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Potentiation by 2,2'-pyridylisatogen tosylate of ATP-responses at a recombinant P_{2Y_1} purinoceptor

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1 2,2'-Pyridylisatogen tosylate (PIT) has been reported to be an irreversible antagonist of responses to adenosine 5'-triphosphate (ATP) at metabotropic purinoceptors (of the P_{2Y} family) in some smooth muscles. When a recombinant P_{2Y_1} purinoceptor (derived from chick brain) is expressed in *Xenopus* oocytes, ATP and 2-methylthioATP (2-MeSATP) evoke calcium-activated chloride currents ($I_{Cl,Ca}$) in a concentration-dependent manner. The effects of PIT on these agonist responses were examined at this cloned P_{2Y} purinoceptor.

2 PIT (0.1-100 μM) failed to stimulate P_{2Y_1} purinoceptors directly but, over a narrow concentration range (0.1-3 μM), caused a time-dependent potentiation (2-5 fold) of responses to ATP. The potentiation of ATP-responses by PIT was not caused by inhibition of oocyte ecto-ATPase. At high concentrations (3-100 μM), PIT irreversibly inhibited responses to ATP with a IC_{50} value of 13 ± 9 μM ($pK_B = 4.88 \pm 0.22$; $n = 3$). PIT failed to potentiate inward currents evoked by 2-MeSATP and only inhibited the responses to this agonist in an irreversible manner.

3 Known P_2 purinoceptor antagonists were tested for their ability to potentiate ATP-responses at the chick P_{2Y_1} purinoceptor. Suramin ($IC_{50} = 230 \pm 80$ nM; $n = 5$) and Reactive blue-2 ($IC_{50} = 580 \pm 130$ nM; $n = 6$) reversibly inhibited but did not potentiate ATP-responses. Coomassie brilliant blue-G (0.1-3 μM) potentiated ATP-responses in three experiments, while higher concentrations (3-100 μM) irreversibly inhibited ATP-responses. The results indicated that potentiation and receptor antagonism were dissociable and not a feature common to all known P_2 purinoceptor antagonists.

4 In radioligand binding assays, PIT showed a low affinity ($pK_i < 5$) for a range of membrane receptors, including: α_1, α_2-adrenoceptors, $5\text{-}HT_{1A}$, $5\text{-}HT_{1B}$, $5\text{-}HT_2$, $5\text{-}HT_3$, D_1, D_2, muscarinic, central benzodiazepine, H_1, μ-opioid, dihydropyridine and batrachotoxin receptors. PIT showed some affinity ($pK_i = 5.3$) for an adenosine (A_1) receptor.

5 In guinea-pig isolated taenia caeci, PIT (12.5-50 μM) irreversibly antagonized relaxations to ATP (3-1000 μM); PIT also directly relaxed the smooth muscle and histamine was used to restore tone. Relaxations to nicotine (10-100 μM), evoked by stimulating intrinsic NANC nerves of taenia caeci preparations in the presence of hyoscine (0.3 μM) and guanethidine (17 μM), were not affected by PIT (50 μM, for 25-60 min).

6 These experiments indicate that PIT causes an irreversible antagonism of ATP receptors but, for recombinant chick P_{2Y_1} purinoceptors, this effect is preceded by potentiation of ATP agonism. The initial potentiation by PIT (and by Coomassie brilliant blue-G) of ATP-responses raises the possibility of designing a new class of modulatory drugs to enhance purinergic transmission at metabotropic purinoceptors.

Keywords: 2,2'-Pyridylisatogen; P_2 purinoceptor; ATP; adenosine; purinergic transmission; recombinant P_{2Y} purinoceptor; metabotropic purinoceptor; *Xenopus* oocyte

Introduction

The classification of ATP receptors (P_2 purinoceptors) is based predominantly on the rank potency of agonists, since there remains a lack of potent and selective antagonists to discriminate between P_2 purinoceptor subtypes (Burnstock, 1978; Burnstock & Kennedy, 1985; Burnstock *et al.*, 1994; Dalziel & Westfall, 1994). Recently, ATP receptors have been reclassified into two extended families, $P_{2X_{1-4}}$ and $P_{2Y_{1-7}}$ purinoceptors, based on agonist activity of novel purine analogues, transduction mechanisms and molecular structure (Abbracchio & Burnstock, 1994).

2,2'-Pyridylisatogen tosylate (PIT) is one of a series of isatogen analogues which, originally, were designed to be selective antagonists of ATP receptors. PIT (10-50 μM) reduced relaxations to adenosine 5'-triphosphate (ATP) in guinea-pig isolated taenia caeci (Spedding *et al.*, 1975), in a time- and concentration-dependent manner consistent with an irreversible antagonism of postjunctional ATP receptors on smooth muscle. Responses to adenosine were unaffected by PIT and, to this extent, this compound provided a pharmacological means to differentiate receptors for ATP and adenosine (Spedding & Weetman, 1976). However, relaxations to the stimulation of intramural non-cholinergic, non-adrenergic (NANC) nerves of taenia caeci were unaffected by PIT (Spedding *et al.*, 1975) and this result raised doubts about PIT as an effective pharmacological tool to test for purinergic transmission.

The early use of PIT to distinguish receptors for ATP and adenosine was complicated by the fact that the compound relaxed smooth muscle directly, an effect secondary to inhibition of oxidative phosphorylation (Foster *et al.*, 1978; Sped-

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ding & Weetman, 1978a,b). This effect meant that spasmogens were needed to recontract the smooth muscle tissues (Spedding & Weetman, 1978b). Furthermore, PIT was not an effective antagonist of ATP responses in all smooth muscles (Spedding & Weetman, 1978a), suggesting its use as an antagonist might be limited to certain subtypes of P_2 purinoceptors. Also, PIT was reported to potentiate, but not antagonize, responses to ATP in the guinea-pig terminal ileum (Kazic & Milosavljevic, 1977).

The present study was designed to re-investigate the pharmacology of PIT on a recombinant P_{2Y} purinoceptor where the actions and efficacy of PIT can be studied in isolation. The P_{2Y_1} purinoceptor derived from chick brain was chosen for this purpose since it is activated by ATP and ADP but by no other naturally-occurring nucleotides or nucleosides (Webb *et al.*, 1993; King *et al.*, 1994; Simon *et al.*, 1995). This recombinant purinoceptor is activated strongly by 2-MeSATP but weakly by α,β-meATP and, therefore, resembles P_{2Y} purinoceptors found on many smooth muscles. Also, the chick P_{2Y_1} purinoceptor is structurally- and pharmacologically-identical to two P_{2Y} purinoceptors cloned recently from vascular endothelial cells (Henderson *et al.*, 1995; Leon *et al.*, 1995). The findings demonstrate that, over a narrow range of concentrations, PIT potentiates the responses to ATP at the P_{2Y_1} purinoceptor expressed in *Xenopus* oocytes, then inhibits responses to ATP and 2-MeSATP at higher concentrations. Other P_2 purinoceptor antagonists were tested and revealed that the potentiating effect is not a feature of these drugs, except for Coomassie brilliant blue-G. The biphasic actions of PIT at a recombinant P_{2Y} purinoceptor might explain some of the discrepancies seen earlier with PIT on ATP receptors in isolated smooth muscle preparations. Part of this study has been communicated to the British Pharmacological Society (King *et al.*, 1994; Spedding *et al.*, 1994).

Methods

Electrophysiology

Defolliculated *Xenopus* oocytes (stages V and VI) were cytosol-injected (40–80 nl, at 2 $\mu g\ \mu l^{-1}$) with a cRNA (transcribed *in vitro* from pSG5-803, capped and polyadenylated, as described in Webb *et al.*, 1993), then stored in a modified Barth's solution and incubated at 18°C for 48 h to express P_{2Y_1} purinoceptors. Thereafter, twin-electrode voltage clamp recordings were carried out on cRNA-injected oocytes, using an Axoclamp 2A (Axon Instruments Inc.) amplifier to control intracellular voltage and measure membrane currents. The voltage-recording microelectrode was filled with K_2SO_4 (0.6 M) and the current-recording microelectrode was filled with KCl (3.0 M). Dual-impaled oocytes were used if their resting potential (E_m) exceeded −40 mV and input resistance (R_{in}) was greater than 0.5 MΩ. Oocytes were superfused (5 ml min^{-1}) with a Ringer solution containing (mM): NaCl 110, KCl 2.5, $CaCl_2$ 1.8, HEPES 5; adjusted to pH 7.4 and maintained at 18±1°C. All drugs tested were dissolved in Ringer solution. Agonists were applied for 1 min followed by a wash period of 20 min with Ringer solution to overcome desensitization of ATP receptors.

Data were stored on magnetic tape (using a Sony-1000ES DAT recorder) and displayed on a two-channel pen-recorder (Gould, 2200S). Experiments were repeated at least three times on oocytes from different donor frogs. Data for concentration-response curves to ATP and 2-MeSATP were normalized to the maximal response obtained for each oocyte; this transformation helped to overcome differences in oocyte size and density of expressed P_{2Y_1} purinoceptors. EC_{50} and EC_{75} values were taken from Hill plots. PIT was applied in cumulative concentrations, for 20 min per dose, followed by application of ATP (given at EC_{75}). IC_{50} values were determined as 50% inhibition of the maximal response to ATP (at EC_{75}), taking into account the potentiation of ATP-responses.

Oocyte ecto-ATPase assays

The enzymatic degradation of ATP by ecto-ATPase was measured by the Fiske/Subbarow (1925) determination of inorganic phosphate (P_i) production, as described for oocytes by Ziganshin and colleagues (1995). Briefly, folliculated oocytes were placed in 24-well dishes, three oocytes per well, in 300 µl of Ringer solution and washed for 30 min, shaking continuously. This wash solution was replaced with 250 µl of Ringer solution containing ATP (100 µM). After 30 min incubation (at 20±1°C), the reaction was halted by removing a sample (100 µl) of the bathing solution and adding it to sodium dodecyl sulphate (0.9 ml, 2.5% w/v solution). Then ammonium molybdate (1 ml, 1.25% w/v solution) and Fiske/Subbarow Reducer (0.1 ml, 0.16% w/v solution) were added to samples and the final solution left at room temperature for 30 min to develop colour. The reaction product was measured spectrophotometrically at 700 nm. KH_2PO_4 solutions were used as phosphate standards to calibrate the spectrophotometer (Beckman Du-65). The resting velocity of P_i production by oocyte ecto-ATPase was compared with the enzyme activity of oocytes incubated with PIT (1–100 µM) for 45 min.

Radioligand binding assays

Binding experiments to study the affinity of PIT for different receptors were carried out as described previously (see Table 1). Inhibition constants (IC_{50} values) were calculated using non-linear regression analysis (Lundon Software) and K_i values were computed according to the Cheng & Prussoff (1973) equation ($K_i = IC_{50}/(1 + K_d)$), where L is the concentration of radioligand and K_d is the apparent dissociation constant. For each receptor, data (pK_i values) are presented for a key reference ligand and for PIT (see Table 1).

Smooth muscle assays

Taenia caeci preparations were taken from female guinea-pigs (weight range: 250–600 g). Isolated preparations were suspended in 10 ml organ baths filled with McEwen's solution (McEwen, 1956) which was maintained at 35±1°C and gassed with 95% O_2 and 5% CO_2. After an equilibration period of 30 min, contractile responses were recorded isotonically (load, 1.5 g) (Spedding *et al.*, 1975).

Cumulative concentration-response curves (Van Rossum, 1963) for purine bases and noradrenaline relaxing taenia caeci strips were determined at 20 min intervals. Each dose of drug was allowed to produce its full effect (5–40 s contact) before the concentration of the drug in the bath was increased. Preparations with low tone (*i.e.*, preparations that failed to contract in the organ bath or were less than 25% of their fully relaxed length) were discarded. The initial concentration-response curve for each agonist was disregarded.

Drugs

All drugs were purchased from Sigma Chemical Co., except for 2-MeSATP which was purchased from Semat (RBI). Suramin was a gift from Bayer plc (U.K.). PIT was synthesized by Dr D. Billington, Institut de Recherches Servier, France. All drugs were dissolved in saline solution except PIT (in 0.1 N HCl, then titrated to pH 7.4).

Results

Oocyte electrophysiology

Under voltage-clamp conditions, ATP (0.01–10 µM) and 2-MeSATP (0.001–1 µM) evoked inward membrane currents ($I_{Cl,Ca}$) in cRNA-injected *Xenopus* oocytes expressing chick P_{2Y_1} purinoceptors. The threshold concentration for activation with ATP (4±2 nM, $n=4$) and 2-MeSATP (≤1 nM, $n=3$) was low,

Table 1 Methodologies employed for evaluation of affinities at various receptor types

Receptors	*References*	*Species*	*Tissues*	*Radioligand concentration Temp/inc time*	*Non-specific* (μM)	*Reference ligand* (p*Ki*)	*PIT* (p*Ki*)
A_1 Adenosine	Hu *et al.*, 1987	Rat	Whole brain	[^{3}H]-**R**(−)-PIA 2 nM, 30°C, 120 min	**R**(−)-PIA 10	**R**(−)-PIA 8.52	5.30
α_1-Adrenoceptor	Glossman *et al.*, 1980	Rat	Cortex	[^{3}H]-prazosin 0.20 nM, 20°C, 60 min	Prazosin 10	Prazosin 9.63	<4
α_2-Adrenoceptor	Renouard *et al.*, 1994	Rat	Cortex	[^{3}H]-RX821,002 0.30 nM, 20°C, 60 min	Adrenaline 10	Adrenaline 7.52	<4
5-HT_{1A}	Hall *et al.*, 1985	Rat	Hippocampus	[^{3}H]-8OH-DPAT 1 nM, 20°C, 30 min	5-HT 10	5-HT 8.69	<4
5-HT_{1B}	Hoyer *et al.*, 1985	Rat	Whole brain	[^{125}I]-cyanopindolol 0.05 nM 37°C, 90 min	5-HT 10	5-HT 7.22	<4
5-HT_2	Leysen *et al.*, 1982	Rat	Frontal cortex	[^{3}H]-ketanserin 2.5 nM, 20°C, 60 min	Ketanserin 10	Ketanserin 8.60	<4
5-HT_3	Nelson *et al.*, 1989	Mouse	Neuro-blastoma	[^{3}H]-granisetron 1 nM, 20°C, 20 min	Ondansetron 10	Ondansetron 8.92	<4
D_1 Dopamine	Hess *et al.*, 1986	Rat	Cortex	[^{3}H]-SCH 23390 0.2 nM, 20°C, 60 min	SCH 23390 10	SCH 23390 9.66	<4
D_2 Dopamine	Köhler *et al.*, 1985	Rat	Striatum	[^{3}H]-raclopride 1 nM, 20°C, 30 min	(+)-Buta-clamol 10	(+)-Buta-clamol 9.30	<4
M (ACh) Muscarine	Roskoski *et al.*, 1985	Rat	Cortex	[^{3}H]-QNB 0.5 nM	Atropine 1	Atropine 10	<4
H_1 Histamine	Hill & Young, 1980	Guinea-pig	Cerebellum	[^{3}H]-mepyramine 0.5 nM 25°C, 30 min	Promethazine 10	Promethazine 9.65	<4
Central Benzodiazepine	Möhler *et al.*, 1981	Rat	Whole brain	[^{3}H]-Ro15-1788 1 nM, 4°C, 60 min	Diazepam 10	Diazepam 8.67	4.15
Mu Opioid	Sharif & Hughes, 1989	Rat	Whole brain	[^{3}H]-DAMGO 0.16 nM, 20°C, 60 min	DAMGO 10	DAMGO 9.79	4.38
Dihydropyridine Calcium Channels	Goll *et al.*, 1983	Rat	Skeletal Muscle	[^{3}H]-isradipine 0.1 nM, 20°C, 60 min	Nifedipine 10	Nifedipine 7.55	<4
Batrachotoxin Sodium Channels	Brown *et al.*, 1986	Rat	Whole brain	[^{3}H]-batrachotoxin 5 nM, 20°C, 60 min	Veratridine 100	Veratridine 6	<4

Temp/Inc time = Temperature/Incubation time.
Results are the means of three independent experiments each made in duplicate.

while maximal responses occurred with ATP ≥ 1 μM ($n=3$) and 2-MeSATP ≥ 0.1 μM ($n=3$). The EC_{50} values for ATP and 2-MeSATP were 155 ± 50 nM ($n=4$) and 10 ± 1 nM ($n=3$), respectively. The agonist activity of 2-MeSATP (1 μM) was $160 \pm 25\%$ ($n=3$) of the full activity of ATP (1 μM, or EC_{100}).

At concentrations up to 100 μM, 2,2′-pyridylisatogen tosylate (PIT) showed no agonist activity at chick P_{2Y_1} purinoceptors (Figure 1a). When superfused for a brief period (60 s), PIT (100 μM) failed either to potentiate or to inhibit the response to a brief superfusion of ATP (10 μM) immediately following the superfusion of PIT (Figure 1a). However, the prolonged superfusion of PIT caused a concentration-dependent potentiation of inward current to ATP (applied at 0.3 μM, the EC_{75}), followed by a concentration-dependent inhibition of inward current to ATP (Figure 1b,c). Thus, low concentrations of PIT (0.1–3 μM) potentiated ATP-responses 2–5 fold, while higher concentrations (3–100 μM) inhibited ATP-mediated inward current with an IC_{50} value of 13.2 ± 9 μM ($pK_B = 4.88 \pm 0.22$, $n=3$). The antagonistic action of PIT did not reverse after washout (for a 2 h period).

Inward current to ATP (0.3 μM) was potentiated in a time-dependent manner by the prolonged superfusion of a single concentration of PIT (0.3 μM, applied for 2 h) (Figure 2a). Under similar conditions, the inward current to 2-MeSATP (10 nM, the EC_{50}) was inhibited in a time-dependent manner by PIT (3 μM) (Figure 2b). The finding that PIT caused a time-dependent potentiation of ATP responses prevented a full study of the concentration-response relationship for ATP in the presence of PIT. Instead, single submaximal concentrations of ATP were selected and PIT (0.1–3 μM) applied cumulatively over a period of several hours to determine the fullest extent ATP-responses were potentiated. These procedures were carried out for four submaximal concentrations of ATP (0.01, 0.03, 0.1 and 0.3 μM), with each concentration tested on separate oocytes. The observed change in ATP responsiveness was compared with the control concentration-response curves for ATP and 2-MeSATP (see Figure 3). This 'concentration-response curve' for ATP (in the presence of PIT) was shifted one $\log_{10}$ unit to the left of the control concentration-response curve (PIT augmenting the potency of ATP 10 fold) but remained to the right of the curve for 2-MeSATP, separated by half a $\log_{10}$ unit. PIT also increased the maximal response of

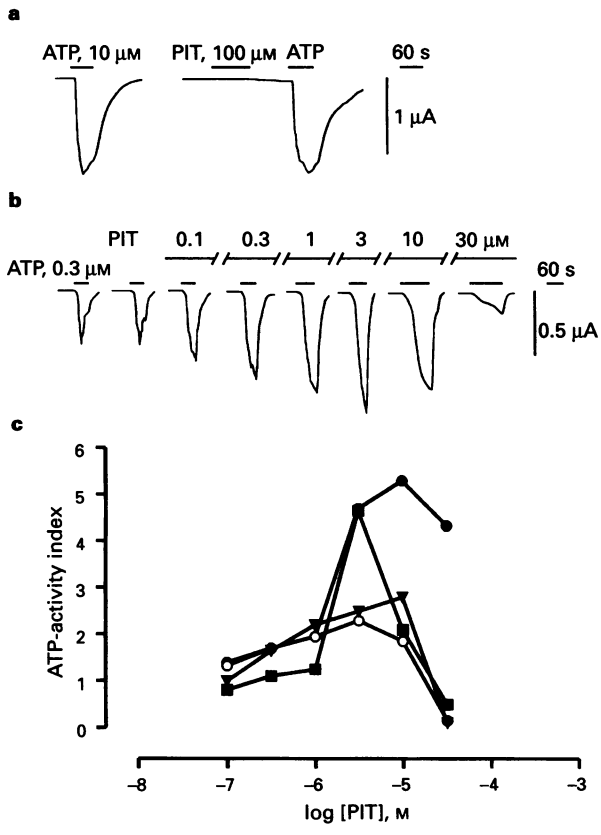

Figure 1 Effects of PIT on membrane current and ATP-mediated currents: in (a), superfused ATP (10 μM, for 60 s) evoked a large (> 1 μA) inward current ($I_{Cl,Ca}$) in oocytes expressing P_{2Y_1} purinoceptors. ATP agonism was not mimicked by PIT (100 μM, for 120 s). A second superfusion of ATP (10 μM, for 60 s), given immediately after PIT, produced an inward current of similar amplitude to the first response to ATP. ($V_h = -40$ mV.) (b) ATP (0.3 μM, the EC_{75}; for 60 s) evoked inward currents of consistent amplitude. PIT (0.1–30 μM), applied cumulatively (20 min per dose, followed by ATP superfusion), failed to alter the holding current but potentiated then inhibited ATP-responses in a concentration-dependent manner. ($V_h = -40$ mV.) (c) The concentration-dependence of potentiation and inhibition of ATP-responses by PIT, in four experiments: ATP-responses (agonist activity, at 0.3 μM) in the absence of PIT were normalized to a value of 1 and the extent of potentiation and inhibition in the presence of PIT was measured relative to the normalized value. Full inhibition of ATP-responses occurred in 3 of 4 experiments. The IC_{50} value for PIT was calculated as 50% inhibition of the maximum response to ATP (in the presence of PIT).

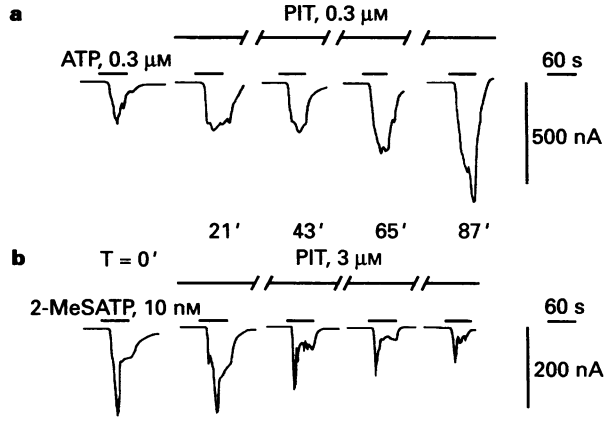

Figure 2 Time-dependent effects of PIT on agonism of P_{2Y_1} purinoceptors: in (a), responses to ATP (0.3 μM, or EC_{75}) were potentiated in a time-dependent manner during the continuous perfusion of PIT (0.3 μM, for 90 min). In (b), responses to 2-MeSATP (10 nM, or EC_{50}) were inhibited in a time-dependent manner by PIT (3 μM). (Note: PIT (3 μM) significantly potentiated ATP-responses in all experiments, see Figure 1c.) ($V_h = -40$ mV).

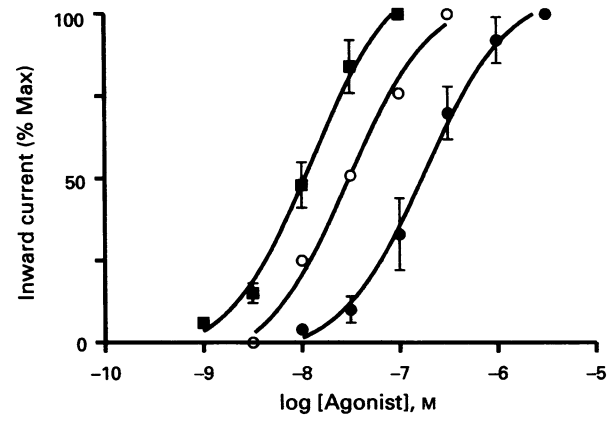

Figure 3 Concentration-response curves for ATP and 2-MeSATP, and for ATP in the presence of PIT: ATP (0.01–1 μM, ●) and 2-MeSATP (0.001–0.1 μM, ■) evoked inward currents in a concentration-dependent manner in oocytes expressing P_{2Y_1} purinoceptors. EC_{50} values were: ATP, 155 ± 50 nM; 2-MeSATP, 10 ± 1 nM. Hill coefficients (n_H) were; ATP, 1.15 ± 0.05; 2-MeSATP, 1.37 ± 0.25. In another series of experiments, PIT (0.1–3 μM) was applied for several hours until the fullest extent of potentiation of ATP-responses were observed. In these experiments, ATP was used at submaximal concentrations (0.01, 0.03, 0.1 and 0.3 μM, ○) and the largest response to these ATP concentrations in the presence of PIT was compared with the response to ATP (10 μM, or > EC_{100}) prior to superfusion of PIT. The resultant PIT-adjusted dose-response curve for ATP (○) lay parallel to the control dose-response curve to ATP (●) and was shifted to the left by 1 $\log_{10}$ unit. Curves fitted by Prism V1.03 (by Graphpad Software Inc.).

ATP (1 μM, the EC_{100}) by 22%, although this raised level failed to match the maximal activity of 2-MeSATP (1 μM) seen under control conditions.

Other known P_2 purinoceptor antagonists were tested for their ability to potentiate ATP-responses at the chick P_{2Y1} purinoceptor. Neither suramin (0.01–100 μM) nor Reactive blue-2 (RB-2, 0.01–100 μM) potentiated ATP-activated inward current although suramin ($IC_{50} = 230 \pm 80$ nM, $n = 5$) and RB-2 ($IC_{50} = 580 \pm 130$ nM, $n = 6$) reversibly inhibited ATP-responses in a concentration-dependent manner. However, the potentiation and antagonistic actions of PIT were mimicked by Coomassie Brilliant blue-G (CBB-G) which is a known antagonist of P_{2Y} purinoceptors in rat parotid acinar cells (Soltoff *et al.*, 1989). CBB-G (0.1–3 μM) potentiated ATP responses in a dose-dependent manner (in 3 experiments) and, thereafter, CBB-G (3–100 μM) irreversibly inhibited ATP responses (in 3 experiments) with an IC_{50} value of 10 μM (see Figure 4a,b).

Oocyte ectoATPase assay

Folliculated oocytes readily dephosphorylated ATP (100 μM), showing a resting velocity of inorganic phosphate (P_i) production of 320 ± 60 pmol min^{-1} per oocyte. This resting velocity of oocyte ectoATPase represented a decrease in the total substrate load (25 nmol) of 1% per min. PIT (1–100 μM) weakly inhibited oocyte ecto-ATPase, but not in a concentration-dependent manner. PIT (1 μM and 100 μM) decreased ecto-enzyme activity by 16%, while other concentrations (3–30 μM) failed to alter the resting rate of ATP breakdown (Figure 5).

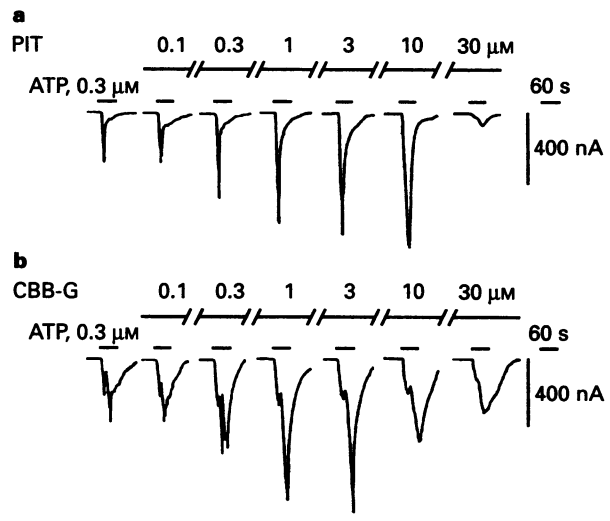

Figure 4 Mimicry of the effects of PIT by Coomassie brilliant blue G: the potentiating and inhibitory effects of PIT (0.1–30 μM, see a) on responses to ATP (0.3 μM) were mimicked by Coomassie brilliant blue-G (0.1–30 μM, see b), potentiating and inhibiting currents to the same extent. ($V_h = -40$ mV).

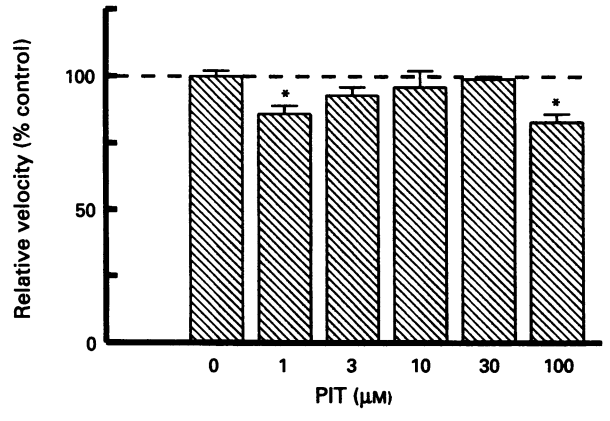

Figure 5 Effect of PIT on ectoATPase activity at *Xenopus* oocytes. Oocytes ectoATPase showed a resting rate of inorganic phosphate production of 320 ± 6 pmol min^{-1} per oocyte. PIT (1–100 μM) inhibited enzyme velocity, but not in a concentration-related manner. PIT (1 μM and 100 μM) significantly inhibited ($^*P < 0.05$) resting ecto-ATPase velocity by 16%, while intermediate concentrations of PIT (3–30 μM) were ineffective.

Radioligand binding assays

PIT had negligible affinity ($pK_i < 5$) for a range of membrane receptors, including: α_1, α_2-adrenoceptors, 5-HT_{1A}, 5-HT_{1B}, 5-HT_2, 5-HT_3, D_1, D_2, muscarinic, central benzodiazepine, H_1, μ-opioid, dihydropyridine and batrachotoxin receptors but showed some affinity ($pK_i = 5.3$) for an adenosine (A_1) receptor, (see Table 1).

Isolated smooth muscle

PIT (12.5–50 μM) antagonized relaxations to ATP (3–1000 μM) in isolated taenia caeci (Figure 6). However, PIT fully relaxed the taenia and it was necessary to restore muscle tone in these experiments with carbachol (0.05–1 μM). Relaxations to nicotine (10–100 μM), which stimulates intrinsic nerves, were studied in the presence of hyoscine (0.3 μM) and guanethidine (17 μM) to obviate the effects of activating cholinergic and adrenergic nerves. Responses to nicotine were unaffected by PIT (50 μM for 25–60 min) although responses

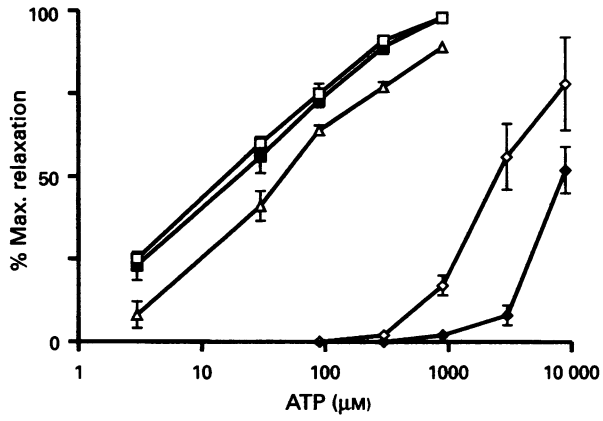

Figure 6 Antagonism of ATP by 2,2′-pyridylisatogen: effects of PIT (control, ■; 12.5 μM, □; 25 μM, △; 50 μM, ◇; 75 μM, ◆) on the cumulative concentration-response curves to ATP in taenia caeci preparations from the guinea-pig caecum. The antagonist was incubated with the tissue for 30 min and the tissues were recontracted with carbachol (0.05–1.0 μM) to within 80–120% of the original tone prior to obtaining the second concentration-response curve to ATP. Data points: mean ± s.e.mean, $n = 8$.

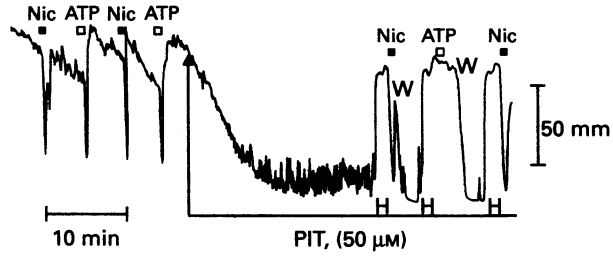

Figure 7 Effects of PIT on ATP and nicotine: the effects of PIT (50 μM, for 25–35 min) on relaxations of taenia caeci preparation to nicotine (Nic, ■, 62 μM) and ATP (□, 20 μM), in the presence of hyoscine (0.3 μM) and guanethidine (17 μM). Histamine (0.9 μM, H) was used to recontract the tissue. Drugs were washed from the isolated tissue bath (at W) and PIT (50 μM) re-applied to the bathing solution.

to ATP were abolished; tone was restored by histamine (0.2–2 μM) in these experiments to counteract the relaxant effects of PIT (Figure 7). The responses to nicotine were abolished by tetrodotoxin (0.33 μM) in three experiments; the relaxant responses to ATP were unaffected by tetrodotoxin (0.33 μM).

Discussion

ATP relaxes the isolated taenia caeci of guinea-pig (Burnstock *et al.*, 1970; Satchell *et al.*, 1972; Spedding *et al.*, 1975; Spedding & Weetman, 1976; Burnstock *et al.*, 1983), by an action at a metabotropic P_{2Y} receptor (Burnstock & Kennedy, 1985; Cusack & Hourani, 1990). In the present study, PIT antagonized the relaxations to ATP and ADP at the taenia in a concentration-dependent manner. There were no overt signs of potentiation of the relaxant response or a leftward shift of the ATP concentration-response curve with low concentrations of PIT. However, the effects of PIT in the taenia differ from its effects in the isolated terminal ileum of guinea-pig where responses to ATP were potentiated (Kazic & Milosavljevic, 1977) and, as seen here, from its effects at chick P_{2Y_1} purinoceptors expressed in oocytes.

PIT caused a time- and concentration-dependent antagonism of the responses to ATP and ADP in the taenia, compatible with irreversible antagonism. Comparison by the dose-

ratio of EC_{50} values for relaxations to purines and catecholamines showed that the inhibitory effects of PIT were selective for only ATP and ADP. In contrast, PIT has little effect on the relaxations to AMP, adenosine, noradrenaline or isoprenaline in the same preparation and at the concentrations used here (Spedding *et al.*, 1975; Spedding & Weetman, 1976). PIT was devoid of affinity for a range of extracellular membrane receptors in radioligand binding experiments, although it did have weak affinity for an adenosine (A_1) receptor. This finding may imply some structural similarity at the site PIT binds to adenosine and ATP receptors, perhaps near a binding site for the adenine ring. Thus, the compound appears to be relatively selective for P_2 purinoceptors in both functional and binding assays. PIT has been shown to bind covalently with some amino acids and will react strongly with sulphydryl groups (Hooper & Robertson, 1971). It was proposed that PIT reacts irreversibly with a nucleophilic site at or near the ATP receptor, causing irreversible antagonism of the response to ATP by alkylating its receptor (Foster *et al.*, 1978).

PIT antagonized the responses to ATP in a guinea-pig isolated stomach preparation (Spedding, 1977), antagonized responses to ATP in rabbit detrusor muscle (Dean & Downie, 1978), guinea-pig uterus (Moritoki *et al.*, 1979), oppossum oesophageal sphincter (Rattan & Goyal, 1980) and the dilator effects of ATP in the pancreatic vascular bed, which are mediated by P_{2Y} receptors (Hillaire-Buys *et al.*, 1991). PIT did not antagonize ATP-induced relaxations in mouse anococygeus muscle (Gibson & Tucker, 1982), or responses to ATP in a series of different vascular preparations from the rabbit (Su, 1978). High concentrations of PIT inhibit oxidative phosphorylation (Foster *et al.*, 1978), an effect which causes the direct relaxant effects of PIT in smooth muscle (Spedding & Weetman, 1978b). Thus, PIT is selective for many but not all P_{2Y} purinoceptors; it may either block or potentiate responses to ATP depending on the tissue and, perhaps, the experimental conditions.

In the present studies, PIT exerted two clear effects on recombinant chick P_{2Y_1} purinoceptors. The compound selectively enhanced the potency and activity of ATP which, normally, is a partial agonist at this cloned purinoceptor (King *et al.*, 1994; Simon *et al.*, 1995). However, PIT irreversibly inhibited responses to 2-MeSATP (which is a full agonist at the P_{2Y_1} purinoceptor) and irreversibly inhibited the responses to ATP secondary to its potentiating effect. It has been suggested (Barnard *et al.*, 1994; Boarder *et al.*, 1995) that the ligand binding pocket for a molecule the size of ATP involves amino acid residues on three transmembrane domains (TM3, TM6 and TM7), with positively-charged amino acids (Arg-262 and Arg-292) binding the phosphate moieties on nucleotides (Erb *et al.*, 1995). As a known alkylating agent of nucleophilic proteins, PIT may bind covalently to extracellular loops (e2, e3 and e4) and/or the amino acid residues of transmembrane domains (TM3, TM6 and TM7) which form the binding pocket of this P_{2Y_1} purinoceptor. It seems plausible that PIT initially enhanced the fit of ATP at the binding pocket by alkylating the receptor, and such an effect would explain the observed increase in affinity and activity for ATP. Under normal circumstances, 2-MeSATP shows a higher affinity than ATP for the binding pocket at the chick, turkey and bovine forms of the P_{2Y_1} purinoceptor and it seems that PIT cannot further enhance its fit and affinity at the binding pocket. Progressive stages of alkylation with PIT appear to denature the receptor to an extent that *either* the access of ATP and 2-MeSATP *or* the necessary conformational change of the receptor during agonist activation are impeded, resulting in an irreversible antagonism. In this context, it was noteworthy that PIT progressively slowed the velocity of inward currents to ATP. It is also noteworthy that other weak alkylating agents (*e.g.*, imidazoline derivatives, antazoline, phentolamine, tolazoline and yohimbine) antagonised relaxations to ATP (and other spasmolytics) in the taenia caeci in a time-dependent and irreversible manner (Rikimaru *et al.*, 1971; Satchell *et al.*, 1973) although, compared to PIT, these agents were not selective for ATP relaxations (Spedding *et al.*, 1975).

The finding that 2-MeSATP possesses a greater potency and activity than ATP at the recombinant P_{2Y_1} purinoceptor holds true for the chick, turkey and bovine homologues of this metabotropic purinoceptor, whether agonism is measured by membrane currents (King *et al.*, 1994; Simon *et al.*, 1995), inositide polyphosphate production (Filtz *et al.*, 1994; Simon *et al.*, 1995) or release of intracelllar calcium (Henderson *et al.*, 1995). The greater agonist activity of 2-MeSATP over ATP is not due to a selective effect of ectoATPase on the breakdown of one nucleotide and not the other. The rates of dephosphorylation of ATP and 2-MeSATP are similar for ecto-ATPase of *Xenopus* oocytes; additionally, the resting velocity of this ecto-enzyme is low, 320 ± 6 pmol min^{-1} per oocyte (Ziganshin *et al.*, 1994; 1995). Instead, synthetic 2-MeSATP has a higher affinity for chick P_{2Y_1} purinoceptors (also for turkey and bovine P_{2Y_1} purinoceptors) than ATP and ADP.

Erb *et al.* (1995) have commented on the potential therapeutic value of novel high-affinity synthetic nucleotides at metabotropic purinoceptors of the P_{2Y} family. However, an enhancement of the potency of ATP represents an alternative approach to novel agonists. This latter approach has been exploited elsewhere, with barbiturates and benzodiazepines at GABA receptors. In this respect, PIT may represent the forerunner of a new class of therapeutic agents designed to modulate the activity of ATP at P_{2Y} purinoceptors. The potentiating effect of PIT was shared by Coomassie brilliant blue-G (which is structurally-unrelated to PIT), raising the possibility of synthesizing more than one class of therapeutic agent capable of augmenting ATP-responses at metabotropic P_2 purinoceptors. The initial observations of a potentiating effect of PIT and Coomassie brilliant blue-G seem sufficiently encouraging to warrant further study of isatogen and brilliant blue derivatives on purinergic control of diverse cellular functions.

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