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Gastric motor responses elicited by vagal stimulation and purine compounds in the atropine-treated rabbit

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1 The effects of vagal inhibitory stimulation and of purine compounds were studied in the rabbit stomach.

2 Gastric motility was assessed by the balloon method. Vagal nerves were electrically stimulated at the neck. Purine compounds were injected intra-arterially.

3 In the atropine-treated rabbit, vagal stimulation caused relaxant motor responses followed by a rebound contraction.

4 Among the purine compounds, only ADP and ATP caused relaxant motor responses similar to the effects of vagal inhibitory stimulation. However, the relaxation produced by ATP was more powerful than that due to ADP, especially at lower infusion rates.

5 Vagal inhibitory responses were recorded during and after infusion of ATP. When relaxation by ATP was fully developed, vagal inhibitory stimulation was ineffective. At the highest infusion rates of ATP, a depression of the vagal inhibitory motility was also observed after cessation of the infusion.

6 Relaxant responses to ATP and vagal inhibitory stimulation were not influenced by theophylline, scarcely affected by α,β-methylene ATP, but were reduced or blocked by reactive blue 2.

7 The results are consistent with ATP being an inhibitory neurotransmitter in the stomach of the rabbit.

Introduction

The nature of the transmitter substance released by vagal postganglionic nerves and responsible for non-adrenergic, non-cholinergic inhibitory motility in the gastrointestinal tract of mammals has not been definitely established. In fact, it is possible that more than one transmitter is directly involved in the relaxant effects (Roman, 1982). Until now, vasoactive intestinal peptide (VIP) and/or adenosine 5'-triphosphate (ATP) have been considered to be the most likely inhibitory neurotransmitters (Burnstock, 1972; 1981; Fahrenkrug, 1979). While VIP may cause slow and long-lasting motor events, the response to ATP resembles the rapid motor responses of the intestine to non-adrenergic inhibitory nerve stimulation (Cocks & Burnstock, 1979; Mackenzie & Burnstock, 1980; Hills *et al.*, 1983). However, in both a variety of nerve-muscle preparations and *in vivo* experiments, ATP or related compounds have been demonstrated not to mimic the non-adrenergic, non-cholinergic motility elicited by neural stimulation (Ohga & Taneike, 1977; Baer & Frew, 1979; Rattan & Goyal, 1980; Daniel *et al.*, 1983; Andrews & Lawes, 1985; Lefebvre, 1986). So the role of ATP as an inhibitory transmitter is still a matter of controversy (Furness & Costa, 1982; Andrews, 1986). Recently, it has been observed that intra-arterially injected ATP causes relaxant responses in the stomach of the rabbit (Brizzi *et al.*, 1984). The following experiments were performed to investigate gastric motor responses evoked in the rabbit by purine compounds and vagal stimulation.

Methods

Fifty-seven male rabbits (2.5–3.5 kg) were fasted for 18–20 h and then anaesthetized, via the ear vein, with urethane and sodium pentobarbitone (nembutal) ($500\,mg\,kg^{-1} + 10\,mg\,kg^{-1}$). A tracheal cannula permitted a free airway. Gastric intubation was performed via the oesophagus. The stomach was washed with physiological saline; the residual

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luminal content was then gently aspirated. Abdominal surgery was not performed as it might have caused relaxation of the stomach (Abrahamsson, 1973). A large flaccid balloon, maximal content 200 ml, introduced via the oesophagus into the gastric lumen, was filled with warm water (37°C) and connected to a wide diameter reservoir. Gastric motility was recorded isotonically (Setekleiv, 1964; Abrahamsson, 1973) with a pressure transducer (Sanborn 228-BC) connected to the reservoir and coupled to a polygraph (Sanborn 7700). Calibration was made by injection of fluid in steps of 1 ml. Gastric motor responses were evoked at low intragastric pressure under an adjustable load of 4–7 cm of water. The vagi, dissected at the neck and cleared of connective tissue for a length of 2–3 cm, were ligated centrally and then crushed. The peripheral ends of the nerves, placed on platinum electrodes bathed in paraffin oil, were electrically stimulated. Square pulse stimulus trains (10–15 V amplitude, 1–3 ms pulse duration, 2–24 Hz pulse frequency, 30 s–1 min train duration) were applied to both nerves by a Grass S8 stimulator equipped with stimulus isolation units. The electrodes were periodically moved caudally to prevent damage due to a prolonged stimulation. A thin polyethylene catheter was inserted into a femoral artery and passed up the aorta to a level of 2–3 cm above the origin of the coeliac artery. The position of the catheter was confirmed by post-mortem examination. Arterial blood pressure was recorded using a pressure transducer (Sanborn 267 BC) and a catheter which had been introduced into a carotid artery. The rectal temperature of the animal was maintained at 37.5°–38°C during the experiment. Motor responses were evoked at basal gastric volume ranging from 60 to 80 ml.

Drugs

Guanethidine sulphate (Ciba) (0.5–1 mg kg^{-1}), propranolol HCl (Sigma) (1–2 mg kg^{-1}), phentolamine (Regitin, Ciba) (1–2 mg kg^{-1}), atropine sulphate (BDH) (1–2 mg kg^{-1} min^{-1}), adenosine (Boehringer) (5–40 μmol kg^{-1} min^{-1}), adenosine 5'-monophosphate, disodium salt (Boehringer) (AMP, 5–40 μmol kg^{-1} min^{-1}), adenosine 5'-diphosphate, disodium salt (Boehringer) (ADP, 5–40 μmol kg^{-1} min^{-1}), adenosine 5'-triphosphate, disodium salt (Sigma) (ATP, 5–60 μmol kg^{-1} min^{-1}), theophylline (BDH) (50–200 μmol kg^{-1} min^{-1}), α,β-methylene adenosine 5'-triphosphate (Sigma) (mATP, 100–600 μg kg^{-1}) and reactive blue 2 (Sigma) (7–10 μmol kg^{-1} min^{-1}) were used. Adenosine, AMP, ADP, ATP, theophylline, (infusion time 1–3 min) and reactive blue 2 (infusion time 5–25 min) were dissolved in physiological saline and administered via the femoral catheter with an infusion pump (Harvard 975). mATP was injected in the femoral artery as bolus injections. Guanethidine, propranolol and phentolamine were administered via a femoral vein (bolus injections). Atropine was also infused in the same way and the infusion was repeated during the experiment when necessary.

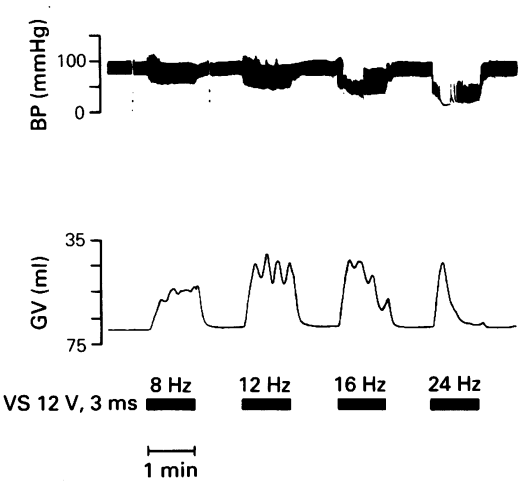

Figure 1 Effects of vagal stimulation on gastric volume in the anaesthetized rabbit before administration of atropine. BP, blood pressure. GV, gastric volume. VS, vagal stimulation.

Results

Excitatory and inhibitory motor responses elicited by vagal stimulation

Before administration of atropine, stimulation of peripheral ends of the cervical vagi caused excitatory motility in the stomach of the rabbit ($n = 15$ rabbits). The amplitude of the motor responses increased with stimulation frequency from 2 to about 12 Hz. The maximal amplitude response was 24.3 ± 0.91 ml (mean ± s.d.). At or above 16 Hz, motor responses were not sustained for the whole period of stimulation: the amplitude rapidly declined after 20–30 s of stimulation (Figure 1).

Infusions of atropine, 1 mg kg^{-1} min^{-1} for 1–2 min, blocked vagal excitatory motility, while at a higher infusion rate (2 mg kg^{-1} min^{-1} for 2 min) the excitatory response was reversed to an inhibitory one.

In the atropine-treated rabbit, the inhibitory response ($n = 10$ rabbits), was maintained during the whole period of stimulation (1 min), from 2 to 24 Hz (Figure 2). Maximal inhibitory responses (1.9 ± 0.5 ml) were generally achieved at a stimu-

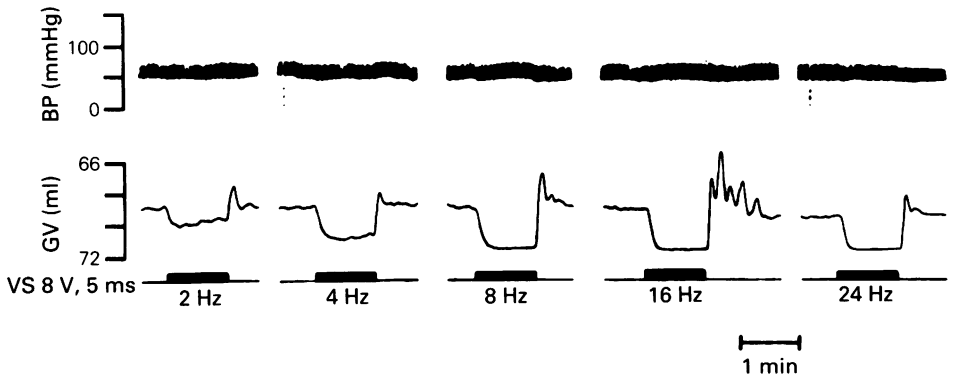

Figure 2 Gastric inhibitory motor responses to vagal stimulation in the anaesthetized rabbit after administration of atropine. BP, blood pressure. GV, gastric volume. VS, vagal stimulation.

lation frequency of about 16 Hz; thus indicating that vagal efferent inhibitory fibres were more fully activated at a higher frequency compared to excitatory ones, but the inhibitory response also developed at a lower frequency (10–12 Hz). After the end of stimulation no depression of gastric tone was observed, but gastric relaxation was generally followed by a rebound contraction which lasted from 30 s to 1–2 min. The maximal amplitude of rebound contraction was obtained after stimulation frequencies of 16–20 Hz.

Basal gastric volume changes, in the above defined range, did not influence inhibitory motility.

Guanethidine, propranolol and phentolamine did not modify the relaxant response ($n = 5$ rabbits).

Motor responses elicited by adenosine

Adenosine, at an infusion rate below 15 μmol kg^{-1} min^{-1}, did not evoke gastric motor responses in 9 rabbits ($n = 15$ animals) treated with atropine and guanethidine. In six animals the motor responses consisted of a brief, slight relaxation which did not last more than a few seconds.

These relaxant responses were generally obtained at a minimum infusion rate of adenosine of 15–20 μmol kg^{-1} min^{-1}. By increasing the infusion rate, the relaxant response tended to be maintained for the total period (1 min) of infusion (Figure 3). Maximal relaxation with adenosine was 0.7 $\pm$ 0.3 ml (mean $\pm$ s.d.). However, consistent and reproducible inhibitory motor responses were not always evoked even at the highest infusion rate (30–40 μmol kg^{-1} min^{-1}). At the end of the infusion, the relaxant response was followed by an increase of gastric tone (a sort of rebound contraction), but this increase was also noted during the infusion of adenosine, especially after relaxation of brief duration. In two experiments adenosine gave a biphasic response: contraction followed by relaxation.

Motor responses elicited by AMP

The inhibitory effect of an arterial infusion of AMP was very similar to that elicited by adenosine ($n = 10$ animals) (Figure 3). The relaxant response was manifested at an infusion rate of about 20 μmol kg^{-1} min^{-1}. The maximal amplitude

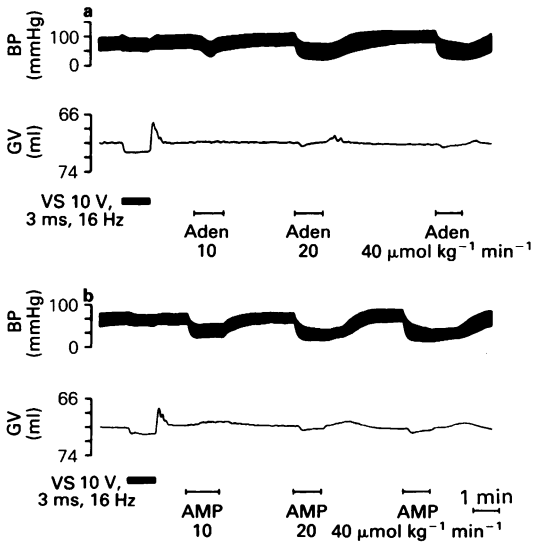

Figure 3 Gastric effects of intra-arterial infusions of (a) adenosine (Aden) and (b) AMP in the anaesthetized atropine-treated rabbit. BP, blood pressure. GV, gastric volume. VS vagal stimulation.

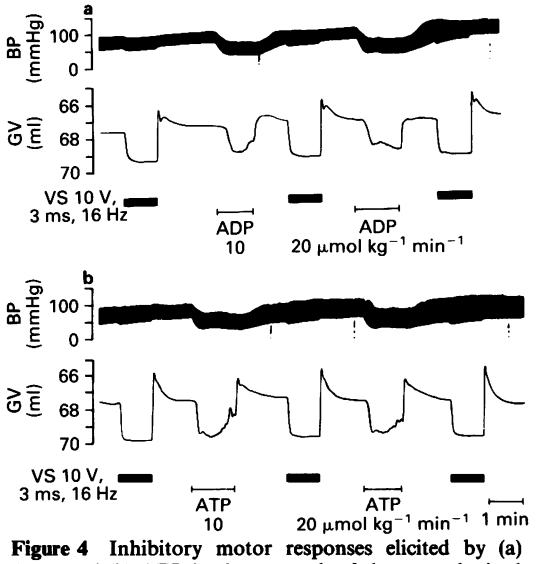

Figure 4 Inhibitory motor responses elicited by (a) ADP and (b) ATP in the stomach of the anaesthetized atropine-treated rabbit. BP, blood pressure. GV, gastric volume. VS, vagal stimulation.

response, for any given concentration, was the same as that evoked by adenosine. However, the relaxation was more reproducible and maintained, during the infusion, with AMP compared to adenosine. Biphasic responses or increase of gastric tone during the infusion were not observed.

Motor responses elicited by ATP

Intra-arterially injected ATP promptly caused a relaxant motor response in the rabbit stomach ($n = 15$ animals). Biphasic or excitatory responses were never observed. Relaxation began 10–15 s after the start of the infusion. At lower infusion rates (5–10 μmol kg^{-1} min^{-1}), after an initial relaxation peak, the motor response was stabilized at a lower level. Generally no decay of the initial amplitude peak was observed at infusion rates at or above 20–25 μmol kg^{-1} min^{-1}. However, decay of this initial amplitude peak was sometimes observed after repeated infusions. Maximal responses (2.1 $\pm$ 0.5 ml) were elicited at infusion rates of 30–35 μmol kg^{-1} min^{-1}. At the end of the infusion, the relaxant response was usually followed by a rebound contraction (Figure 4). No rebound contraction was noted in only two experiments. After prolonged infusion (3 min) at infusion rates of 30–40 μmol kg^{-1} min^{-1} a reduction of gastric tone which lasted several minutes was observed. The gastric volume slowly returned to the basal level in about 3–8 min.

Motor responses elicited by ADP

Gastric motility evoked by ADP ($n = 10$ animals) strictly resembled that elicited by ATP. The only difference noted was that the relaxation caused by ADP was less intense than that induced by ATP at lower infusion rates (5–15 μmol kg^{-1} min^{-1}) (Figure 4).

The inhibitory responses evoked by ADP and ATP were not influenced by guanethidine, adrenoceptor blockers and basal volume changes.

Effects of ATP on inhibitory vagal motor responses

Basal gastric volume changes do not affect vagal inhibitory motor responses; therefore, if ATP and

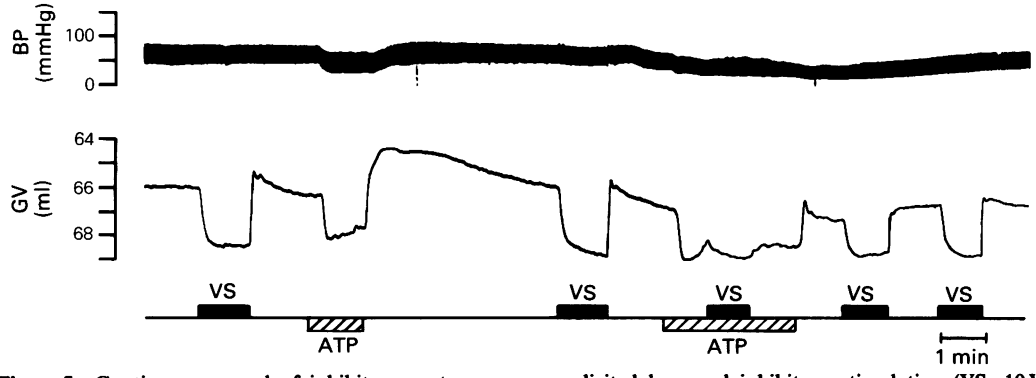

Figure 5 Continuous record of inhibitory motor responses elicited by vagal inhibitory stimulation (VS; 10 V, 3 ms, 16 Hz) and ATP (25 μmol kg^{-1} min^{-1}). During relaxation by ATP a slight vagal inhibitory response was still present. BP, blood pressure. GV, gastric volume.

vagal inhibitory stimulation act on different relaxant mechanisms, during relaxation induced by ATP the inhibitory vagal responses must be unaffected. To verify this, the effect of ATP on vagal inhibitory responses was studied in 7 rabbits.

At infusion rates below 30 μmol kg^{-1} min^{-1}, for 3 min, vagal stimulation still evoked relaxant responses during the inhibitory effects of ATP. At infusion rates of 30–35 μmol kg^{-1} min^{-1}, vagal inhibitory responses were not present and the rebound contraction was absent (Figures 5 and 6). After the end of the infusion, motor responses to vagal stimulation were immediately restored (Figure 5). At the highest infusion rates (50–60 μmol kg^{-1} min^{-1}) vagal stimulation was ineffective, even after the end of infusion (Figure 7). The absence of relaxant effects to vagal stimulation lasted 7 ± 1.7 min (mean ± s.d.); the rebound contraction was greatly reduced or abolished. Gastric volume returned slowly (after about 10 min or more) to the basal level. The reappearance of vagal motor responses after the infusion was independent of the gastric volume. The rebound contraction tended to reappear and recover earlier than the vagal relaxation. The full recovery of inhibitory motor responses occurred about 15–20 min after the end of the infusion.

Effects of theophylline, α,β-methylene ATP and reactive blue 2 on relaxations evoked by vagal stimulation and ATP

Since in these experiments ATP seems to mimic vagal inhibitory stimulation, the effects of theophylline, α,β-methylene ATP and reactive blue 2 on gastric inhibitory motility were studied.

Theophylline Theophylline, a P_1-purinoceptor antagonist (Burnstock, 1978), was tested in 5 rabbits. Bolus injections or infusions of theophylline (50–100 μmol kg^{-1} min^{-1} for 1 min) had no effect on inhibitory responses: relaxation by vagal stimulation and ATP was not affected after theophylline. Following repeated infusions, theophylline caused marked and sustained gastric relaxation.

α,β-Methylene ATP This ATP analogue has been demonstrated to cause desensitization of P_2-purinoceptors (Kasakov & Burnstock, 1983) and, in the cat stomach, abolition of relaxation to vagal stimulation and ATP (Delbro & Fändriks, 1984). These effects were not confirmed in the ferret (Andrews & Lawes, 1985). In our experiments ($n = 5$ rabbits) α,β-methylene ATP, at doses of 200–300 μg kg^{-1}, evoked gastric relaxation, but no

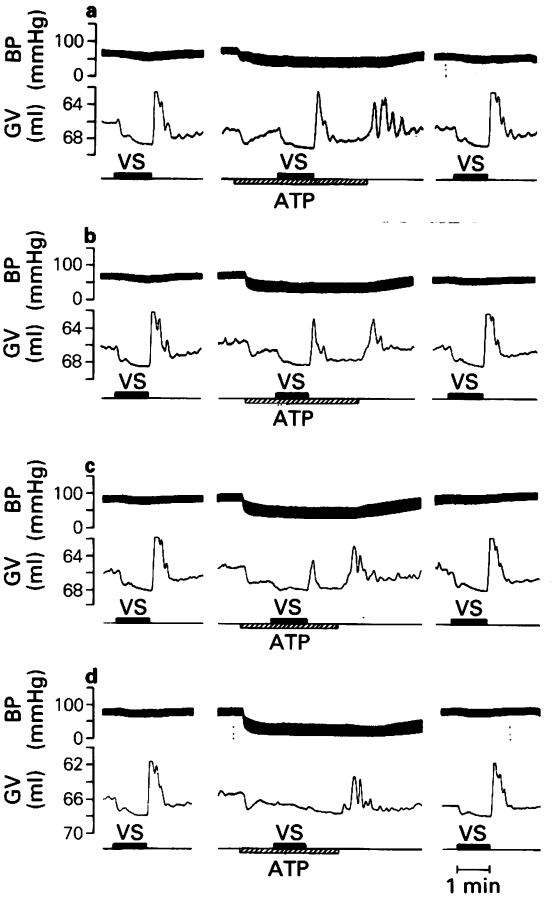

Figure 6 Inhibitory motility elicited by vagal stimulation (VS; 10 V, 3 ms, 16 Hz) during the relaxation induced by different concentrations of ATP; (a) 5, (b) 10, (c) 20 and (d) 40 μmol kg^{-1} min^{-1}. Vagal inhibitory responses were also recorded 5 min before and 5 min after the infusion of ATP. Note that in (d), after repeated infusions, the slackness of the relaxant responses elicited by ATP and the absence of vagal relaxation during the infusion; 5 min after the infusion, gastric volume had not returned to the basal level. BP, blood pressure. GV, gastric volume.

reduction of gastric responses to vagal stimulation and ATP was observed. Higher doses, up to 600 μg kg^{-1} (single or repeated injections), could also cause a reduction of these motor responses. However, the influence of α,β-methylene ATP on inhibitory motor responses was inconsistent (present only in 30% of observations) and of brief duration. The responses to ATP and vagal inhibitory stimulation were fully restored 3–4 min after the end of the infusion of the ATP analogue (Figure 8). Further-

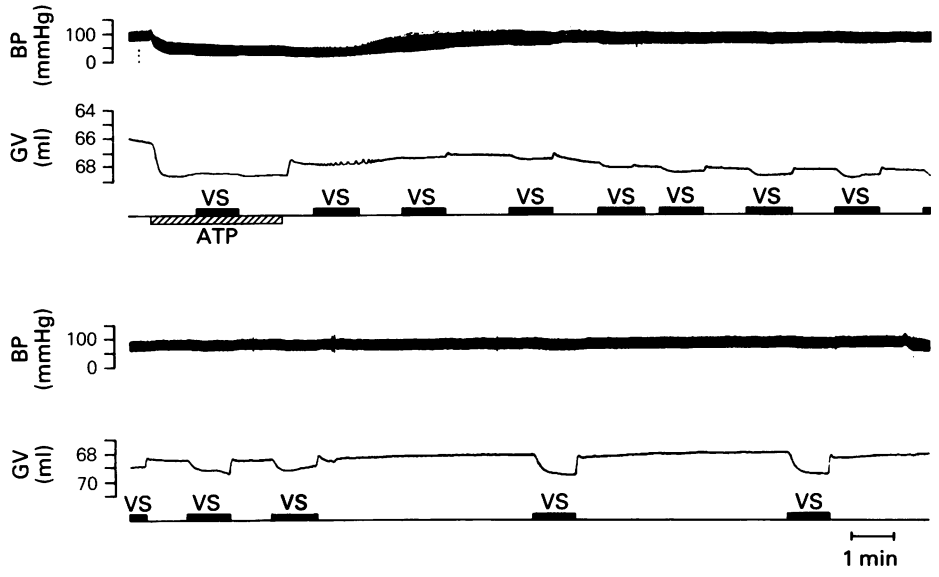

Figure 7 Depression of inhibitory responses elicited by vagal stimulation (VS; 10 V, 3 ms, 16 Hz) caused by arterial infusion of ATP 55 μmol kg^{-1} min^{-1}. BP, blood pressure, GV, gastric volume.

more, high doses of α,β-methylene ATP, especially as a single bolus injection, were difficult to handle since they could have deleterious effects on arterial blood pressure: after an initial rise there could be a relentless decline in blood pressure itself.

Reactive blue 2 Reactive blue 2, a P_{2Y}- purinoceptor antagonist (Kerr & Krantis, 1979; Burnstock *et al.*, 1986; Manzini *et al.*, 1986a), was injected into 5 rabbits. At an infusion rate of 7–10 μmol kg^{-1} min^{-1}, for 5–10 min, reactive blue 2 caused a progressive reduction and finally (about 15–20 min from the end of the infusion) a concomitant disappearance of relaxant responses elicited by vagal stimulation and ATP (Figure 9, upper trace). The block of gastric relaxation could persist up to 30 min and then tended to be slowly reversed. The influence of reactive blue 2 was also studied on vagal excitatory motor responses (n = 5 rabbits). Doses which blocked inhibitory motor responses did not influence excitatory ones (Figure 9, lower trace). A depression of excitatory motility was observed after about 20 min of infusion of reactive blue 2 (8–10 μmol kg^{-1} min^{-1}, continuous or interrupted infusions) (Figure 10). The reduction of the maximal amplitude of the excitatory response reached 70% of the control value. Excitatory motor responses could also recover after about 10 min, but generally longer periods were needed.

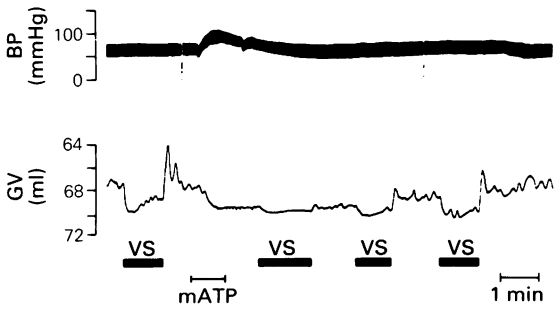

Figure 8 Effects of α,β-methylene ATP (mATP, 400 μg kg^{-1}) on gastric volume and relexation induced by vagal stimulation (VS; 13 V, 3 ms, 16 Hz). Note also the pressor action of α,β-methylene ATP (see Delbro & Burnstock, 1987). BP, blood pressure. GV, gastric volume.

Discussion

In the rabbit treated with atropine, gastric inhibitory motor responses were easily obtained by vagal stimulation. These relaxant motor responses were evoked in the same range of stimulation frequency and at the same stimulus intensity as those elicited by excitatory motor responses. Therefore, no marked differences exist between electrical activation of cholinergic excitatory and non-adrenergic, non-cholinergic inhibitory efferent vagal fibres. However, the decay of excitatory motility and the maximal

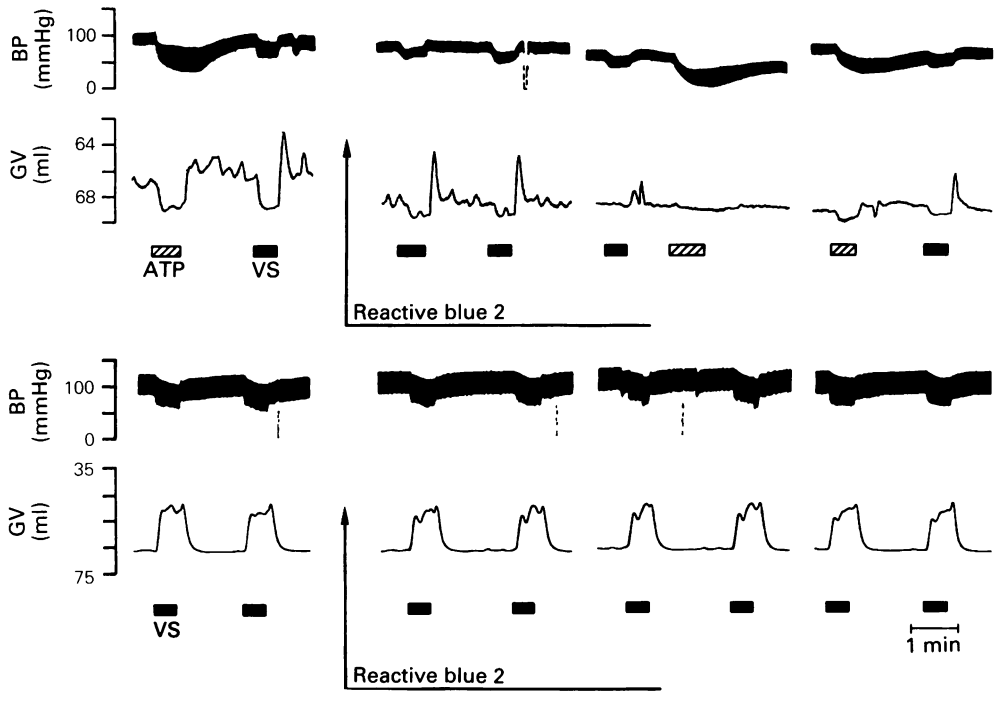

Figure 9 Upper record: blockade by reactive blue 2 (8 μmol kg^{-1} min^{-1}, infused for 10 min) of gastric inhibitory motor responses elicited by vagal stimulation (VS; 14 V, 3 ms, 16 Hz) or ATP (20 μmol kg^{-1} min^{-1}). Lower record: excitatory responses to vagal stimulation (14 V, 3 ms, 12 Hz). Upper and lower records, from left to right: inhibitory and excitatory responses 2 min before and 7 min, 15 min and 30 min after the infusion of reactive blue 2 (records from two different experiments). BP, blood pressure. GV, gastric volume.

amplitude of the inhibitory response were obtained at a stimulation frequency of 16 Hz or more, indicating that the full activation of inhibitory vagal fibres occurs at frequencies higher than those required for excitatory vagal fibres. The lack of inhibitory vagal gastric responses in the atropine-treated rabbit found by Gustafsson (1981) may be explained by different operating and recording methods.

Relaxant responses were unaffected by guanethidine, which causes inhibition of responses to adrenergic nerves (Chang *et al.*, 1965), and by adrenoceptor blocking agents (propranolol and phentolamine), thus excluding adrenergic motor effects.

At the end of stimulation the relaxant response was followed by a rebound contraction, further indicating the involvement of non-adrenergic, non-cholinergic nerves (Burnstock *et al.*, 1975).

In the atropine-treated rabbit, purine compounds (particularly ATP and ADP) caused gastric inhibitory motility. Adenosine and AMP, even at the highest infusion rates, elicited only inconsistent relaxant responses which were not comparable with those evoked by vagal inhibitory stimulation. So it is unlikely that adenosine and AMP are involved as neurotransmitters in gastric inhibitory motility. Furthermore, with adenosine, excitatory motility was also observed, thus excluding a direct inhibitory role. Clear inhibitory motor responses were evoked only by arterial infusions of ADP and ATP. These responses were unaffected by guanethidine, phentolamine and propranolol, and resembled the effects of vagal inhibitory stimulation. However, at low infusion rates, the relaxation induced by ADP was less intense than that induced by ATP. This implies a lower gastric relaxation threshold for ATP and so the latter, rather than ADP, closely mimics vagal inhibitory stimulation. In our experimental conditions, excitatory motility induced by ATP or ADP was never observed and our results are in accordance with the inhibitory effects of ATP observed in the stomach of the cat (Delbro & Fändriks, 1982). Species differences may explain different results *in vivo* (Andrews & Lawes, 1985; Andrews, 1986).

Methodological considerations must also be taken into account. In these experiments, gastric motility was recorded from the whole stomach and we were not able to detect if ATP had excitatory effects on

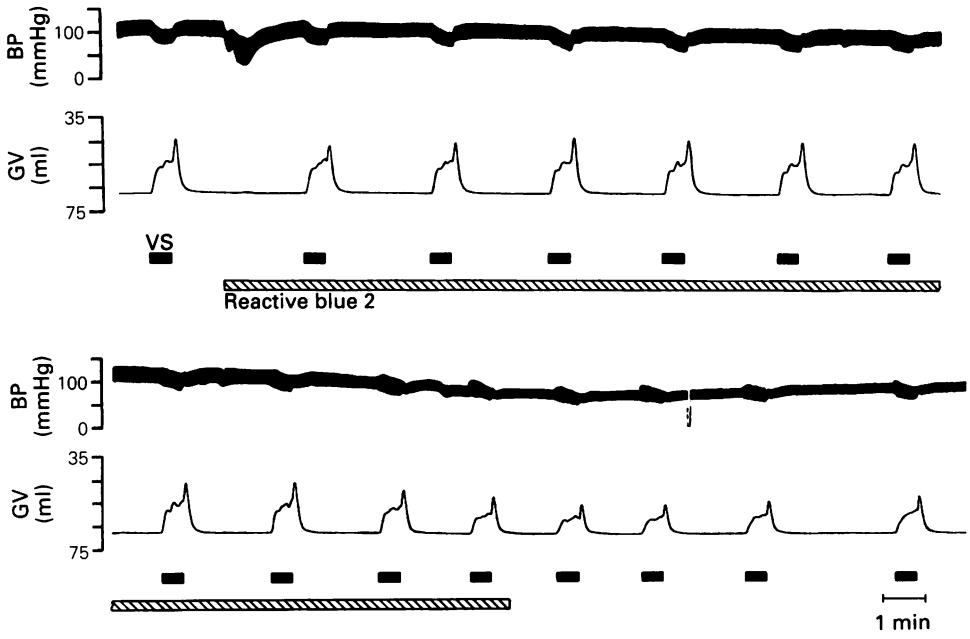

Figure 10 Influence of a continuous prolonged infusion of reactive blue 2 ($8\,\mu mol\,kg^{-1}\,min^{-1}$) on excitatory motor responses elicited by vagal stimulation (VS; 14 V, 3 ms, 12 Hz). Five to six minutes after the end of the infusion, which lasted 26 min, excitatory motor responses began to recover. BP, blood pressure. GV, gastric volume.

certain parts of the stomach as observed *in vitro* on fundic strips of pig, guinea-pig and rat (Ohga & Taneike, 1977; Baer & Frew, 1979; Lefebvre, 1986). Further, in different regions of the gastrointestinal tract, in the same animal species, ATP may cause excitatory or inhibitory motility (Delbro & Fändriks, 1982; Hedlund *et al.*, 1983; Andrews, 1986; Manzini *et al.*, 1986b). Thus, we cannot exclude that, in the stomach of the rabbit, ATP may have some local excitatory action, especially considering also the different motility patterns and functions of the proximal and distal stomach (Haffner, 1971; Haffner & Stadaas, 1972; Kelly, 1981). However, even if theoretically this action might occur, the effect *in toto* is a constant clear gastric relaxation.

ATP seems to play a direct role as an inhibitory transmitter in the stomach of the rabbit since: (1) excitatory motility after arterial infusions of ATP was never observed; (2) ATP promptly caused relaxant responses of the stomach of the same amplitude as those evoked by vagal stimulation; (3) relaxation by ATP was sustained for the whole period of infusion; (4) at the end of the infusion the inhibitory response was followed by a rebound contraction as occurred after vagal stimulation.

Furthermore, during infusion of ATP, and when the inhibitory effect of ATP was fully developed, the inability of vagal stimulation to evoke further relaxation (in a range of gastric volumes in which the amplitude of the inhibitory response, caused solely by vagal stimulation, was unaffected by basal gastric volume changes) suggests that vagal stimulation acts on a relaxant mechanism previously engaged by ATP. This is also supported by the absence of vagal relaxant effects after the infusion of ATP at the highest rate. This effect might resemble the desensitization phenomenon already described *in vitro* (Burnstock *et al.*, 1970; Maggi *et al.*, 1984).

With regard to the mechanism underlying the effects of ATP, it has been suggested that ATP might act on purinoceptors and particularly on P_2-purinoceptors (Burnstock, 1978; 1981). Recently it has been proposed that there are two subclasses of the ATP receptor; P_{2X}-purinoceptors mediate excitation of smooth muscle which can be blocked by selective desensitization with α,β-methylene ATP; P_{2Y}-purinoceptors mediate relaxation of smooth muscle which can be antagonized by reactive blue 2 (Burnstock & Kennedy, 1985; Manzini *et al.*, 1986a). The influence of reactive blue 2 demonstrated in this study suggests that P_{2Y}-purinoceptors might be the basis for the action of ATP and vagal inhibitory activity in the rabbit stomach. The brief and inconstant influence of α,β-methylene ATP compared with the effect of ATP on reducing vagal inhibitory responses of the stomach, indicating an order of

potency of ATP > α,β-methylene ATP, is consistent with this view. Doses of reactive blue 2 that blocked the inhibitory responses did not affect the excitatory cholinergic responses of the stomach. However, with higher doses, reactive blue 2 reduced the excitatory motor responses too, indicating that this compound can act on sites other than purinoceptors, as observed *in vitro* (Choo, 1981). To determine whether this is a non-specific phenomenon requires further investigation. More *in vivo* experiments need to be done to study the effects of purinoceptor agonists and antagonists and the role of purinoceptors on gastric inhibitory motility.

In conclusion, our experiments demonstrate that, in the rabbit stomach, ATP closely mimics vagal inhibitory stimulation and indicate that, presumably, ATP and vagal inhibitory stimulation act on the same relaxant mechanism.

To confirm the role of ATP in non-adrenergic, non-cholinergic transmission it would be useful to see if, when vagal excitatory motor responses are maximally developed, arterial infusions of ATP cause a decay in this motility such as that observed at the highest stimulation frequency when inhibitory vagal fibres are also fully activated). Ongoing experiments support this idea.

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