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EFFECTS OF DESENSITIZATION TO ADENOSINE 5′-TRIPHOSPHATE AND ADENOSINE ON NON-ADRENERGIC INHIBITORY RESPONSES IN THE CIRCULAR MUSCLE OF RABBIT COLON

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1 An approximate eight fold desensitization of the circular coat of the distal rabbit colon to adenosine 5′-triphosphate (ATP) and adenosine could be achieved by repeatedly exposing the organ to relatively low concentrations (10–100 μM) of these compounds.

2 The desensitization was specific and reversible after prolonged washing. It could be overcome by increasing the concentrations of the purine agonists.

3 Dipyridamole potentiated the non-adrenergic inhibition in response to transmural stimulation but failed to influence the caudad relaxation evoked by radial distension.

4 Desensitization to ATP and adenosine (and to ATP + adenosine simultaneously) did not affect the non-adrenergic inhibition in response to radial distension or transmural stimulation.

5 These results suggest that neither ATP nor adenosine are the final transmitters mediating the non-adrenergic inhibitory responses in the distal colon of the rabbit.

Introduction

It has been suggested that adenosine 5′-triphosphate (ATP), or a related compound, may act as a neurotransmitter at non-adrenergic inhibitory synapses in the gut (Burnstock, 1972). To assess the validity of this hypothesis and in the absence of a selective antagonist of purine action (Small & Weston, 1979; Coleman, 1980; Huizinga & Den Hertog, 1980), desensitization has been widely used as an investigational tool. However, this experimental approach has yielded conflicting results. In separate studies, desensitization to the muscular action of ATP has been found either virtually to abolish the electrically induced non-adrenergic inhibition (Burnstock, Campbell, Satchell & Smythe, 1970; Okwuasaba, Hamilton & Cook, 1977) or to leave it essentially unchanged (Weston, 1973; Ohga & Taneike, 1977; Bartlett, Stewart & Nakatsu, 1979). These discrepancies are likely to result in part from differences in the experimental procedures used to induce desensitization. In some studies, for example, only relatively low concentrations of purine agonists were used to desensitize the intestinal muscle. In others, no quantitative evaluations of the degree of desensitization were obtained.

The present study was designed to investigate the non-adrenergic relaxation of the circular coat of rabbit colon in response to radial distension and electrical stimulation after the musculature had been desensitized to purine agonists. Of the latter compounds, ATP and its metabolite adenosine were selected because these substances relax intestinal muscle by acting on at least two separate purinoceptors (Burnstock 1978; Bartlett *et al.*, 1979). An advantage of including adenosine in the study is that this compound possesses only some of the properties typical of ATP. Pharmacological actions of ATP not shared by adenosine include the induction of prostaglandin biosynthesis (Needleman, Minkes & Douglas, 1974; Kamikawa, Serizawa & Shimo, 1977), the chelation of Ca^{2+} ions (Kahn & Martell, 1962; Daniel, 1965) and the ability to provide energy by rapid degradation of the polyphosphate.

Methods

Rabbits of either sex (2.4–2.6 kg) were killed by stunning and bleeding. Segments of distal colon, 10 cm long, were removed with the aboral end, cut 1 cm above the symphysis pubis. From this specimen two separate segments, 0.3 and 6 cm long, were dissected and transferred to organ baths containing Tyrode solution maintained at 35°C and gassed with a mixture of 95% O_2 and 5% CO_2. The preparations

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were allowed to equilibrate for 30 min before any drug was added.

The 6 cm long segment was mounted horizontally in a 100 ml bath with its mesenteric border sewn on a perspex rod placed immediately below the organ (Costa & Furness, 1976). Relaxation of the circular coat was recorded by connecting the serosa on the superior surface of the organ to an isotonic type transducer under a tension of 300–400 mg. Prior to desensitization, the muscular response was assessed by constructing concentration-response curves to ATP (10, 25, 50 μM) and adenosine (25, 50, 100 μM). Since it was not possible to maintain any given state of desensitization for longer than 3 min (see below), each individual preparation was exposed to one dose only (test dose). The degree of relaxation was expressed as a percentage of the response induced by 100 μM ATP (taken as 100% response). This was determined 30 min after the initial test dose had been given and was followed by a prolonged wash-out (20 min). In preliminary experiments, attempts to desensitize the organ by using high (>200 μM) initial concentrations of ATP and adenosine invariably resulted in incomplete recovery of the muscular tone. In order to circumvent this problem, desensitization was achieved by adding repeatedly to the bath a dose of ATP or adenosine identical to the initial test dose. The latter was repeated every 3 min without washing and until the response had disappeared. Under these conditions, desensitization to ATP or adenosine could be consistently achieved with 4–6 repeated administrations, usually without loss of the muscular tone. Those preparations in which tone was altered after exposure to ATP or adenosine were discarded. The need for maintaining an interval of 3 min between doses was determined by the fact that longer periods had been found to result in partial loss of desensitization. After the circular coat had been desensitized (i.e., no response was evoked after addition of the test dose), a dose of ATP or adenosine eight times greater than the initial test dose was added to the bath. This procedure was justified by the observation that in preliminary experiments an eight fold increase in dose was required to produce the same degree of relaxation after desensitization had developed. A quantitative assessment of the degree of muscular desensitization to ATP or adenosine was obtained by comparing the concentration-response curves constructed before and after desensitization, respectively (Figure 1).

To study the reflex relaxation of the circular coat, a 6 cm long segment of colon was mounted in a 100 ml organ bath according to the same procedure used for assessing the response to ATP. The reflex was elicited by inflating a rubber balloon fixed inside the organ. The muscular response was measured at a single site 3 cm aborally to the site of distension. The neurogenic nature of the reflex was confirmed by the ability of tetrodotoxin (TTX 0.7 μM) to block the response. Distension-relaxation curves before and after desensitization to ATP or adenosine were obtained by inflating the balloon with 0.3, 0.6, 0.9 ml water. In preliminary experiments, consistent reflex responses could be achieved only if at least 15 min were allowed to elapse between two consecutive inflations. In order to maintain the desensitization of the circular coat ATP or adenosine had to be administered every 3 min; therefore in any individual preparation only one degree of distension was tested, twice before (the mean of the two responses was used for the analysis) and once after desensitization had developed (Figure 2). The degree of relaxation was expressed as a percentage of the response produced by distending the balloon with 1.2 ml water (taken as 100% response). The 100% response was determined 5 min before adding the first dose of ATP (50 μM) or adenosine (100 μM). Desensitization was induced by repeating the same dose of ATP or adenosine without washing and until disappearance of the response. Preparations in which the tone was altered after exposure to ATP or adenosine were discarded. The effect of desensitization on the reflex relaxation was assumed by re-inflating the balloon 1 min after the last dose of ATP or adenosine had been added.

In order to assess the effect of desensitization to ATP or adenosine relaxation induced by transmural stimulation, a segment of colon 0.3 cm long (ring preparation) was mounted in a 50 ml bath connected to an isometric transducer under a tension of 1–1.5 g. The bath solution (Tyrode) contained hyoscine (2.2 μM), piperoxan (3.7 μM) and propranolol (3.3 μM). Unlike the longer segment, the ring preparation usually was markedly hypotonic (Tucker, Snape & Cohen, 1979). Therefore, the tone had to be raised by doubling the concentration of Ca^{2+} in the medium. Transmural stimulation was performed by means of 2 silver electrodes placed 1 cm apart with square wave pulses of 0.5 ms duration at supramaximal potential difference. Stimulations were delivered every 20 min and lasted for 15 s. The responses evoked were abolished by TTX (0.7 μM). Frequency-response curves before and after desensitization to ATP or adenosine were constructed with frequencies of stimulation of 1, 1.5 and 3 Hz. The experimental procedure was similar to that used to investigate the reflex relaxation. In any preparation, only one frequency of stimulation was tested, twice before and once 1 min after desensitization to ATP or adenosine developed. The electrically-induced inhibitory responses were calculated as percentage of the response induced by a 10 Hz stimulus (taken as 100% response). The 100% response was elicited 5 min before the first dose of ATP (50 μM) or adenosine

(100 μM). Desensitization was achieved by repeatedly adding the same dose of ATP or adenosine without washing and until disappearance of the response. Preparations in which the tone was altered after exposure to ATP or adenosine were discarded.

The composition of the Tyrode solution was (mM): NaCl 136.89, KCl 2.68, $CaCl_2$ 1.80, $MgCl_2$ 1.04, $NaHCO_3$ 11.90, NaH_2PO_4 0.41, glucose 5.55. The drugs used were: adenosine (BDH), adenosine-5'-triphosphoric acid (disodium salt) (BDH), indomethacin (MSD), (−)-isoprenaline bitartrate (Cilag Chemie), carbachol (BDH), hyoscine hydrobromide (BDH), piperoxan hydrochloride (Rhône-Poulenc), (±)-propranolol hydrochloride (ICI), dipyridamole (Boehringer Ingelheim) and tetrodotoxin (Sankyo).

In all preparations the volume of drug solutions injected did not exceed 2% of the bath volume.

Results

Both ATP and adenosine induced concentration-dependent, transient relaxation of the circular muscle. On a molar basis, adenosine was found to be consistently less potent than ATP (Figure 1). The onset of relaxation was rapid, the maximal response being reached within 30 s for both substances. In most experiments the muscle spontaneously resumed its original tone within 160 s after the addition of ATP and 120 s after the addition of adenosine. With the higher concentrations of ATP (25–50 μM), a transient after-contraction was occasionally seen, particularly after washout. With adenosine, 'rebound' contractions were never seen.

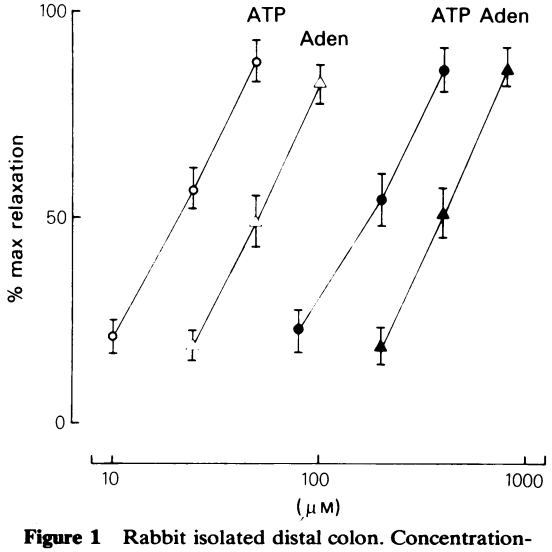

Figure 1 Rabbit isolated distal colon. Concentration-response curves to ATP and adenosine (Aden) before and after desensitization of the circular coat to these compounds. Responses are expressed as a percentage of the relaxation induced by 100 μM (maximal response). Each individual preparation was exposed to one concentration only, selected to elicit a submaximal response. Desensitization to each dose was achieved according to the procedure described in the text. Each point represents the mean of 8 experiments; vertical lines show s.e.mean. The open symbols refer to the curves obtained prior to desensitization and the closed symbols to the curves obtained after desensitization.

In separate experiments, the inhibitory response of the circular muscle to ATP and adenosine remained unaffected by tetrodotoxin (0.7 μM), indicating that the action of these purine agonists was not nerve-mediated.

Desensitization to ATP or adenosine usually without modification of the baseline tone could be consistently achieved by repeatedly adding the same dose of either compound at 3 min intervals and without washing. Complete muscular inresponsiveness to ATP or adenosine was usually obtained after no more than 6 administrations. A similar loss of responsiveness was also seen in 5 preparations in which the tone, having not recovered completely after ATP, had to be restored to its original level by carbachol (0.05 μM). Comparison of the concentration-response curves before and after desensitization indicates that, under the experimental conditions used, the depression in muscle sensitivity was approximately eight fold (Figure 1). A similar degree of desensitization was obtained when 4 experiments for each purine compound were carried out in the presence of tetrodotoxin (0.7 μM). The desensitization was probably relatively specific, since in 4 separate experiments the inhibitory effect of isoprenaline (2–10 μM) persisted unchanged after unresponsiveness to both ATP (50 μM) and adenosine (100 μM) had developed. Complete recovery of muscle responsiveness to both purines was observed after prolonged washing (30–50 min) in all preparations tested ($n = 12$).

A prostaglandin-mediated contraction of intestinal smooth muscle in response to ATP has been described (Burnstock, Cocks, Paddle & Staszewska-Barczak, 1975; Kamikawa *et al.*, 1977). In order to exclude the possibility that the development of unresponsiveness to ATP in our preparations could be explained as the result of two opposing actions, eight experiments were carried out in the continuous presence of indomethacin (10 μM added to the bath 60 min before the experiment). In 6 out of 8 preparations, indomethacin caused a slight reduction in tone accompanied by an enhancement of segmental motility. Indomethacin, however, did not prevent the development of desensitization to ATP (50 μM), which occurred in all preparations after no more than 6 administrations of the latter compound. In the pres-

ence of indomethacin after-contractions were never observed.

The loss of responsiveness of the musculature to repeated administration of ATP might be explained by either a chelating effect of ATP on Ca^{2+} ions or an intracellular uptake of adenosine (released from the hydrolysis of ATP) limiting by a feed-back mechanism the primary effect of ATP (Maguire & Satchell, 1979). In order to examine these possibilities, some experiments were carried out after trebling the concentration of Ca^{2+} ions in the medium or in the presence of dipyridamole (2 μM). Dipyridamole added to the bath, at least 30 min beforehand potentiated the relaxation in response to 25 μM ATP by $23.5 \pm 3.2\%$ ($n = 5$). On the other hand, the response to 50 μM ATP ($n = 5$) was not affected by raising the Ca^{2+} concentration of the medium. Neither dipyridamole nor the rise in Ca^{2+} concentration modified the time course or the degree of desensitization to ATP.

Radial distension of the gut produced by filling the balloon invariably elicited a reflex relaxation of the circular muscle aborally to the site of distension. The onset of relaxation was rapid, the maximal response being observed within 10 s. The magnitude of relaxation was directly proportional to the degree of radial distension (Figure 3). In order to evaluate the effect of desensitization to purine compounds on the reflex, 52 preparations were exposed to repeated doses of ATP (50 μM, $n = 27$) or adenosine (100 μM, $n = 25$). All exhibited full refractoriness to the inhibitory effect of these compounds after no more than 6 administrations (Figure 2). However, 6 preparations exposed to ATP and 4 exposed to adenosine, had to be discarded because of insufficient recovery of the muscular tone. In the preparations in which the tone was preserved, desensitization to either ATP or adenosine did not influence the degree of reflex relaxation in response to radial distension (Figure 3). Since the possibility of inducing cross desensitization to ATP and adenosine is still controversial (Weston, 1973; Spedding & Weetman, 1976; Frew & Baer, 1979) in 5 experiments the organ was desensitized simultaneously to ATP (50 μM) and adenosine (100 μM) added alternately every 90 s until the response to both substances had disappeared. Under these conditions, the degree of relaxation of the circular coat in response to a 0.6 ml distension was not significantly different before and after desensitization ($62 \pm 4\%$ of the maximal response before desensitization compared to $61 \pm 4\%$ of the maximal response after desensitization).

In 5 separate experiments, we found that dipyridamole itself (2 μM) had no significant effect on the relaxation-distension relationship. Transmural electrical stimulation caused a frequency-dependent relaxation of the ring preparation (Figure 4). In order to evaluate the effect of desensitization to purine compounds on the electrical response, 45 preparations were exposed to repeated doses of ATP (50 μM, $n = 23$) and adenosine (100 μM, $n = 22$). Although all preparations exhibited complete loss of responsiveness after no more than 6 administrations, 5 preparations treated with ATP and 4 treated with adenosine, had to be discarded because of incomplete recovery of the muscular tone. In the preparations in which tone was preserved, the frequency-relaxation relationship after desensitization was virtually identical to that observed in the baseline state.

In 9 separate experiments dipyridamole itself (2 μM) potentiated the relaxation in response to transmural stimulation (Figure 5).

Discussion

The present experiments demonstrate that ATP and adenosine relax the circular coat of rabbit colon by an action which is neither under nervous control, since it is not abolished by TTX, nor prostaglandin-

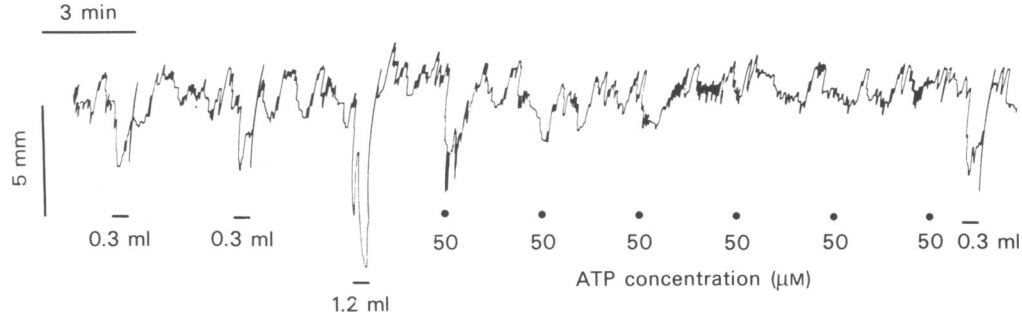

Figure 2 Rabbit isolated distal colon. Effect of desensitization to ATP on the reflex relaxation of the circular coat in response to radial distension. Horizontal bars below the tracing indicate the 30 s distension of the balloon. Note that the relaxation of the circular coat following distension of the balloon remains unchanged after the response to ATP had disappeared.

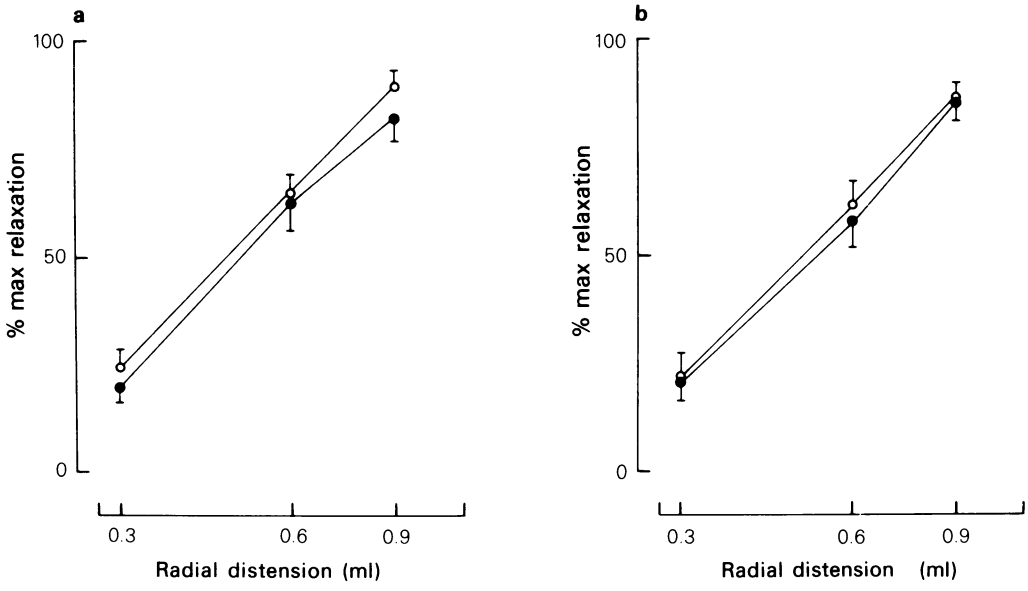

Figure 3 Rabbit isolated distal colon. Effect of desensitization to ATP or adenosine on the relaxation of the circular coat in response to radial distension. Relaxations are expressed as a percentage of the inhibitory response observed after a 1.2 ml distension of the balloon (maximal distension). Only one degree of submaximal distension was tested in each preparation. Desensitization was produced by repeatedly adding 50 μM ATP (a) or 100 μM adenosine (b) until the response had disappeared. The open symbols refer to the curves obtained before desensitization and the closed symbols to the curves obtained after desensitization. Note that the relaxation-distension relationship remained unchanged after desensitization had been achieved. Each point represents the mean of 7 experiments; vertical lines show s.e.mean.

dependent, since it is not prevented by indomethacin. These findings are in agreement with those of McKenzie, Frew & Baer (1977) and of Kamikawa *et al.* (1977), respectively. In previous observations (Tonini, Onori, Frigo, Lecchini, d'Angelo & Crema, 1981), we have shown that repeated administrations of purine agonists at short intervals results in desensitization of the longitudinal coat of the distal colon to these compounds. The findings obtained in the present study demonstrate that a similar effect may also be produced in the circular coat.

Under the experimental conditions used, the sensitivity of the circular muscle to the inhibitory effect of both ATP and adenosine was reduced approximately eight fold. Attempts to produce a greater degree of desensitization by repeatedly adding larger doses of ATP (or adenosine) or by continuing the administration after loss of responsiveness had developed, were hampered by the occurrence of an incomplete recovery of the muscle tone. Development of desensitization was not prevented by pretreating the organ with TTX or indomethacin, suggesting that the mechanism involved is neither nerve-mediated nor dependent on the concomitant opposing action of prostaglandins released by ATP. The possibility that the desensitization to ATP was mediated by chelation of Ca^{2+} ions is unlikely since a similar loss of responsiveness was observed in a Ca^{2+} enriched medium. The experiments in which dipyridamole was used to prevent adenosine uptake suggests that the unresponsiveness to ATP was due to true desensitization of the purinoceptor and not to the action of its metabolite adenosine limiting by a feed-back mechanism at intracellular level the action of ATP at the receptor site (Maguire & Satchell, 1979). Our results indicate that desensitization to purine compounds was relatively specific since the response of the circular coat to isoprenaline was not affected.

The desensitization could be overcome by higher concentrations of purinoceptor agonists and was reversed by prolonged washing. These results provide indirect evidence that in the absence of a specific antagonist of purine compounds desensitization of the circular coat to ATP and adenosine could be a valuable experimental tool in physiological studies.

In previous studies desensitization to ATP had been investigated in respect to an unphysiological stimulus such as transmural stimulation. An advantage of the design used in our experiments is that of operating under conditions closer to the normal intestinal propulsive activity.

It is generally accepted that the reflex relaxation elicited by distension of the colon is dependent on

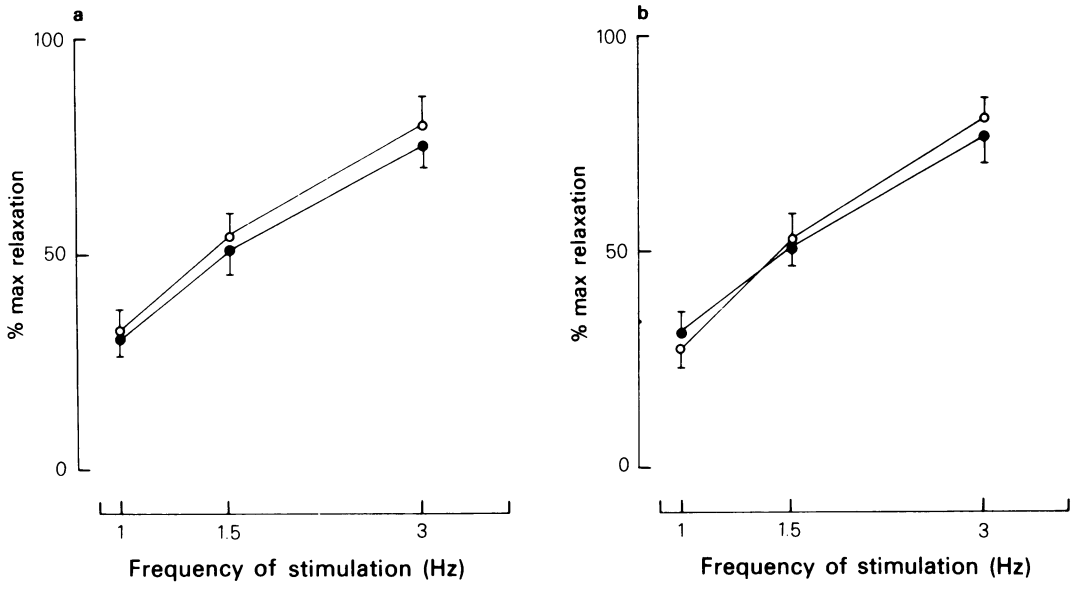

Figure 4 Rabbit isolated distal colon (ring preparation). Failure of desensitization to ATP (a) or adenosine (b) to modify the relaxation of the circular coat in response to transmural stimulation in the presence of hyoscine (2.2 μM), piperoxan (3.7 μM) and propranolol (3.3 μM). Relaxations are expressed as a percentage of the inhibitory response observed after stimulation at 10 Hz (maximal stimulation). Each preparation was tested at only one frequency of submaximal stimulation. The open symbols refer to the curves obtained after desensitization. Desensitization was produced by repeatedly adding 50 μM ATP or 100 μM adenosine until the response had disappeared. Each point represents the mean of 6 experiments; vertical lines show s.e.mean.

activation of non-adrenergic, non-cholinergic intramural inhibitory neurones (Bianchi, Beani, Frigo & Crema, 1968; Costa & Furness, 1976; Julé, 1980). In our experiments, desensitization of the circular musculature to ATP, adenosine, and ATP plus adenosine simultaneously did not affect the distension-reflex relaxation relationship. Similarly, the relaxation of the circular musculature elicited by transmural stimulation of the enteric inhibitory nerves in the presence of hyoscine and α- and β-adrenoceptor blocking agents remained unaltered when desensitization of the musculature to ATP or adenosine was achieved.

Potentation of the inhibitory responses to transmural stimulation by dipyridamole was originally thought to provide evidence in support of the purinergic hypothesis (Satchell & Burnstock, 1975) but more recent evidence has pointed to the non-specific nature of such potentiations (Baer, Frew & Burnstock 1977). Our inability to provide evidence of a potentiation of muscular inhibition in response to radial distension in the presence of dipyridamole could be accounted for by a difference in the type and/or degree of the stimulus or to the lack of specificity of dipyridamole as inhibitor of adenosine uptake (Dowdle & Maske, 1980). Hence, some doubt must be cast on the interpretation of the results obtained with dipyridamole.

It is known that ATP, in addition to its direct effect on smooth muscle, may have a modulatory action on neurotransmitter release (Enero & Saidman, 1977; Kazic & Milosavljevic, 1980; Silinsky, 1980) and may be released together with neurotransmitters (Burnstock, 1976). Therefore, it would not be surprising if some ATP was released during transmural stimulation of the colon. However, the fact that desensitization of the circular musculature to ATP did not affect the relaxation of this coat in response to transmural stimulation argues against the putative role of ATP as a neurotransmitter at the muscular level.

Alternatively, it could be argued that the evidence against the mediation of the inhibition by ATP is not critical because the desensitization could be overcome by increasing the concentration of the nucleotide and there is no evidence of the local concentration of the possible physiological mediators. Nevertheless, the results argue against a role for ATP as the final mediator of the non-adrenergic inhibitory responses in the distal colon of the rabbit.

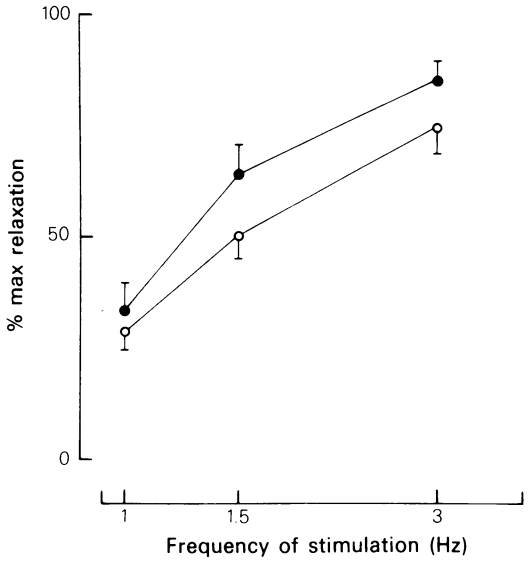

Figure 5 Rabbit isolated distal colon (ring preparation). Effect of dipyridamole on the relaxation of the circular coat induced by transmural stimulation in the presence of hyoscine (2.2 μM), piperoxan (3·7 μM) and propranolol (3.3 μM). Relaxations are expressed as a percentage of the inhibitory response observed after stimulation at 10 Hz (maximal stimulation). Each preparation was tested at three different frequencies of submaximal stimulation before (○) and after addition of 2 μM dipyridamole (●). Each point represents the mean of 9 experiments; vertical lines show s.e.mean. Dipyridamole caused a significant increase ($P < 0.05$) in the response to 1.5 Hz stimulation.

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