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THE MECHANISM OF THE RELAXANT EFFECT OF 2-2'-PYRIDYLISATOGEN ON THE ISOLATED TAENIA OF THE GUINEA-PIG CAECUM

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1 2-2'-Pyridylisatogen tosylate (PIT) slowly relaxed taenia caeci preparations of the guinea-pig in a concentration-dependent manner (threshold 2.5 μM). The relaxant effect did not show tachyphylaxis.

2 The relaxation was not affected by tetrodotoxin (0.3 μM), guanethidine (17 μM) nor by a combination of phentolamine (36 μM) and propranolol (4 μM)

3 In taenia caeci preparations suspended in K^+-depolarizing, Ca^{2+}-free Ringer, addition of Ca^{2+} (0.1 to 30 mM) resulted in a slow contraction. PIT (50 μM) and papaverine (15 μM) antagonized these contractions, whereas indomethacin (28 μM) was ineffective.

4 Although PIT (50 μM for 30 min) caused a relaxation of the taenia, and, when the tone of the preparations was restored with carbachol, antagonized adenosine 5'-triphosphate (ATP)-induced relaxations, relaxation of the taenia with papaverine (30 μM for 5 min) did not antagonize ATP-induced relaxations. It is concluded that the relaxant and ATP-receptor blocking actions of PIT are independent properties of the compound.

Introduction

2-2'-Pyridylisatogen tosylate (PIT) is an antagonist of the relaxant effect of adenosine 5'-triphosphate (ATP) on the isolated taenia of guinea-pig caecum (Hooper, Spedding, Sweetman & Weetman, 1974). However, PIT does not antagonize the relaxant effects of adenosine (Spedding & Weetman, 1976), nor are the relaxant effects of non-adrenergic inhibitory nerves antagonized (Spedding, Sweetman & Weetman, 1975). From this latter evidence it is unlikely that the inhibitory neurotransmitter is ATP.

The use of PIT as an antagonist of the inhibitory effects of ATP is complicated by a loss of tone in the smooth muscle preparations, necessitating the use of histamine or carbachol to restore tone before the relaxant effects of ATP can be evaluated in the presence of the antagonist. This paper investigates the mechanism whereby taenia caeci preparations lose tone following incubation with PIT.

Methods

Taenia caeci preparations were taken from female guinea-pigs (250 to 600 g). Preparations were set up in 10 ml isolated organ baths filled with McEwen's solution (McEwen, 1956) maintained at 35 ± 1°C and gassed with 95% O_2 and 5% CO_2. Responses were recorded isotonically on a smoked drum (magnification 4:1, load 1.5 g). Tissues were allowed to equilibrate for 30 min before each experiment. Preparations with low tone, i.e. which did not contract by more than 25% of their relaxed length, were discarded.

In some experiments, tissues were gassed with nitrogen; as bubbling McEwen's solution with nitrogen drives off CO_2 rather easily and may cause pH changes, these tissues were maintained in Locke solution.

Potassium depolarized preparations

In order to obtain Ca^{2+}-induced contractions, preparations were set up as described above, but maintained in K^+-depolarizing, Ca^{2+}-free Locke solution (Ferrari & Carpenedo, 1968) and gassed with compressed air. Tissues were incubated in this solution for at least 30 min, during which time the bathing solution was changed a minimum of ten times. After the 30 min incubation period, preparations exhibited no tone, but contracted in response to addition of calcium chloride (0.1–30 mM).

Cumulative concentration-response curves

Cumulative concentration-response curves to Ca^{2+} were constructed by increasing the concentration of Ca^{2+} in the isolated organ bath logarithmically (van Rossum, 1963). The tissue was allowed to contract fully to each addition of Ca^{2+} (1 to 3 min contact) before the concentration of Ca^{2+} in the bath was increased.

Cumulative concentration-response curves were obtained to the relaxant effects of ATP in an analogous fashion (Spedding *et al.*, 1975) except that tissues were maintained in McEwen's solution and the contact period between additions of ATP was 8 to 20s.

Evaluation of data

The concentration producing a 50% maximal effect (EC_{50}) was estimated graphically from concentration-response curves. Dose-ratios were estimated as the ratio of the EC_{50} in the presence of a modifying drug compared with the control EC_{50}. The slopes of the concentration-response curves were calculated as the ratios of the concentrations producing 80% and 20% maximal effects (Stephenson, 1956).

Preliminary experiments showed that some drugs, including PIT, relaxed tissues slowly (>10 min to maximal relaxation). Cumulative concentration-response curves to these drugs could not be constructed because spontaneous variations in the tone of the preparations occurred over a 10 min period. The relaxant effects of these drugs were measured as the time (min) taken to relax the initial tone of taenia caeci preparations by 50% (t_{50}).

Values in the text refer to mean ± s.e. mean. Differences in means were determined by Student's *t* test, after checking the homogeneity of the variances (Snedecor & Cochran, 1967).

Drugs

2-Phenylisatogen and PIT were synthesized by Dr M. Hooper of the Department of Pharmaceutical Chemistry at the School of Pharmacy, Sunderland Polytechnic. Other drugs used were: adenosine, adenosine 5'-triphosphate disodium salt, (−)-ascorbic acid, carbachol, histamine acid phosphate, theophylline (BDH); dipyridamole (Boehringer); guanethidine monosulphate, phentolamine mesylate (CIBA); labetalol (Allen & Hanbury); (±)-propranolol hydrochloride (ICI); (−)-isoprenaline sulphate (K & K Labs California.); hexamethonium bromide, (−)-noradrenaline bitartrate (Koch-Light); indomethacin (MSD); papaverine hydrochloride B.P. (Mawson & Proctor); prostaglandin E_2 (Upjohn); 2,4-dinitrophenol, dicyclohexylcarbodiimide (Sigma); tetrodotoxin (Sankyo). The catecholamines were protected from oxidation by the inclusion of approximately 100 μg/ml (−)-ascorbic acid in each dilution.

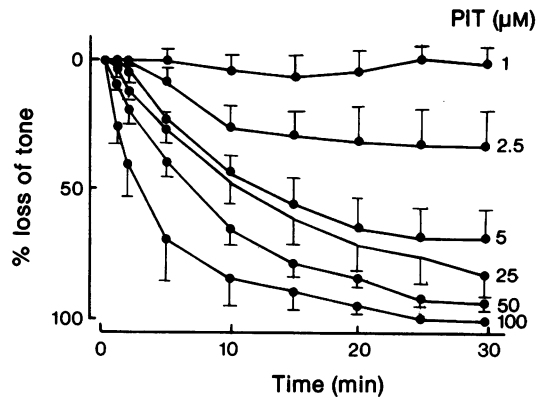

Figure 1 The relaxant effects of different concentrations of 2-2'-pyridylisatogen (PIT) on the taenia caeci preparation of the guinea-pig. PIT was added to the taenia at time zero and the resultant loss of tone expressed as % of the initial tone. Vertical bars represent s.e. mean. n = 4 to 27.

Indomethacin was dissolved in 5 μg/ml sodium carbonate before it was added to the bath solution. Prostaglandin E_2 was dissolved by the method of Bennett & Posner (1971).

The salt solutions had the following compositions (mM): McEwen's solution: NaCl 130, KCl 5.6, $CaCl_2$ 2.2, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 11.1 and sucrose 13.2; Locke solution: NaCl 154, KCl 5.6, $CaCl_2$ 2.2, $NaHCO_3$ 5.9 and glucose 5.6; K^+-depolarizing, Ca^{2+}-free Locke solution: KCl 154; $NaHCO_3$ 5.9 and glucose 5.6.

All concentrations in the text refer to the final concentration of drug in the isolated organ bath.

Results

The relaxant effect of 2-2'-pyridylisatogen

Taenia caeci preparations were slowly relaxed by PIT in a concentration-dependent manner (Figure 1). The threshold concentration of PIT required to cause a significant ($P < 0.05$) relaxation of the taenia after 10 min exposure was 2.5 μM. The relaxation was accompanied by an increase in the frequency of spontaneous contractions. Preparations slowly regained tone when PIT was washed from the organ bath. Following low concentrations of PIT (< 10 μM for 30 min), tone could be restored by a single change of McEwen's solution; after higher concentrations (50 μM for 30 min) 10 to 15 changes of bathing fluid were required before tone was fully re-established.

Tachyphylaxis did not occur following addition of PIT. In four experiments, the t_{50} following the addition of PIT (50 μM) was 13.3 ± 1.8 min and 13.5 ± 2.1 min after the second administration 90 min later ($P > 0.1$), when the tone of the preparations had been restored to the original level by washing.

Effects of drugs on 2-2'-pyridylisatogen-induced relaxations

The time taken by PIT (50 μM) to relax the taenia was not significantly ($P > 0.1$) prolonged by prior incubation with labetalol (15 μM, $n = 4$), guanethidine (17 μM, $n = 4$), tetrodotoxin (0.33 μM, $n = 6$), nor by a combination of phentolamine (36 μM) and propranolol (4 μM, $n = 4$). Preincubation with theophylline (500 μM, $n = 4$) in an attempt to inhibit phosphodiesterase enzymes did not significantly reduce the t_{50} following incubation with PIT (50 μM, $P > 0.1$).

Preparations which had been pretreated with indomethacin (2.8 to 28 μM) in an attempt to inhibit endogenous prostaglandin production were also relaxed by PIT. Incubation with indomethacin relaxed the smooth muscle, so tone was induced with carbachol (0.02 to 0.1 μM) in these preparations. Increasing the concentration of indomethacin in the organ bath to 140 μM did not relax carbachol-induced tone *per se*, indicating that the inhibition of prostaglandin synthetase was maximal (Figure 2). However, there was no significant difference between the time taken by PIT (50 μM) to relax carbachol-induced tone (t_{50}, 10.5 ± 1 min, $n = 4$) and endogenous tone (t_{50}, 9.2 ± 2 min, $n = 27$; Figure 2). This suggested that the relaxation produced by PIT was not being mediated by inhibition of prostaglandin synthesis.

Comparison with other relaxant drugs

The relaxant effects of PIT (50 μM t_{50}:9 ± 2 min, $n = 27$), were compared with those produced by several metabolic inhibitors. 2-4-Dinitrophenol (55 μM, t_{50} 9 ± 3 min, $n = 3$), dicyclohexylcarbodiimide (20 μM, t_{50} 4 ± 2 min, $n = 3$), 2-phenylisatogen (25 μM, t_{50} 4 ± 1 min, $n = 4$) and papaverine (15 μM, t_{50} 5 ± 2 min, $n = 4$) relaxed the taenia, the relaxation being accompanied by an increase in the frequency of spontaneous contractions. Similar relaxations were obtained by gassing with nitrogen (t_{50} 9 ± 4 min, $n = 4$). The tone of the preparations could be restored by washing with 10 to 15 changes of McEwen's solution, by the addition of carbachol (0.01 to 0.1 μM) or, in the experiments with nitrogen, by gassing with compressed air. Apart from the rate of relaxation produced, there was no discernible difference between the ability of PIT on the one hand and that of each of the metabolic inhibitors or gassing the tissue with nitrogen on the other, to produce relaxation of the tissue.

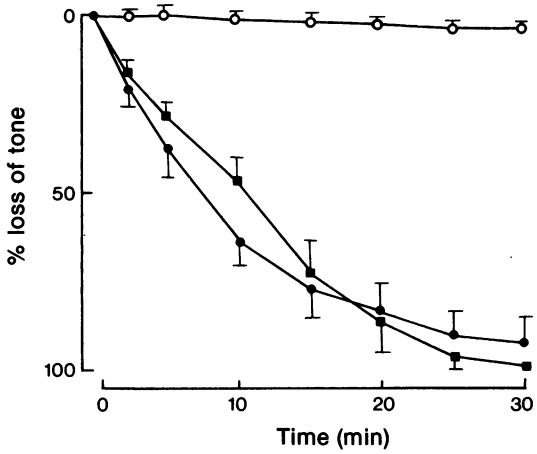

Figure 2 Effects of 2-2'-pyridylisatogen (PIT, 50 μM) on endogenous tone (●, $n = 27$) and on tone induced by carbachol (0.02 to 0.2 μM) in indomethacin-pretreated preparations (28 μM, ■, $n = 4$) of guinea-pig taenia caeci. The presence of indomethacin did not affect the relaxation caused by PIT. In 4 experiments, the carbachol-induced tone was challenged by increasing the concentration of indomethacin (140 μM, ○) instead of adding PIT; there was no reduction in carbachol-induced tone.

Effects of relaxant drugs on sensitivity to ATP

Taenia caeci preparations were fully relaxed by various drugs or by gassing with nitrogen. Tone was then restored to within 20% of the original level with carbachol. Concentrations of the relaxant drugs and incubation times were chosen so that the concentrations of carbachol (0.05 to 0.4 μM) used did not differ significantly ($P < 0.05$) between the different relaxant techniques. PIT (50 μM) was the only drug that appreciably antagonized the inhibitory responses to ATP (Table 1).

Effects of 2-2'-pyridylisatogen on K^+-depolarized taenia

In an attempt to determine whether PIT interfered with excitation-contraction coupling, tissues were depolarized by substituting K^+ for Na^+ in the salt solutions, thereby depolarizing the membrane of the smooth muscle cells.

Taenia caeci preparations which were maintained in K^+-depolarizing Ca^{2+}-free solution for 30 min exhibited no tone and did not contract in response to carbachol (1 μM to 1 mM). However, preparations contracted slowly in response to addition of Ca^{2+}

(threshold 0.1 mM, maximum contraction 30 mM; onset 8 to 25s, maximum effect 45s to 3 min). After 30 min incubation, PIT displaced cumulative concentration-response curves to the right in parallel (25 μM, dose-ratio 5.9 ± 3.4, $n = 5$; 50 μM, dose-ratio 16 ± 5, $n = 6$, $P < 0.05$ compared with control; Figure 3). Papaverine (15 μM) was also an effective antagonist of the Ca^{2+}-induced contractions of the depolarized taenia, although the investigation of this effect was confined to the single concentration of the drug. Thus in addition to PIT, 2-phenylisatogen and papaverine were able to antagonize the Ca^{2+}-induced contractions, whereas noradrenaline, isoprenaline, ATP, adenosine and indomethacin, in concentrations that fully relaxed taenia caeci preparations maintained in McEwen's solution, failed to produce any significant antagonism (Table 2).

Discussion

It is likely that the relaxation caused by PIT was a direct effect on the smooth muscle cells, by a mechanism independent of adrenoceptor activation, because the rate of relaxation of the taenia when exposed to PIT was not altered by tetrodotoxin, labetalol nor by a mixture of phentolamine and propranolol. There is a significant correlation between the

Table 1 The effects of relaxant drugs on the sensitivity of taenia caeci preparations to ATP

Relaxant (incubation time)	*Dose-ratio ± s.e. mean* (n)
Bubble with nitrogen (30 min)	0.8 ± 0.3 (3)
Noradrenaline 2.2 μM (2 min)	3.6 ± 1.7 (4)
Isoprenaline 0.5 μM (2 min)	2.4 ± 1.4 (3)
Papaverine 30 μM (4 min)	0.9 ± 0.3 (5)
PIT 50 μM (30 min)	47 ± 7 (26)

Taenia caeci preparations were fully relaxed by incubation with drugs or gassing with nitrogen for the times indicated. Tone was restored to within 20% of the control level with carbachol (0.05 to 0.4 μM) Dose-ratios were obtained from cumulative concentration-response curves as the ratio of the concentrations of ATP producing 50% inhibition of carbachol-induced tone after and before the smooth muscle was relaxed.

Table 2 Effects of 2-2'-pyridylisatogen (PIT) and other relaxant drugs on the responses to addition of Ca^{2+} in guinea-pig taenia caeci preparations maintained in K^+-depolarizing Ca^{2+}-free solution

Drug	μM	*Dose ratio ± s.e. mean*	n	P
Papaverine	15	33 ± 14	4	< 0.05
PIT	50	16 ± 5	6	< 0.05
2-Phenylisatogen	25	27 ± 8	4	< 0.05
Indomethacin	28	0.9 ± 0.1	4	> 0.1
Noradrenaline	0.9	0.9 ± 0.2	4	> 0.1
Isoprenaline	0.4	3.1 ± 1.5	4	> 0.1
ATP	200	1.4 ± 0.3	4	> 0.1
	600	1.9 ± 0.4	4	> 0.1
Adenosine	400	1.7 ± 0.4	4	> 0.1
	1200	6.4 ± 3.8	4	< 0.1 > 0.05

Cumulative concentration-response curves to Ca^{2+} were obtained either after 2 min (adenosine, ATP, noradrenaline, isoprenaline) or 30 min incubation and compared with control concentration-response curves obtained from untreated preparations taken from the same animal.

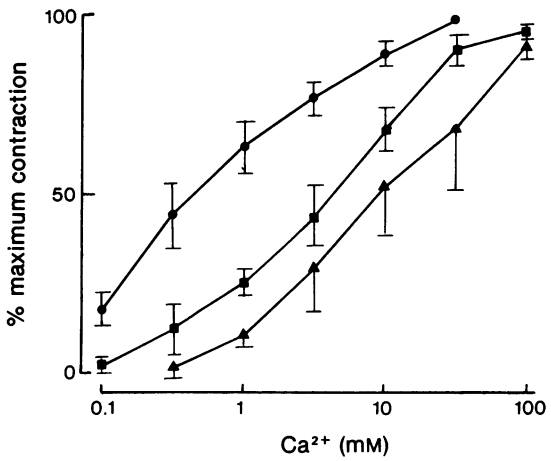

Figure 3 Displacement to the right of cumulative concentration-response curves to addition of Ca^{2+} by 2-2′-pyridylisatogen (PIT, 50 μM, ■, $n = 6$) and papaverine (15 μM, ▲, $n = 4$). Guinea-pig taenia caeci preparations were maintained in K^+-depolarizing Ca^{2+}-free solution and cumulative concentration-response curves obtained following addition of Ca^{2+}. PIT and papaverine were each allowed to act for 30 min. Control concentration-response curves to Ca^{2+} are represented by (●). Vertical bars represent s.e. mean.

relaxant effects of 2-substituted isatogens and their ability to inhibit mitochondrial oxidative processes (Foster, Hooper, Spedding, Sweetman & Weetman 1978).

It has been established that any interference with oxidative processes by drugs or by gassing with nitrogen inhibits prostaglandin production in smooth muscle (Splawinski, Nies, Sweetman & Oates, 1973; Ferreira, Herman & Vane, 1976), so it was pertinent to consider whether or not PIT relaxed the taenia by this mechanism. Yamaguchi, Hitzig & Coburn (1976) demonstrated that indomethacin (0.28 μM) abolished the efflux of prostaglandins from the taenia. Apparent maximal inhibition of prostaglandin synthetase was presumed to have been achieved in our experiments by incubating taenia caeci preparations with indomethacin (2.8 to 28 μM) because when the tone was restored with carbachol, increasing the concentration of indomethacin did not further relax the taenia. The ability of PIT to relax carbachol-induced tone in indomethacin-treated preparations suggests that the relaxation was not primarily caused by inhibition of prostaglandin biosynthesis.

The relaxant effect of PIT is not accompanied by a hyperpolarization of the smooth muscle cells (Spedding & Small, 1978), thus it is unlikely that PIT relaxes the taenia by acting on ATP receptors because ATP causes a hyperpolarization of the taenia (Tomita & Watanabe, 1974). We therefore investigated whether PIT inhibited the events leading to activation of the contractile proteins. Addition of Ca^{2+} to taenia maintained in K^+-depolarizing, Ca^{2+}-free media resulted in a contraction, presumably caused by a direct activation of the contractile proteins. Cheng (1976) has shown that exogenous Ca^{2+} may activate an intracellular pool of Ca^{2+} in depolarized mouse rectum preparations, resulting in a rapid, phasic contraction. This Ca^{2+} pool could also be activated by muscarinic agonists. However, in the taenia the Ca^{2+}-induced contraction is slow and tonic and carbachol has no effect. Entry of Ca^{2+} is facilitated in K^+-depolarized taenia (Chujyo & Holland, 1963, see van Breeman, Wuytack & Casteels, 1975). Papaverine displaces Ca^{2+} concentration-response curves to the right in parallel (Ferrari & Carpenedo, 1968; Simonis, Ariëns & Van den Broeke, 1971; Tomiyama, Takayanagi & Takagi, 1973), whereas noradrenaline, isoprenaline, ATP, adenosine and indomethacin have little effect on Ca^{2+}-induced contractions despite the use of concentrations which fully relaxed taenia in normal Ringer (Spedding *et al.*, 1975; Spedding & Weetman, 1976). The mechanism of the papaverine inhibition is not yet fully established. There is strong evidence that papaverine inhibits phosphodiesterase enzymes (Ferrari, 1974; Pöch & Umfahrer, 1976), thereby increasing intracellular levels of cyclic AMP and cyclic GMP (Miyamoto, Takayanagi, Ohkubo & Takagi, 1976), but Honda, Katsuki, Miyahara & Shibata (1977) have been unable to demonstrate increased intracellular cyclic AMP levels in the taenia after incubation with papaverine. In the present experiments, both PIT and 2-phenylisatogen antagonized Ca^{2+}-induced contractions and thus these drugs resemble papaverine.

However, PIT was the only relaxant drug found to antagonize the inhibitory effects of ATP. Furthermore, PIT antagonized the effects of ATP in the few taenia caeci preparations where PIT did not relax the smooth muscle (Spedding *et al.*, 1975). Close analogues of PIT (for example 2-phenylisatogen, 2,4′-methoxyphenylisatogen) have no affinity for ATP receptors and do not antagonize ATP-induced relaxations, yet these drugs are at least as potent as PIT in relaxing the taenia (Foster *et al.*, 1978). Thus PIT exerts two apparently independent actions on smooth muscle: a direct smooth muscle relaxant effect and an antagonism of the inhibitory effects of ATP.

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