Br. J. Pharmac. (1978), **63**, 309–314

ANTAGONISM OF THE INHIBITORY EFFECTS OF ADENOSINE 5'-TRIPHOSPHATE ON THE ISOLATED TAENIA OF THE GUINEA-PIG CAECUM: STRUCTURE-ACTIVITY RELATIONSHIPS WITHIN A SERIES OF ISATOGEN DERIVATIVES

H.E. FOSTER, M. HOOPER, M. SPEDDING*, A.J. SWEETMAN[1]* & D.F. WEETMAN*

Department of Pharmaceutical Chemistry and Department of Pharmacology*, School of Pharmacy, Sunderland Polytechnic, Sunderland SR1 3SD, Tyne and Wear

1 A series of eight isatogen derivatives was studied on isolated tissues taken from guinea-pigs. The ability of the compounds to relax the taenia, to inhibit adenosine 5'-diphosphate (ADP)-stimulated respiration in mitochondria and to antagonize the inhibitory effects of adenosine 5'-triphosphate (ATP) on smooth muscle was measured.

2 All the derivatives inhibited ADP-stimulated respiration in mitochondria and relaxed the smooth muscle. These two effects were found to be significantly correlated.

3 Only half the compounds blocked the inhibitory effects of ATP, and all of these had a chemically reactive group (nitro, methoxy or pyridyl) in the 2-2'-position. 2-2'-Pyridylisatogen was the most effective blocking agent.

4 The blockade of the inhibitory effects of ATP is not related to the other actions of the derivatives.

Introduction

2-2'-Pyridylisatogen (PIT) has been shown to exert two actions on the isolated taenia of the guinea-pig caecum. Initially there is a relaxation of the smooth muscle which develops slowly (over 10–30 min) and cannot be reduced by autonomic blocking agents (Spedding & Weetman, personal communication). If the tone of the smooth muscle is restored, either by washing or by the addition of a spasmogenic agent, it can be shown that the tissue has become specifically blocked to the inhibitory effects of adenosine 5'-triphosphate (ATP) (Hooper, Spedding, Sweetman & Weetman, 1974; Spedding, Sweetman & Weetman, 1975).

Congeners of PIT, particularly 2-phenylisatogen and the related 2-phenylindolone, have been shown to inhibit oxidative reactions in rat mitochondria (Sweetman, Green & Hooper, 1971). We have therefore studied a series of 2-substituted isatogens with a view to answering the following questions. First, are the three effects of the isatogens related? Second, is PIT the most effective and specific ATP receptor antagonist within this series of compounds?

[1] Present address: Department of Life Sciences, Leeds Polytechnic.

Methods

Smooth muscle

Taenia caeci preparations were obtained from female guinea-pigs (250–600 grams). The preparations were suspended 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. The composition of McEwen's 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. After an equilibration period of 30 min, responses were recorded isotonically on a smoked drum (magnification 1:4, load 1.5 grams).

Cumulative concentration-response curves were obtained for ATP at 20 min intervals, the tissue being washed at least four times between curves (Van Rossum, 1963). Each dose of ATP was allowed to produce its full relaxant effect (5–10 s contact) before the concentration in the bath was increased (see Figure 1). The occasional preparation with low tone, i.e. that did not contract in the isolated organ bath so that it was 25% or less of the fully relaxed length, was not used.

The initial concentration-response curve for ATP on each preparation was disregarded because this did not give a reliable index of the responsiveness of the tissue. Subsequent curves were measured and the EC_{50} value determined on each tissue (i.e. the concentration of ATP producing 50% maximal relaxation). Dose-ratios (DR) were estimated as the ratios of the EC_{50} values after and before the modifying drug, each preparation receiving only one exposure to an isatogen.

A concentration of isatogen, which was selected in preliminary experiments, was allowed to act on the taenia for 30 minutes. In most cases a direct relaxation of the smooth muscle occurred; the degree of this effect was measured as a T_{50} value (i.e. the time taken for the selected concentration of drug to relax the taenia to 50% of the maximum). Carbachol (0.05–1.0 μM) was routinely used to recontract the taenia to within 20% of the initial length, so the measurement of the dose-ratios (see above) was performed in the presence of the cholinomimetic agent.

Isolated mitochondria

The conditions employed to prepare mitochondria from guinea-pig liver were those previously used for the rat (Chappell & Hansford, 1969). Respiration was measured polarographically with a Clark oxygen electrode, the reaction volume being 3 ml and the temperature 32 ± 1°C. Mitochondrial respiration was stimulated by the addition of adenosine 5′-diphosphate (ADP, 0.5 μmol), and the degree of inhibition of this stimulation produced by the isatogens was determined. The inhibitors were added to the reaction chamber 2 min before ADP, and from a series of determinations of this type, the IC_{50} was obtained for each isatogen (i.e. the concentration of inhibitor reducing by 50% the stimulation of respiration induced by ADP). The protein content of the mitochondrial preparations was determined by the Biuret reaction (Gornall, Bardawill & David, 1949) and the volume was adjusted with medium so that it was constant (4 mg) in all experiments. The medium for these experiments consisted of (mM): sucrose 217, Tris-HCl 2.8, sodium glutamate 3.3, sodium hydrogen malate 3.3, phosphate buffer 3.3; pH was 7.4.

Drugs

Six phenylisatogen derivatives were synthesized by established chemical procedures (Bond & Hooper, 1969: see Table 1 for the structures). PIT and its 5-chloro analogue (5-Cl-PIT) were prepared by the method of Ruggli & Cuenin (1944) as modified by Robertson (1973). When the tosylate salt was used, it was prepared by treating the isatogen with *p*-toluensulphonic acid in ether.

The other drugs were: adenosine 5′-triphosphate disodium salt, adenosine 5′-diphosphate disodium salt, carbachol chloride, Tris-HCl and dimethylformamide (BDH); ethylene-glycolbis-(β-aminoethylether)-N,N′-tetracetic acid (EGTA) (Sigma).

Statistics

Values in the text refer to the mean ± s.e. mean. Differences in means were determined by Student's *t* test, after checking the homogeneity of the variances (Snedecor & Cochran, 1967). Correlation coefficients were determined by the method of Daniel (1974).

Results

Relaxation of the taenia

All the isatogens relaxed the taenia, 2-phenylisatogen and its 2′-nitro derivative being the most active (Table 1). The only compound with a substitution in the A-ring, 5-Cl-PIT, was less active than the other derivatives; in half the experiments it did not produce a 50% maximal relaxation of the taenia within 30 min (the T_{50} values were: >30, >30, >30, 24, 15 and 12 minutes).

Antagonism of ATP-induced relaxations of the taenia

Of the eight compounds in Table 1, only PIT, 5-Cl-PIT and the 2′-nitro and 2′-methoxy derivatives of 2-phenylisatogen exerted an appreciable effect. PIT was the most potent of the derivatives: see Figure 1.

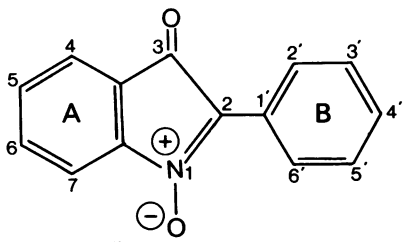
Inhibition of ADP-stimulated respiration

All the derivatives were able to reduce ADP-stimulated respiration. The IC_{50} value for PIT, which was determined on five occasions, showed only a small variation (7.0 ± 0.96 nmol/mg mitochondrial protein). Only single estimates of the IC_{50} values were made for the other compounds (Table 1).

Relationship between the three action of the isatogens

As it was not possible to obtain exact T_{50} values for 5-Cl-PIT (see above), and because this was the only derivative with a substitution in the A-ring, it was omitted from the statistical analysis. Table 2 contains the correlation coefficients for the three properties with the remaining isatogens. The relaxant effect was significantly correlated with the inhibition of the mitochondrial respiratory function, but not with the ATP-receptor antagonism. Indeed, the ATP-receptor

Table 1 Structure-activity relationships of the isatogens



.2-Phenylisatogen

Derivative	Conc (μM)	Antagonism of ATP-receptors (DR ± s.e. mean)	Relaxant activity T_{50} ± s.e. mean (min)	Inhibition of ADP-stimulated respiration IC_{50} (nmol/mg protein)
*2-Phenylisatogen	25	3 ± 1 (4)	4 ± 1	0.6
*2-2'-Nitro-phenylisatogen	25	37 ± 29 (4)	6 ± 1	3
*2-2'-Methoxy-phenylisatogen	50	34 ± 11 (6)	15 ± 6	28
*2-4'-Methoxy-phenylisatogen	50	2 ± 1 (5)	11 ± 2	2
*2-2'-Biphenyl-isatogen	50	2, 1, 1 (3)	15, 18, 25	26
*2-2'-Ethoxycar-bonylphenylisatogen	50	1, 1, 1 (3)	16, 30, 25	31
2-2'-Pyridyl-isatogen	50	61 ± 7 (13)	12 ± 2	7
5-Chloro-2-2'-pyridylisatogen	50	13 ± 2 (6)	see text	5

The derivatives were dissolved in either dimethylformamide (*) or water.
DR represents the ratio of the concentrations of ATP producing a 50% maximal relaxation of the taenia after and before the addition of the isatogen. T_{50} is the time for the tissue to relax by 50% under the influence of the selected concentration of the isatogen; the number of experiments in which the T_{50} and DR were determined is shown in parentheses (see Figure 1). Carbachol (0.05–1.0 μM) was used to restore the tone of the smooth muscle after it had been relaxed by an isatogen derivative. IC_{50} is the concentration of the isatogen that produced a 50% reduction in the stimulation of respiration that followed the addition of ADP to the reaction vessel containing mitochondria.
Note that the range of T_{50} values for the isatogens (4- > 30 min) is much greater than is the value for ATP (for example 20 μM ATP produces a 50% relaxation of the taenia in 2–8 seconds).

blocking effect appears to be independent of the other two actions.

Discussion

These results show that there is no stringent structural requirement within this series of isatogens for either the relaxant action or for the inhibition of ADP-stimulated respiration. In contrast with this, distinct chemical groupings are necessary for the ATP-receptor blocking activity. Consequently, this will be considered first.

The pyrrole ring. Spedding (1977) has shown that the nitrone moiety must be intact for activity, because replacement of either the dative oxygen or the nitrogen yielded inactive derivatives. Similarly, the 3-carbonyl group was also essential.

The A-ring. Although we have not replaced the phenyl ring with others, the activity of 5-Cl-PIT suggests that this may be worthwhile. This compound, at least in some experiments, was much less active than PIT in relaxing the taenia and yet retained a substantial degree of the ATP-receptor blocking activity. However, to date we have been unsuccessful in our attempts to synthesize naphthyl and other derivatives.

The B-ring. Substitutions in this ring determines ATP-receptor blocking activity in a crucial way. As

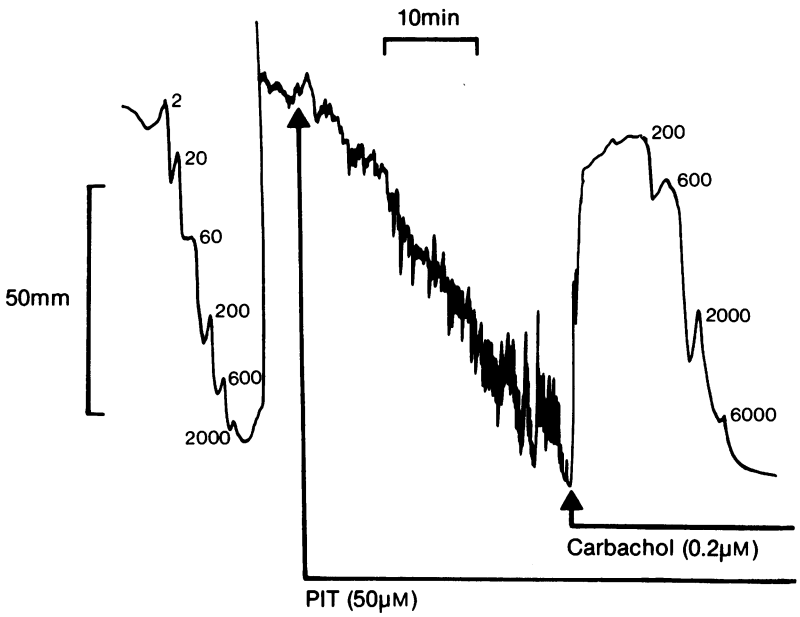


Figure 1 The effect of 2-2'-pyridylisatogen (PIT) on the response of the taenia to ATP. The left hand part of the trace shows the concentration-response curve to ATP (the values are μM). After a 20 min period, during which the kymograph was switched off and the preparation washed four times, PIT (50 μM) was added and the taenia relaxed. During the relaxation the drum speed was a quarter of that employed to record the effects of ATP. After 30 min exposure to PIT, carbachol (0.2 μM) was added to restore the tone of the preparation. The drum speed was increased to that used originally and a second concentration-response to ATP was obtained in the presence of carbachol and PIT. The dose-ratio (DR) was calculated as the ratio of the concentrations of ATP producing a 50% maximal relaxation of the taenia in the second relative to the first curve. The DR in this experiment was 13.5, which was the lowest value for PIT in this series of experiments. The T_{50} (time for 50% maximal relaxation) was 16 minutes.

the 2'-methoxy derivative of 2-phenylisatogen is active but its 4'-methoxy isomer is not, the 2'-position can be seen to be important. This is confirmed by the present finding that all the active ATP-receptor blocking agents have a reactive group in this position. However, there is no simple correlation between the electro-reactivity of the 2'-substituents and the ATP-receptor blocking action: pyridyl and nitro groups lead to electron withdrawal, whereas methoxy is electron donating. There is also no simple correlation between steric factors and the possession of ATP-receptor blocking activity.

It has been shown previously with PIT that the antagonism of ATP-induced relaxation of the taenia was not competitive when the conditions were similar to those described in this paper (Spedding *et al.*, 1975). If either the duration of the exposure or the concentration of PIT is increased (>30 min, >50 μM), the antagonism eventually becomes irreversible (Spedding *et al.*, 1975). Thus it is possible that PIT forms an irreversible complex with or near the ATP-receptor. From the range of isatogen derivatives considered in this paper, only those with a 2'-substituent were active, and all of these would be capable of forming

Table 2 Statistical relationships between the properties of a series of isatogens

First effect	*Second effect*	*r*	P
Respiration (IC_{50})	ATP-receptors (DR)	−0.20	>0.05
Respiration (IC_{50})	Relaxation (T_{50})	+0.88	<0.01
ATP-receptors (DR)	Relaxation (T_{50})	−0.21	>0.05

The values in this table were derived from the data in Table 1. The 5-chloro derivative of 2-2'-pyridylisatogen was omitted (see the text). For other details see footnotes to Table 1. Note that only the mitochondrial action and relaxant effects are significantly correlated.

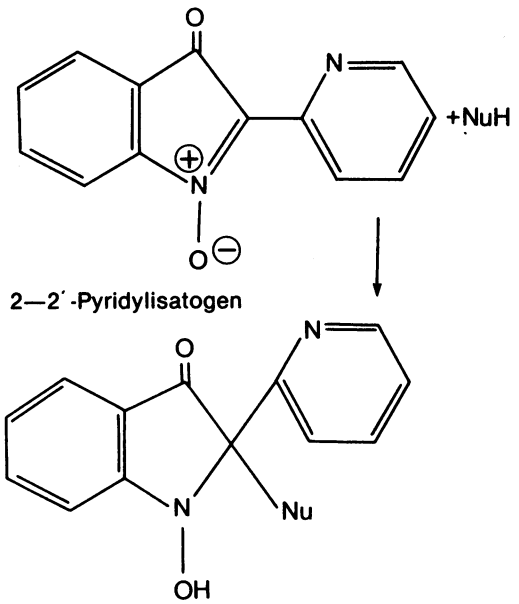


Figure 2 A suggested scheme for the interaction between 2-2′-pyridylisatogen (PIT) and ATP-receptors. A chemically reactive group in the 2.2′-position, in this case pyridyl, reduces the conjugation of this ring with the nitrone group and consequently increases the chemical reactivity of the 2-carbon atom in the pyrrole ring. A covalent bond can then be formed with a nucleophilic group (represented as NuH) at or near the ATP-receptor. Reactions of this type between PIT and the amino group in aminoacids were taken to completion by refluxing at 80°C for 1 h in alcohol. PIT has also been shown to bind irreversibly to albumin at room temperature (Robertson, 1973).

a covalent bond with nucleophilic groups at or near the receptor. The 2′-substituent on the B-ring will reduce the conjugation of this ring with the nitrone group and thereby increase the chemical reactivity of the 2-carbon atom in the pyrrole ring, facilitating the formation of covalent bonding to amino and possibly other groups on the tissue (Robertson, 1973; Hooper & Hiremath, 1977). The possible reaction is depicted in Figure 2. The process can be considered to be analogous to the alkylation of receptor protein by phenoxybenzamine and related compounds (Goodman & Gilman, 1965).

The significant correlation between the relaxant activity and inhibition of ADP-stimulated respiration may indicate that a disturbance of mitochondrial function causes the loss of smooth muscle tone. It is well known that substances that inhibit mitochondrial respiration cause a loss of smooth muscle tone (Bülbring & Lüllman, 1957; Chujyo & Holland, 1963; Weetman & Turner, 1974). The mechanism of the relaxation is not yet established, but it may be caused by an inhibition of prostaglandin biosynthesis, which is at least in part responsible for the tone (Bennett & Posner, 1971; Ferreira, Herman & Vane, 1972; Eckenfels & Vane, 1972), and tissues generate less prostaglandin when their oxidative reactions are inhibited (Nugteren, Beerthuis & Van Dorp, 1967; Splawinski, Nies, Sweetman & Oates, 1973; Ferreira, Herman & Vane, 1976; Samuelsson, Granstom & Hamberg, 1976). However, the correlation between the loss of tone and the inhibition of ADP-stimulated respiration in the present experiments provides only circumstantial evidence of a causative relationship. In other experiments (Spedding & Weetman, unpublished observations), evidence has been obtained that the relaxation of the taenia induced by PIT is due to a 'papaverine-like' action, in which the combination of Ca^{2+} with the contractile proteins is inhibited.

Correspondence to D.F.W. please.

References

BOND, C.C. & HOOPER, M. (1969). Isatogens. Part VI. Synthesis of isatogens *via* tolan (diphenylacetylene) intermediates. *J. Chem. Soc.*, 2455–2460.

BENNETT, A. & POSNER, J. (1971). Studies on prostaglandin antagonists. *Br. J. Pharmac.*, **42**, 584–594.

BÜLBRING, E. & LÜLLMANN, H. (1957). The effect of metabolic inhibitors on the electrical and mechanical activity of the smooth muscle of the guinea-pig's taenia coli. *J. Physiol.*, **136**, 310–323.

CHAPPELL, J.B. & HANSFORD, R.G. (1969). Preparation of mitochondria from animal tissues and yeasts. In *Subcellular Components, Preparation and Fractionation.* ed. Birnie, G.D. & Fox, S.M., pp. 43–56. London: Butterworths.

CHUJYO, N. & HOLLAND, W.C. (1963). Potassium-induced contracture and calcium exchange in the guinea-pig's taenia coli. *Am. J. Physiol.*, **205**, 94–100.

DANIEL, W.W. (1974). *Biostatistics: A Foundation for Analysis in the Health Sciences*, p. 257. London: Wiley.

ECKENFELS, A. & VANE, J.R. (1972). Prostaglandins, oxygen tension and smooth muscle tone. *Br. J. Pharmac.*, **45**, 451–462.

FERREIRA, S.H., HERMAN, A. & VANE, J.R. (1972). Prostaglandin generation maintains the smooth muscle tone of the rabbit isolated jejunum. *Br. J. Pharmac.*, **44**, 328–330P.

FERREIRA, S.H., HERMAN, A.G. & VANE, J.R. (1976). Prostaglandin production by rabbit isolated jejunum and its relationships to the inherent tone of the preparation. *Br. J. Pharmac.*, **56**, 469–477.

GOODMAN, L.S. & GILMAN, A. (1965). *The Pharmacological Basis of Therapeutics.* 3rd edition, pp. 547–548. New York: Macmillan Co.

GORNALL, A.G., BARDAWILL, C.J. & DAVID, M.M. (1949). Determination of serum proteins by means of the biuret reaction. *J. biol. Chem.*, **177**, 751–766.

HOOPER, M. & HIREMATH, S.P. (1977). Isatogens and indolones. In *Advances in Heterocyclic Chemistry*, vol. 22. ed. Katritzky, A.R. & Boulton, A.J. London: Academic Press (in press).

HOOPER, M., SPEDDING, M., SWEETMAN, A.J. & WEETMAN, D.F. (1974). 2-2'-Pyridylisatogen tosylate: an antagonist of the inhibitory effects of ATP on smooth muscle. *Br. J. Pharmac.*, **50**, 458–459P.

McEWEN, L.M. (1956). The effect on the isolated rabbit heart of vagal stimulation and its modification by cocaine hexamethonium and ouabain. *J. Physiol.*, **131**, 678–689.

NUGTEREN, D.H., BEERTHUIS, R.K. & VAN DORP, D.A. (1967). Biosynthesis of prostaglandins. In *Proceedings II Nobel Symposium*. ed. Bergstrom, S. & Samuelsson, B., pp. 45–50. Stockholm: Interscience Publishers.

ROBERTSON, J.W. (1973). Isatogens: further studies. *Ph.D. Thesis, C.N.A.A.*

RUGGLI, P. & CUENIN, H. (1944). Nitrogen heterocycles. LII. Pyridylisatogen. *Helv. Chim. Acta*, **27**, 649–662.

SAMUELSSON, B., GRANSTRÖM, E. & HAMBERG, M. (1967). On the mechanism of biosynthesis of prostaglandins. In *Proceedings II Nobel Symposium*. ed. Bergstrom, S. & Saumelsson, B., pp. 31–34. Stockholm: Interscience Publishers.

SNEDECOR, G.W. & COCHRAN, W.G. (1967). *Statistical Methods*. p. 258. Iowa: Iowa State College Press.

SPEDDING, M. (1977). Studies on the Pharmacological Actions of Isatogens. *Ph.D. Thesis, C.N.A.A.*

SPEDDING, M., SWEETMAN, A.J. & WEETMAN, D.F. (1975). Antagonism of adenosine 5'-triphosphate-induced relaxation by 2-2'-pyridylisatogen in the taenia of guinea-pig caecum. *Br. J. Pharmac.*, **53**, 575–583.

SPLAWINSKI, J.A., NIES, A.S., SWEETMAN, B. & OATES, J.A. (1973). The effects of arachidonic acid, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ on the longitudinal stomach strip of the rat. *J. Pharmac. exp. Ther.*, **187**, 501–510.

SWEETMAN, A.J., GREEN, A.P. & HOOPER, M. (1971). The effect of 2-phenylisatogen on oxidative phosphorylation in rat liver mitochondria. *FEBS Lett.*, **14**, 306–308.

VAN ROSSUM, J.M. (1963). Cumulative dose-response curves. II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Archs int. Pharmacodyn. Thér.*, **143**, 299–330.

WEETMAN, D.F. & TURNER, N. (1974). The effects of inhibitors of adenosine 5'-triphosphate biosynthesis on the responses of the guinea-pig isolated bladder and some other preparations to nerve stimulation. *Archs int. Pharmacodyn. Thér.*, **210**, 12–26.

(Received November 18, 1977.
Revised December 14, 1977.)