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ANTAGONISM OF ADENOSINE 5'-TRIPHOSPHATE-INDUCED RELAXATION BY 2-2'-PYRIDYLISATOGEN IN THE TAENIA OF GUINEA-PIG CAECUM

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1 2-2'Pyridylisatogen tosylate (PIT) (>2.5 μM) relaxed the guinea-pig isolated taenia caeci by an unknown mechanism.

2 With higher concentrations of PIT (>12.5 μM) subsequent applications of adenosine 5'-triphosphate (ATP) (2-600 μM) revealed a blockade of the ATP receptors. The antagonism was characterized by a delayed onset of action (>10 min incubation with 50 μM PIT) and eventually became irreversible (>50 μM PIT for >30minutes). The antagonism was specific for ATP, was not competitive, and was not dependent upon the relaxant effect.

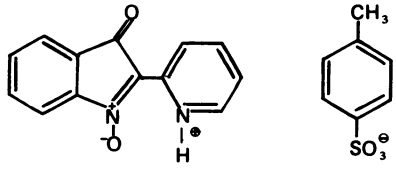
3 The presence of either acetylcholine (0.05-1.0 μM) or carbachol (0.05-1.0 μM) increased the antagonistic effect of PIT (50 μM) approximately five-fold.

4 Following prolonged exposure, PIT (50 μM for 90 min) did not block the inhibitory effects of field stimulation (2 Hz, 10 s) of the taenia caeci in the presence of hyoscine (0.33 μM). These results do not support the purinergic nerve hypothesis.

Introduction

Atropine does not completely block the effects of parasympathetic nerve stimulation to many organs; in the gut, for example, contractions are often converted to relaxations (McSwiney & Robson, 1929; McSwiney, 1931; Paton & Vane, 1963; Campbell, 1966; Burnstock, Campbell, Satchell & Smythe, 1970; Furness, 1970; Beani, Bianchi & Crema, 1971; Burnstock, 1972). In many situations, adenosine 5'-triphosphate (ATP) mimics these atropine-resistant effects, a finding which led Burnstock and his colleagues to propose that ATP or a related purine nucleotide is the chemical transmitter released when the nerves are stimulated. Much evidence supports this claim (see Burnstock *et al.*, 1970; Burnstock, 1972), but the lack of a specific ATP receptor antagonist has led to the use of very high concentrations of some drugs to provide support for the hypothesis. Phentolamine, tolazoline, antazoline and yohimbine have been used by Satchell, Burnstock & Dann (1973) in an attempt to show a similar blockade of exogenous ATP and field stimulation on the guinea-pig taenia caeci. All these drugs have a diversity of pharmacological actions (Boyd, Chang & Rand, 1960; Goodman & Gilman, 1970).

During routine testing of compounds synthesized in our department we discovered that the water soluble tosylate salt of 2-2'pyridylisatogen (PIT) blocked the inhibitory action of ATP on isolated taenia caeci (Hooper, Spedding, Sweetman & Weetman, 1974). In the present paper we report the actions of this compound on the isolated taenia caeci of the guinea-pig, stimulated with drugs and via the nerves.



2-2'Pyridylisatogen tosylate (PIT)

Methods

Taenia caeci preparations were obtained from female guinea-pigs in the weight range 250-600 g. The preparations were arranged in 10 ml or 100 ml organ baths filled with McEwen's (1956) solution maintained at 35 ± 1° C and oxygenated with 95% O_2 and 5% CO_2. After an equilibration period of 30 min, responses were recorded isotonically on a smoked drum (magnification 1 : 4, load 1.5 g).

In some experiments, preparations were stimulated transmurally by rectangular pulses from an SRI 6051 stimulator, by means of electrodes similar to those described by Birmingham &

Wilson (1963). Stimulation characteristics were as follows: pulse duration 0.5 ms; train duration 10 s; interval between pulse trains 2 or 4 min; 150 V setting on the stimulator was used.

Some preparations were stimulated via the perivascular nerves (Burnstock, Campbell & Rand, 1966). Shielded platinum electrodes (Palmer, type H87) were used, the stimulation characteristics being as described above except that the train duration was 15 s and the voltage supramaximal.

Cumulative concentration-response curves were obtained for noradrenaline and ATP at 20 min intervals (Van Rossum, 1963). Each dose of agonist was allowed to produce its full effect (5-13 s contact) before the concentration in the bath was increased. Preparations with low tone, i.e. those which did not contract to 25% of their relaxed length, were discarded.

Drugs

PIT was synthesized by the method of Patterson & Wibberley (1965) and the tosylate salt prepared. Other drugs used were: acetylcholine chloride, (−)-ascorbic acid, carbachol chloride and histamine acid phosphate (B.D.H.); guanethidine sulphate (Ismelin–CIBA); hyoscine hydrobromide B.P., quinidine sulphate B.P. (Mawson and Proctor); imidazole (Sigma); (−)-isoprenaline sulphate (K and K Labs. Inc.); (−)-noradrenaline bitartrate (Koch-Light); phentolamine mesylate B.P. (CIBA); prostaglandin E_2 (Upjohn); tetrodotoxin (Sankyo); adenosine 5'-triphosphate (B.D.H. or Sigma). The catecholamines were protected from oxidation by the inclusion of approximately 100 μg/ml of (−)-ascorbic acid in each dilution. Prostaglandin E_2 was dissolved by the method of Bennett & Posner (1971). Solutions of ATP and imidazole were adjusted to pH 7.4 by the addition of 1.0 M NaOH and 1.0 M HCl respectively. The composition of the McEwen's (1956) solution was as follows (mM): NaCl 130; KCl 5.6; $CaCl_2$ 2.2; $NaHCO_3$ 25; NaH_2PO_4 1.2; glucose 11.1 and sucrose 13.2.

Statistical analyses and other procedures

When complete concentration-response or frequency-response curves were obtained, responses were calculated as percentage maximum relaxation. Relaxations in the presence of an antagonist were expressed as percentage of the control relaxations. Dose-ratios were calculated as the number of times the agonist concentration had to be increased to maintain the 50% maximal response in the presence of the antagonist, the 50% maximal response being calculated from the individual curves. The slopes of the concentration-response curves were calculated as the ratios of the concentrations producing 80% and 20% maximal effects (Stephenson, 1956). Student's *t*-test was used for comparisons; homogeneity of variance was checked by a variance ratio test (Snedecor & Cochran, 1967).

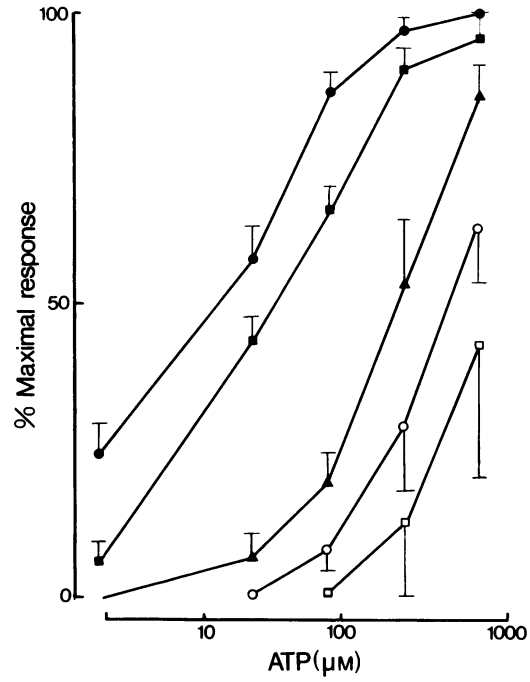


Figure 1 The influence of the duration of exposure on the antagonistic effect of 2-2'pyridylisatogen (PIT) to adenosine 5'-triphosphate (ATP) on taenia caeci. Cumulative concentration response curves to ATP were obtained in the absence (●) and in the presence of PIT (25 μM) after 30 (■), 60 (▲), 90 (○) and 120 (□) min contact. Each point is the mean of eight determinations; vertical bars show s.e.mean. Carbachol (0.05-1.0 μM) was used to restore the tone of the preparations to within 20% of the original level.

Results

Antagonism of ATP-induced relaxations by PIT

ATP (2-600 μM) produced a concentration-dependent, rapid relaxation of the guinea-pig isolated taenia caeci. The relaxation was probably a direct effect on the smooth muscle because tetrodotoxin (0.33 μM) did not modify the responses ($n = 6$). Concentration-response curves, constructed from experiments in which the tissue

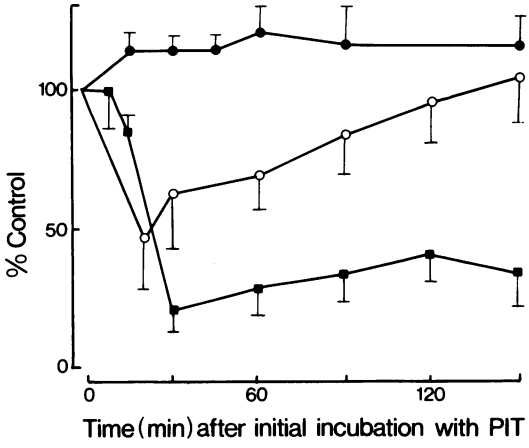


Figure 2 The effect of washing on the antagonism of submaximal responses to adenosine 5'-triphosphate (ATP) by 2-2'pyridylisatogen (PIT) on the taenia caeci. The curves are as follows: control ATP (10 μM) responses (•), ATP after exposure to PIT (50 μM) for 20 (○) or 30 (■) min, starting at time zero. The values represent the mean of five determinations. Vertical bars show s.e.mean. Carbachol (0.05-0.5 μM) was used to restore the tone of the preparations to within 20% of the original length in the initial stages, but was not required for later determinations. Preparations were washed with McEwen's solution (1 litre/hour). Note the delayed onset of the antagonism.

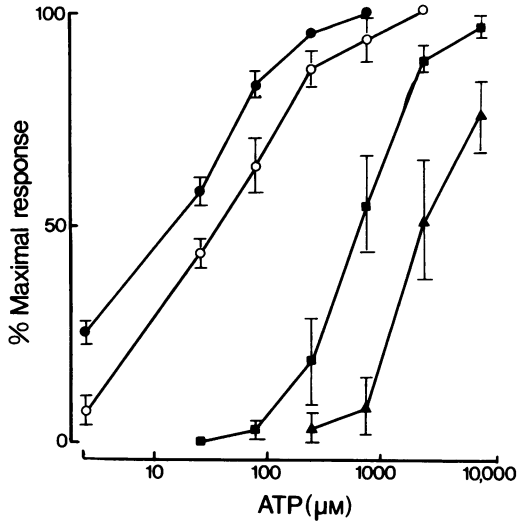


Figure 3 The effect of different concentrations of 2-2'pyridylisatogen (PIT) on the cumulative concentration-response curves to adenosine 5'-triphosphate (ATP). The curves are as follows: control (•), 25 (○), 50 (■) and 75 (▲) μM PIT. The antagonist was incubated with the tissue for 30 min and carbachol (0.05-1.0 μM) was used to restore tone. The values represent the mean of eight determinations. Vertical bars show s.e.mean.

was dosed cumulatively, showed that there was no tachyphylaxis to ATP, provided that the initial curve was disregarded (5 curves over 100 min) (n = 5). In all subsequent experiments the effect of the first cumulative exposure of the tissue to ATP was not measured.

PIT exerted two actions on the taenia caeci; first, low concentrations (threshold at 2.5 μM) slowly relaxed the preparation. The second effect, a blockade of the inhibitory action of ATP, was seen with higher concentrations of PIT (25 μM or more). In order to detect the antagonism, the taenia, which had been relaxed by the PIT, had to be re-contracted. Acetylcholine (0.05-1.0 μM) and carbachol (0.1-1.0 μM) were routinely used for this purpose (see below). Only preparations that could be restored to within 20% of their length during the control part of the experiment were used.

Both the magnitude and specificity of the ATP antagonism depended upon the duration of incubation of the tissue with PIT. Figure 1 shows how the antagonism of ATP-induced relaxations increased with prolonged exposure of the taenia caeci to PIT 25 μM. If equilibrium between the antagonist and the ATP receptors had been established in these experiments, the curves would not have been progressively displaced to the right; so it can be concluded that PIT did not reach equilibrium within 120 minutes.

The combination of PIT with the ATP receptors on the taenia caeci was reversible in the initial stages, but later became irreversible. Figure 2 depicts a series of five experiments in which the submaximal responses of the taenia caeci to single doses of ATP were investigated after either 20 or 30 min exposure to 50 μM PIT. It can be seen that the response to ATP was restored to the control level following 20 changes of bathing solution when the incubation had been for 20 min, but there was little restoration of sensitivity to ATP following a 30 min exposure. With lower concentrations of PIT (12.5 μM) the antagonism could be reversed after a longer exposure, but this too eventually became irreversible (n = 2). A concentration-dependent delay in the onset of the block characterized all the experiments.

The antagonism of ATP-induced relaxations of the taenia caeci by PIT was concentration-dependent (Figure 3). In a series of eight experiments the concentration-response curves for ATP were displaced to the right to an extent

determined by the concentration of the PIT. The slopes of these curves were not significantly different from the control ($P > 0.05$), except at the highest concentration tested (75 μM). The data in Figure 3 were analysed by the method of Arunlakshana & Schild (1959), and yielded an apparent pA_2 of 4.8, the slope being 2.6.

The effect of the presence of spasmogenic agents on the antagonism

Table 1 shows the activities of PIT when different spasmogens were used to restore the tone of the taenia caeci. The antagonism of ATP by PIT was significantly greater when the cholinomimetics were used to restore tone to the taenia caeci than occurred with histamine, prostaglandin E_2, or in those rare preparations that did not appreciably relax in response to PIT (3 out of 70). If acetylcholine (0.05-1.0 μM) or carbachol (0.05-1.0 μM) were used to recontract the tissue after PIT, small regular spontaneous contractions which were similar to those seen before the addition of drugs were obtained. In contrast with this histamine (0.2-2.5 μM) induced much larger spontaneous contractions (see Figure 5).

A second method was used to investigate the antagonism of ATP by PIT in the absence of exogenous spasmogenic agents. In these experiments, preparations were incubated with PIT 50 μM for 30 min and then washed in drug-free solution at the rate of 1 litre/h for 90 minutes. The tone of the preparations returned to within 20% of the original level. The dose-ratio for ATP was then found to be 6.7 ± 3.0 ($n = 6$). In another series of experiments, similar to these except that carbachol was included for the final 10 min of the exposure of the tissue to PIT, the dose-ratio for ATP 90 min after PIT was removed from the bath was 24.6 ± 9.7 ($n = 6$), this dose-ratio being significantly higher than in the former series ($P < 0.05$). The presence of spasmogens may therefore determine the degree of the antagonism of ATP by PIT, but is not a prerequisite for the antagonism to occur.

Specificity of the antagonism

In preliminary experiments, the specificity of the ATP receptor blocking action of PIT was assessed by the use of single doses of ATP, noradrenaline and isoprenaline (Hooper *et al.*, 1974). This work has been extended to include full concentration-response curves for the three agonists before and after a 30 min exposure to PIT (50 μM). The curves for noradrenaline and ATP were obtained by cumulative dosing; with isoprenaline, single doses were used and the curves constructed from the results with a large number of tissues. Carbachol (0.1-0.8 μM) was used to restore the smooth muscle tone to within 20% of the control level. The results of the experiments are shown in Figure 4. When assessed by comparison of the dose-ratios, PIT was 30 times more effective in blocking ATP-induced relaxations than noradrenaline relaxations, and 60 times more effective against ATP than against isoprenaline.

Comparison with other ATP receptor antagonists

Imidazole, phentolamine and quinidine have been used to block the inhibitory actions of ATP on isolated intestinal muscle (Bueding, Bülbring, Gercken, Hawkins & Kuriyama, 1967; Bowman & Hall, 1970; Burnstock *et al.*, 1970; Rikimaru, Fukushi & Suzuki, 1971; Satchell *et al.*, 1973; Sneddon, Smythe, Satchell & Burnstock, 1973). PIT has been compared to these other antagonists:

Table 1 The effect of spasmogens, used to restore tone, on the antagonism of adenosine 5'-triphosphate (ATP) by 2-2'pyridylisatogen (PIT)

Spasmogen	*Concentration (μM)*	*Dose-ratio (± s.e.mean)*	n
Acetylcholine	0.05- 1.0	42 ± 11	6
Carbachol	0.05- 1.0	49 ± 3	24
Histamine	0.2 - 2.0	10 ± 3	5
Prostaglandin E_2	0.3 -33.0	5 ± 2	4
None (preparations with little relaxation)		8,6,8	3

Taenia caeci preparations were incubated with PIT 50 μM for 30 min and the tone was restored to within 20% of the control level. Dose-ratios were obtained from cumulative concentration-response curves for ATP before and after exposure to the antagonist. The level of contraction was not maintained when prostaglandins were used to restore tone; thus the value in the table may be an underestimate of the potency of PIT.

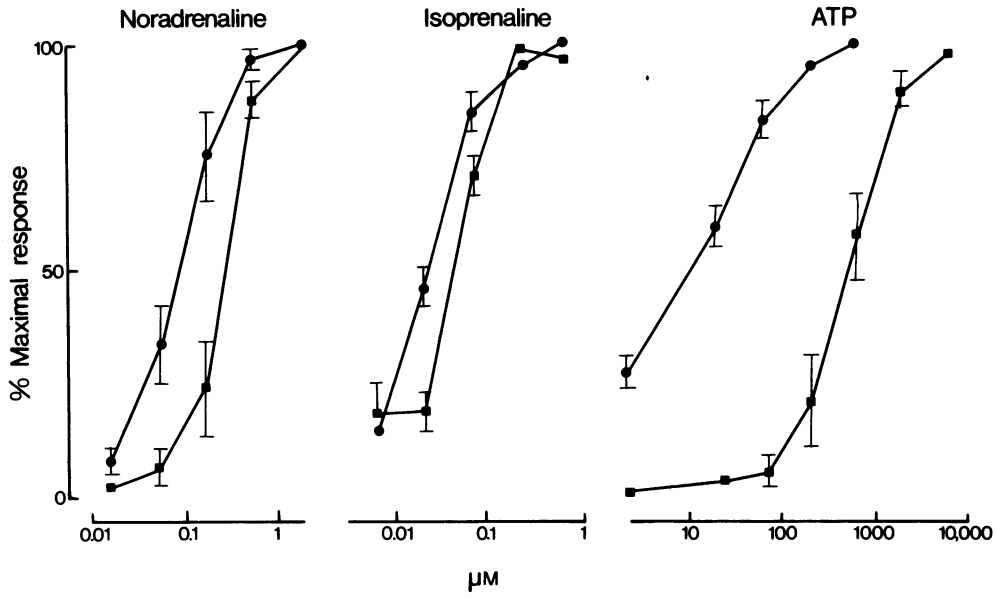


Figure 4 Specificity of the antagonism of adenosine 5'-triphosphate (ATP) by 2-2'pyridylisatogen (PIT). Concentration-response curves for ATP (8 experiments) and noradrenaline (7 experiments) were obtained by cumulative dosing and those for isoprenaline (10 experiments) by constructing concentration-response curves from single responses, one preparation being used for each response. Curves were obtained before (•) and after (▪) 30 min incubation with PIT (50 μM): vertical bars show s.e.mean. Carbachol (0.05-1.0 μM) was used to restore tone. Dose-ratios calculated from the curves were: 2.7 (noradrenaline), 1.6 (isoprenaline) and 92.6 (ATP). Relaxations induced by isoprenaline were slowed in the presence of PIT.

the results constitute Table 2. Only PIT and phentolamine significantly reduced the responses to ATP. The high concentration of phentolamine required to block the ATP-induced relaxations resulted in non-specific effects, the responses to noradrenaline, isoprenaline and ATP being similarly blocked. In the eight experiments, PIT completely blocked ATP and reduced the responses to noradrenaline (to 42% of the control) and isoprenaline (70%). Imidazole and quinidine were not antagonists of ATP at the concentrations tested.

Table 2 Specificity of drugs used to block the inhibitory effects of adenosine 5'-triphosphate (ATP)

Antagonist	*Concentration (μM)*	n	*Responses as % control (s.e.mean)* *ATP*	*Noradrenaline*	*Isoprenaline*
Quinidine	140	6	83(7)	1(2)	53(9)
Phentolamine	180	6	28(9)	20(12)	52(10)
Imidazole	50000	5	80(15)	43(15)	54(19)
2-2'Pyridyl-isatogen (PIT)	50	8	2(1.5)	42(13)	70(9)

Single submaximal relaxations were induced by ATP (10-40 μM), noradrenaline (0.08-2.0 μM) or isoprenaline (0.02-0.05 μM). Agonists were added to the bath, on a 5 min cycle, before and after incubation of the antagonists with the preparation for 30 minutes. Imidazole initially contracted the taenia, but the tone returned to the control level within the 30 min incubation. Carbachol (0.1-0.8 μM) was used to restore the tone with PIT and in two experiments with phentolamine.

Note that PIT was the only drug to antagonize preferentially ATP-induced relaxations.

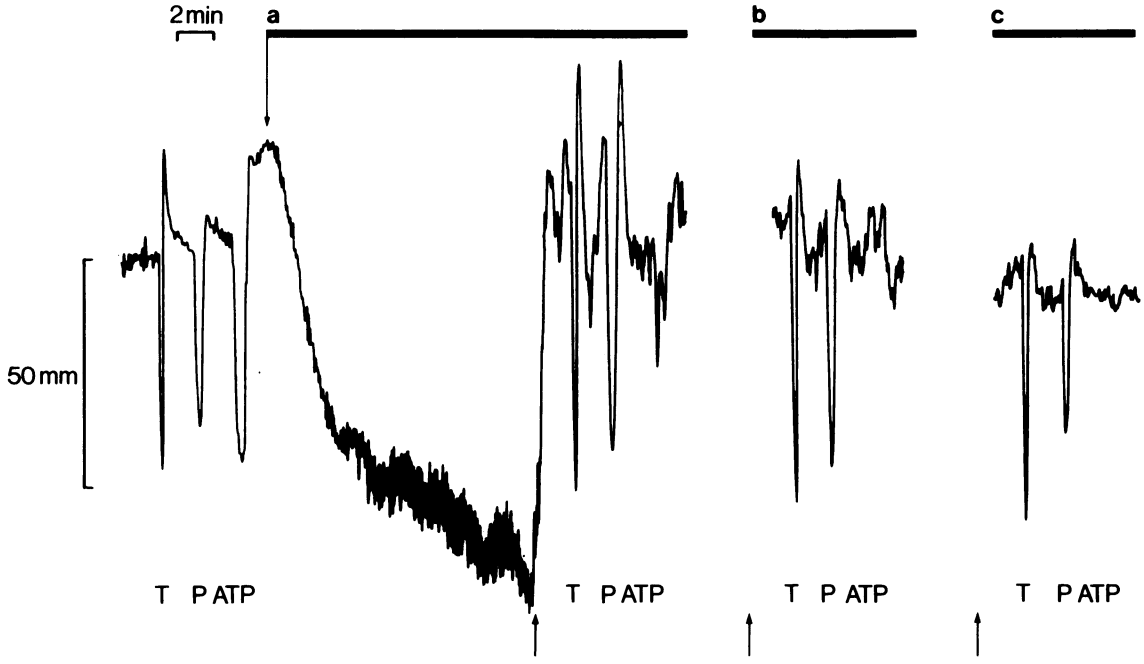


Figure 5 The effect of 50 μM 2-2'pyridylisatogen (PIT) on relaxations induced by transmural stimulation (2 Hz; 10 s; T), perivascular stimulation (50 Hz; 15 s; P) and exogenous adenosine 5'-triphosphate (20 μM; ATP). PIT was added to the bathing fluid, for the duration of the experiment, at the upper arrow; the drum speed was halved to show the resulting relaxation. Histamine was added at the lower arrows in panels (a), (b) and (c) (0.6, 0.8 and 2.5 μM respectively) and washed out between panels. There is a time interval of 30 min between panels. Hyoscine (0.33 μM) was present throughout.

The effect of PIT on transmural and perivascular nerve stimulation

The taenia caeci relaxes in response to either perivascular nerve or field stimulation in the presence of hyoscine. Figure 5 shows an experiment in which the effects of PIT were studied on both forms of stimulation and exogenous ATP. ATP was the most easily blocked, neither type of nerve stimulation being affected until the antagonist had been in contact with the tissue for at least one hour. In six experiments, after the tissue had been in contact with PIT for 30 min, the effects of perivascular nerve stimulation were 97% ± 14 of the control, transmural nerve stimulation was 114% ± 7 of the control, and ATP was 48 ± 10% (mean ± s.e.mean).

In a separate series of experiments the effects of PIT on different rates of field stimulation were compared to those on the concentration-response curves to ATP, using adjacent strips of taenia caeci. The bathing solution contained hyoscine (0.33 μM) and guanethidine (17 μM) to exclude effects on cholinoceptors or adrenergic neurones. PIT (50 μM for 30 min) blocked exogenous ATP but had little effect on the relaxations following field stimulation (Figure 6).

Discussion

In addition to blocking the effects of ATP on the guinea-pig taenia caeci, PIT relaxed the preparation. The mechanism of this relaxation is unknown, but preliminary experiments indicate that it is unlikely to be due to an action on nerves, adrenoceptors, ATP receptors or by inhibition of prostaglandin biosynthesis. These results and those of experiments with analogues of PIT (Hooper & Spedding: unpublished observations) show that independent mechanisms are involved in these two actions of PIT.

The relaxation of the taenia caeci by PIT necessitated the use of spasmogenic agents in most experiments. Although the degree of the ATP receptor blockade was influenced by the individual spasmogen, the presence of a smooth muscle

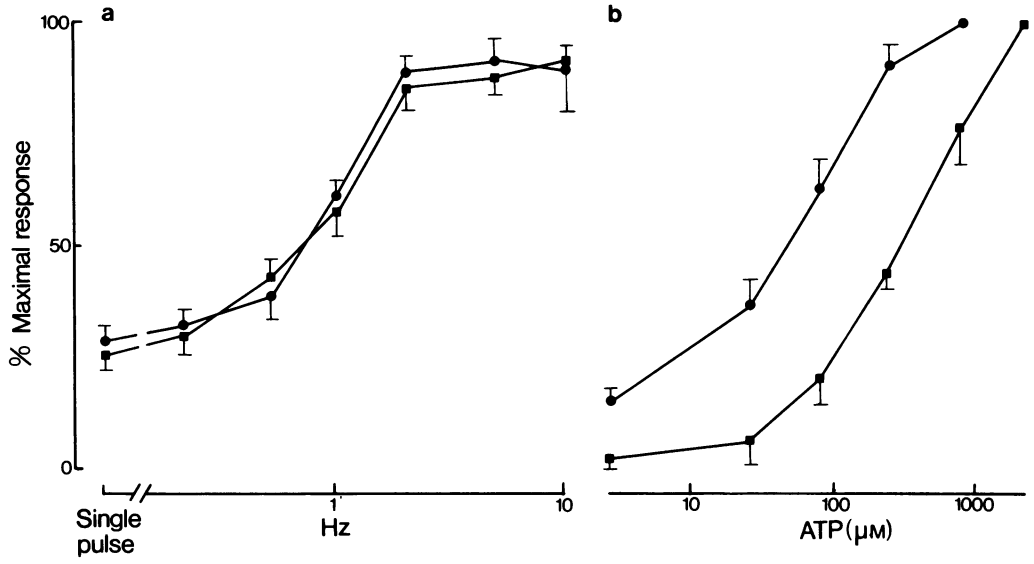


Figure 6 The effects of (a) transmural stimulation of adjacent strips of guinea-pig taenia caeci and (b) of exogenous adenosine 5'-triphosphate (ATP). The McEwen's solution contained hyoscine (0.33 μM) and guanethidine (17 μM). Responses to ATP were obtained cumulatively; adjacent strips were stimulated transmurally on an ascending series from 0.2-10 Hz every 2 minutes. Responses before (•) and after (▪) incubation of the preparations with 2-2'pyridylisatogen (50 μM) for 30 minutes. Vertical bars show s.e.mean (n = 6). Histamine was used to restore tone. Note that although responses to ATP were diminished, the responses to transmural stimulation were unchanged.

stimulant was not a prerequisite for the antagonism to occur.

The antagonism of ATP by PIT was reversible in the early stages, so it is possible that PIT initially combines with ATP receptors in a competitive manner. However, after a period of time that depended upon the concentration of PIT, the antagonism became irreversible. Thus, although it may seem feasible, the data obtained from concentration-response curve experiments should not be used to calculate a pA_2 value for PIT because these constants depend upon measurements made during a reversible antagonism with equilibria established between the antagonist and the receptors and the agonist and the receptors. Nevertheless, using the Arunlakshana & Schild (1959) method, PIT has an apparent pA_2 of 4.8, but the slope of the plot ($\log_{10}$ agonist dose-ratio−1 against $\log_{10}$ reciprocal of the molar concentration of the agonist) was 2.6, and not the hypothetical value for a competitive antagonist of 1.0. Thus, under these conditions, PIT was not a competitive antagonist of ATP. Hooper & Robertson (1971) have shown that PIT is capable of forming covalent bonds with certain amino acids, so it is possible that PIT could be reacting with such an amino acid located at, or near, the ATP receptors in the taenia caeci to produce the irreversible blockade.

PIT was clearly a better antagonist of the inhibitory effects of ATP than were those drugs used in previous studies. Quinidine and imidazole did not significantly block the effects of ATP. Phentolamine, although it did block the effects of ATP, also blocked the inhibitory effects of noradrenaline and isoprenaline. PIT was the only drug to antagonize differentially the ATP-induced relaxations of the smooth muscle. In this series of experiments, quinidine was found to possess appreciable α-adrenoceptor antagonist activity, as had been reported previously (Hiatt, 1950; Bowman & Hall, 1970; Schmid, Nelson, Mark, Heistad & Abboud, 1974).

Relaxation of the taenia caeci induced by field stimulation in the presence of hyoscine and guanethidine, a procedure which should specifically excite the hypothetical purinergic nerves, was not reduced by PIT. Even after an extended incubation of the taenia caeci with several concentrations of PIT and with a range of stimulation frequencies, there was no reduction in the effects of field stimulation. If ATP is the

mediator of the hyoscine- and guanethidine-resistant inhibitory effects of field stimulation, why was the response to field stimulation unaffected? One explanation is that ATP may be released into the synaptic cleft in such high local concentrations that PIT is ineffective. This is improbable when one considers that PIT produces an irreversible, non-competitive inhibition. However, Tomita & Watanabe (1973), using the taenia caeci in a double sucrose gap assembly, have reported that injections of ATP 1 mM matched the hyperpolarization produced by field stimulation with a single pulse. If such high concentrations of ATP occur in the synaptic cleft under physiological conditions one would expect to find 'close-contact' varicosities on electronmicrographs of the tissue so that the ATP is used efficiently. These have rarely been observed in the taenia caeci (Bennett & Rogers, 1967). Also, the long latency of the inhibitory junction potential (Bennett, Burnstock & Holman, 1966) suggests that the neurotransmitter diffuses over a considerable distance (100-300 nm) before reaching the receptors on the smooth muscle cells. Thus it would appear unlikely that ATP would reach such high concentrations in the synaptic cleft. However, it must be acknowledged that the highest concentration of the transmitter would occur in the centre of the muscle bundle where, presumably, the concentration of PIT is lowest.

The results in this paper are at variance with the purinergic nerve hypothesis. However, the acceptance or rejection of the hypothesis must be postponed until there is more evidence about the release of purine nucleotides from the taenia caeci and other tissues.

We are grateful to our colleague Dr M. Hooper, for synthesizing the 2-2'pyridylisatogen tosylate; to Dr R.T. Brittain, Allen and Hanburys Ltd., Ware, Herts., for stimulating discussions throughout the course of this work; and to Mr D.W. Snowdon for photographing the figures. We would also like to thank Dr J.E. Pike, The Upjohn Co., Kalamazoo, Michigan, for a gift of prostaglandin E_2.

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