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Evidence that tachykinins are the main NANC excitatory neurotransmitters in the guinea-pig common bile duct

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1 Application of electrical field stimulation (EFS; trains of 10 Hz, 0.25 ms pulse width, supramaximal voltage for 60 s) to the guinea-pig isolated common bile duct pretreated with atropine (1 μM), produced a slowly-developing contraction ('on' response) followed by a quick phasic 'off' contraction ('off peak' response) and a tonic response ('off late' response), averaging 16 ± 2, 73 ± 3 and 20 ± 4% of the maximal contraction to KCl (80 mM), $n=20$ each, respectively. Tetrodotoxin (1 μM; 15 min before) abolished the overall response to EFS ($n=8$).

2 Neither *in vitro* capsaicin pretreatment (10 μM for 15 min), nor guanethidine (3 μM, 60 min before) affected the excitatory response to EFS ($n=5$ each), showing that neither primary sensory neurons, nor sympathetic nerves were involved. N^{ω}-nitro-L-arginine (L-NOARG, 100 μM, 60 min before) or naloxone (10 μM, 30 min before) significantly enhanced the 'on' response (294 ± 56 and 205 ± 25% increase, respectively; $n=6-8$, $P<0.01$) to EFS. The combined administration of L-NOARG and naloxone produced additive enhancing effects (655 ± 90% increase of the 'on' component, $n=6$, $P<0.05$).

3 The tachykinin NK_2 receptor-selective antagonist MEN 11420 (1 μM) almost abolished both the 'on' and 'off late' responses ($P<0.01$; $n=5$ each) to EFS, and reduced the 'off-peak' contraction by 55 ± 8% ($n=5$, $P<0.01$). The subsequent administration of the tachykinin NK_1 receptor-selective antagonist GR 82334 (1 μM) and of the tachykinin NK_3 receptor-selective antagonist SR 142801 (30 nM), in the presence of MEN 11420 (1 μM), did not produce any further inhibition of the response to EFS ($P>0.05$; $n=5$ each). At 3 μM, GR 82334 significantly reduced (by 68 ± 9%, $P<0.05$, $n=6$) the 'on' response to EFS.

4 The contractile 'off peak' response to EFS observed in the presence of both MEN 11420 and GR 82334 (3 μM each) was abolished ($P<0.01$; $n=6$) by the administration of the P_2 purinoceptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 30 μM). PPADS (30 μM) selectively blocked (75 ± 9 and 50 ± 7% inhibition, $n=4$ each) the contractile responses produced by 100 and 300 μM ATP.

5 Tachykinin-containing nerve fibres were detected by using immunohistochemical techniques in all parts of the bile duct, being distributed to the muscle layer and lamina propria of mucosa. In the terminal part of the duct (ampulla) some labelled ganglion cells were observed.

6 In conclusion, this study shows that in the guinea-pig terminal biliary tract tachykinins, released from intrinsic neuronal elements, are the main NANC excitatory neurotransmitters, which act by stimulating tachykinin NK_2 (and possibly NK_1) receptors. ATP is also involved as excitatory neurotransmitter. Nitric oxide and opioids act as inhibitory mediators/modulators in this preparation.

Keywords: Guinea-pig common bile duct; biliary tract; tachykinins; tachykinin receptors; tachykinin receptor antagonists; ATP; nitric oxide; endogenous opioids

Introduction

The tachykinins substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), are a family of neuropeptides distributed in the mammalian central and peripheral nervous system. They produce a wide range of biological effects through the stimulation of at least three distinct receptor types, termed NK_1, NK_2 and NK_3 (Regoli *et al*, 1989; Guard & Watson, 1991; Maggi *et al*., 1993).

Tachykinin-immunoreactivity (TK-IR) has been identified throughout the gastrointestinal tract of several species, including humans. The bulk of extractable TK-IR originates from enteric (intrinsic) neurons (Holzer & Holzer-Petsche, 1997, for review), while the remainder is contributed by the peripheral endings of capsaicin-sensitive primary afferent neurons (Maggi, 1995, for review) and by immune cells (Maggi, 1997; De Giorgio *et al*., 1998). In the smooth muscle of the mammalian gastrointestinal tract tachykinins almost invariably produce contraction, either if applied exogenously, or if released from intrinsic neurons and/or from peripheral endings of capsaicin-sensitive primary afferents (Maggi *et al*., 1993; Maggi, 1995; Holzer & Barthó, 1996). Moreover, tachykinins play a role as non-cholinergic excitatory transmitters in the mammalian intestine, as mediators of both the atropine-resistant ascending enteric reflex and peristalsis (Barthó & Holzer, 1985; Costa *et al*., 1985; Barthó *et al*., 1989; Maggi *et al*., 1994a).

In the gallbladder TK-IR has been detected in neurons of ganglionated plexuses and in nerve fibres innervating blood vessels, smooth muscle, lamina propria and mucosa (Goehler *et al*., 1988; Talmage *et al*., 1992; Sand *et al*., 1993; De Giorgio *et*

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al., 1995). Functional studies performed on the isolated gallbladder from different species have shown that tachykinins released by capsaicin from sensory nerve terminals (Maggi *et al*., 1989b) or applied exogenously (Feeley *et al*., 1987; Shook and Burks, 1987; Meldrum *et al*., 1987; Guo *et al*., 1989; Maggi *et al*., 1989a; Patacchini & Maggi, 1992), produce smooth muscle contraction. In contrast, little is known on the distribution of tachykinins in the biliary tract, as well as their effect on the smooth muscle of the biliary duct. We have recently shown that exogenously-applied tachykinins produce contraction of the guinea-pig isolated common bile duct, by stimulating all three tachykinin receptor types (Patacchini *et al*., 1997).

In the present work we studied the possible contribution of endogenous tachykinins to the non-cholinergic contractile response elicited by electrical field stimulation (EFS) in the guinea-pig isolated common bile duct. To characterize the receptor type(s) mediating the response to endogenous tachykinins, we used the following tachykinin receptor-selective antagonists: GR 82334 (NK_1 receptor-selective; Hagan *et al*., 1991), MEN 11420 (NK_2 receptor-selective; Santicioli *et al*., 1997; Catalioto *et al*., 1998) and SR 142801 (NK_3 receptor-selective; Emonds-Alt *et al*., 1995; Patacchini *et al*., 1995). All the above mentioned tachykinin receptor antagonists have been shown to produce a selective antagonism toward exogenously-administered tachykinins in the guinea-pig isolated common bile duct (Patacchini *et al*., 1997). Also we investigated the occurrence and distribution of TK-IR in the common bile duct, by using immunohistochemical techniques.

The second aim of our study was to investigate whether other NANC neurotransmitter(s), notably ATP, nitric oxide (NO) and endogenous opioids, could be involved as mediator(s)/modulator(s) of the overall motor response to electrical nerve stimulation.

Methods

Functional experiments

General Male albino guinea-pigs (300–350 g) were stunned and bled. The common bile duct, including the terminal enlarged segment (*ampulla*) was carefully dissected from the surrounding tissue and excised from the outer surface of the duodenum to the junction with the hepatic duct, as described previously (Patacchini *et al*., 1997). The bile duct was opened along its longitudinal axis, and cut in two parallel strips which were tied at each end and placed in 5 ml organ baths, filled with warmed (37°C) and oxygenated (96% O_2 and 4% CO_2) Krebs-Henseleit buffer solution of the following composition (in mM): NaCl 119; $NaHCO_3$ 25; KH_2PO_4 1.2; $MgSO_4$ 1.5; $CaCl_2$ 2.5; KCl 4.7 and glucose 11. The strips were connected to isotonic transducers (load 1.0–1.5 mN) for recording mechanical activity. Unless indicated otherwise, one strip was treated, and the other served as control. Atropine (1 μM) was added to the buffer solution from the beginning, and left in contact with the tissue throughout the experiment, with the exception of some preliminary experiments in which the response to EFS was recorded in untreated preparations. All the experiments commenced after an equilibration period of 90–120 min.

Experimental protocol The preparations were exposed to electrical field stimulation (EFS; trains of stimuli of 10 Hz, 0.25 ms pulse width, supramaximal voltage, for 60 s) by means of two platinum wire electrodes placed at the top and the bottom of the organ bath, and connected to a Grass S88 stimulator. Repetition of EFS at 30–60 min intervals produced responses undergoing a progressive decay. Thus, the effects produced by a number of drugs, including tachykinin NK_1, NK_2 and NK_3 receptor-selective antagonists (see below), PPADS (see below), guanethidine (3 μM; 60 min before), capsaicin (10 μM; contact time 15 min), N^{ω}-nitro-L-arginine (L-NOARG; 100 μM; 60 min before) and naloxone (10 μM; 30 min before) on EFS-induced responses, were evaluated on one strip, and compared to responses obtained in parallel on a matched strip from the same animal. In particular, GR 82334, MEN 11420 and SR 142801 were tested at concentrations reportedly selective for the tachykinin NK_1, NK_2 and NK_3 receptors (Patacchini *et al*, 1997) following a consecutive procedure: 10 min after an initial control response to EFS had been obtained in a pair of matched strips, MEN 11420 (0.1 μM) was given to one preparation and left in contact with this latter for 20 min, while the other strip received the vehicle. Thereafter, a second EFS-induced response was elicited in both preparations, and the two responses were compared. After a thorough washout, a higher concentration of MEN 11420 (1 μM, 20 min incubation) was administered to the strip previously pretreated with this antagonist, and a third response to EFS was elicited 30 min later. GR 82334 and SR 142801 were added afterwards to the preparation pretreated with MEN 11420, following a similar procedure. In another series of experiments, SR 142801 (30 nM; 60 min before) or GR 82334 (1 or 3 μM; 20 min before) were added as first to preparations, to be tested against EFS-induced responses. The preparations receiving GR 82334 (3 μM) were subsequently challenged with MEN 11420 (3 μM), in the presence of GR 82334 (3 μM), and with PPADS (30 μM), in the presence of both GR 82334 and MEN 11420, following a procedure similar to that detailed above for MEN 11420. The maximal contractile response to KCl (80 mM) was used as the internal standard in all experiments.

Immunohistochemistry

For morphological studies, the common bile duct including the terminal ampulla was rapidly removed as described above, thoroughly rinsed with saline, stretched gently and pinned on wax plates. Specimens were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) for 6–8 h at 4°C, and subsequently placed in 25% sucrose in 0.1 M phosphate buffer for cryoprotection until sectioning. Tissue specimens were cut with a cryostat at 10 μM, mounted onto chrome-alum gelatin coated slides, and stored at −30°C until processed for immunohistochemistry.

Tissue sections were processed with the avidin-biotin-peroxidase complex (ABC) method, as previously described (De Giorgio *et al*., 1992). Briefly, sections were washed in 0.1 M phosphate buffer, pretreated for 30 min at room temperature with 10% normal goat serum, and incubated in rabbit polyclonal antibody directed against the C-terminal portion of the tachykinin sequence (NKA8701; working dilution 1 : 5000) (De Giorgio *et al*., 1992) overnight at 4°C, in a humid chamber. Sections were then washed in phosphate buffer and incubated for 2 h at room temperature in affinity purified goat anti-rabbit biotinylated IgG (dilution 1 : 100) (Vector Laboratories, Burlingame, CA, U.S.A.), followed by ABC solution (30 min) and then exposed to 3,3′-diaminobenzidine with 0.01% H_2O_2. Sections were finally dehydrated and coverslipped with mounting medium. In order to reduce non specific staining due to endogenous peroxidase, at the beginning of each experiment tissue sections were first dehydrated, placed in 100% methanol and then in a solution

composed of 98% methanol, 1% acetic acid and 1% sodium nitroferricyanide (Sigma, St. Louis, U.S.A.) for 15 min, followed by 100% methanol, rehydrated and then incubated in the primary antibody. Both primary and secondary antibodies were diluted in 0.5% Triton X-100 in 0.1 M phosphate buffer. Sections were analysed with a Leitz Dialux microscope using bright field optics. Specificity studies were performed as follows (De Giogio *et al*., 1995): (a) omission of the primary antibody, (b) substitution of the primary antibody with commercially available normal rabbit serum used at a dilution of 1 : 50, and (c) incubation with the primary antibody preabsorbed for 12–16 h at 4°C with synthetic homologous or heterologous peptides (Bachem, Torrance, CA, U.S.A.), including SP, NKA, NKB, α-rat calcitonin gene-related peptide (1–37), α-rat calcitonin gene-related peptide (23–37) and vasoactive intestinal polypeptide (VIP), at concentrations of 10 μM. The above mentioned control tests showed that SP immunostaining was abolished by preabsorption of the primary antibody with either SP, or NKA or NKB, thus indicating that the antiserum employed (NKA8701) is a generalized marker for all the mammalian tachykinin peptides. Therefore, the term TK-IR was used to refer to the staining obtained with the mentioned SP antiserum. On the other hand, preabsorption of the SP antibody with other structurally-unrelated peptides did not modify the immunostaining.

Statistical analysis

The values in the text, tables or figures are expressed as means ± 95% confidence limits, or ± s.e.mean. Statistical analysis was performed by means of Student's *t* test for paired or unpaired data or by means of two-way analysis of variance (ANOVA), when applicable.

Drugs

MEN 11420 (or: c{[(β-D-GlcNAc)Asn-Asp-Trp-Phe-Dpr-Leu]c(2β-5β)}) was synthesized at Menarini laboratories, Florence, Italy, by conventional solid-phase methods. Atropine was purchased from Serva (Heidelberg, Germany), tetrodotoxin from Sankyo (Japan), GR 82334 from Neosystem (Strasbourg, France), guanethidine from ICFI (Milan, Italy), pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) from RBI (Natick, U.S.A.), N^{ω}-nitro-L-arginine (L-NOARG), naloxone hydrochloride and capsaicin from Sigma (St. Louis, U.S.A.). The nonpeptide antagonist SR 142801 (or[(S)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylipiperidin-4-yl)-N-methylacetamide]) was kindly provided by Drs X. Emonds-Alt and G. Le Fur, Sanofi (Montpellier, France).

Results

Functional experiments

General In a preliminary series of experiments performed in the absence of atropine, the application of trains of electrical stimuli (0.25 ms pulse width for 60 s, supramaximal voltage) to the guinea-pig isolated common bile duct produced a frequency-related (range 1–30 Hz) phasic contraction followed by a sustained tonic response. At 10 Hz, the peak response averaged 65 ± 5% of the maximal response to KCl 80 mM ($n = 12$) (Figure 1). Also a rebound contraction was observed in untreated strips, developing immediately after the end of the electrical stimulus: however, the rebound contraction was often hardly recognizable, since it rarely exceeded the tonic response to EFS (not shown). In the presence of atropine (1 μM) the response to EFS was markedly inhibited, while the rebound contraction was apparently unchanged (Figure 1). Thus, two distinct non-cholinergic contractile responses were observed in the presence of atropine: a slowly-developing contraction, starting at about 10–15 s from the beginning of the EFS ('on' response) followed by a phasic contraction ('off peak' response) developing immediately after the end of the EFS. A third component ('off late' response) was also evaluated by measuring the tonic contraction at 30 s from the end of the electrical stimulus. At a frequency of 10 Hz, the 'on', 'off peak' and 'off late' responses averaged 16 ± 2, 73 ± 3 and 20 ± 4% of the response to KCl 80 mM, respectively ($n = 20$) (Figure 1). The application of repetitive cycles of EFS (every 30–60 min) produced responses undergoing a progressive decay. For this reason, the responses to EFS obtained in strips pretreated with drugs (see below) were compared to control responses evoked in parallel on matched strips. Tetrodotoxin (1 μM, 15 min before) completely abolished all the above described contractile responses to EFS. In the presence of tetrodotoxin, a small (18 ± 3% of the response to KCl 80 mM, $n = 8$) direct contraction developed soon after the application of EFS, and faded to baseline within a few s; therefore, there was no overlap of this response with the previously defined 'on' response to EFS.

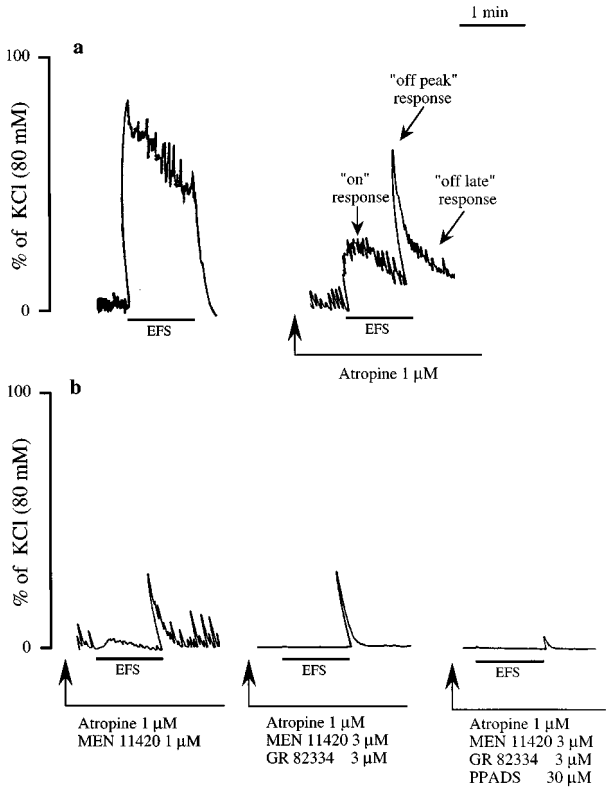

Figure 1 Typical tracings showing the contractile response of the guinea-pig isolated common bile duct to electrical field stimulation (EFS: trains of stimuli of 10 Hz, 0.25 ms pulse width, supramaximal voltage, for 60 s) in the absence or in the presence of atropine (a), and in the presence of atropine plus the tachykinin NK_2 and NK_1 receptor-selective antagonists MEN 11420 and GR 82334, respectively, and the P_2 purinoceptor antagonist PPADS (b). In the presence of atropine, two distinct responses could be observed: a slowly-developing contraction, starting at about 10–15 s from the beginning of the EFS ('on' response) followed by a phasic contraction ('off-peak' response) developing immediately after the end of the EFS. A third component ('off late' response) was measured at 30 s from the end of EFS.

Effects of 'in vitro' *capsaicin desensitization of sensory nerves and of guanethidine on the non-cholinergic response to EFS*

In order to obtain a blockade of the efferent function of primary afferent neurons in the common bile duct, an *in vitro* protocol of sensory nerves blockade was adopted, by applying capsaicin (10 μM) for 15 min to the preparations. In the presence of atropine (1 μM), capsaicin (10 μM) produced either no effect, or a fast contraction not exceeding 15% of the maximal response produced by KCl (80 mM), which quickly faded to baseline and was followed by a prolonged inhibition of the spontaneous motility of the duct ($n=6$). After washout of capsaicin, EFS was applied again: all three components of the contractile response to EFS (i.e. 'on', 'off peak' and 'off late' responses) appeared slightly increased by capsaicin treatment, but this effect did not reach statistical significance (Table 1).

Guanethidine (3 μM, 60 min before) did not produce any motor response *per se*, nor did it significantly affect the contractile responses to EFS, although there was a slight tendency to an increase of the 'on' component (Table 1).

Effect of tachykinin NK_1, NK_2 and NK_3 receptor antagonists and of the P_2 purinoceptor antagonist PPADS on the non-cholinergic response to EFS

The tachykinin NK_2 receptor-selective antagonist MEN 11420 (0.1 μM) strongly inhibited the 'on' response to EFS, while producing no significant reduction of the other two components (Table 2). At 1 μM, MEN 11420 inhibited all phases of the response to EFS, the inhibition of the 'on' response being practically complete (Table 2; Figure 1). The subsequent applications of GR 82334 (1 μM) in the presence of MEN 11420 (1 μM), and of SR 142801 (30 nM) in the presence of GR 82334 (1 μM) and MEN 11420 (1 μM), did not further inhibit the responses to EFS (Table 2).

In a separate series of experiments, GR 82334 (1 μM) failed to inhibit the contractile responses to EFS ($n=4$, not shown). However, at 3 μM GR 82334 significantly reduced the 'on' response to EFS, while leaving the other two components unaffected (Table 3). The subsequent administration of MEN 11420 (3 μM) in the presence of GR 82334 (3 μM), practically

Table 1 Effect of capsaicin (10 μM for 15 min) pretreatment and effect of guanethidine (3 μM, 60 min before) on non-cholinergic contractile responses of the guinea-pig isolated common bile duct to electrical field stimulation

Treatment	*'on' response* (% of control)	*'off peak' response* (% of control)	*'off late' response* (% of control)
Vehicle	82±6	93±7	91±16
Capsaicin	107±30	110±12	115±11
Vehicle	61±9	80±10	69±14
Guanethidine	102±18	93±2	72±11

All values are mean±s.e.mean of five experiments. Definition of 'on', 'off peak' and 'off late' response is reported in the text. Electrical field stimulation (EFS: trains of 60 V, 0.25 ms pulse width, supramaximal voltage, of 60 s) was applied every 30 (experiments with capsaicin) or 65 min (experiments with guanethidine). Capsaicin (10 μM) was left in contact with preparations for 15 min, then it was washed out before repeating the EFS. A pair of preparations obtained from the same animal was used: one preparation was pretreated with capsaicin or treated with guanethidine, while the other received the vehicle. Control responses were those obtained before incubation with the drug or vehicle.

Table 2 Effect of MEN 11420 alone or combined with GR 82334 or with GR 82334 and SR 142801 on non-cholinergic contractile responses of the guinea-pig isolated common bile duct to electrical field stimulation

Treatment	*'on' response* (% of control)	*'off peak' response* (% of control)	*'off late' response* (% of control)
Vehicle (30 min)	81±3	85±5	53±9
MEN 11420 (0.1 μM)	24±5**	61±12	29±11
Vehicle (60 min)	77±8	75±4	43±8
MEN 11420 (1 μM)	5±5**	31±9**	9±9*
Vehicle (90 min)	48±7	59±4	15±5
MEN 11420 (1 μM)	0**	20±9**	4±4*
GR 82334 (1 μM)			
Vehicle (120 min)	42±8	54±6	4±4
MEN 11420 (1 μM)	0**	22±6**	4±4
GR 82334 (1 μM)			
SR 142801 (30 nM)			

All values are mean±s.e.mean of five experiments, and are % of control responses. Control responses were obtained at time=0, before administering the antagonists or vehicle. Definition of 'on', 'off peak' and 'off late' response is reported in the text. Electrical field stimulation (EFS: trains of 60 V, 0.25 ms pulse width, supramaximal voltage, for 60 s) was applied every 30 min. A pair of preparations obtained from the same animal was used: on preparation was consecutively treated with the antagonists (incubation time=20 min), while the other received the corresponding vehicle. *Significantly different from the corresponding value obtained in the time-matched strip treated with vehicle, $P<0.05$ and **$P<0.01$.

Table 3 Effect of GR 82334 alone or combined with MEN 11420 or with MEN 11420 and PPADS on non-cholinergic contractile responses of the guinea-pig isolated common bile duct to electrical field stimulation

Treatment	*'on' response* (% of control)	*'off peak' response* (% of control)	*'off late' response* (% of control)
Vehicle (30 min)	58±9	74±4	65±11
GR 82334 (3 μM)	18±9*	63±8	62±9
Vehicle (60 min)	37±8	54±6	27±10
GR 82334 (3 μM)	2±2**	24±4**	0±0**
MEN 11420 (3 μM)			
Vehicle (90 min)	28±7	52±5	20±8
GR 82334 (3 μM)	0±0**	1±1**,§§	0±0**
MEN 11420 (3 μM)			
PPADS (30 μM)			

All values are mean±s.e.mean of six experiments and are % of control responses. Control responses were obtained at time=0, before administering the antagonists or vehicle. Definition of 'on', 'off peak' and 'off late' response is reported in the text. Electrical field stimulation (EFS: trains of 60 V, 0.25 ms pulse width, supramaximal voltage, for 60 s) was applied every 30 min. A pair of preparations obtained from the same animal was used: one preparation was consecutively treated with the antagonists (incubation time=20 min), while the other received the corresponding vehicle. *Significantly different from the corresponding value obtained in the time-matched strip treated with vehicle, $P<0.05$ and **$P<0.01$. §§Significantly different from the preceding value, $P<0.01$.

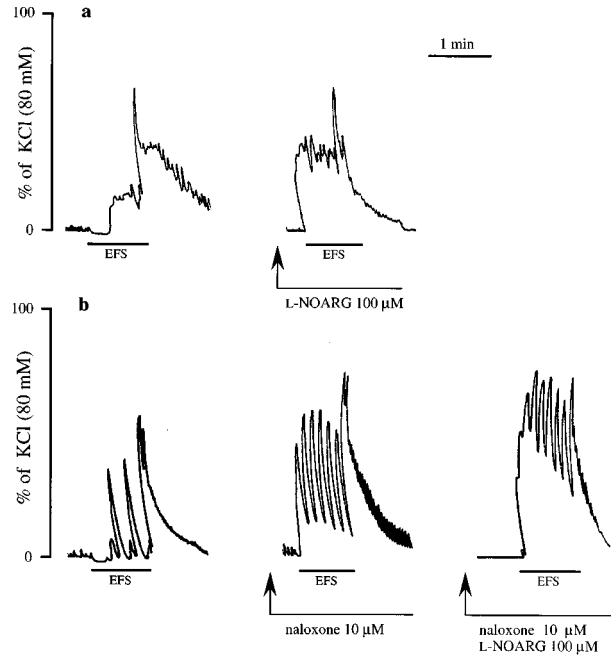

Figure 2 Typical tracings showing the enhancing effects produced by N^{ω}-nitro-L-arginine (L-NOARG: (a), by naloxone (b) and by the combined pretreatment with L-NOARG and naloxone (b) on the contractile response to electrical field stimulation (EFS) of the guinea-pig isolated common bile duct.

abolished both the 'on' and 'off late' responses, while a residual 'off peak' response was still present; it is noteworthy that the inhibition of the 'off peak' response produced by the combination of MEN 11420 and GR 82334 was no larger than that produced by MEN 11420 (1 μM) alone (55 ± 8 vs $59\pm6\%$ inhibition, $n=6$ each, respectively; cf. Tables 2 and 3) (Figure 1). In the presence of MEN 11420 and GR 82334, the administration of the P_2 purinoceptor antagonist PPADS (30 μM) abolished the residual 'off peak' response to EFS (Table 3; Figure 1).

In separate experiments we observed that, in the presence of atropine (1 μM), ATP (100 and 300 μM) evoked reproducible contractions of the common bile duct, averaging 12 ± 2 and $25\pm4\%$ of KCl (80 mM), respectively ($n=4$ each). PPADS (30 μM, 20 min before) inhibited the contractions induced by 100 and 300 μM ATP by 75 ± 9 and $50\pm7\%$, respectively ($n=4$ each), while leaving the response to submaximal concentration of KCl (24 mM) unaffected (26 ± 5 vs $24\pm5\%$ of Emax, in the absence and presence of PPADS, respectively, $n=4$). In a further series of experiments SR 142801 (30 nM), given alone, failed to affect the responses to EFS ($n=4$; not shown). Higher concentrations of SR 142801 were not tested because of loss of selectivity of this antagonist among tachykinin receptors (Patacchini *et al*., 1995).

Effect of L-NOARG and naloxone on the non-cholinergic response to EFS

The complex shape of the atropine-resistant contractile response to EFS suggested us that also some inhibitory transmitter(s) could be released in the common bile duct; we therefore studied the effects of an inhibitor of NO generation, L-NOARG, and of the opioid receptor antagonist naloxone, administered either alone or in combination. As shown in Table 4, L-NOARG (100 μM, 60 min before) greatly enhanced the 'on' response to EFS (Figure 2). Also the 'off peak' response was somewhat increased, while the 'off late' response remained unchanged by L-NOARG (Table 4).

Likewise, naloxone (10 μM, 30 min before) greatly enhanced both the 'on' response and, to a lesser extent, the 'off peak' contraction, while the 'off late' component was left unchanged (Table 4; Figure 2). To investigate whether the two drugs exert additive effects, the preparations pretreated with naloxone (10 μM) were subsequently incubated with L-NOARG (100 μM) for 60 min. As shown in Table 4 the 'on' response underwent a further increase, so that in the presence

Table 4 Effects of N^{ω}Nitro-L-arginine (L-NOARG: 100 μM, 60 min before) and naloxone (10 μM, 30 min before), alone or in combination, on non-cholinergic contractile responses of the guinea-pig isolated common bile duct to electrical field stimulation

Treatment	*'on' response* (% of control)	*'off peak' response* (% of control)	*'off late' response* (% of control)
Vehicle	79 ± 9	87 ± 5	57 ± 7
L-NOARG	311 ± 54**	112 ± 6**	60 ± 19
Vehicle (30 min)	72 ± 8	84 ± 6	67 ± 9
Nalaxone	219 ± 21**	113 ± 4**	133 ± 32
Vehicle (90 min)	57 ± 8	68 ± 6	41 ± 8
Naloxone L-NOARG	431 ± 80**,§	104 ± 9**	60 ± 12

All values are mean$\pm$s.e.mean of six to eight experiments, and are % of control responses. Control responses were obtained at time = 0, before administering the drugs or vehicle. Definition of 'on', 'off peak' and 'off late' response is reported in the text. Electrical field stimulation (trains of 60 V, 0.25 ms pulse width, supramaximal voltage, for 60 s) was applied every 30 or 60 min. A pair of preparations obtained from the same animal was used: one preparation was treated with L-NOARG and/or naloxone, while the other received the corresponding vehicle. **Significantly different from the corresponding value obtained in the time-matched strip treated with vehicle, $P<0.01$. §Significantly different from the preceding value obtained in the same strip treated with naloxone alone: $P<0.05$.

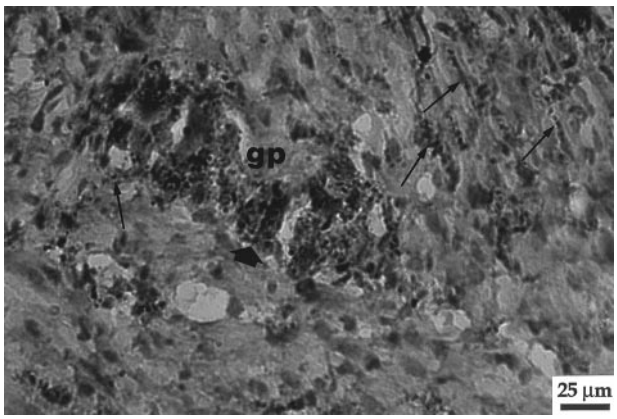

Figure 3 Representative photomicrograph showing the tachykinin (TK)-containing innervation of the guinea-pig common bile duct. TK-IR varicose nerve processes (arrows) were identified throughout the muscle layer of the terminal portion of the common bile duct. At this level, TK-containing varicose fibres encircled labeled (arrowhead) or unlabeled perikarya of ganglionated plexuses (gp). Calibration bar: 25 μm.

of both drugs it practically approached the size of the 'off peak' response (Figure 2).

Immunohistochemistry

The presence of TK-IR was investigated by using an anti-SP rabbit polyclonal antibody, fully reacting with all mammalian tachykinins (see Methods). Nerve fibres containing TK-IR were found in the upper and lower parts of the common bile duct. In particular, in the terminal enlarged portion (or *ampulla*) TK-IR was identified in varicose fibres, running singly or in small fascicles, in the muscle layer as well as in ganglionated plexuses encircling unstained or, more often, stained perikarya (Figure 3). In the upper region of the duct, TK-IR varicose fibres were observed targeting the muscle layer and, occasionally, the lamina propria of the mucosa. At this level no TK-IR labelled ganglion cells were identified.

Discussion

We have undertaken the present study to investigate the role of endogenous tachykinins in the control of the motility of the terminal biliary tract. In order to induce the release of tachykinins from neuronal elements, we applied a prolonged EFS (trains of 1 min duration) which, on the basis of our previous studies performed in the guinea-pig colon (Maggi *et al*., 1994b, 1997) and rat urinary bladder (Meini & Maggi, 1994), was expected to fully activate a putative tachykininergic neurotransmission.

Our data show that EFS, applied to the guinea-pig isolated common bile duct in the presence of atropine, produces a complex, neurogenic NANC contractile response. In this response, 'on' and 'off' contractile components can clearly be distinguished and, as revealed by the pharmacological analysis, seem to have different neurotransmitter backgrounds. By the use of specific and selective antagonists/inhibitors we have shown that at least two different excitatory and two inhibitory neurotransmitters are responsible for the NANC responses, as discussed below.

The role of tachykinins

The present study provides functional and morphological evidence that tachykinins are present in nerve fibres and cell bodies throughout the guinea-pig common bile duct. They are released by EFS and produce smooth muscle contraction by activating specific receptors, mostly of the NK_2 type. This conclusion is supported by the following observations: (1) the contractile response obtained in atropine-pretreated preparations is almost completely abolished by the tachykinin NK_2 receptor-selective antagonist MEN 11420, a novel bicyclic peptide antagonist with a high selectivity towards tachykinin NK_2 receptors and of a great metabolic stability *in vivo* (Santicioli *et al*, 1997; Catalioto *et al*., 1998). It is noteworthy that the concentrations of MEN 11420 (0.1 – 1 μM) used in the present study were previously shown to selectively block tachykinin NK_2 receptors in this preparation (Patacchini *et al*., 1997); (2) Part of the response to endogenous tachykinins could be mediated by NK_1 receptors, as suggested by the inhibitory effect produced by GR 82334 (Hagan *et al*., 1991) on the EFS-induced 'on' response of the duct. The possibility that GR 82334 produced its effect by blocking tachykinin NK_2 receptors seems unlikely, since GR 82334 did not inhibit the 'off' responses which are MEN 11420-sensitive. We interpret the present findings as indication that both NK_1 and NK_2 receptor-preferring ligands (probably SP and NKA) contribute to the 'on' response induced by EFS, while mostly NKA (along with ATP, see below) mediates the 'off' response. A different temporal contribution of SP and NKA to the atropine-resistant contraction would be in keeping with a model whereby the two tachykinins are co-released during EFS and cooperate to produce the overall 'on' response to EFS. A cooperation between endogenous tachykinin NK_1 and NK_2 receptor ligands in producing 'on' contraction in response to EFS was previously proposed in the circular muscle of the guinea-pig duodenum (Zagorodnyuk *et al*, 1995). On the other hand, the resistance of NKA to degradation by peptidases in the guinea-pig biliary tract (Maggi *et al*., 1989a) may enable this ligand to activate NK_2 receptors even after the completion of EFS, thus producing the 'off late' response.

Despite the presence of tachykinin NK_3 receptors in this preparation (Patacchini *et al*., 1997), these receptors do not seem to play a significant role in mediating the contractile response to endogenous tachykinins, since SR 142801 (Emonds-Alt *et al*, 1995; Patacchini *et al*., 1995) had no effect on the response to EFS. This finding could be explained by the fact that NKB – the NK_3 receptor-preferring natural tachykinin – is not expressed in peripheral tissues of mammals, or, if present, its tissue levels are extremely low as compared to SP or NKA (see Maggi, 1995, for review). Thus, NKB probably is not present among the tachykinins released by EFS in the common bile duct. Furthermore, all the present experiments were performed in the presence of atropine which, as stated previously (Patacchini *et al*., 1997), largely reduced NK_3 receptor-mediated contractions in this preparation.

With regard to the source of tachykinins released upon application of EFS, this seems independent from extrinsic sensory nerve terminals, as shown by the experiments with capsaicin. The protocol of *in vitro* capsaicin treatment adopted in this study is known to produce a functional blockade of capsaicin-sensitive primary afferent neurons (Szolcsányi & Barthó, 1978; Maggi & Meli, 1988; Maggi, 1995); this treatment did not influence the contractile response to EFS in the common bile duct.

The role of ATP

ATP has been considered a major inhibitory NANC transmitter in the mammalian intestine (Burnstock *et al*., 1970; Burnstock, 1990). In recent years, evidence has been presented indicating additional role for ATP in enteric physiology; ATP has been shown to act as neuromodulator in the enteric nervous system (LePard *et al*., 1997; Barthó *et al*., 1997) and, in some preparations, ATP can also act as an excitatory neuromuscular transmitter (Zagorodnyuk *et al*., 1995; 1996; Zagorodnyuk & Maggi, 1997). In particular, our group has shown that, in the presence of atropine and tachykinin receptor antagonists, a residual NANC neuromuscular excitation can be demonstrated in the circular muscle of the guinea-pig stomach, duodenum and colon and that this residual response, typically fast in character, is abolished by PPADS (Lambrecht *et al*., 1992), a P_2 purinoceptor antagonist (Zagorodnyuk *et al*., 1995; 1996; Zagorodnyuk & Maggi, 1997). The present results show that, on a similar basis, ATP could act as excitatory transmitter in the guinea-pig biliary tract, being responsible for the 'off' response to EFS. This is shown by the experiments in which the residual contractile response to EFS, obtained in the presence of MEN 11420 and GR 82334, was completely and selectively prevented by PPADS. However, the relative contribution of ATP to the overall response to EFS seems lower as compared to that given

by tachykinins. With regard to the source of the released ATP, the most probable seems to be intrinsic neurons of the bile duct. The contribution of ATP-containing sympathetic nerves is less likely, as shown by our experiments with guanethidine, which failed to modify the EFS-induced response of this preparation. There is ample evidence that guanethidine, besides preventing noradrenaline release in pretreated tissues, also blocks 'purinergic' responses to sympathetic nerve stimulation (see e.g. Kohno *et al*., 1995; Morris, 1994) as well as ATP release from sympathetic neurons (Todorov *et al*., 1996) in a concentration range (up to 10 μM) close to that used in our study (3 μM). Likewise, the present experiments with guanethidine provide evidence that neuropeptide Y, stored in sympathetic nerves, is not involved in the EFS-induced response, since also the release of this transmitter is prevented by guanethidine (see e.g. Donoso *et al*., 1997; Lundberg *et al*., 1989).

Inhibitory neurotransmitters

The observation that the contraction of the common bile duct obtained during the electrical stimulus ('on' response) was exceeded by a phasic contraction developing immediately after the end of the EFS ('off' responses), prompted us to investigate whether some inhibitory transmitter(s), possessing shorter time course of action than that of the excitatory transmitters, could act to limit the size of the 'on' response to EFS. Among the possible candidates, we chose to investigate the possible role of NO and endogenous opioids by studying the effects of suitable blockers of these mediators. NO synthase has been detected in the gallbladder and in the sphincter of Oddi of several species, including man (Grozdanovic *et al*., 1994; Wells *et al*., 1995; Thune *et al*., 1995; Uemura *et al*., 1997; Salomons *et al*., 1997), and NO itself is responsible for nerve-mediated NANC relaxations and for tonic inhibitory control of motility at this level (Mourelle *et al*., 1993; Lonovics *et al*., 1994; McKirdy *et al*., 1994; Konturek *et al*., 1995; Thune *et al*., 1995; Salomons *et al*., 1997). Opioid peptide-immunoreactive nerve fibres have been detected in both the gallbladder and biliary ducts (Polak *et al*., 1977; Melander *et al*., 1991; Wells *et al*., 1995). In particular, in the guinea-pig isolated common bile duct and terminal ampulla dynorphin(1-13), Met-enkephalin or β-endorphin, applied exogenously, have been shown to inhibit smooth muscle contractions elicited by EFS (Oouchi *et al*., 1983).

Our data show that either the inhibition of NO generation by L-NOARG (Moncada *et al*., 1991) or the administration of the opioid receptor antagonist naloxone greatly enhances the 'on', and, to a minor extent, the 'off' contractile responses to EFS. These results, along with the demonstration that the effects of L-NOARG and naloxone are additive, provide pharmacological evidence for the involvement of both NO and endogenous opioids as inhibitory mediators/modulators of smooth muscle motility in the guinea-pig common bile duct.

Other two endogenous peptides worthy of consideration as possible inhibitory transmitters released during EFS of the common bile duct are VIP and calcitonin gene-related peptide (CGRP). Both peptides are present in nerves distributing to the mammalian gallbladder and biliary tract (Alumets *et al*., 1979; Mawe & Gershon, 1989; Talmage *et al*., 1992; Sand *et al*., 1993; De Giorgio *et al*, 1995; Maggi, 1995). However, neither VIP (which is not able to relax precontracted common bile duct strips; unpublished observation), nor CGRP (because of the lack of capsaicin desensitization to affect the EFS-induced responses) seem to be involved.

In conclusion, this study provides functional and morphological evidence that tachykinins, released from intrinsic neuronal elements, are the main NANC excitatory neurotransmitters in the guinea-pig terminal biliary tract. They act by stimulating tachykinin NK_2 (and possibly NK_1) receptors. ATP is another excitatory neurotransmitter involved, although of minor importance as compared to tachykinins, while NO and opioids act as inhibitory mediators/modulators at this level.

Furthermore, our data suggest that tachykinins might play a role as excitatory NANC transmitters in both physiological and pathological conditions affecting the human biliary pathways. Since the understanding of biliary tract dysfunction, which is known to be involved in several gallbladder and pancreatic disorders (Toouli, 1993), is far from being understood, tachykinin NK_2 receptor antagonists, such as MEN 11420, could be proposed for experimental treatment/prevention of such disorders.

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