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Purine receptors in the guinea-pig internal anal sphincter

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1 In the isolated internal anal sphincter of the guinea-pig, adenosine 5′-triphosphate (ATP) and adenosine induced a concentration-dependent and tetrodotoxin-insensitive relaxation.

2 Pretreatment with theophylline (25–50 μM) had no significant effect on the concentration-response curves obtained with either purine compound.

3 Reactive blue 2 (25–100 μM) shifted the curve to ATP to the right in a dose-dependent fashion leaving that to adenosine unaltered. The antagonism appeared to be non-competitive.

4 Neither reactive blue 2 nor purine receptor occupation by ATP or adenosine altered the electrically-induced non-adrenergic, non-cholinergic inhibitory response.

5 The actions of ATP and adenosine in the guinea-pig internal anal sphincter appear to be mediated by separate receptors. These receptors are not involved in the nerve-mediated relaxation.

Introduction

The existence of intrinsic inhibitory neurones in the mammalian gastro-intestinal tract that are neither adrenergic nor cholinergic is well established. Burnstock, Campbell, Satchell & Smythe (1970) proposed that a purine compound (the most likely candidate being ATP) was the final mediator involved in such responses and that the non-adrenergic, non-cholinergic nerves should be regarded as purinergic nerves (Burnstock, 1972). Results continue to appear both in support and against the involvement of purine compounds in enteric inhibition (see Stone, 1981), this uncertainty being essentially derived from the lack of specific ATP antagonists (Burnstock *et al.*, 1970; Satchell, Burnstock & Dann, 1973; Spedding, Sweetman & Weetman, 1975; Muller & Baer, 1980). Recently, the presence of different types of purine receptors (purinoceptors) both in extra-intestinal (Van Calker, Muller & Hamprecht, 1979; Londos, Cooper & Wolff, 1980; Burnstock & Meghji, 1981) and intestinal preparations (Spedding & Weetman, 1976; Burnstock, 1976; Bartlett, Stewart & Nakatsu, 1979) has been demonstrated. Burnstock (1978) proposed two types of purinoceptor (P_1-receptors most sensitive to adenosine and competitively blocked by theophylline and P_2-receptors most sensitive to ATP and unaffected by methylxanthines). In the present work, theophylline and reactive blue 2 (an anthraquinone-sulphonic acid derivative recently proposed as an ATP antagonist, Kerr & Krantis, 1979; Choo, 1980) were used to define the type of receptors subserving the muscular action of both ATP and adenosine in the guinea-pig internal anal sphincter. Moreover, the involvement of purinoceptors in the electrically-induced nerve-mediated relaxation was studied by employing either reactive blue 2 or full purinoceptor occupation by both ATP and adenosine, as recently described by Romano (1981) in rat colon.

Methods

Guinea-pigs of either sex (400–500 g) were killed by cervical dislocation and bled. A segment of terminal rectum plus the external and internal anal sphincters was removed and immersed rapidly in Tyrode solution (composition mM: NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.04, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, and glucose 5.55). The internal anal sphincter was dissected from the surrounding tissues and cut transversely to obtain a strip 6–8 mm long (unstretched) and about 2 mm wide. After removal of the mucosa the internal anal sphincter strip was set up isometrically (tension applied 1–1.5 g) in a 10 ml

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organ bath containing Tyrode solution kept at 36 °C and bubbled continuously with a mixture of 95% O_2 and 5% CO_2. The preparations were equilibrated for 90 min the bathing solution being changed at 15 min intervals. Since ATP and adenosine did not show tachyphylaxis in this preparation, the concentration-response curves for the relaxant effects were obtained by the stepwise cumulative increase in the concentration of the agents. Each concentration was added immediately after a steady response had been obtained to the preceding dose. Concentration-response curves were constructed in the absence or in the presence of either theophylline (25–50 μM) or reactive blue 2 (25–50–100 μM), which were added for 30 and 60 min, respectively, before the agonists. The antagonists were replaced after each wash during the period of incubation. Responses to both purine compounds were expressed as percentages of the maximum response induced by ATP (250 μM). In preparations used to study the electrically-induced inhibition, transmural stimulation (at supramaximal voltage, 0.5, 1, 2, or 3 Hz, with a pulse duration of 0.5 ms) was applied in the presence of hyoscine (2.2 μM) and guanethidine (5 μM) via two platinum loop electrodes. The interval between two consecutive stimulations was 3 min and the duration of stimulation was 6 s. Responses were expressed as a percentage of that induced by 5 Hz stimulation (taken as 100% response). Frequency-response curves were obtained in the absence of drugs, in the presence of reactive blue 2 (50–100 μM for 60 min) and in conditions of purinoceptor occupation with either ATP or adenosine. Saturation of purinoceptors was accomplished by adding concentrations of ATP or adenosine which induced maximum and persistent relaxations (each at 250 μM). In the continued presence of ATP or adenosine, once tone had been restored (and when necessary maintained) to the original level by noradrenaline (15–25 μM), the purinoceptor occupation was confirmed by the absence of any response to further additions of purine compounds given 0.5–1 min before eliciting electrical stimulation. In some experiments, this procedure allowed a final concentration of ATP or adenosine of 1 mM to be attained, which did not affect submaximal relaxations induced by isoprenaline (0.1–3 μM). The drugs used were: adenosine 5'-triphosphate (BDH), adenosine (BDH), hyoscine hydrobromide (BDH), guanethidine sulphate (Ciba), theophylline (BDH), (−)-noradrenaline hydrochloride (Sigma), (−)-isoprenaline bitartrate (Sigma), reactive blue 2 (Sigma) and tetrodotoxin (Sankyo). Doses refer to the bath concentrations.

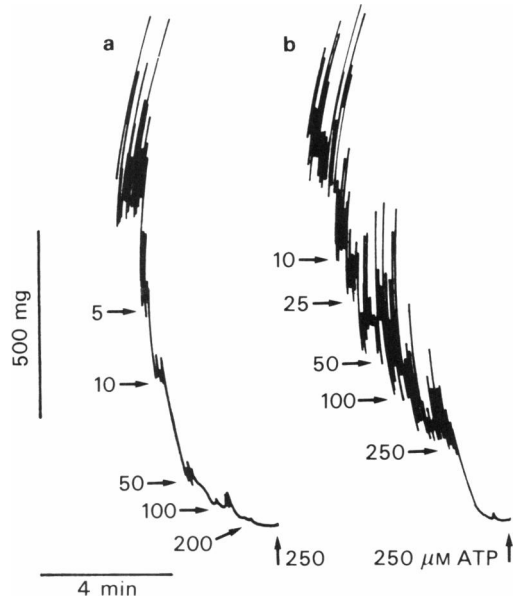

Figure 1 Cumulative concentration-response curves for ATP (a) and adenosine (b) in relaxing the guinea-pig internal anal sphincter. Each purine concentration (μM) was added as soon as the previous concentration had produced a steady response (indicated by the arrows). In both curves the maximal inhibitory response was attained after ATP 250 μM. Vertical bar: tension of the preparation in mg. Horizontal bar: time in min.

Results

Both ATP and adenosine relaxed the internal anal sphincter in a concentration-related manner. On a molar basis, adenosine appeared less potent than ATP. Furthermore, the rate of response to adenosine was often slower than that to ATP (Figure 1). The ineffectiveness of tetrodotoxin (0.7 μM for 10 min) in preventing the relaxation revealed a direct action of both purines on the smooth muscle cells.

Pretreatment for 30 min with theophylline (25–50 μM) failed to modify the concentration-response curves to either ATP or adenosine (Figure 2). Theophylline itself (especially at 50 μM) caused some temporary loss of tone which was usually regained within 15 min. Higher theophylline concentrations were not used since they caused a persistent fall in tone. Incubation with reactive blue 2 (50–100 μM) caused a slow, persistent and concentration-dependent relaxation of sphincter strips; no modification of tone was observed at lower concentrations. To avoid the use of a spasmogen which might interfere with the degree of antagonism (Hooper, Spedding, Sweetman & Weetman, 1979), preparations in which relaxation appeared to be particularly marked were discarded. Reactive blue 2 (25–50 μM) did not modify the concentration-response curve to adenosine, but 100 μM slightly

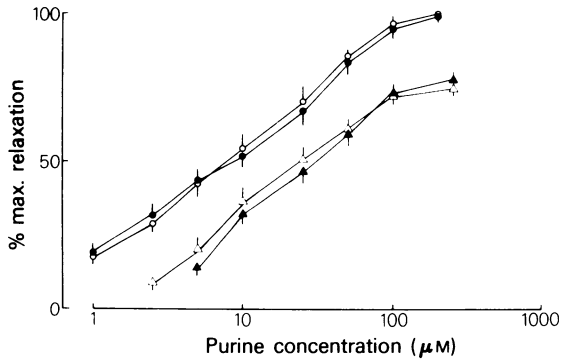

Figure 2 Inhibition of sphincter tone produced by ATP (○) and adenosine (△); responses to ATP (●) and adenosine (▲) after 30 min exposure to theophylline (50 μM). Each point represents the mean of at least 5 experiments; vertical lines show s.e.mean. Responses are expressed as a percentage of the relaxation induced by ATP (250 μM).

reduced the sensitivity of the strip to the agonist (dose ratio ± s.e.mean.: 1.49 ± 0.14, $n = 4$). On the contrary, reactive blue (25–50–100 μM) dose-dependently shifted the curve to ATP to the right (Figure 3) and caused, at the highest concentration, some depression of the maximum ATP response, thus confirming the data obtained by Kerr & Krantis (1979) in guinea-pig distal colon, and by Choo (1980) in the guinea-pig detrusor. However, at variance with the results obtained by Choo, the degree of antagonism was much less pronounced (about a 10 fold rather than a 70 fold shift in the presence of 100 μM reactive blue). The antagonist effect of reactive blue was not reversed following washout for 1 h. The Arunlakshana-Schild plot (Arunlakshana & Schild, 1959), using dose-ratios for ATP calculated at the ED_{50} level, yielded a slope of 1.52 with a

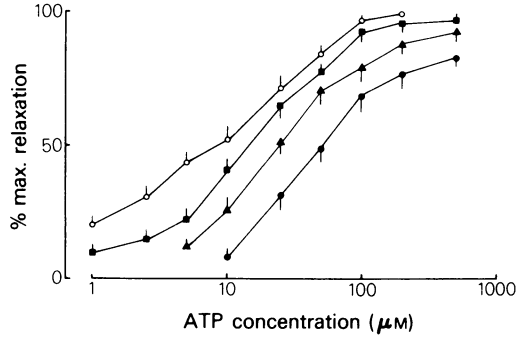

Figure 3 Inhibition of sphincter tone produced by ATP. (○) Initial responses; responses after 60 min exposure to 25 μM (■), 50 μM (▲) and 100 μM (●) reactive blue 2. Responses are expressed as a percentage of the initial maximal ATP response (250 μM) and are means of 6 experiments; vertical lines show s.e.mean.

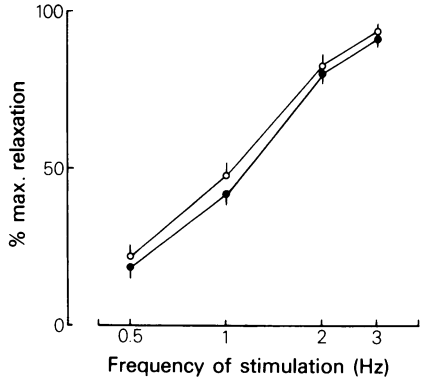

Figure 4 Non-adrenergic, non-cholinergic inhibitory responses obtained by stimulating electrically (at 0.5, 1, 2 and 3 Hz) sphincter strips in the presence of hyoscine (2.2 μM) and guanethidine (5 μM). Responses are expressed as a percentage of the response induced by 5 Hz stimulation. (○) Initial responses; (●) responses after 60 min exposure to reactive blue 2 (100 μM). Each point represents the mean of 6 experiments; vertical lines show s.e.mean.

correlation coefficient of 0.99. The slope was significantly different ($P < 0.05$) from that (1), expected for competitive antagonism. Determination of pA_2 and its 95% confidence limits gave a value of 4.61 ± 0.06. Reactive blue (100 μM) also caused a slight antagonism of the noradrenaline-induced contraction and isoprenaline-induced relaxation (dose-ratios: 1.67 ± 0.12 and 1.55 ± 0.1, respectively, $n = 4$).

Electrical stimulation in the presence of hyoscine (2.2 μM) and guanethidine (5 μM) induced a frequency-dependent relaxation of the sphincter strip. These responses were prevented completely by pretreatment with tetrodotoxin (0.7 μM) for 10 min. In contrast, incubation with reactive blue (50–100 μM) failed to antagonize the electrically-induced non-adrenergic, non-cholinergic relaxations (Figure 4). Similar results were obtained in 3 preparations in which the tone was raised with noradrenaline (20 μM) both in the control and in the presence of reactive blue (100 μM) before eliciting electrical stimulation. Moreover, no significant modification of the non-adrenergic, non-cholinergic responses was observed in the presence of occupation of purinoceptors with either ATP or adenosine (Figure 5).

Discussion

Although the presence of intramural non-adrenergic inhibitory neurones has been demonstrated in the internal anal sphincter of both humans (Parks, Fishlock, Cameron & May, 1969; Frenckner & Ihre,

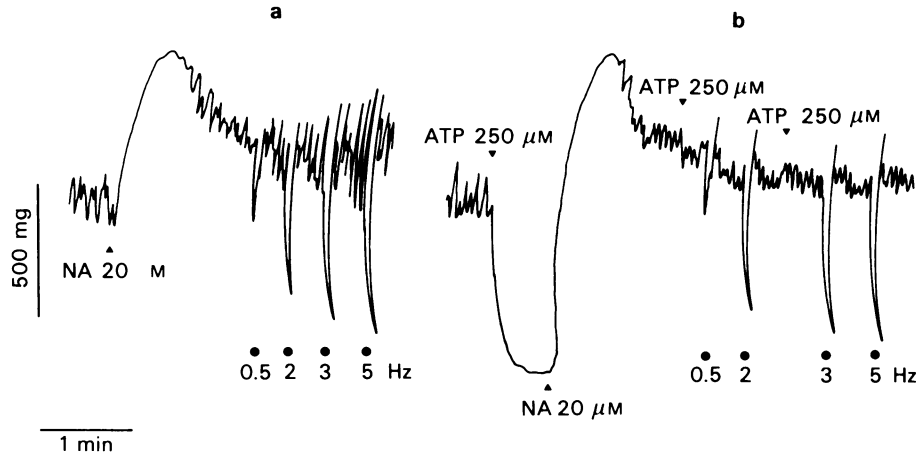

Figure 5 Non-adrenergic, non-cholinergic inhibitory responses elicited by electrical stimulation of sphincter strips in the presence of hyoscine (2.2 μM) and guanethidine (5 μM) after the muscular tone was raised by 20 μM noradrenaline (NA). (a) Control responses; (b) same as (a) but in the presence of a concentration of ATP capable of inducing maximal inhibitory response (250 μM) and occupying purinoceptors. The responses for electrical stimulation were tested after the preparation had lost its responsiveness to ATP (250 μM). Vertical bar: tension of the preparation in mg. Horizontal bar: time in min.

1976) and animals (Costa & Furness, 1974; Garrett & Howard, 1975), to our knowledge, evidence in support of or against the purinergic nature of these nerves has never been presented.

In our experiments ATP and adenosine each relaxed the internal anal sphincter by a direct action on the smooth muscle cells. An attempt to differentiate adenosine receptors from those which interact with ATP by theophylline (Brown & Burnstock, 1981) failed since this drug did not modify the concentration-response curves to either purine compound. Resistance of adenosine receptors to theophylline in some intestinal preparations has already been described (Small & Weston, 1979; Huizinga & Den Hertog, 1980); thus theophylline should not be regarded as a general tool for distinguishing purinoceptors in smooth muscle. On the contrary, reactive blue 2 antagonized the action of ATP (Kerr & Krantis, 1979) leaving that of adenosine essentially unaltered. Even though the antagonism appeared to be non-competitive (at variance with the results obtained by Choo (1980) in guinea-pig detrusor), and non-specific at higher concentrations, our findings suggest the presence of two separate purinoceptors on the smooth muscle cells of the guinea-pig internal anal sphincter. Desensitization to the muscular action of purine compounds has been used as a mean of assessing the purinergic nerve hypothesis both in the longitudinal (Tonini, Onori, Frigo, Lecchini, d'Angelo & Crema, 1981) and circular muscle of rabbit colon (Crema, d'Angelo, Frigo, Lecchini, Onori & Tonini, 1982). The present preparation did not show tachyphylaxis after repeated exposure to purine compounds, thus the nerve-mediated relaxation was studied in the presence of reactive blue 2 and after saturation of the purinoceptors by either ATP or adenosine. In these latter experiments, since the agonist was present throughout at concentrations which elicited the maximum effect, one would expect all functional receptor sites to be occupied. In our experiments, neither reactive blue 2 pretreatment nor occupation of functional receptor sites by ATP or adenosine significantly modified the electrically-induced nerve-mediated relaxation in the presence of hyoscine and guanethidine. The latter results are in agreement with those of Romano (1981), who studied the non-adrenergic inhibition induced by nicotine in rat colon. Although, only a ten fold shift of the concentration-response curve to ATP was obtained with reactive blue 2, nevertheless, it is likely that a transmitter other than ATP or adenosine mediates the non-adrenergic, non-cholinergic response of the guinea-pig internal anal sphincter. Similar conclusions have been recently reached by Bauer, Matusak & Kuriyama (1982) and by Frew & Lundy (1982) for the small intestine and the stomach of the guinea-pig, respectively. However, since there is abundant evidence that nerve activity is accompanied by the release of purines (Fredholm & Hedquist, 1980) these substances are more likely to modulate neurotransmission (Stone, 1981) than to behave as final inhibitory transmitters. This hypothesis is further supported by our findings (Tonini, Onori, Lecchini, Frigo, Perucca & Crema, 1982) that, in the rabbit colon, ATP decreases the velocity of propulsion mainly by modulating the cholinergic pathway without affecting the descending inhibition.

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