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DMPP causes relaxation of rat distal colon by a purinergic and a nitrergic mechanism

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Abstract

The non-adrenergic relaxation of carbachol precontracted longitudinal muscle of the rat distal colon was investigated. Intrinsic nerves were activated by the nicotinic, ganglionic receptor agonist, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP). DMPP at 1 and 4 μM caused a relaxation that was markedly antagonized by the nerve blocker tetrodotoxin (1 μM) or the nicotinic receptor antagonist, hexamethonium (1 mM). The response to DMPP was significantly antagonized by apamin (an inhibitor of ATP-sensitive K^+-channels), by reactive blue 2 (a blocker of P_{2y} purinoceptors) and by an inhibitor of nitric oxide (NO)-synthase (N^G-nitro-L-arginine, L-NNA). The combined treatment with reactive blue 2 and L-NNA reduced the relaxatory response to 1 μM DMPP by 77 ± 8% and to 4 μM DMPP by 58 ± 4% of control, but left a residual component. Our results indicate that ATP and NO, together with at least one additional (hitherto unidentified) substance may be inhibitory neurotransmitters in rat distal colon. © 1997 Elsevier Science B.V.

Keywords: Apamin; Colon; DMPP (1,1-dimethyl-4-phenylpiperazinium iodide); Nitric oxide (NO); P_{2y} purinoceptor; (Rat); Reactive blue 2

1. Introduction

Since the early 1960s, a large number of studies of the muscularis propria of the gastrointestinal tract have demonstrated the presence of intrinsic, inhibitory motoneurons that utilize a non-adrenergic, non-cholinergic (NANC) neurotransmitter. The quest to define the identity of such a mediator has resulted in three major hypotheses: The purinergic, the peptidergic, and, most recently, the nitrergic hypothesis. Thus, adenosine 5′-triphosphate (ATP), vasoactive intestinal peptide (VIP) and nitric oxide (NO), respectively, have been proposed being the responsible transmitter (see Hoyle and Burnstock, 1989; Stark and Szurszewski, 1992 for references). It became eventually evident that inhibition of the gut muscle may be elicited via different mechanisms which appear to be expressed with a regional heterogeneity, making it likely that several compounds may serve as inhibitory NANC transmitters (Costa et al., 1986; Manzini et al., 1985). Moreover, it was demonstrated that at least two different neural mechanisms may contribute to inhibition of the muscle at one and the same site (Manzini et al., 1986; Knudsen and Tøttrup, 1991; Maggi and Giuliani, 1993; Crist et al., 1992; Keef et al., 1993; He and Goyal, 1993). In support of these notions, there are reports of neuronal co-localization of the above-mentioned transmitter candidates, for instance VIP and NO (Furness et al., 1992) or ATP and NO (Belai and Burnstock, 1994).

With particular emphasis on the *longitudinal* muscle layer of the colon, Suthamnatpong et al. (1993a) demonstrated in rat that NO may mediate electrically induced relaxation of the proximal part. In the distal colon, on the other hand, VIP appeared to be the most likely candidate to transmit NANC inhibition.

The principal aim of the present investigation was to further analyze the pharmacology of the NANC inhibitory neurotransmission of the longitudinal muscle of the rat distal colon. The specific aim was to analyze purinergic and nitrergic mechanisms. In order to activate enteric nerves, we utilized *pharmacological* nerve stimulation by 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP; Trendelenburg, 1967), which is an agonist at nicotinic (ganglionic) cholinoceptors.

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2. Materials and methods

2.1. General

The study design was approved by the Ethics Committee of the Göteborg University. Colon tissues were obtained from male Sprague–Dawley rats (B&K Universal AB, Sollentuna; 210–400 g b.w.) The animals were killed by cervical dislocation or by exsanguination during pentobarbital anaesthesia (60 mg/kg, i.p.). A segment of the distal colon (40 mm length; anal end situated at the level of the vertex of the urinary bladder, some 40 mm proximal to the anal orifice) was removed and immediately placed in chilled oxygenated (95% O_2, 5% CO_2) Krebs solution, containing (mM): NaCl 115.5, KCl 4.6, KH_2PO_4 1.2, $NaHCO_3$ 21.9, $MgSO_4$ 1.2, glucose 11.5 and $CaCl_2$ 2.5. All concentrations reported refer to the final ones in the organ baths. Since DMPP is known to stimulate adrenergic neurons at postsynaptic sites causing local depolarization and release of noradrenaline (Holbach et al., 1977), the Krebs solution also contained guanethidine (3.4 μM) in order to block noradrenergic neurotransmission (Broadley, 1996). Each segment was gently flushed with the Krebs solution and divided in two halves of 20 mm length. A silk thread was tied around either end of each preparation. In all tissues except for 6 ('open' preparations; see below), the lumen was occluded by the ligature ('closed' preparations); the tissues were mounted vertically in 15 ml organ chambers, and were immersed in gassed Krebs solution at 38°C. The segments were allowed to equilibrate under an initial load of 15 mN for 30 min with a wash-out every 15 min. Isometric contractions were recorded using a Grass FT 03 transducer on a Grass polygraph.

2.2. Experimental protocol

2.2.1. Basal contractile activity

Basal contractile activity was evaluated during a 5 min period commencing 20 min subsequent to the last wash-out. This period was digitized at a frequency of 1/s using a computer program (FlexiTrace 1.02, Tree Star, Santa Barbara, CA), and the distribution of the amplitude of the spontaneous contractions occurring was determined, using a statistical package (StatView 4.0, Abacus Concepts, Berkeley, CA).

2.2.2. Effects of papaverine or DMPP on basal tone

The muscle relaxant, papaverine (Huddart and Saad, 1980; 4.5, 18, 63 and 198 μM), or DMPP (1 and 4 μM) were cumulatively added at 5 min intervals. The former drug was given to investigate whether or not the tissue could relax below basal tone, even if DMPP failed in this action.

2.2.3. Effect of carbachol

In order to determine a concentration of carbachol to obtain a submaximal level of pre-contraction, this compound was administered at cumulatively increasing concentrations (1, 11, 111 and 1111 μM) at 3 min intervals.

The reproducibility of the effects of carbachol (1 μM) and of DMPP (1 and 4 μM, at 5 min intervals; first concentration given 5 min upon carbachol-induced tone) were investigated. Ten min after the addition of the second concentration of DMPP, wash-outs (5 complete changes of the Krebs solution at 5 min intervals) were performed during 30 min. Thereafter, carbachol and DMPP were again administered to the bath, as described above.

2.2.4. Effect of DMPP in the presence or absence of tetrodotoxin or hexamethonium on carbachol induced tone

The concentration–effect relationship for DMPP (added cumulatively at 5 min intervals and resulting in the following final concentrations: 1, 4, 14, 44 and 144 μM) was investigated on carbachol induced tone (1 μM). After wash-outs, the nerve-blocking agent, tetrodotoxin (1 μM; Ritchie, 1980), or the nicotinic ganglionic receptor blocker, hexamethonium (1 mM; Taylor, 1996), was administered. After 20 min equilibration, carbachol was added followed by DMPP, as above.

2.2.5. Effect of DMPP on tone induced by lidocaine

The local anaesthetic, lidocaine (Catterall and Mackie, 1996), utilized both as a *spasmogen* (cf. Wali, 1985) and as a nerve blocking agent, was administered on basal tone (0.38 mM). After 15 min equilibration, DMPP was added (1–144 μM), as above.

2.2.6. Effect of inhibitors of ATP and/or NO on relaxations by DMPP on carbachol induced tone

After an initial challenge with DMPP (1 and 4 μM at 5 min intervals) on carbachol (1 μM) precontracted tissues and subsequent wash-outs, the following compounds were added to the baths and after 30 min again followed by carbachol and DMPP, as above:

(1) Apamin (0.5 μM, a blocker of a class of low conductance Ca^{2+}-dependent K^+-channels in smooth muscle (Castle et al., 1989), and assumed to function as a selective blocker of ATP-induced effects (Maas et al., 1980).

(2) Reactive blue 2 (20 or 50 μM), a putative P_{2y} purinoceptor antagonist (see Dalziel and Westfall, 1994 for references).

(3) N^G-nitro-L-arginine (L-NNA; 100 μM), an inhibitor of NO-synthase (Moore et al., 1990; Tøttrup et al., 1991), or its biologically inactive D-enantiomer (D-NNA; Tøttrup et al., 1991; 100 μM).

(4) The combination of reactive blue 2 (50 μM) and L-NNA (100 μM).

2.2.7. Effect of sodium nitroprusside on carbachol induced tone

The 'NO-donor', sodium nitroprusside (Feelisch and Noack, 1987) was added either cumulatively (1, 4, 14, 44 and 144 μM at 3 min intervals) to preparations precon-

tracted with carbachol. After wash-outs carbachol was again added followed by DMPP (1–144 μM), as above ($n = 4$); alternatively, after carbachol, a single concentration of sodium nitroprusside (30 μM) was administered, followed after 5 min by DMPP (4 μM; $n = 4$).

2.2.8. Comparison between 'closed' and 'open' preparations

Twelve segments from a total of 6 rats were either occluded or left open at both ends (for each type: $n = 6$, of which 3 were taken from the proximal, and 3 from the distal end, of the distal colon). The segments were mounted and subjected to initial load as above and after 30 min equilibration, carbachol (1 μM) was administered, followed after 5 min by DMPP (1 and 4 μM at 5 min intervals). After wash-out (as above) this series (carbachol + DMPP) was repeated once.

2.3. Evaluation of results

From each animal, two segments from the distal colon were obtained. Usually these were used in different investigations. In preliminary experiments it was found that there were neither qualitative nor quantitative differences between the two segments of the distal colon with regard to contractile activity being either spontaneous, or resulting from the administration of drugs to the tissue. The *n*-values denote the number of segments investigated. It should be mentioned that in investigations where $n \leq 6$, all segments were taken from different animals, while when $n > 6$, the number of segments used sometimes exceeded the total number of animals (always 6 or more, though) used for that investigation.

An excitatory or inhibitory response of the tissue was defined as a deflection of the graph from the baseline, corresponding to ≥ 0.5 mN. Data are presented as mean ± S.E.M. Relaxations were calculated as the difference between the prevailing resting tension immediately prior to DMPP administration, and the nadir of the resulting response (cf. Manzini et al., 1985), and expressed as % of the prevailing tone, as estimated immediately prior to each DMPP-administration. The effect of a blocking drug was always compared with a control response obtained on the same segment, prior to the administration of the drug in question, i.e. a paired investigation.

Non-parametric statistical analyses were employed; Wilcoxon's signed rank test, and Mann–Whitney *U*-test, were used for paired, and unpaired data, respectively, and the Kruskal–Wallis one-way analysis of variance for group analyses (Siegel and Castellan, 1988). A *P*-value of less than 0.05 was considered significant.

2.4. Drugs

The following drugs were used:

Apamin, N^G-nitro-L-arginine, carbamylcholine chloride (carbachol), 1,1-dimethyl-4-phenylpiperazinium iodide, guanethidine monosulphate, hexamethonium chloride and tetrodotoxin were obtained from Sigma Chem. (St. Louis, MO). N^G-nitro-D-arginine was purchased from Serva Feinbiochemica (Heidelberg, FRG). Reactive blue 2 was obtained from Research Biochemicals (Natick, MA); pentobarbital sodium (pentobarbitalnatrium) and papaverine sulphate from Apoteksbolaget (Umeå) and sodium nitroprusside from May and Baker, Dagenham. The solid drugs were dissolved in isotonic saline or distilled water. L-NNA and D-NNA demanded sonification in order to dissolve.

3. Results

3.1. Basal contractile activity

Mean basal tone was 5.9 ± 3.5 mN (range 2–24.5 mN, $n = 85$). Spontaneous activity was scarce, as shown in Fig. 1A. The computerized analysis ($n = 6$, selected at random) revealed a mean and maximal amplitude of 1.4 ± 0.2 and 5.0 ± 1.8 mN, respectively. The distribution of the contractile activity is presented in Fig. 2, showing that a major part of the contractions occurred within a low range. The contractile pattern observed was similar to that reported by Laniyonu et al. (1989).

3.2. Effect of drugs

3.2.1. Effects of papaverine or DMPP on basal tone

Papaverine ($n = 6$) was administered cumulatively at increasing concentrations (4.5–198 μM), in order to inves-

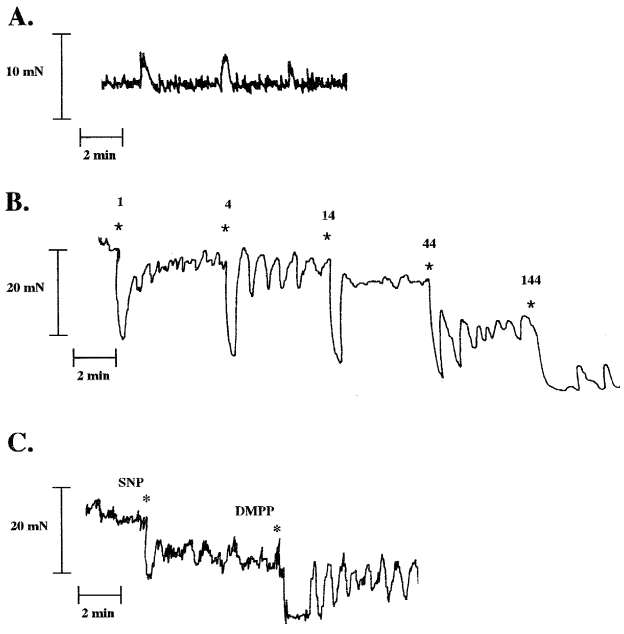

Fig. 1. (A) Spontaneous contractile activity of the preparation of rat distal colon representing activity of the longitudinal muscle layer. (B) Effect of cumulative administration of 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP (μM), at *), on segments of rat distal colon, precontracted with carbachol (1 μM). (C) Effects of the administration of the 'nitric oxide donor', sodium nitroprusside (SNP, 30 μM, at *), and of DMPP (4 μM, at *) on a carbachol precontracted (1 μM) segment of rat distal colon.

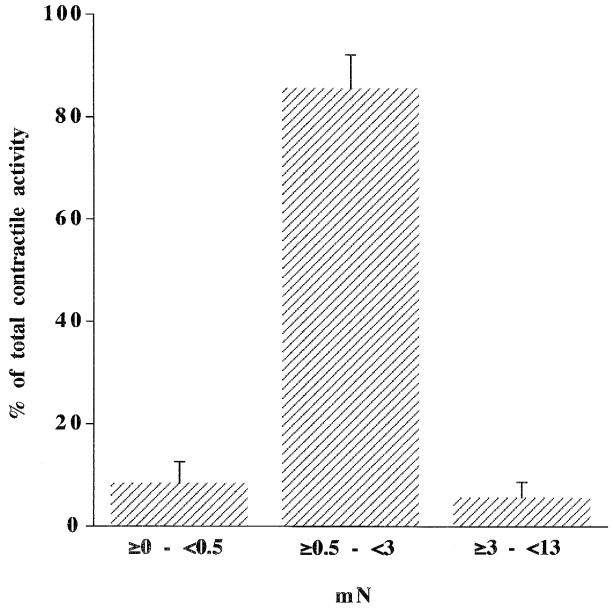

Fig. 2. Frequency-distribution of the mechanical activity of the longitudinal muscle of rat distal colon. In 6 randomly chosen experiments, the last 5 min of the equilibration period were analyzed with regard to the force exerted by the tissue once every s (a total number of 300 consecutive points of the graph examined). The analysis is presented as percentage of points examined (means ± S.E.M.). The contractile activity is grouped into three intervals: ≥ 0 but < 0.5 mN (representing 'zero-activity', cf. methods); ≥ 0.5 but < 3 mN and ≥ 3 but < 13 mN.

tigate whether basal tone and/or phasic activity could be inhibited. In 5/6 experiments, there was a clear-cut inhibition of either variable, but in the remaining one, there was instead an unexpected excitatory response. Excitations to papaverine were observed in other tissues (cf. Kenakin, 1984) but this phenomenon was not further analyzed in the present study.

DMPP (1 and 4 μM) was administered on basal tone ($n = 6$). In 5/6 experiments there was no effect on either basal tone or phasic activity; in the remaining one, a clear-cut transient excitation was obtained in response to either concentration. Papaverine, on the other hand, administered to the tissues when DMPP was still present in the bath, caused an inhibition of basal tone or phasic activity, as described above ($n = 6$).

3.2.2. Effect of carbachol

Carbachol (1–1111 μM) increased tone in a concentration-dependent fashion, while phasic activity was unchanged or diminished ($n = 2$). Maximal contraction was obtained at 11 μM in one of the experiments and 111 μM in the other. A concentration of 1 μM induced a contraction of about 55 mN, being approximately 70% of the maximum, also reported by Bailey and Hourani (1992).

Investigations of the reproducibility of the excitatory effect of carbachol at 1 μM showed that the basal tone of the segment was significantly lowered by 28 ± 7%, as estimated immediately prior to the second administration of carbachol ($P < 0.05$; $n = 6$). Carbachol elicited a contractile response, consisting of a peak of 48.0 ± 3.9 mN (first challenge) vs. 54.9 ± 4.9 mN (second challenge; $P > 0.05$; $n = 6$) which during 5 min observation period levelled off to a stable plateau, 80 ± 10% vs. 80 ± 4.7% of the peak value ($P > 0.05$). It is therefore evident, that carbachol at 1 μM elicited a reproducible tone of the tissue. In the following, all further investigations of DMPP were conducted with preparations precontracted with carbachol at 1 μM.

3.2.3. Effect of DMPP in the absence or presence of tetrodotoxin or hexamethonium on carbachol induced tone

DMPP, at 1 μM, caused a relaxatory response, whereupon tone usually resumed to pre-DMPP level. Sometimes, however, a slight increase or decrease in tone of ± 5 mN was induced by this concentration of DMPP (Fig. 1B).

At higher concentrations (44 and 144 μM), DMPP invariably caused a relaxation as a primary effect. Sometimes, during the 'downstroke' of the recording pen, there was a tendency to a brief 'rebound' contraction. A second rebound contraction could usually be observed at ≥ 14 μM whereupon tone did not return to the level noted prior to this concentration (Fig. 1B).

In the quantitative evaluation, only the force recorded at the *nadir* of the DMPP induced relaxation was estimated. Fig. 3 shows the concentration–effect relationship of DMPP produced relaxations (cumulatively administered (1–144 μM); $n = 29$).

Tetrodotoxin (1 μM; $n = 12$) or hexamethonium (1

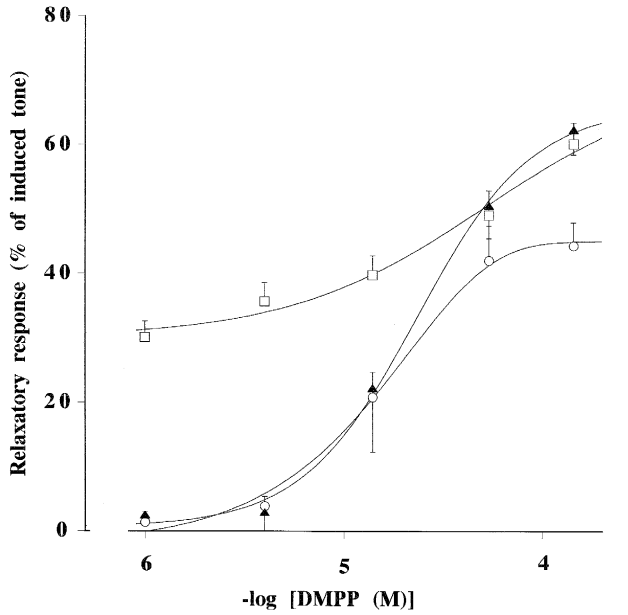

Fig. 3. Concentration–effect relationships for DMPP induced relaxations of carbachol precontracted segments of rat distal colon. DMPP was administered cumulatively. Squares: Control responses ($n = 29$). Triangles: DMPP administered in the presence of tetrodotoxin (1 μM; $n = 12$). Circles: DMPP administered in the presence of hexamethonium (1 mM; $n = 14$).

mM; $n = 14$) induced an increase in the phasic activity of the colonic muscle, suggesting a prevailing, neurogenic inhibition of this variable (cf. Laniyonu et al., 1989). Tetrodotoxin induced a decrease in basal tone by $40 \pm 7\%$ (indicating an ongoing neurogenic excitation of this variable) and an increase in carbachol induced tone by 20% ($P < 0.05$ for either effect), while hexamethonium did not affect basal or carbachol induced tone. The relaxatory response to DMPP was antagonized by tetrodotoxin or hexamethonium as shown in Fig. 3. Thus, the response to 1 μM was abolished by either tetrodotoxin or hexamethonium, whereas the response to 4 μM was blocked by $66 \pm 15\%$ and $88 \pm 4{,}3\%$, respectively. There was a considerable residual response to DMPP at 144 μM. Thus, tetrodotoxin was completely inefficient and hexamethonium reduced the response only by $10 \pm 7\%$. Therefore, we assume that the effect to DMPP at 1 and 4 μM was predominantly neurogenic, mediated by an activation of nicotinic receptors. In the further analysis, DMPP only at 1 and 4 μM (added at 5 min intervals) were investigated.

Analysis of the reproducibility of DMPP, when given after the second administration of carbachol showed that the response to the second addition of DMPP was $90 \pm 8\%$ (1 μM) and $104 \pm 6\%$ (4 μM) of that obtained by the first challenge with this compound ($P > 0.05$; $n = 6$ for either concentration). Therefore, we assume that DMPP at these two concentrations cause relaxations in a reproducible fashion.

3.2.4. Effect of DMPP on tone induced by lidocaine

Lidocaine (0.38 mM; $n = 4$) caused a stable increase in tone of 46.1 ± 4.9 mN, an effect investigated by Wali (1985). Phasic activity was abolished by lidocaine. DMPP (1–144 μM; $n = 4$) had no effect while papaverine (45 μM, $n = 4$) relaxed the tone of the preparation to, or slightly below, basal tone.

3.2.5. Effect of inhibitors of ATP and / or NO on relaxations to DMPP on carbachol induced tone

Apamin (0.5 μM, $n = 6$) elicited an increase in phasic activity of the tissues (cf. Laniyonu et al., 1989) but did not significantly influence basal tone of the preparations. Tone induced by carbachol was reduced by $15 \pm 8\%$ ($P < 0.05$). DMPP induced relaxations were reduced by apamin to $32.1 \pm 9\%$ (1 μM; $P < 0.05$) and $49.0 \pm 5\%$ (4 μM; $P < 0.05$) of control, respectively.

According to Bailey and Hourani (1992), ATP relaxes the longitudinal muscle of the rat distal colon (precontracted with carbachol) probably via P_{2y} purinoceptors. Therefore, we investigated the effect of the P_{2y} antagonist, reactive blue 2 (20 or 50 μM; $n = 6$ for either concentration), to further corroborate an involvement of ATP in the DMPP induced relaxation. This compound caused a minor increase in the phasic activity. Basal tone was lowered by $31 \pm 6\%$ (20 μM; $P < 0.05$) and by $30 \pm 8\%$ (50 μM; $P < 0.05$). Neither concentration of the drug changed the tone induced by carbachol while at 50 μM, the DMPP induced relaxations were significantly reduced (Fig. 4).

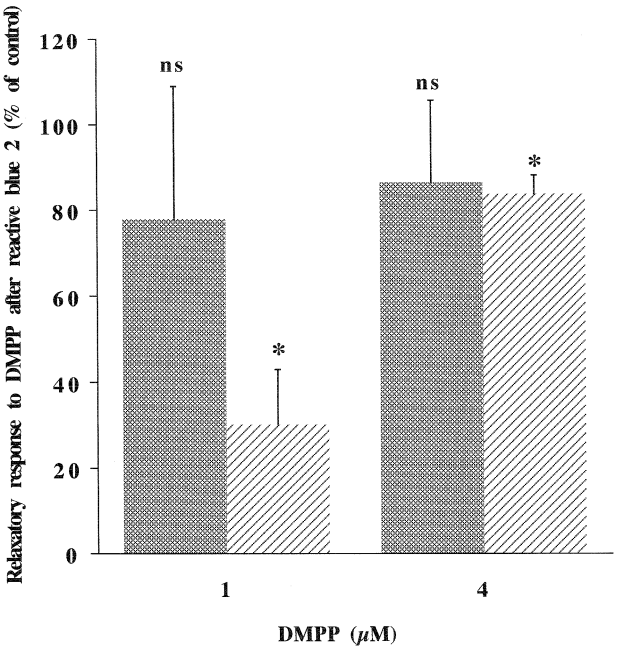

Fig. 4. Relaxation of carbachol precontracted segments of rat distal colon induced by DMPP (1 and 4 μM) in the presence of reactive blue 2 (checked bars: 20 μM; hatched bars: 50 μM; $n = 6$ for either concentration). $^{*}P < 0.05$ vs. control (Wilcoxon's signed rank test); ns, not significant.

A possible contribution of NO to the non-adrenergic relaxation elicited by DMPP was analyzed by the use of L-NNA (100 μM; $n = 10$) This compound elicited a marked increase in phasic activity suggesting an ongoing, nitrergic inhibition of this variable, in concert with recent (unpublished) findings from this laboratory. Neither basal tone, nor the tone induced by carbachol were affected by L-NNA. DMPP induced relaxations were reduced by L-NNA to $30.6 \pm 12\%$ (1 μM; $P < 0.05$) and $70.2 \pm 15\%$ (4 μM; $P < 0.05$) of control, respectively. Conversely, the biologically inactive D-enantiomer, D-NNA (100 μM; $n = 4$) affected neither phasic contractile activity, basal tone, induced tone nor DMPP induced relaxations (data not shown).

When both reactive blue 2 (50 μM) and L-NNA (100 μM) were added to the tissues ($n = 6$) there was a marked increase in phasic activity while basal tone, and tone induced by carbachol were unchanged. DMPP induced relaxations were reduced by the combined treatment to $22.6 \pm 8\%$ (1 μM; $P < 0.05$) and $41.7 \pm 4\%$ (4 μM; $P < 0.05$) of control, respectively. Moreover, the combined treatment was significantly more efficient in blocking the effect of DMPP (4 μM, but *not* 1 μM) than L-NNA (100 μM; $P < 0.05$) or reactive blue 2 (50 μM; $P < 0.005$), when given separately.

In all experiments evaluating the effect of inhibitors of putative neurotransmitters (above), papaverine (45 μM)

was given at the end of the experiment (the gut segment still being precontracted). This agent was able to relax the tissues consistently to a level even below the initially prevailing basal tone.

3.2.6. Effect of sodium nitroprusside on carbachol induced tone

Sodium nitroprusside (1, 4, 14, 44 and 144 μM at 3 min intervals; $n = 4$), caused relaxation of the precontracted preparations but there was no clearcut concentration-dependent effect, but, instead there was a definite tendency to 'tachyphyllaxis' of the resulting response. After wash-out, DMPP produced (on precontracted segments) concentration-dependent relaxations that qualitatively did not differ from those observed above (see Section 3.2.3). In 4 additional experiments, a single concentration of sodium nitroprusside was instead administered followed by a single concentration of DMPP without wash-out in-between. The response to either agent did not differ qualitatively (Fig. 1C).

3.2.7. Comparison between 'closed' and 'open' preparations

There were no differences between 'closed' and 'open' preparations with respect to: spontaneous contractions prior to carbachol (as estimated by inspection); basal tone (estimated immediately prior to each administration of carbachol in the individual experiments); the amplitude of the contraction to carbachol (twice in each individual experiment); as well as relaxatory response to DMPP (1 and 4 μM; twice in each individual experiment) (data not shown). Therefore, we conclude that there are seemingly no qualitative or quantitative differences between the two types of preparations.

4. Discussion

4.1. Non-adrenergic relaxation of rat distal colon

NANC relaxation of the longitudinal muscle layer of rat distal colon was previously elicited by electrical stimulation on basal tone (Al-Dhahir and Zeitlin, 1981; Gillan and Pollock, 1980; Suthamnatpong et al., 1993a) or on the peak of rhythmic activity induced by morphine (Gillan and Pollock, 1980; Laniyonu et al., 1989). Romano (1981) investigated the effect of DMPP on precontracted segments of rat distal colon. This compound caused a relaxatory response (although the effect of adrenoceptor antagonists was not investigated) that exhibited a concentration–effect relationship similar to that of the present study.

The reason for us to choose 'closed' instead of 'open' colon segments was that we wanted to avoid a release of substances from the mucosa by e.g. DMPP, that could interfere with muscle activity (cf. Coupar, 1987). Moreover, the present results will be compared with similar ones obtained in rats with acute chemically induced colitis (unpublished data). According to Van der Vliet et al. (1992), substances released from the inflamed mucosa could markedly influence contractility. Therefore, it seemed most appropriate to close off the mucosal compartment.

Our findings suggest that DMPP activates non-adrenergic, relaxatory motoneurons. Basal tone, on the other hand, was seemingly too low to reveal relaxations to DMPP. The results obtained with hexamethonium could indicate that DMPP is an agonist at nicotinic cholinoceptors; at the two concentrations of the compound used for further analyses (viz. 1 and 4 μM) the resulting relaxation was blocked by $> 85\%$ (cf. Holbach et al., 1977). Moreover, at these concentrations, the response was antagonized by $> 65\%$ by tetrodotoxin, while at higher concentrations of DMPP, there was a clearcut tetrodotoxin-resistant component, noted also by Romano (1981). Also in other preparations, tetrodotoxin-resistant responses to DMPP have been noted (e.g. Maggi et al., 1985; Kannan and Johnson, 1992). Although extra-neuronal effects of DMPP have been suggested (Romano, 1981; Kannan and Johnson, 1992), studies on aganglionic segments of the piebald mouse indicate an exclusively *neuronal* site of action of this compound (Wood and Brann, 1986). Speculatively, the tetrodotoxin-resistant component could be due to an activation of nicotinic receptors on terminal varicosities, a process being independent of (tetrodotoxin-sensitive) action potentials (cf. Romano, 1981). Such a suggestion is strongly supported by functional studies undertaken on guinea-pig myenteric varicosities (White, 1982). Since, however, nicotinic receptors consistently have been demonstrated also on *sensory* neurons of various tissues (cf. Maggi, 1991), one cannot exclude that part (or even all!) of the effect caused by DMPP resulted from an antidromic activation of afferents within the gut segment under investigation.

Interestingly, in contrast to TTX, the local anaesthetic, lidocaine, considerably increased basal tone. This response was observed even in the presence of TTX (Wali, 1985) but could not be observed in a calcium-free medium (Delbro and Engberg, 1993). On such lidocaine precontracted preparations, the relaxatory response to DMPP (≤ 144 μM) was abolished. The mechanism for this latter effect may involve not only blockade of voltage gated sodium channels (Catterall and Mackie, 1996) but also ion channels of nicotinic cholinoceptors (cf. Boeckxstaens et al., 1990); should this latter suggestion be valid, lidocaine may be more potent in this action than hexamethonium.

4.2. Purinergic neurotransmission

There are several lines of evidence in favor of a role for ATP as a transmitter in the gastrointestinal tract (although this hypothesis is still controversial). Thus, ATP appears to be localized (together with NO) in a subpopulation of myenteric neurons (Belai and Burnstock, 1994). ATP was

early found to mimic neurogenic (NANC) relaxatory effects on gut muscle in some parts (Burnstock, 1972; Burnstock, 1981) but not in others (Manzini et al., 1986; Serio et al., 1990; Serio et al., 1992). Electrophysiologically, ATP mimicked the fast inhibitory junction potential (IJP) in some preparations (Crist et al., 1992) while not in others (Serio et al., 1992).

Membrane receptors for adenine compounds have been termed purinoceptors, of which there are P_1, and P_2 subtypes. Adenosine and ATP are the 'natural' ligands at P_1, and P_2 purinoceptors, respectively (Dalziel and Westfall, 1994). While originally, ATP induced relaxation of smooth muscle was ascribed to be mediated via the P_{2y} purinoceptor subtype (Kennedy, 1990), recent findings add to the complexity of ATP actions. Thus, P_{2y} purinoreceptor mediated contraction of the muscularis mucosae of rat colon was reported (see Bailey and Hourani, 1992). Moreover, these authors, in the longitudinal muscle of rat distal colon (the tissue being precontracted with 1 μM carbachol, i.e. a preparation being similar to that used in the present study), observed that this tissue contains a mixture of P_1, and P_2 (presumably P_{2y}) purinoceptors, the activation of which leads to relaxation. An interesting conclusion of that study is that a definite classification of the P_2 purinoceptor involved was difficult since the various nucleotides investigated rapidly degraded to adenosine. Furthermore, as a consequence of the findings of Bailey and Hourani (1992), we assume that using *desensitization* to ATP by repeated administration of agonist does not by necessity elicit a blockade of the receptor subtype (e.g. P_{2y} purinoceptors) under investigation (cf. Tonini et al., 1981; Manzini et al., 1986; Serio et al., 1990; Smits and Lefebvre, 1996).

Perhaps the most convincing evidence in favor of purinergic neurotransmission is reports of pharmacological blockade of neurogenic (NANC) relaxatory responses of the gut muscle. In particular, the two compounds, apamin and reactive blue 2, appear to be suitable antagonists. The bee venom, apamin, is a peptide that blocks a class of low conductance, Ca^{2+}-dependent K^+-channels in smooth muscle, which are opened by either ATP or adrenaline (see He and Goyal, 1993 for references). Moreover, this compound blocked the inhibitory response in various parts of the gut to electrical stimulation of NANC nerves (e.g. He and Goyal, 1993; Boeckxstaens et al., 1993; Keef et al., 1993; Maggi and Giuliani, 1993), or to the administration of ATP (Boeckxstaens et al., 1993; Keef et al., 1993, see also Briejer et al., 1995 and Qian and Jones, 1995, for earlier references). Electrophysiological studies have shown that apamin is an inhibitor of the fast component of the IJP in some gut muscle (He and Goyal, 1993; Zagorodnyuk and Maggi, 1994).

Reactive blue 2 is a putative P_{2y} purinoceptor antagonist (Kennedy, 1990; Dalziel and Westfall, 1994) that antagonized inhibitory responses in some gut muscle to electrical stimulation of NANC nerves (see Kennedy, 1990, for earlier references; Soediono and Burnstock, 1994) or to the administration of ATP (Manzini et al., 1985, Soediono and Burnstock, 1994). Reactive blue 2 can be used only in a narrow concentration-range above which it displays non-specific effects (Kennedy, 1990, see also Bültmann and Starke, 1994). The contractile effect to carbachol was unchanged by reactive blue 2 at the concentration used in the present study (50 μM), and, moreover, on such a precontracted preparation, papaverine (45 μM) caused a full relaxation. Therefore, we rule out undue effects of reactive blue 2 on the tissue used in the present study.

A purinergic component of the non-adrenergic relaxation to DMPP may be inferred, since either apamin or reactive blue 2 antagonized this response at most by about 70%.

4.3. Nitrergic neurotransmission

L-NNA, at a concentration known to efficiently antagonize neurogenic relaxation of gastro–intestinal smooth muscle (Boeckxstaens et al., 1993) inhibited the relaxation to DMPP at most by about 70%, which indicates that NO is a possible transmitter of this response.

Our findings in this respect are at variance with those of Suthamnatpong et al. (1993a). These authors could not affect, by a NO-synthase blocker, relaxations elicited by electrical field stimulation on the basal tone of the tissue. Neither did gaseous NO affect the basal tone of this preparation, while we observed a relaxation in response to the administration of the 'NO-donor', sodium nitroprusside (Feelisch and Noack, 1987), to the precontracted tissue. The cGMP content in response to NO administration or electrical field stimulation, however, was found to be elevated to the same extent as in the proximal colon, where a clear relaxatory response to such stimuli was observed (Suthamnatpong et al., 1993b). In addition, we have demonstrated the existence of nitrergic neurons to this tissue causing *tonic inhibition* of the phasic spontaneous activity of the muscle (unpublished observation; see also Section 3.2.5). Therefore, NO should be taken into consideration as a putative NANC relaxatory transmitter also in distal colon. The discrepancy between our data and those of Suthamnatpong et al. (1993a,b) could in part be explained by differences in experimental conditions, viz. whether or not precontracted preparations were investigated (cf. Serio et al., 1992).

4.4. Purinergic–nitrergic interaction

Interestingly, the combination of reactive blue 2 (50 μM) and L-NNA (100 μM) caused an inhibition of the DMPP (4 μM) induced relaxation that was significantly greater than by either drug alone at the same concentrations. Such a summation of the effect of antagonists of putative NANC neurotransmitters was reported by Maggi and Giuliani (1993) in the circular muscle of the guinea pig proximal colon, but could not be demonstrated by

Smits and Lefebvre (1996) in the longitudinal muscle of rat distal ileum. More studies are required to elucidate the mechanism of action of these two neurotransmitter candidates; a coexistence of which was proposed in rat gut on the basis of findings by histochemistry (Belai and Burnstock, 1994) and by functional studies (Soediono and Burnstock, 1994; Smits and Lefebvre, 1996).

The residual response to DMPP obtained after the combined treatment with reactive blue 2 and L-NNA may indicate that the concentration used of either or both of these blockers may not be sufficient for a full antagonism of the response to DMPP. Alternatively, the residual effect could be due to another NANC, relaxatory transmitter. Vasoactive intestinal peptide was proposed by Suthamnatpong et al. (1993a), although these authors found that VIP did not affect the basal tone of the tissue. Moreover, in guinea-pig, and mouse colon, VIP (when added to the basal tone) *contracted* the longitudinal muscle layer (Bennett et al., 1984; Fontaine et al., 1986). The mechanism for the residual response remains to be elucidated.

4.5. Conclusion

In the present study, we demonstrated that DMPP at concentrations that elicited predominantly tetrodotoxin-sensitive responses via nicotinic receptors (1 and 4 μM), caused (non-adrenergic) relaxation of the carbachol precontracted longitudinal rat distal colon. This effect was significantly antagonized by either of two compounds that interact with purinergic neurotransmission, or by an inhibitor of NO-synthase. The combined antagonism of these two (hypothesized) neuronal pathways resulted in a further inhibition of the response to DMPP (at 4, but not 1 μM), but a residual relaxation persisted. Our results indicate that ATP and NO may be inhibitory neurotransmitters in this part of the gut.

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