of the stimulus train applied) and 5 min for the long acting peptides VIP and PACAP. As ATP, NO and capsaicin induced a temporary interruption of phasic activity followed by a progressive recuperation of phasic activity, the end of the response was taken when the troughs of phasic activity exceeded the mean trough level of phasic activity during the 5 min before administration. In some cases with administration of 10^{-3} M ATP, the mean trough level was not achieved after 5 min; the duration was then fixed at 5 min. In the second part of the experiment, inhibitory responses in the presence of interfering drugs were analysed for the same duration as determined in the first part. The inhibitory response was quantified by calculating the area under the curve (AUC) of the registered activity during the determined duration, and subtracting it from the AUC of the registered activity for 5 min before applying the relaxant stimulus, normalised to the same duration. The value for the inhibitory response was then expressed as the percent of the normalised reference AUC.

The influence of interfering drugs on basal contractility was assessed as follows: the AUC during the 15 min after application of a given drug was normalised to 5 min and compared to the AUC of the 5 min period before administration of the drug. This value was then compared to the corresponding value obtained in a parallel control strip (unpaired *t*-test).

All results are expressed as means ± s.e.m. *n* refers to tissues obtained from different animals. Inhibitory responses before and in the presence of interfering substances were compared by a paired *t*-test. The change in inhibitory responses induced by interfering substances was compared to the spontaneous change in the control tissues by an unpaired *t*-test. The responses to PACAP in parallel tissues were also compared by an unpaired *t*-test. A *P*-value less than or equal to 0.05 was considered to be statistically significant (GRAPHPAD, San Diego, CA, U.S.A.).

Drugs used

ATP (Sigma, Belgium), apamin (Alomone labs, Israel), bacitracin (Sigma), bovine serum albumin (Sigma), capsaicin (Sigma), ω-conotoxin GVIA (Alomone labs, Israel), guanethidine (Sigma), ODQ (Tocris Cookson, U.K.), isoprenaline hydrochloride (isoprenaline; Abbott S.A./n.v., Belgium), *O*-acetyl-β-methyl-cholinchloride (methacholine; Schuchardt, Munchen, Germany), L-NAME (Sigma), PACAP(1–38) (Alexis; Switzerland), PACAP(6–38) (Alexis; Switzerland), RB2 (RB2; Sigma), TTX (Alomone labs, Israel), VIP (Sigma). PG 97-269 and PG 99-465 were synthesised in the lab of P. Robberecht. All drugs except ODQ and capsaicin were dissolved in deionised water. ODQ was dissolved in 100% ethanol up to a concentration of 10^{-2} M. Capsaicin was dissolved in 100% ethanol and further diluted with an equal volume of distilled water to give a stock solution of 10 mg ml^{-1}.

Further dilutions were made with distilled water. Saturated NO solution was prepared from gas (Air Liquide, Belgium) as described by Kelm & Schrader (1990).

Results

General observations

The length of the strips was measured after equilibration and determination of optimal load, while the tissue wet weight was measured immediately after the experiment. To give an idea, the length and weight of the control strips used for the study of NO-induced responses was 6.9 ± 0.3 mm and 3.03 ± 0.27 mg ($n = 32$). At L_o, a variable amount of phasic activity on top of the basal tone was displayed.

Methacholine induced an increase in tone as well as an increase of phasic activity (Figure 1). When comparing the response to methacholine in the second part of the experiment to the response in the first part, the response to methacholine was generally significantly increased. To illustrate this, the AUC of the methacholine-induced contractile activity, as measured for 5 min just before a first inhibitory stimulus was applied, significantly increased during the second part of the experiment, in four of the five series of control strips, used for the study of NO-induced responses (AUC in g·s: $201 \pm 20 \rightarrow 225 \pm 17$, $P<0.01$; $272 \pm 18 \rightarrow 308 \pm 24$, $P<0.05$; $391 \pm 54 \rightarrow 419 \pm 53$, $P<0.05$; $292 \pm 15 \rightarrow 347 \pm 26$, $P<0.05$, paired t-test;

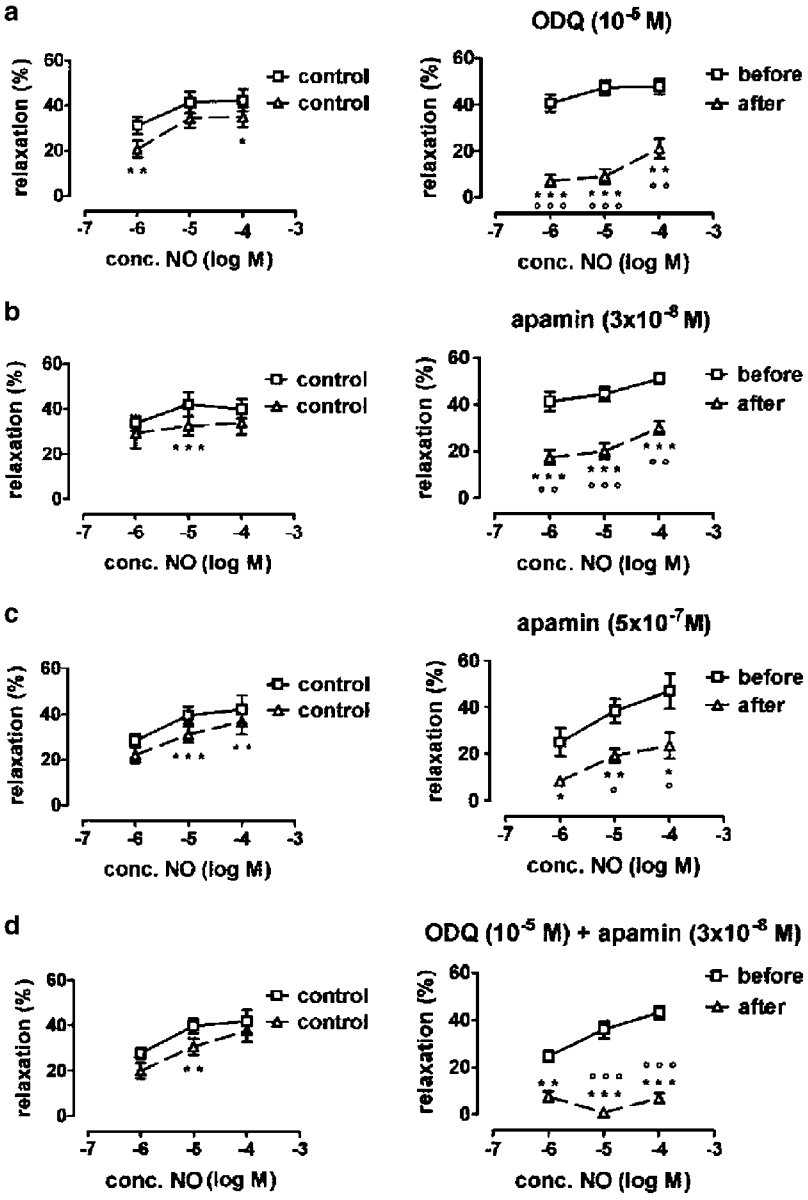

Figure 2 Consecutive concentration–response curves of NO (10^{-6}–10^{-4} M) in control strips (left) and in parallel strips (right) before and after incubation with ODQ (10^{-5} M; a), apamin (3×10^{-8} M; b), apamin (5×10^{-7} M; c) and ODQ (10^{-5} M) plus apamin (3×10^{-8} M; d). Data are expressed as the percent relaxation of the precontracted circular muscle strip. Values are means ± s.e.m. of $n = 6$–7 experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$: paired t-test. $^\circ P<0.05$, $^{\circ\circ}P<0.01$, $^{\circ\circ\circ}P<0.001$: unpaired t-test *versus* control.

$n=6$–7 per series). In the fifth series, the AUC was 210 ± 20 and $224\pm26\,g\cdot s$ for the first and second application of methacholine. The response to methacholine was not systematically altered by any of the interfering drugs.

When interfering agents were administered to study their effects on the responses to NO, ATP, VIP, PACAP and EFS, their possible influence on basal motility during their incubation period was studied (see Methods). None of the agents tested had a consistent influence on basal activity.

Inhibitory responses to exogenously applied NO

Application of exogenous NO (10^{-6}–10^{-4} M) to precontracted muscle strips induced TTX (3×10^{-6} M)-insensitive inhibitory responses that were longer in duration upon increasing concentrations of NO (Figure 1). In control tissues, the inhibitory responses to NO tended to decline (Figure 2). Therefore, the reduction in the NO-induced responses by interfering drugs was always compared to the spontaneous decline in the control tissues. ODQ (10^{-5} M) and apamin (3×10^{-8} M) significantly inhibited the NO-induced responses at all concentrations (Figures 1 and 2). Apamin (5×10^{-7} M) did not have a more pronounced inhibitory effect *versus* NO than 3×10^{-8} M apamin. The reduction of the NO-induced responses by 10^{-5} M ODQ plus 3×10^{-8} M apamin was not more pronounced than that of ODQ alone, except for the response induced by 10^{-4} M NO (Figure 2). RB2 (3×10^{-4} M) only inhibited the relaxant responses of 10^{-4} M NO. L-NAME (3×10^{-4} M), the $VPAC_1$ receptor antagonist PG 97-269 (3×10^{-7} M), the $VPAC_2$ receptor antagonist PG 99-465 (3×10^{-7} M) and $PACAP_{6-38}$ (3×10^{-6} M) had no influence on the NO-evoked inhibitory responses.

Inhibitory responses to exogenously applied ATP, VIP and PACAP

Exogenously applied ATP evoked concentration-dependent inhibitory responses (Figure 3) that were well maintained if studied for the second time in control tissues. The responses to both concentrations of exogenous ATP were nearly abolished by RB2 (3×10^{-4} M): from 22.9 ± 5.3–1.2 ± 1.8% for 10^{-4} M ATP and from 28.2 ± 4.4–3.1 ± 0.8% for 10^{-3} M ATP ($n=7$). Apamin (3×10^{-8} M) had no significant influence; apamin (5×10^{-7} M) reduced the relaxant response to 10^{-4} M ATP from 21.8 ± 4.1% before to 5.7 ± 1.2% ($n=7$), but did not significantly influence the relaxant response to 10^{-3} M ATP. ODQ (10^{-5} M), L-NAME (3×10^{-4} M) and TTX (3×10^{-6} M) had no significant effect on the ATP-evoked relaxations.

The inhibitory responses elicited by the peptides VIP (10^{-7} M or 3×10^{-7} M) and $PACAP_{1-38}$ (3×10^{-8} M) were slow in onset and long lasting (Figure 3). The responses to VIP were reproducible when studied again in control strips (results not shown). However, the response to $PACAP_{1-38}$ could not be reproduced at all in control tissues. Therefore, the influence of antagonists *versus* $PACAP_{1-38}$ was studied in parallel tissues, where $PACAP_{1-38}$ was applied only once. The inhibitory responses to 3×10^{-7} M VIP were significantly decreased by RB2 (3×10^{-4} M; Figure 4), but not by the $VPAC_1$ receptor antagonist PG 97-269 (10^{-7} M) and the $VPAC_2$ receptor antagonist PG 99-465 (10^{-7} M). The $VPAC_2$ receptor antagonist PG 99-465 (3×10^{-7} M) significantly reduced the inhibitory response to 10^{-7} M VIP, while the

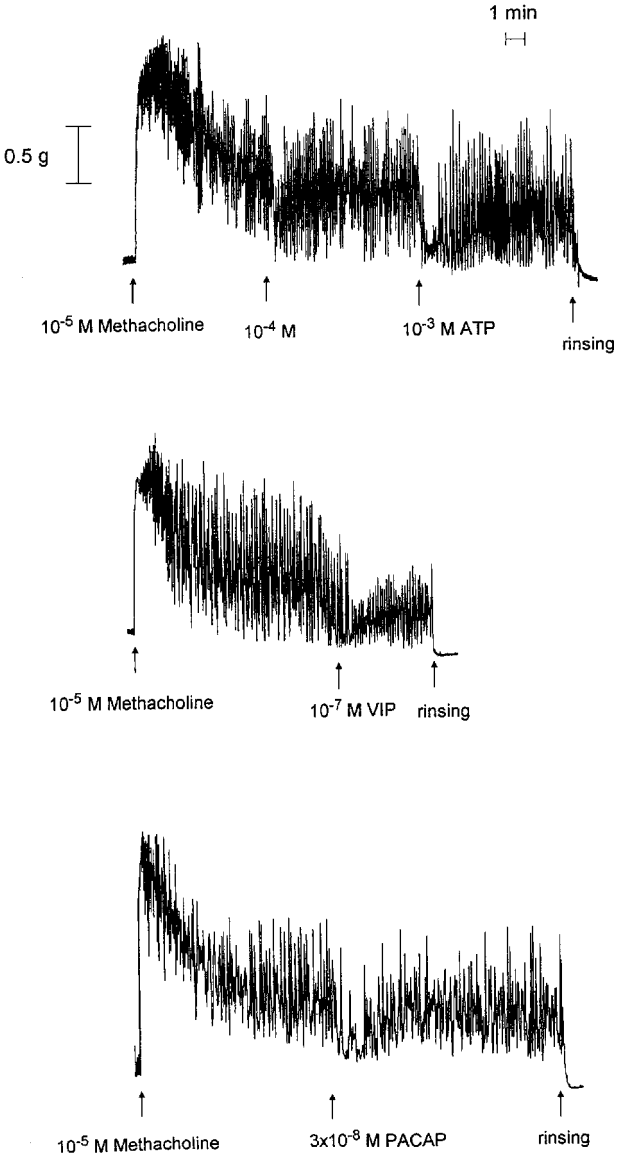

Figure 3 Traces showing the inhibitory responses of mucosa-free precontracted (methacholine; 10^{-5} M) circular muscle strips of Wistar–Han rat jejunum to exogenously applied ATP (10^{-4}–10^{-3} M) (top), VIP (10^{-7} M; middle) and $PACAP_{1-38}$ (3×10^{-8} M; bottom). Arrows indicate the moment of administration.

$VPAC_1$ receptor antagonist PG 97-269 (3×10^{-7} M) still did not; the response to 10^{-7} M VIP was also reduced by 3×10^{-8} M apamin and $PACAP_{6-38}$ (3×10^{-6} M), but not by L-NAME (3×10^{-4} M) and TTX (3×10^{-6} M). The inhibitory response to 3×10^{-8} M $PACAP_{1-38}$ was significantly reduced by apamin (3×10^{-8} M), RB2 (3×10^{-4} M) and $PACAP_{6-38}$ (3×10^{-6} M) (Figure 5), but not by TTX (3×10^{-6} M), the $VPAC_1$ receptor antagonist PG 97-269 (3×10^{-7} M) and the $VPAC_2$ receptor antagonist PG 99-465 (3×10^{-7} M).

RB2 (3×10^{-4} M) was also tested on isoprenaline (3×10^{-7} M)-induced inhibitory responses, but had no significant effect (percent relaxation by isoprenaline in control tissues: 40.4 ± 1.8 and 38.7 ± 1.7, $n=6$; percent relaxation by isoprenaline before and after RB2: 44.0 ± 3.3 and 41.8 ± 3.9, $n=6$).

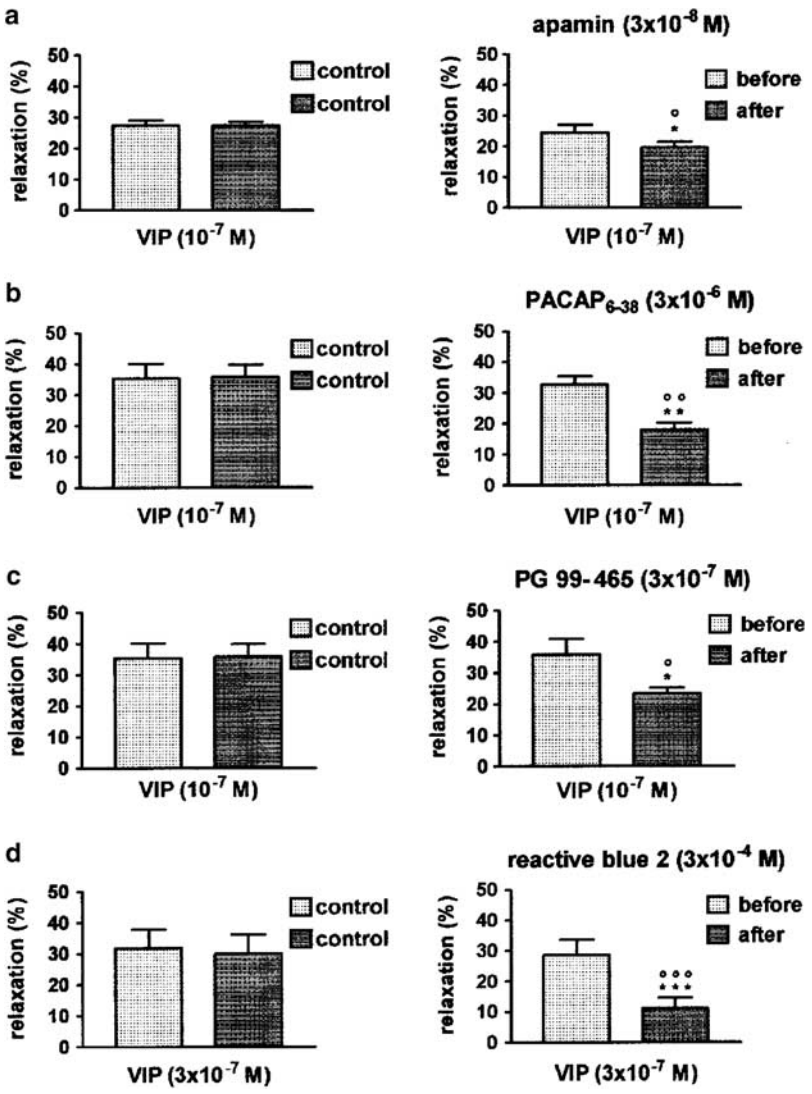

Figure 4 Consecutive responses to VIP (10^{-7}–3×10^{-7} M) in control strips (left) and in parallel strips (right) before and after incubation with apamin (3×10^{-8} M; a), $PACAP_{6-38}$ (3×10^{-6} M; b), the VPAC2 receptor antagonist PG 99-465 (3×10^{-7} M; c) and RB2 (3×10^{-4} M; d). Data are expressed as the percent relaxation of the precontracted circular muscle strip. Values are means ± s.e.m. of $n=5$–7 experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$: paired *t*-test. °$P<0.05$, °°$P<0.01$, °°°$P<0.001$: unpaired *t*-test *versus* control.

Inhibitory responses to EFS

EFS (40 V; 0.1 ms; 1–8 Hz for 20 s) induced frequency-dependent inhibitory responses (Figure 6) that tended to decline when studied for the second time in some series of control strips. As for NO, the effect of an interfering drug on EFS-evoked responses was always compared to the spontaneous decrease in the control strips. The responses elicited by the electrical stimulations were significantly decreased by TTX (3×10^{-6} M), except at 8 Hz (Figure 7). ω-Conotoxin GVIA (10^{-6} M) had a similar influence on the EFS-induced responses as TTX (Figure 7). When capsaicin (10^{-6} M) was added to the tissues, contracted with methacholine, it induced an inhibitory response of 31.5 ± 5.2% ($n=8$); the effect of 10^{-5} M capsaicin was not more pronounced (25.4 ± 3.8%, $n=6$). After washout and recontracting the tissues with methacholine, a second application of capsaicin (10^{-6} M) had no influence, illustrating desensitisation to capsaicin. Desensitisation to capsaicin did not influence significantly the EFS-induced responses, except at 1 Hz (Figure 7). All inhibitory responses were significantly reduced or abolished by L-NAME (3×10^{-4} M), ODQ (10^{-5} M) and apamin (3×10^{-8} M), except for the response at 1 Hz (Figures 6 and 8). In the presence of L-NAME and ODQ, stimulation at 4 and 8 Hz resulted even in contractions, while in the presence of apamin only the highest stimulation frequency resulted in a contraction (Figure 8). The reduction of EFS-evoked responses by ODQ (10^{-5} M) plus apamin (3×10^{-8} M) was not more pronounced than by ODQ (10^{-5} M) alone, except for responses induced by EFS at 4 Hz (from 41.9 ± 6.5% relaxation to 2.9 ± 3.6% contraction, $n=6$, in the presence of ODQ, and from 49.3 ± 3.8% relaxation to 10.1 ± 3.6% contraction, $n=6$, in the presence of ODQ and apamin). RB2 (3×10^{-4} M) increased the relaxation at 1 Hz, but had no significant effect on the inhibitory responses induced by the other frequencies. $PACAP_{6-38}$ (3×10^{-6} M), the $VPAC_1$

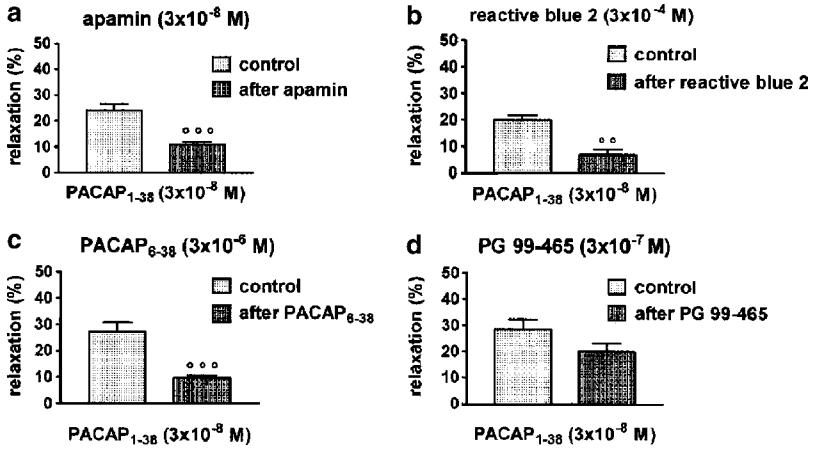

Figure 5 Responses to $PACAP_{1-38}$ (3×10^{-8} M) in the absence (control) and presence of apamin (3×10^{-8} M; a), RB2 (3×10^{-4} M; b), $PACAP_{6-38}$ (3×10^{-6} M; c) and the VPAC2 receptor antagonist PG 99-465 (3×10^{-7} M; d). Responses to $PACAP_{1-38}$ were studied in parallel tissues (one administration per tissue), in the absence (control) or presence of an antagonist. Data are expressed as the percent relaxation of the precontracted circular muscle strip. Values are means ± s.e.m. of $n = 6$–7 experiments. $^{\circ\circ}P<0.01$, $^{\circ\circ\circ}P<0.001$: unpaired *t*-test *versus* control.

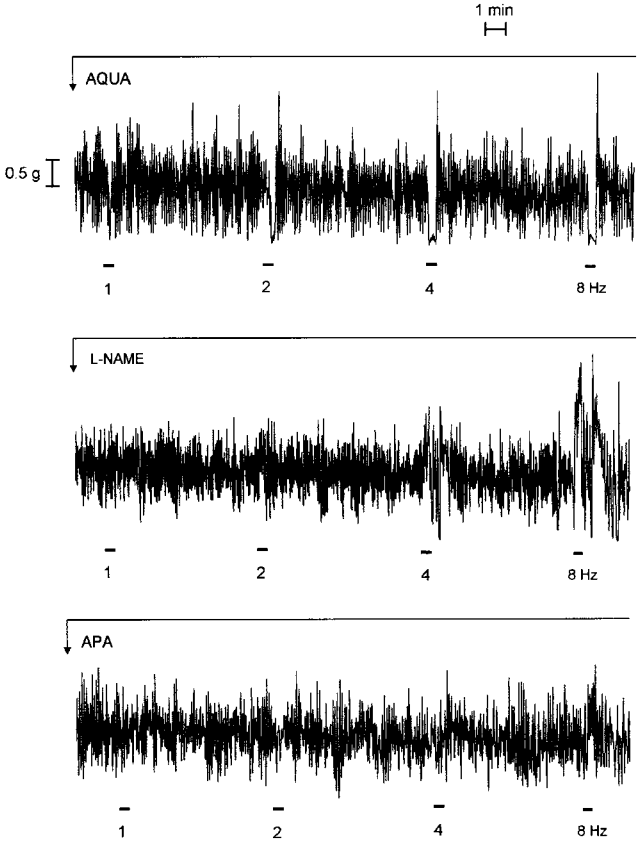

Figure 6 Traces showing the inhibitory responses of mucosa-free precontracted (methacholine;10^{-5} M) circular muscle strips of Wistar–Han rat jejunum to EFS (40 V, 0.1 ms, 1–8 Hz, 20 s trains) in control conditions (top), in the presence of L-NAME (3×10^{-4} M; middle) and apamin (APA; 3×10^{-8} M; bottom). Bars represent EFS.

receptor antagonist PG 97-269 (3×10^{-7} M) and the $VPAC_2$ receptor antagonist PG 99-465 (3×10^{-7} M) had no influence on the EFS-evoked responses.

As the effects of VIP and $PACAP_{1-38}$ developed slowly and are maintained for a longer period, their receptor antagonists were also tested on EFS trains of 100 s at 4 and 8 Hz. The inhibitory responses by EFS for 100 s were not maintained during the whole stimulation period and were still insensitive to $PACAP_{6-38}$ (3×10^{-6} M), the $VPAC_1$ receptor antagonist PG 97-269 (3×10^{-7} M) and the $VPAC_2$ receptor antagonist PG 99-465 (3×10^{-7} M).

Discussion

The aim of our study was to investigate the effect and mechanism of action of NO, ATP, VIP and PACAP in the circular muscle of Wistar–Han rat jejunum, as well as to investigate systematically their contribution to inhibitory neurotransmission. Exogenous NO, ATP, VIP, $PACAP_{1-38}$ or EFS induced an inhibitory response, consisting of a reduction of tone as well as an inhibition of phasic motility, in methacholine precontracted muscle strips. When comparing the response to methacholine in the second part of the experiment to the response in the first part, the response to methacholine was generally moderately but significantly increased, suggesting some increase in reactivity towards a cholinergic agonist with time. Inhibitory responses were therefore expressed as the percent of the methacholine-induced activity, immediately before the stimulus. None of the interfering agents tested, such as L-NAME and TTX, had a consistent influence on basal activity. This illustrates that a tonic neurogenic release of one of the studied inhibitory neurotransmitters is not probable.

Inhibitory responses to exogenously applied NO

The primary transduction pathway reported for NO-induced relaxation results from the stimulation of sGC activated by NO binding to the haeme group of this enzyme with a subsequent production of cGMP (Tanovic *et al*., 2001). The increased cGMP levels lead to relaxation through the activation of cGMP-dependent protein kinases that induce

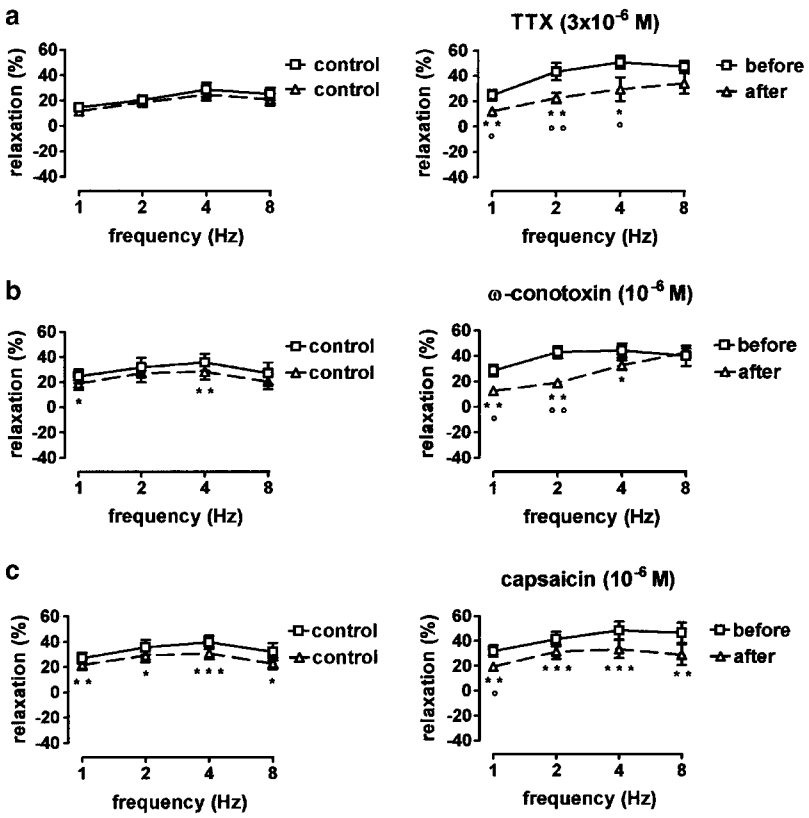

Figure 7 Consecutive frequency–response curves of EFS (40 V, 0.1 ms, 1–8 Hz, 20 s trains) in control strips (left) and in parallel strips (right) before and after incubation with TTX (3×10^{-6} M; a), ω-conotoxin (10^{-6} M; b) and capsaicin (10^{-6} M; c). Data are expressed as the percent relaxation of the precontracted circular muscle strip. Values are means ± s.e.m. of $n = 5$–8 experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$: paired *t*-test. $^{\circ}P<0.05$, $^{\circ\circ}P<0.01$: unpaired *t*-test *versus* control.

the activation of apamin-sensitive small conductance Ca^{2+}-dependent potassium (SK_{Ca}) channels, leading to hyperpolarisation, inhibition of voltage-operated calcium channels and subsequent relaxation by a decrease in Ca^{2+} influx (Keef *et al*., 1993; Koh *et al*., 1995; Nelson & Quayle, 1995; Cayabyab & Daniel, 1996; Shuttleworth *et al*., 1999). The direct activation of ion channels by cGMP has been reported, so it is also possible that cGMP directly activates the SK_{Ca} channels (Gold & Nakamura, 1987; Koh *et al*., 2000). A second possible transduction pathway does not require an increase in cGMP levels but involves a direct activation by NO or NO derivates of other cellular structures such as enzymes and proteins (Tanovic *et al*., 2001). It has been reported that SK_{Ca} channels can be directly activated by NO through covalent modifications without interference of cGMP (Bolotina *et al*., 1994; Koh *et al*., 1995).

ODQ was reported to be a potent and selective inhibitor of sGC (Garthwaite *et al*., 1995). In our study, ODQ (10^{-5} M) reduced the NO-induced responses at all concentrations. This suggests that the activation of sGC and the subsequent production of cGMP is involved in NO-evoked inhibitory responses in the circular muscle of rat jejunum. To assess the possible involvement of SK_{Ca} channels in the effect of NO, the small conductance Ca^{2+}-activated K^{+}-channel blocker (SK_{Ca} channel) apamin was tested on NO-induced relaxations. SK2 and SK3 channels are reported to be apamin sensitive, while SK1 and SK4 channels are apamin insensitive (Vergara *et al*., 1998; Bond *et al*., 1999; Warth *et al*., 1999). However, Ca^{2+}-activated K^{+} currents from SK1 channels were inhibited by 5×10^{-7} M apamin in a study by Grunnet *et al*. (2001). In our study, apamin (3×10^{-8} M) significantly inhibited the NO-induced responses at all concentrations, suggesting the involvement of apamin-sensitive SK_{Ca} channels (i.e. SK2 and SK3) in NO-induced responses. Moreover, 5×10^{-7} M apamin did not have a more pronounced inhibitory effect than 3×10^{-8} M apamin, suggesting no obvious role for SK1 channels. Adding 3×10^{-8} M apamin to 10^{-5} M ODQ did not produce a more pronounced inhibitory effect than ODQ alone, except at 10^{-4} M NO. For the lower concentrations of NO, there is thus a serial link between the rise of cGMP and the activation of SK_{Ca} channels. Whether there is an intermediary activation of cGMP-dependent protein kinase or a direct activation of the SK_{Ca} channels by cGMP cannot be differentiated from our results. At 10^{-4} M, NO might activate SK_{Ca} channels directly to some extent. The activation, by NO or a related compound, of apamin-sensitive SK_{Ca}-channels *via* the generation of cGMP has already been demonstrated for NO-induced slow ijp's in circular smooth muscle of hamster ileum (Matsuyama *et al*., 1999).

As there has been reports that NO is able to induce neuronal VIP release in the circular muscle of hamster jejunum (Matsuyama *et al*., 2002) and in rat intestinal synaptosomal preparations (Kurjak *et al*., 2001), the influence of TTX and the $VPAC_1$, $VPAC_2$ and PAC_1 receptor antagonists was tested *versus* NO. Although the $VPAC_2$ and PAC_1 receptor antagonists reduced the inhibitory effect of VIP (see below),

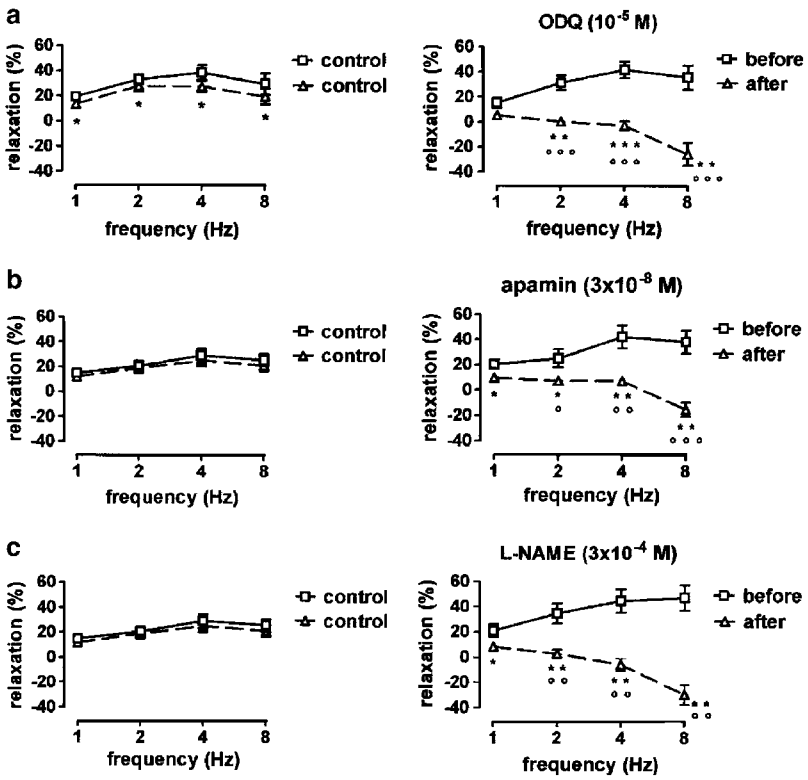

Figure 8 Consecutive frequency–response curves of EFS (40 V, 0.1 ms, 1–8 Hz, 20 s trains) in control strips (left) and in parallel strips (right) before and after incubation with ODQ (10^{-5} M; a), apamin (3×10^{-8} M; b) and L-NAME (3×10^{-4} M; c). Data are expressed as the percent relaxation of the precontracted circular muscle strip. Values are means ± s.e.m. of $n = 6$ experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$: paired *t*-test. $^{\circ}P<0.05$, $^{\circ\circ}P<0.01$, $^{\circ\circ\circ}P<0.001$: unpaired *t*-test *versus* control.

they did not influence the effect of NO. No evidence for NO-induced VIP release was thus obtained for rat jejunal circular muscle.

Inhibitory responses to exogenously applied ATP, VIP and PACAP

ATP is a ligand for P2 purinoceptors existing in two main subtypes: (1) the P2X receptors that are ligand-gated ion channels and (2) the P2Y receptors that are coupled to G proteins (Ralevic & Burnstock, 1998). Inhibitory responses are assumed to be mediated mainly through P2Y receptors (Dalziel & Westfall, 1994; Kennedy, 2000), although some reports suggest the involvement of P2X receptor subtypes in relaxant responses (De Man *et al*., 2003). The main transduction pathway activated by the binding of ATP to P2Y purinoceptors involves the activation of phospholipase C, production of inositol trisphosphate and release of Ca^{2+} from intracellular stores (Von Kugelgen & Wetter, 2000). This local Ca^{2+} transient activates Ca^{2+}-sensitive ion channels such as SK_{Ca} channels, leading to hyperpolarisation, inhibition of voltage-operated calcium channels and subsequent relaxation (Koh *et al*., 1997). P2Y purinoceptors are also able to activate adenylate cyclase leading to the production of cyclic adenosine monophosphate (Von Kugelgen & Wetter, 2000).

RB2, commonly described as a P2Y receptor antagonist (Dalziel & Westfall, 1994; Von Kugelgen & Wetter, 2000), nearly abolished the responses to both concentrations of exogenous ATP, suggesting that ATP interacts with P2Y purinoceptors. The observation that 3×10^{-8} M apamin did not influence the effects of 10^{-4} M ATP suggests that the main transduction pathway of ATP in rat jejunum circular muscle is not linked to the activation of the SK_{Ca} channels that are involved in the effect of NO, although some role of SK_{Ca} channels sensitive to higher concentrations of apamin cannot be excluded in view of the effect of 5×10^{-7} M apamin *versus* 10^{-4} M ATP. Some studies reported that RB2 does not discriminate between P2Y and P2X receptors when used in high concentrations ($>3 \times 10^{-5}$ M; Connolly & Harrison, 1994; Trezise *et al*., 1994). If the effect of RB2 *versus* ATP in the rat jejunum were due to an interaction at relaxant P2X receptors, this would also explain why apamin (3×10^{-8} M) does not influence the effect of ATP, as P2X receptors are ligand-gated ion channels not linked to phospholipase C and SK_{Ca} channel activation. In contrast to what our group observed in rat distal colon circular muscle (Van Crombruggen & Lefebvre, 2004), the responses to ATP were not influenced by ODQ, L-NAME and TTX, so that no evidence for ATP-induced neuronal NO release was obtained.

$PACAP_{1-38}$ has a much higher affinity than VIP at PAC_1 receptors and a similar affinity at $VPAC_1$ and $VPAC_2$ receptors (Harmar *et al*., 1998). The principle transduction pathway of these receptors starts with activation of adenylate cyclase (Laburthe & Couvineau, 2002; Lecci *et al*., 2002), although also phospholipase C and phospholipase D activation have been reported (MacKenzie *et al*., 2001; Laburthe & Couvineau, 2002). In addition, $PACAP_{1-38}$ was reported to lead to the activation of apamin-sensitive SK_{Ca} channels *via*

stimulation of tyrosine kinase, inducing membrane hyperpolarisation and relaxation in longitudinal muscle of rat distal colon (Takeuchi *et al*., 1999; Lecci *et al*., 2002). The inhibitory response to $PACAP_{1-38}$ was not influenced by the $VPAC_1$ and $VPAC_2$ receptor antagonist, but it was by the PAC_1 receptor antagonist $PACAP_{6-38}$, suggesting that it mainly interacts with PAC_1 receptors in rat jejunum circular muscle. The inhibitory response to $PACAP_{1-38}$ was significantly reduced by apamin, suggesting that opening of SK_{Ca} channels is to some extent involved in the effect of $PACAP_{1-38}$. The observation that $PACAP_{6-38}$ and apamin also reduced the inhibitory response to VIP also suggests that VIP is able to interact with the PAC_1 receptors as already suggested in rat colon (Ekblad, 1999). An inhibitory effect of $PACAP_{6-38}$ on VIP-induced relaxations has been reported in different gastrointestinal smooth muscle preparations such as guinea-pig *Taenia caeci* (Lenard *et al*., 2000) and small intestine (Lazar *et al*., 2001). $PACAP_{6-38}$, with an IC_{50} value of 1.4×10^{-8} M for PAC_1 receptors in COS 7 cells, has also affinity ($IC_{50} = 1.7 \times 10^{-7}$ M) for the $VPAC_2$ receptors in the same system (Dickinson *et al*., 1997); at the concentration we used (i.e. 3×10^{-6} M), its influence on the effect of VIP might thus to some extent be related to antagonism of $VPAC_2$ receptors. Indeed, the results with the VPAC receptor antagonists illustrate that VIP also interacts with $VPAC_2$ receptors.

PG 97-269 is a selective $VPAC_1$ receptor antagonist with an IC_{50} value of 10^{-8} M for the rat $VPAC_1$ receptor and 2×10^{-6} M for the rat $VPAC_2$ receptor, suggesting that at the concentration we used (i.e. 3×10^{-7} M) PG 97-269 is selective for the rat $VPAC_1$ receptor (Gourlet *et al*., 1997). PG 99-465 is an antagonist with an IC_{50} value of 2×10^{-9} M for inhibition of VIP binding at the human $VPAC_2$ receptor. It is a partial agonist at the human $VPAC_1$ receptor with an IC_{50} value of 2×10^{-7} M for inhibition of VIP binding (Moreno *et al*., 2000). On the rat $VPAC_2$ recombinant receptor expressed in CHO cells, PG 99-465 was a weak partial agonist displaying a maximal adenylate cyclase activation of 10% of that elicited by VIP with an EC_{50} value of 3×10^{-8} M. The IC_{50} value calculated from concentration–response curves of VIP, obtained in the presence of increasing concentrations of the dualist, was 4×10^{-8} M. On the rat $VPAC_1$ recombinant receptor, PG 99-465 was also a partial agonist, but the EC_{50} value was 1×10^{-6} M (unpublished results). The inhibitory responses to 3×10^{-7} M VIP were not altered by 10^{-7} M PG 97-269 and 10^{-7} M PG 99-465, but 3×10^{-7} M PG 99-465 reduced the inhibitory response to 10^{-7} M VIP, suggesting an interaction with $VPAC_2$ receptors. Messenger RNA (mRNA) for $VPAC_1$ receptors and $VPAC_2$ receptors was indeed found in the rat small intestine, but the $VPAC_1$ receptor mRNA was reported to be more abundant, as demonstrated by a Northern blot (Usdin *et al*., 1994). In human colon, $VPAC_1$ receptors were found to be located in the superficial layers of the mucosa, while $VPAC_2$ receptor expression was detected in the smooth muscle layers (Rettenbacher & Reubi, 2001).

Surprisingly, RB2 clearly reduced the effect of $PACAP_{1-38}$ and VIP. Prejunctional neuronal release of ATP by VIP and $PACAP_{1-38}$ has been reported in the circular muscle of hamster proximal urethra (Pinna *et al*., 1998) and mouse distal colon (Serio *et al*., 2003). However, this seems unlikely in rat jejunum as TTX, which reduced PACAP-induced ATP release in mouse distal colon (Serio *et al*., 2003), did not influence the responses to VIP and $PACAP_{1-38}$ in the actual study and 3×10^{-8} M apamin, which did not significantly influence the responses to exogenous ATP, reduced the responses to VIP and $PACAP_{1-38}$. The effect of RB2 on the peptide-induced responses might be due to a nonspecific effect. As RB2 had no effect on the response to isoprenaline (a beta-adrenoceptor agonist inducing relaxation *via* activation of adenylate cyclase; Kondo *et al*., 1979; Abraham *et al*., 2003), a nonspecific effect of RB2 involving the adenylate cyclase pathway is unlikely. Interestingly, suramin, another P2 receptor antagonist, has been suggested to act as a competitive antagonist at VIP receptors in rat gastric fundus (Jenkinson & Reid, 2000). The response to VIP was not influenced by L-NAME so that no evidence was obtained for VIP-induced muscular NO release, as has been suggested in rabbit and rat gastric muscle (Jin *et al*., 1996).

Inhibitory responses to EFS

EFS, delivered in 20 s trains, induced frequency-dependent inhibitory responses, which were reduced but not abolished by the nerve conduction blocker TTX. This illustrates that nerve activation is involved, but we have no explanation as to why TTX is not able to abolish the electrically induced responses. The N-type Ca^{2+} channel blocker ω-conotoxin GVIA had a similar effect as TTX, excluding the activation of mechanisms at the varicosities of the NANC inhibitory neurons that require N-type Ca^{2+} channels, but not action potential propagation *via* TTX-blocked Na^+ channels. The influence of *in vitro* capsaicin-induced desensitisation on the EFS-induced responses was also studied. In the concentration used, capsaicin is expected to stimulate neuropeptide-containing primary sensory neurons. The neuropeptide release from the sensory nerve endings induced by capsaicin is TTX resistant (Maggi & Meli, 1988), and a similar mechanism induced by EFS might contribute to the partial TTX resistance of the EFS-induced responses. Desensitisation is a feature of the specific action of capsaicin on sensory nerves (Maggi *et al*., 1990). A second application of a maximally effective concentration of capsaicin also illustrated the desensitisation in the rat jejunum. In this condition, the EFS-induced responses were hardly influenced excluding an important contribution of capsaicin-sensitive sensory nerves with TTX-resistant Na^+ channels.

L-NAME, ODQ and apamin nearly abolished the inhibitory responses to EFS, while RB2 had no influence. This excludes the involvement of ATP but suggests the release of NO, leading to sGC and SK_{Ca} channel activation. The reduction of the EFS-evoked responses by ODQ plus apamin was not more pronounced than by ODQ alone, except at a stimulation frequency of 4 Hz. This illustrates that cGMP and SK_{Ca} channels involved are generally linked in a serial way. Direct activation of SK_{Ca} channels by higher concentrations of NO released at a higher stimulation frequency cannot be excluded, see for example, the responses to 10^{-4} M exogenous NO.

In the presence of L-NAME, ODQ and apamin, EFS induced contractile responses at a stimulation frequency of 4 and still more at 8 Hz, illustrating the release of contractile neurotransmitters not longer counteracted by an inhibitory one. Possible candidates as contractile neurotransmitters in rat jejunum are acetylcholine and substance P (Bauer & Matusak, 1986). Further investigation of this contractile neurotransmitter was not the aim of our study.

PG 99-465 and $PACAP_{6-38}$ had no influence on the EFS-evoked responses, suggesting that VIP and PACAP are not involved in inhibitory neurotransmission. As the inhibitory responses to exogenous VIP and PACAP developed slowly and were maintained for a longer period, and as VIP in cotransmission with NO has been shown to be released upon longer stimulation trains at higher frequencies (Boeckxstaens *et al*., 1992), we tested the peptide receptor antagonists also on stimulation trains of 100 s at 4 and 8 Hz. The electrically induced inhibitory responses observed in the beginning of the stimulation train were not maintained, in contrast to the responses to exogenous VIP and PACAP, and the peptide antagonists had no influence, again illustrating that VIP and PACAP are not involved in inhibitory neurotransmission in the circular muscle of rat jejunum.

In conclusion, these results suggest that NO is the main inhibitory neurotransmitter in the circular muscle of Wistar–Han rat jejunum; it induces inhibition *via* a rise in cGMP levels and the activation of SK_{Ca} channels in a serial way. ATP induces inhibition *via* P2Y receptors that are not linked to apamin-sensitive SK_{Ca} channels. VIP induces inhibition by interaction with both $VPAC_2$ and PAC_1 receptors, while $PACAP_{1-38}$ only interact with PAC_1 receptors. It should be fully realised that different results might be obtained in other rat strains.

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