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Effect of papaverine on synaptic transmission in the guinea-pig ileum

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1 The effect of papaverine, a well known smooth muscle relaxant, was investigated on neural transmission within the enteric nervous system. Segments of guinea-pig ileum were placed in a partitioned bath to enable drugs, including papaverine, to be applied to enteric nerve pathways without interfering with the recording of the smooth muscle contraction. Ascending excitatory enteric nerve pathways were activated by electrical field stimulation in the anal compartment (10 Hz for 2 s, 45 mA, 0.5 ms pulse duration) and the resulting contraction of the intestinal circular muscle in the oral compartment was recorded isotonically.

2 Tetrodotoxin (0.6 μM) and hexamethonium (100 μM) both abolished, or greatly reduced, the contractions when applied to either compartment indicating that nicotinic synapses are involved in this pathway.

3 Papaverine (0.3–30 μM) applied independently to each compartment depressed in a concentration-dependent manner, the nerve-mediated contractions. The IC_{50} of this inhibitory effect was 3.53 μM for the oral and 4.76 μM for the anal compartments, respectively. Two other phosphodiesterase (PDE) inhibitors, 3-isobutyl-l-methylxanthine (IBMX 10–300 μM) and theophylline (30–1000 μM) added to the anal compartment also inhibited the nerve mediated contractions. Papaverine applied to the anal bath, after IBMX 100 μM (or theophylline 300 μM) further inhibited the nerve-mediated contractions, but was less effective than when applied alone.

4 Phentolamine (1 μM), an α-adrenoceptor antagonist, reduced the inhibitory effect of papaverine, but not that of IBMX (100 μM) or theophylline (300 μM). A combination of phentolamine and IBMX (or theophylline) prevented the inhibitory effect of papaverine.

5 Tetrodotoxin, but not papaverine or hexamethonium, inhibited the contraction elicited by electrical stimulation just anal to the partition indicating that papaverine did not affect the generation or conduction of nerve action potentials.

6 Verapamil (1 μM) and nifedipine (1 μM), two smooth muscle relaxants which act by blocking L-type calcium channels, only inhibited the contractions when applied directly to the recording (oral) compartment. This indicates that L-type Ca^{2+} channels are probably not involved in synaptic transmission in these ascending pathways and thus that the PDE inhibitors do not inhibit synaptic transmission by acting on these channels. ω-Conotoxin GVIA (10 nM), a potent inhibitor of the N-type Ca^{2+} channels, blocked the nerve-mediated contractions applied to either compartment. Whether the PDE inhibitors exert their inhibitory actions via these channels remains to be established.

7 The results indicate that the PDE inhibitors, papaverine, IBMX and theophylline inhibit excitatory enteric neural pathways by depressing synaptic transmission. The inhibitory effect of papaverine (but not IMBX or theophylline) involves, at least in part, the release of noradrenaline from sympathetic nerves acting on α-adrenoceptors on enteric neurones.

Keywords: Enteric nervous system; intestinal motility; phosphodiesterases; electrical stimulation; nicotinic transmission; calcium channels; noradrenaline

Introduction

Phosphodiesterases (PDE) are a family of enzymes which regulate the levels of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) and therefore the functions of these nucleotide second messengers (Beavo, 1995). These enzymes are found in several mammalian tissues, including the nervous system (Ludvig *et al*., 1991; Yan *et al*., 1993). Among numerous and potent biological effects PDE inhibitors enhance the release of acetylcholine in the enteric nervous system (Takeuchi *et al*., 1992) and influence airways function by modulating the neural control of the airway smooth muscle (Aikawa *et al*., 1992; Barlinski *et al*., 1992).

Papaverine is an inhibitor of PDE (Walker *et al*., 1983; Weishaar *et al*., 1986; Beavo, 1995) and of calcium transport (Kimura *et al*., 1984) and is often used in pharmacological experiments to relax fully smooth muscle preparations (Needleman *et al*., 1985). In addition administration of papaverine or other PDE inhibitors increases the release of noradrenaline in peripheral sympathetically innervated tissues (Cubeddu *et al*., 1974; Celuch *et al*., 1978; Boehm *et al*., 1994).

In this work, we have investigated the effects of papaverine and other PDE inhibitors on neural transmission in the enteric nervous system. We have used a preparation of guinea-pig small intestine in which nerve pathways were stimulated electrically to activate excitatory motor neurones to the circular muscle, employing a partitioned bath in which drugs could be applied to enteric nerve pathways without interfering with the recording of the smooth muscle contraction.

Preliminary accounts of some of these studies have been communicated to the 3rd Joint Meeting of French and Italian Pharmacological Societies (Izzo *et al*., 1996).

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Methods

Male guinea-pigs weighing between 300 and 350 g were used. The animals were killed by being stunned and bled via the carotid arteries. Segments (40–60 mm) of ileum were removed, flushed of luminal contents and placed in a Krebs solution (composition in mM: NaCl 119, KCl 4.75, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.5, $CaCl_2$ 2.5 and glucose 11). The middle part of each segment was cut open for 10–15 mm along the mesenteric border and pinned flat to strip off the mucosa and submucosa. The segment was placed horizontally in a bath filled with warm (37°C) aerated (95% O_2: 5% CO_2) Krebs solution. The experimental set up is shown in Figure 1 and represents a modification of a similar partitioned bath developed by Tonini & Costa (1990). A removable partition with a soft edge was pressed on the flat part of the preparation to divide the bath into an oral and anal compartment which contained the oral and the anal end of the intestinal segment respectively. The mechanical activity of the circular muscle at the oral end was recorded isotonically (load 0.5 g) with a transducer connected to a 'Gemini' recording apparatus (Ugo Basile, Varese, Italy). The enteric nerve pathways were activated by supramaximal electrical field stimulation (10 Hz for 2 s, 45 mA, 0.5 ms pulse duration) via a pair of platinum electrodes placed around the intestine in the anal compartment, 30 mm from the partition. The distance between the stimulating electrodes and the recording of the circular muscle was 35 mm. Stable and reproducible contractions were obtained with stimulations every 5 min and were expressed as % of contraction produced by 10 μM carbachol. This concentration of carbachol produced a circular muscle contraction corresponding to total occlusion of the ileal lumen.

The effect of papaverine on these nerve-mediated contractions was evaluated by adding papaverine to a single compartment in each preparation. Cumulative concentration-effect curves (0.3–30 μM papaverine, contact time 5 min for each concentration) were constructed. The other PDE inhibitors, 3-isobutyl-1-methylxanthine (IBMX 10, 30, 100 and 300 μM) and theophylline (30, 100, 300 and 1000 μM) were added to the anal compartment in single concentrations and the time course of their effect evaluated. Papaverine was also tested after either IBMX 100 μM or theophylline 300 μM. In some experiments the effect of papaverine was evaluated after IBMX 100 μM (or theophylline 300 μM) plus phentolamine 1 μM.

In order to test the seal between the two compartments, in some experiments ($n=20$) tetrodotoxin (1 μM) was applied to the anal bath while the circular muscles motorneurones were activated by electrical stimulation in the oral bath (single pulses, 45 mA, 0.5 ms pulse duration). The absence of inhibition by tetrodotoxin applied to the anal compartment was taken as evidence of an effective seal between the two compartments. Atropine (10 μM) which inhibits cholinergic transmission from the circular muscle motorneurones was also used to test the seal ($n=91$).

Single concentrations of tetrodotoxin 0.6 μM, hexamethonium 100 μM, verapamil 1 μM, nifedipine 1 μM, phentolamine 1 μM and ω-conotoxin GVIA (conotoxin) 10 nM were also evaluated (contact time: 10, 10, 20, 20, 20 and 30 min, respectively). These concentrations were selected on the basis of previous findings (Tonini & Costa, 1990; Aikawa *et al*., 1992; Poli *et al*., 1994; Thornbury *et al*., 1995).

Drugs

Drugs used were: papaverine hydrochloride, atropine sulphate, hexamethonium bromide, carbachol chloride, tetrodotoxin, verapamil hydrochloride, nifedipine, theophylline, phentolamine hydrochloride (Sigma, Milan, Italy), 3-isobutyl-1-methylxanthine (IBMX) and ω-conotoxin GVIA (RBI, Milan, Italy). The drugs were dissolved in distilled water (warmed for IBMX) with the exception of nifedipine which was dissolved in DMSO. All drugs were added in volumes less than 0.1% of the bath volume. DMSO (0.01%) had no effect on the responses under study.

Statistical analysis

Results are given as mean ± s.e.mean (or confidence limits (c.l.) of the IC_{50} values). Comparisons between two sets of data were made by Student's t test for paired data. When multiple comparisons against a single control were made, analysis of variance was used. Analysis of covariance was used to compare different cumulative concentration-effect curves. Probability less than 0.05 was regarded as significant. The potency of papaverine in both compartments was expressed by the IC_{50} values (geometric mean ± 95% confidence limits) calculated from the individual concentration-response curve.

Results

Electrical field stimulation (10 Hz for 2 s) in the anal compartment elicited a single contraction of the circular muscle in the oral compartment which lasted about 2–3 s. These contractions were 34 ± 3% of the contraction produced by 10 μM carbachol applied to the oral compartment ($n=111$). The contractions elicited by electrical field stimulation were abolished by tetrodotoxin (0.6 μM, $n=4$) added to either compartment (Figure 2a and b) indicating that they were nerve mediated. Hexamethonium (100 μM) abolished the nerve mediated contractions when applied to the anal bath and greatly reduced them (92 ± 8% inhibition, $n=4$, $P<0.05$) when applied to the oral bath (Figure 2c and d) indicating the presence of nicotinic synapses in both chambers.

Papaverine produced a significant ($P<0.01$) concentration-dependent inhibition of the nerve-mediated contractions when applied to either compartment (Figure 3). Cumulative concentration-effect curves to papaverine were not significantly different when administered to the oral or anal compartments. IC_{50} (c.l.) values were 3.38 (2.84–4.38) μM for the oral compartment and 4.76 (1.67–13.57) μM for the anal compartment.

IBMX (10–300 μM, Figure 4a) and theophylline (30–1000 μM, Figure 4b) applied to the anal compartment reduced the nerve-mediated excitatory responses within 10 and 40 min, respectively. In contrast to papaverine (data not shown), the nerve-mediated contractions recovered with time even in the presence of these drugs. Maximum and stable recovery was achieved within 30 min and 60 min after IBMX (Figure 4a) or theophylline (Figure 4b) application, respectively. Papaverine, when added after IBMX or theophylline was significantly ($P<0.01$) less effective than when added alone (Figure 3). Phentolamine (1 μM), applied in the anal compartment, did not modify the nerve-mediated contractions (1 ± 5% inhibi-

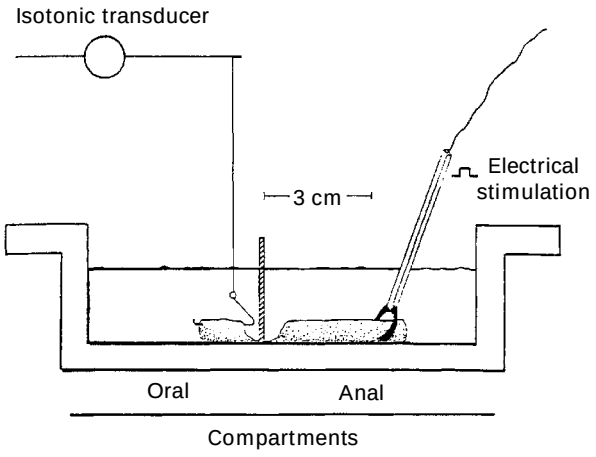

Figure 1 Diagram showing the partitioned bath for the separate application of drugs to enteric nerve pathways and the recording of the resulting contraction of the circular muscle.

tion, $P>0.2$, $n=15$) but significantly ($P<0.01$) reduced the inhibitory effect of papaverine (Figure 3) without modifying the action of IBMX 100 μM (% of control response after 10 min: IMBX 21 ± 7, phentolamine + IBMX 24 ± 8; % of control response after 30 min: IBMX 89 ± 7, phentolamine + IMBX 99 ± 10) or theophylline 300 μM (% of control response after 40 min: theophylline 47 ± 9, phentolamine + theophylline 49 ± 7; % of control response after 60 min: theophylline 95 ± 20, phentolamine + theophylline 100 ± 10). When papaverine was added after phentolamine 1 μM plus IBMX 100 μM or phentolamine 1 μM plus theophylline 300 μM it had no inhibitory effect (% of control response after phentolamine plus IBMX: 0.3 μM papaverine 100 ± 11; 1 μM papaverine 92 ± 8, 3 μM papaverine 104 ± 10, 10 μM papaverine 103 ± 9, 30 μM papaverine 97 ± 8, $n=5$; % of control response after phentolamine plus theophylline: 0.3 μM

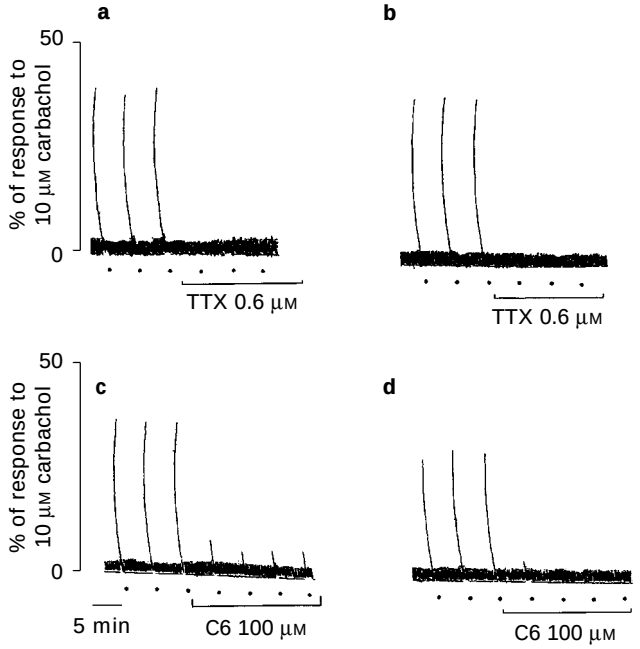

Figure 2 Traces showing the ascending excitatory response elicited by electrical stimulation of enteric pathways. The electrodes were placed on the intestine in the anal compartment about 30 mm from the partition. Trace (a) shows the effect of tetrodotoxin (TTX, 0.6 μM) added to the oral compartment, trace (b) shows the effect of TTX added to the anal compartment, trace (c) shows the effect of hexamethonium (C6, 100 μM) added to the oral compartment and trace (d) shows the effect of hexamethonium added to the anal compartment.

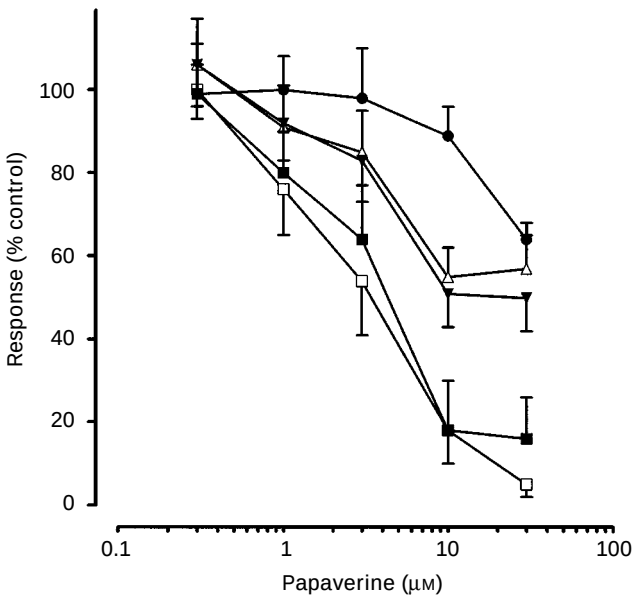

Figure 3 The inhibitory effect of papaverine added either to the oral (□) or the anal (■) compartment, on the ascending excitatory response elicited by electrical stimulation of enteric pathways. In the anal compartment, the effect of papaverine was also evaluated 30 min after 3-isobutyl-1-methylxantine (IBMX) 100 μM (△), 60 min after theophylline 300 μM (▼) or 20 min after phentolamine 1 μM (●). The electrodes were placed on the intestine in the anal compartment about 30 mm from the partition. The ordinates show the percentage of control response. Each point represents the mean of 5 experiments; vertical lines show s.e.mean.

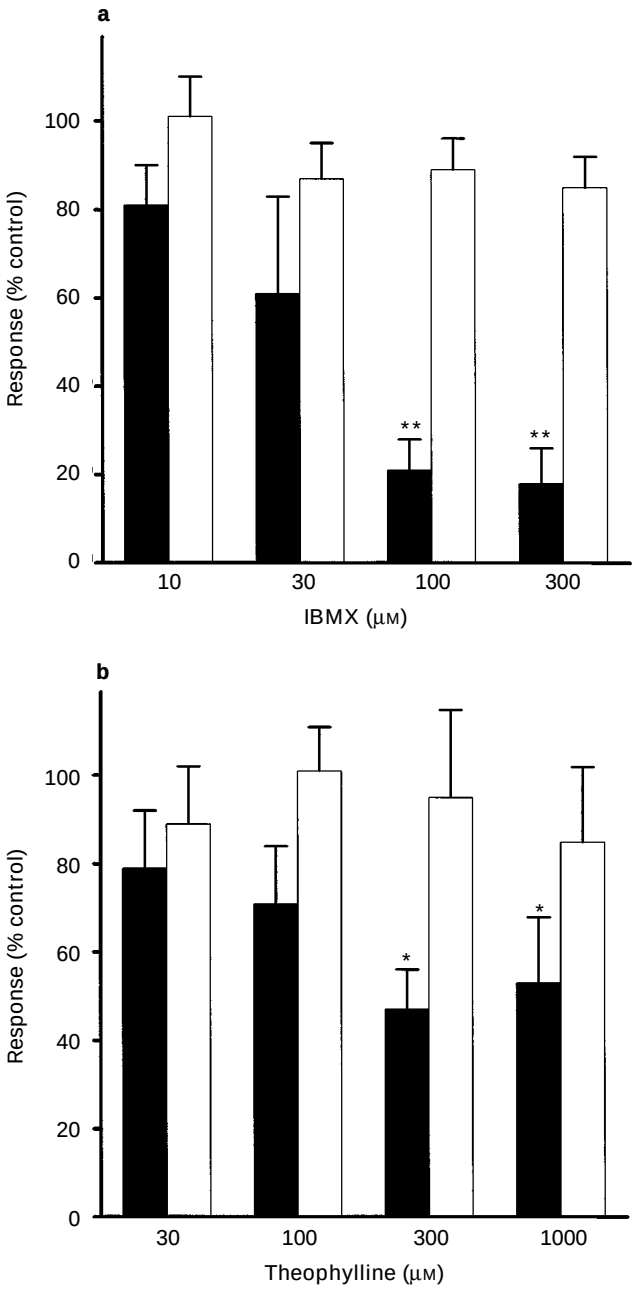

Figure 4 Ascending excitatory response elicited by electrical stimulation of enteric pathways after (a) 10 min (solid columns) or 30 min (open columns) exposure to 3-isobutyl-1-methylxanthine (IBMX) 10–300 μM and (b) after 40 min (solid columns) or 60 min (open columns) exposure to theophylline 30–1000 μM, both added independently to the anal compartment. The electrodes were placed on the intestine in the anal compartment about 30 mm from the partition. Each column represents the mean ± s.e.mean of 5–7 experiments. $^*P<0.05$ and $^{**}P<0.01$ vs control.

papaverine 101±11, 1 μM papaverine 93±8, 3 μM papaverine 89±10, 10 μM papaverine 103±8, 30 μM papaverine 97±8, $n=5$).

Verapamil (1 μM) and nifedipine (1 μM), two blockers of L-type calcium channels, inhibited the nerve-mediated contractions when applied to the oral compartment but not when applied to the anal compartment (Figure 5). The potent N-type calcium channel blocker ω-conotoxin GVIA (10 nM) practically abolished the nerve-mediated contractions when applied to either the oral or anal compartment (Figure 5).

When the electrode was placed 2 mm just anal to the partition the contraction in the oral compartment was abolished by tetrodotoxin ($n=4$) applied to the anal compartment, indicating that the responses were nerve-mediated. These contractions were unaffected by hexamethonium (5±4% inhibition, $P>0.2$, $n=5$), by 30 μM papaverine (11±6% inhibition, $P>0.2$, $n=5$) or by 300 μM IBMX and 1000 μM theophylline (data not shown) when these drugs were applied to the anal compartment.

Discussion

Within the intestinal wall there are orally directed nerve pathways which mediate the ascending excitatory reflex (Furness & Costa, 1987). Such reflex pathways involve a chain of orally directed interneurones as demonstrated by Tonini & Costa (1990). The electrical field stimulation used in this study is likely to have activated this ascending pathway. This conclusion is based on the similarity of actions of drugs. Tetrodotoxin abolished the oral contractions elicited by the electrical field stimulation in the anal compartment. This indicates that nerve pathways at least 35 mm long were activated. Along these electrically activated ascending pathways there are multiple nicotinic synapses as hexamethonium added to either compartment practically blocked the contractions. Thus it is likely that the ascending pathways activated in our experiments are similar to the pathways involved in the ascending excitatory reflex in which there are two or more nicotinic synapses (Tonini & Costa, 1990). Indeed the longest orally directed interneurones are less than 10 mm long (Brookes *et al*., 1991).

The inhibition of the nerve-mediated ascending contractions by papaverine when added to the oral compartment reflects its well known powerful smooth muscle relaxant action (Needleman *et al*., 1985). However, the inhibition of the nerve-mediated contraction recorded in the oral compartment by papaverine added to the anal compartment suggests that it has an inhibitory effect on enteric nerves. This inhibitory effect could be due to the action of papaverine on nerve action potentials. However tetrodotoxin, but not papaverine abolished the contraction elicited by the electrical stimulation applied just anal to the partition. Hexamethonium also had no effect on the contraction elicited by stimulation just anal to the partition. These results indicate that electrical stimulation just anal to the partition activates axons of ascending enteric neurones that do not make nicotinic synapses within the anal compartment. Thus the most likely interpretation of these results is that papaverine does not affect sodium channels.

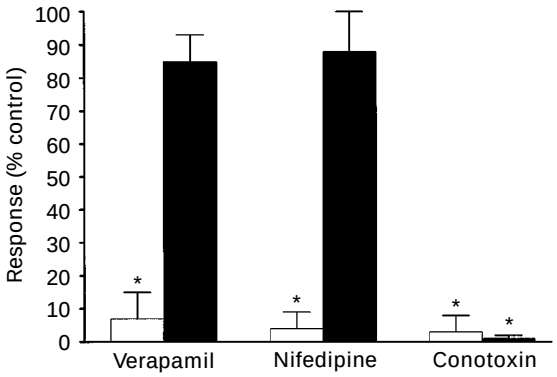

Figure 5 Effect of verapamil 1 μM, nifedipine 1 μM and ω-conotoxin GVIA (conotoxin) 10 nM added either to the oral (open columns) or the anal (solid columns) compartment, on the ascending excitatory response elicited by electrical stimulation of enteric pathways. The electrodes were placed on the intestine in the anal compartment about 30 mm from the partition. Each point represents the mean± s.e.mean of 5 experiments. *$P<0.01$ vs control.

Conversely when the electrical stimulation was applied at some distance from the partition papaverine inhibited the contraction. This pathway, as discussed above, involves nicotinic synapses. Therefore, it is most likely that the inhibitory action of papaverine on this nerve pathway is due to an action on nicotinic synaptic transmission. Also the PDE inhibitors, IBMX and theophylline (Smellie *et al*., 1979; Beavo, 1995), applied to the anal compartment inhibited the ascending excitatory responses. It is possible that in this preparation they also inhibit synaptic transmission. The partial recovery of the ascending contractions in the continuing presence of IBMX and theophylline probably reflects adaptative changes within the enteric neural pathways. It is unlikely that the effect of these two PDE inhibitors is due to their known action as adenosine antagonists as adenosine itself inhibits neural transmission (Wood, 1987).

The effect of these PDE inhibitors on enteric neural pathways may not be due to a single and common site of action. It is possible that the PDE inhibitors release, from some neural or non neural cells, an endogenous substance which in turn acts on the nerve terminals of the enteric neurones involved in the ascending excitatory pathways.

It has been demonstrated that papaverine and other PDE inhibitors increase the release of noradrenaline from sympathetic neurones (Cubeddu *et al*., 1974; Celuch *et al*., 1978; Boehm *et al*., 1994) and that cyclic AMP facilitates noradrenergic transmission to submucous neurones (Zafirov *et al*., 1993). Noradrenaline is the main neurotransmitter released from postganglionic sympathetic neurones acting on enteric neurones (Wood, 1987). Within myenteric ganglia noradrenaline acts presynaptically to prevent acetylcholine release (Wood, 1987). We have shown that phentolamine, an α-adrenoceptor antagonist (Hoffman & Lefkowitz, 1995), strongly reduces the inhibitory effect of papaverine indicating that noradrenaline is involved in the depressant effect of papaverine on synaptic transmission. Therefore it is most probable that the effect of papaverine may not be mainly due to PDE inhibition. However, it is unlikely that the inhibitory action of IMBX and theophylline involves the release of noradrenaline, since phentolamine failed to modify the depressant effect of the two PDE inhibitors. Indeed Celuch *et al*. (1978) showed that IBMX (500 μM) produced only a weak increase in noradrenaline release in the cat perfused spleen.

Other studies have shown that theophylline (Aikawa *et al*., 1992; Barlinski *et al*., 1992) and phosphodiesterase type IV inhibitors (Undem *et al*., 1994; Qian *et al*., 1994; Spina *et al*., 1995) reduce non-adrenergic non-cholinergic (but not cholinergic) nerve responses in the airways. In addition, cyclic AMP mimics slow synaptic excitation in the myenteric plexus (Palmer *et al*., 1987; Tamura & Wood, 1989) and enhances the release of acetylcholine from enteric neurones and noradrenaline from sympathetic neurones (Yau *et al*., 1987). Furthermore prostaglandin E_2 stimulates the release of acetylcholine in myenteric neurones through a cyclic AMP-dependent mechanism (Takeuchi *et al*., 1992; Mulholland & Simeone, 1993).

The inhibition of synaptic transmission could be due to an action of PDE inhibitors used on the calcium channels involved in synaptic transmission. Three different blockers of L-type Ca^{2+} channels (Spedding & Paoletti, 1992), i.e. verapamil and nifedipine in this work and nicardipine in Tonini & Costa (1990) were tested on the synaptic transmission in the ascending excitatory pathways and found to have no effect. Thus it is unlikely that neuronal L calcium channels are involved in

these neural pathways. In contrast the N-type Ca^{2+} channels are essential in the synaptic transmission in these pathways as conotoxin, a blocker of N-type Ca^{2+} channels (McCleskey *et al*., 1987), when applied to either compartment, blocked the nerve-mediated excitatory response.

In conclusion our study demonstrates that the PDE inhibitors papaverine, IBMX and theophylline, in addition to their smooth muscle relaxants effect, also inhibit synaptic transmission in the enteric nervous system. The inhibitory effect of papaverine (but not IBMX or theophylline) could involve, at least in part, the release of noradrenaline from sympathetic nerves acting on α-adrenoceptors on enteric neurones. In addition, this work suggests that enteric nerve pathways represent a useful model to investigate the mechanisms of action of drugs which affect intracellular messengers on synaptic transmission.

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