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Responses of the longitudinal muscle and the muscularis mucosae of the rat duodenum to adenine and uracil nucleotides

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1 Previous studies have shown that the rat duodenum contains P_1 and P_{2Y} purinoceptors via which it relaxes to adenosine and adenosine 5'-triphosphate (ATP) respectively. It has also been shown to contract to uridine 5'-triphosphate (UTP) and adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S), and based on their differential inhibition by the P_2 antagonist suramin it has been suggested that they act via two separate receptors. In addition, the rat duodenum has been shown to dephosphorylate ATP rapidly via ectonucleotidases and adenosine deaminase. In this study the responses of two preparations from the rat duodenum, the longitudinal muscle and the muscularis mucosae, were investigated using a series of nucleotides and suramin.

2 2-Methylthioadenosine 5'-triphosphate (2-MeSATP), ATP, ATP-γ-S and adenosine 5'-α,β-methylene-triphosphonate (AMPCPP) each relaxed the longitudinal muscle, with an agonist potency order of 2-MeSATP > ATP = ATP-γ-S > AMPCPP, while UTP and uridine 5'-diphosphate (UDP) were not observed to elicit relaxation. This indicates the presence of a relaxant P_{2Y}-purinoceptor on the longitudinal muscle. The longitudinal muscle did not contract to any of the agonists at concentrations of 300 μM, apart from ATP-γ-S which caused very weak contractions.

3 ATP-γ-S, adenosine 5'-methylenediphosphonate (AMPCP), AMPCPP, ATP, UTP, adenosine 5'-diphosphate (ADP), UDP and 2-MeSATP each contracted the muscularis mucosae with an agonist potency order of ATP-γ-S $\geqslant$ AMPCP $\geqslant$ AMPCPP = ATP = UTP = ADP = UDP > > 2-MeSATP, although maximal responses were not obtained at concentrations of 300 μM. The muscularis mucosae did not relax to any of the agonists at concentrations of 300 μM.

4 Suramin (1 mM) inhibited relaxations induced by ATP on the longitudinal muscle, shifting the relaxation concentration-response curve to the right. This further supports the presence of a P_{2Y}-purinoceptor on this muscle layer. Suramin (1 mM) inhibited contractions induced by AMPCPP, but not those induced by ATP, UTP or ATP-γ-S, in the muscularis mucosae. Desensitization of the muscularis mucosae was seen with AMPCPP, but not with UTP or ATP-γ-S, and no cross-desensitization between AMPCPP and UTP or ATP-γ-S was observed. This suggests there are two receptors which mediate contraction on the rat duodenum muscularis mucosae, one suramin-sensitive and the other suramin-insensitive.

5 ATP was rapidly degraded by the muscularis mucosae to ADP, adenosine 5'-monophosphate (AMP) and inosine, with no adenosine being detected. A similar rate of degradation was seen for UTP with UDP, uridine 5'-monophosphate (UMP) and uridine being formed and for 2-MeSATP with 2-methylthioadenosine 5'-diphosphate (2-MeSADP), 2-methylthioadenosine 5'-monophosphate (2-MeSAMP) and 2-methylthioadenosine being formed. AMPCPP and ATP-γ-S were both degraded more slowly, AMPCPP being degraded to AMPCP, and ATP-γ-S to ADP, AMP and inosine. Suramin (1 mM), did not significantly affect the rate and pattern of degradation of these nucleotides, apart from AMPCPP which was degraded slightly more slowly in the presence of suramin.

6 These results show that there is a P_{2Y}-purinoceptor which mediates relaxation in the rat duodenum longitudinal muscle. They also show that there is a contraction-mediating suramin-sensitive receptor on the rat duodenum muscularis mucosae which is desensitized by AMPCPP, and thus is probably of the P_{2X} subtype. In addition, there is a contraction-mediating suramin-insensitive receptor on the rat duodenum muscularis mucosae which is not desensitized by UTP or ATP-γ-S, and at which ATP and UTP show equal potency, and is thus probably of the P_{2U} subtype. In addition, the rat duodenum muscularis mucosae contains ectonucleotidases and adenosine deaminase, which rapidly degrade nucleotides, although the inhibition by suramin of this degradation is unlikely to explain the differential antagonism by suramin of the nucleotides.

Keywords: Rat duodenum longitudinal muscle; rat duodenum muscularis mucosae; P_2 purinoceptors; ectonucleotidases; ATP; UTP; suramin

Introduction

The contraction or relaxation of smooth muscle elicited by adenosine and adenosine 5'-triphosphate (ATP) are mediated via receptors termed purinoceptors. P_1 purinoceptors at which adenosine act are subdivided into A_1, A_{2a}, A_{2b} and A_3 subtypes (reviewed by Collis & Hourani, 1993; and Fredholm *et al.*, 1994). P_2-purinoceptors on smooth muscle at which ATP act are subdivided into P_{2X}, P_{2Y} and P_{2U} ('nucleotide receptor') subtypes, and agonist potency orders have been used to distinguish them (Fredholm *et al.*, 1994). While the observed agonist potency order at the P_{2X} subtype in smooth muscle preparation is adenosine 5'-α,β-methylenetriphosphonate (AMPCPP) > ATP = 2-methylthioadenosine 5'-triphosphate (2-MeSATP) (Burnstock & Kennedy, 1985), there is now evidence from cloning data that this potency order does not re-

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flect the true potency at the receptor, and that there is more than one type of P_{2X} receptor. ATP, 2-MeSATP and AMPCPP are approximately equipotent at one type (cloned from the rat vas deferens), whereas ATP and 2-MeSATP are approximately equipotent while AMPCPP is inactive at the other type (cloned from PC12 cells) (reviewed by Kennedy & Leff, 1995; and Surprenant *et al.*, 1995). The agonist potency order at the P_{2Y} subtype is 2-MeSATP > ATP > AMPCPP (Burnstock & Kennedy, 1985), whereas at the P_{2U} subtype ATP and the pyrimidine nucleotide uridine 5'-triphosphate (UTP) show equal potency, but AMPCPP and 2-MeSATP show little or no activity and thus the agonist potency order is UTP = ATP > > 2-MeSATP = AMPCPP (O'Connor *et al.*, 1991; Dubyak & El-Moatassim, 1993). However, cross-desensitization is seen between ATP analogues and UTP in the rat colon muscularis mucosae (P_{2Y}) (Hourani *et al.*, 1993) and in the mouse vas deferens (P_{2X}) (Von Kügelgen *et al.*, 1990), suggesting that in some tissues UTP may act at these receptors. In addition, separate UTP receptors termed pyrimidinoceptors at which purines are not active have been proposed to exist in a number of tissues, including several blood vessels (Von Kügelgen *et al.*, 1987; Saïag *et al.*, 1987; 1990; Von Kügelgen & Starke, 1990; reviewed by Seifert & Schultz, 1989), the rat superior cervical ganglion (Connolly, 1994) and C6-2B rat glioma cells (Lazarowski & Harden, 1994).

The action of ATP and its analogues at P_2-purinoceptors can be complicated by the presence of ectonucleotidases on smooth muscle which sequentially dephosphorylate them to their corresponding nucleosides which may then act at P_1-purinoceptors (Bailey & Hourani, 1990; 1992). Dephos-phorylation of ATP and some of its analogues including 2-MeSATP has been shown to result in reduced potency in some tissues such as the guinea-pig bladder (P_{2X}), in which ATP and adenosine elicit opposing responses (Welford *et al.*, 1987), but not in others such as the taenia caeci (P_{2Y}) in which ATP and adenosine elicit the same response (Welford *et al.*, 1986). Indeed, the discrepancy between the potency order at the cloned P_{2X} receptors and that expected from studies in smooth muscle preparations is likely to be due to this factor (Kennedy & Leff, 1995). The effect of ectonucleotidases on potency of nucleotides has recently been confirmed using an ectonucleotidase inhibitor (Crack *et al.*, 1995).

Although no truly selective antagonists exist for either the P_{2X}, P_{2Y} or P_{2U} subtypes, suramin has been widely used, although it does not discriminate between the P_{2X} or P_{2Y} subtypes for which it has pA_2 values of around 5 (Hoyle *et al.*, 1990). Suramin has also been shown to antagonize P_{2U} receptors in some tissues such as rat PC12 cells (Murrin & Boarder, 1991) and the rat aorta (Dainty *et al.*, 1994), but not in others such as bovine aortic endothelial cells (Wilkinson *et al.*, 1993) and aortic collateral arteries (Wilkinson *et al.*, 1994a) or canine tracheal epithelium (Dainty *et al.*, 1994), suggesting there may be subclasses of the P_{2U} receptor. In addition to antagonism of some P_2 subtypes, suramin has also been shown to be an inhibitor of several enzymes including ectonucleotidases (Hourani & Chown, 1989; Crack *et al.*, 1994), which may complicate the interpretation of results.

In the absence of truly selective antagonists for the P_2 subtypes, desensitization has been used to discriminate between subtypes. P_{2X}-purinoceptors readily desensitize to AMPCPP and other slowly degradable analogues, and indeed this desensitization is characteristic of this subtype (Burnstock & Kennedy, 1985). P_{2Y}- and P_{2U}-purinoceptors in many tissues do not show marked desensitization, but 2-MeSATP has been shown to desensitize P_{2Y}-purinoceptors in some tissues such as the rat colon muscularis mucosae (Hourani *et al.*, 1993) and bovine aortic endothelial cells (Wilkinson *et al.*, 1994b). P_{2U} receptors have also been shown to desensitize to UTP in bovine aortic endothelial cells (Wilkinson *et al.*, 1994b).

The rat duodenum contains P_1 (A_1 and A_2 subtypes) (Nicholls *et al.*, 1992a) and P_{2Y} purinoceptors (Nicholls *et al.*, 1990) which mediate relaxation to adenosine and ATP respectively. In addition it contains receptors at which UTP and adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S) act to cause contraction (Johnson & Hourani, 1994). In the rat colon the response to adenosine and to nucleotides on the longitudinal muscle (Bailey & Hourani, 1992) and the muscularis mucosae (Bailey *et al.*, 1992; Bailey & Hourani, 1990) are different, and it seemed likely that in the duodenum the different receptors might also be located on different muscle layers. We therefore investigated the response of the longitudinal muscle and the muscularis mucosae of the rat duodenum to a series of adenine and uracil nucleotides and the effect of suramin. In addition, the rate of degradation of some of these nucleotides in the rat duodenum muscularis mucosae was investigated.

Methods

Pharmacological studies

Non-starved adult male Wistar Albino rats (University of Surrey strain) (200–250 g) were killed by cervical dislocation. The duodenum was dissected out by cutting at the base of the pyloris and at 1.5 cm from this point, cleared of any connective tissue and washed thoroughly with Krebs solution, and a glass pipette (external diameter 5 mm) place inside the tissue. A scalpel was gently run down the length of the duodenum cutting only the outer longitudinal muscle layer, leaving the inner muscularis mucosae intact. The longitudinal muscle was then removed as a sheet from the muscularis mucosae by gentle rubbing with moist cotton wool. The wet weights of the whole duodenum, muscularis mucosae and longitudinal muscle were approximately 300, 250 and 50 mg respectively. For each of the muscle layers cotton thread was tied round each end of the segment of tissue, so that in the case of the muscularis mucosae the lumen was sealed. The tissues were then mounted in 4 ml organ baths containing Krebs solution aerated with 95% O_2/5% CO_2 and maintained at 35–36°C. The Krebs solution had the following composition (mM): NaCl 118, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. A resting tension of 1 g was applied to the tissues and isometric responses were recorded with a Grass FT03 transducer and displayed on a Grass 79D polygraph. Tissues were allowed to equilibrate for 45 min, with washes at 15 min intervals, before the addition of drugs.

The potency of the agonists was expressed as the EC_{50} for the longitudinal muscle, calculated by regression analysis of the linear portions of the individual log concentration-response curves. For statistical analysis of results the pEC_{50} values (the negative log of these EC_{50} values) were compared by Student's t test and a value of $P<0.05$ considered to be significant. Concentration-response curves to only one agonist were carried out on any single tissue. Relaxations were quantified by pre-contracting the tissue with carbachol (0.3 μM) with a contact time of 1.5 min and challenging with agonist (contact time 1 min). Contractions to carbachol were measured from the centre of spontaneous baseline activity to the highest point of carbachol contraction. Relaxations were measured as the reduction in the height of the rhythmic contractions, relative to the time immediately before addition of the relaxant agonist, and expressed as % reversal of carbachol contraction. Contractions were observed by adding agonist (contact time 1 min) without pre-contracting the tissue and contractions were expressed as % of contraction caused by a concentration of carbachol which gave a maximal response (10 μM), determined as the mean value of 3 doses of carbachol given before addition of any other drugs. A 10–15 min dose-cycle was used and a 30–60 s washout following each dose of test agonist. Concentration-response curves were obtained non-cumulatively using tissues from 4–8 animals, and results were expressed graphically as the mean and standard error of the mean (s.e. mean) of the responses obtained.

Relaxant and contractile responses of the muscularis mucosae to a series of nucleotides were obtained using a protocol similar to that used for the longitudinal muscle (see above).

However, for relaxant responses 10 μM carbachol was used to pre-contract the muscularis mucosae, and for contractile responses a concentration of 100 μM carbachol gave a maximal response. The potency of the agonists was expressed as the EC_{50} for the muscularis mucosae, calculated by regression analysis of the linear portions of the individual log concentration-response curves. For statistical analysis of results the pEC_{50} values were compared using Student's t test and a value of $P<0.05$ considered to be significant.

On the longitudinal muscle relaxation concentration-response curves to ATP were also determined in the presence of suramin (1 mM) pre-incubated for 1 h. On the muscularis mucosae contraction concentration-response curves to ATP, UTP, ATP-γ-S and AMPCPP were also determined in the presence of suramin (1 mM) pre-incubated for 1 h. For AMPCPP, the EC_{50} value in the presence of suramin was extrapolated by linear regression analysis of each of the individual log concentration-response curves, as contractile responses fell just below this value.

UTP, ATP-γ-S and AMPCPP were used in an attempt to desensitize the receptors in the muscularis mucosae. A 100 μM concentration of each agonist was administered repeatedly over 90 min with a contact time of 5 min followed by washout of the agonist and a time of 10 min before readdition of the agonist to allow the tension to return to baseline values. The agonists were tested on separate tissues, and the percentage contraction for each dose of agonist was determined as a percentage relative to the initial dose of the agonist given. Cross-desensitization between AMPCPP and UTP or ATP-γ-S was also investigated. A 100 μM concentration of UTP or ATP-γ-S (control response) was first administered followed after 10 min by the addition of the desensitizing dose of AMPCPP (100 μM) for 5 min, after which the tissue was again challenged with UTP, ATP-γ-S or AMPCPP (100 μM). The percentage contraction for UTP, ATP-γ-S or AMPCPP was determined relative to the control response.

Degradation studies in the rat duodenum muscularis mucosae

The degradation of ATP, UTP, 2-MeSATP, AMPCPP and ATP-γ-S by the muscularis mucosae was investigated in the presence and absence of suramin (1 mM). The muscularis mucosae was mounted in 4 ml organ baths and allowed to equilibriate for 45 min with regular washings as described above. Suramin (1 mM) or water (40 μl) was then added to the organ baths and incubated for 1 h, with regular washes and readministration of the suramin. ATP, UTP, 2-MeSATP, AMPCPP or ATP-γ-S (100 μM) were then added to the organ baths and 150 μl aliquots were taken at 0, 5, 10, 15 and 20 min time points, stored at -20°C, and analysed by high performance liquid chromatography (h.p.l.c.) at a later date. Percentage degradation of the nucleotides were compared at the 5 min time points in the presence and absence of suramin by Student's t test, and a value of $P<0.05$ considered to be significant. H.p.l.c. analysis was carried out with a Perkin Elmer Integral 4000 h.p.l.c. with an Apex II 5 μm ODS C_{18} column eluted with 0.1 M KH_2PO_4/8 mM tetrabutylammonium hydrogen sulphate (pH 6.0) (solvent A) and a 60:40 mixture of solvent A and acetonitrile (pH 6.73) (solvent B), using a non-linear gradient (0–2.5 min 0% B, 2.5–5 min 0–20% B, 5–10 min 20–40% B, 10–13 min 40–100% B, 13–18 min 100% B), at a flow rate of 1.3 ml min^{-1}. The purines were detected by u.v. absorbance at 259 nm and quantified from the height of their absorbance peaks, which was linearly related to the concentration.

Materials

ATP, UTP, uridine 5'-diphosphate (UDP), adenosine 5'-diphosphate (ADP), adenosine 5'-methylenediphosphonate (AMPCP), AMPCPP, carbachol and tetrabutylammonium hydrogen sulphate were obtained from Sigma Chemical Co., Poole, Dorset, 2-MeSATP was obtained from Research Biochemicals, Semat Ltd, St. Albans, Herts, ATP-γ-S was obtained from Boehringer Mannheim, Lewes, East Sussex and buffer salts and solvents were of analytical or h.p.l.c. grade and were obtained from BDH, Poole, Dorset. Suramin was a generous gift from Bayer, UK.

Results

Rat duodenum longitudinal muscle

2-MeSATP, ATP, ATP-γ-S and AMPCPP each relaxed the longitudinal muscle in a concentration-dependent manner with mean pEC_{50} values of 5.96 ± 0.11 (EC_{50} = 1.1 μM), 5.51 ± 0.29 (EC_{50} = 3.1 μM), 5.49 ± 0.04 (EC_{50} = 3.2 μM) and 5.21 ± 0.09 (EC_{50} = 6.2 μM) respectively. UDP and UTP were inactive. The agonist potency order for relaxation was 2-MeSATP > ATP = ATP-γ-S > AMPCPP, and the concentration-response curves to ATP and 2-MeSATP appeared to be biphasic (Figure 1). Pretreatment with suramin (1 mM) shifted the relaxation concentration-response curve for ATP to the right, and this shift was statistically significant ($P<0.001$) resulting in a mean pEC_{50} value of 3.90 ± 0.20 (EC_{50} = 130 μM), and a dose ratio of 42 (Figure 2). Relaxant responses to the agonists were reversible and occurred immediately upon their administration reaching a maximum response after 10–15 s. The responses were therefore qualitatively similar to those seen in the whole duodenum (see representative traces in Johnson & Hourani, 1994; Nicholls *et al.*, 1990). The longitudinal muscle contracted very weakly to high concentrations of ATP-γ-S (100

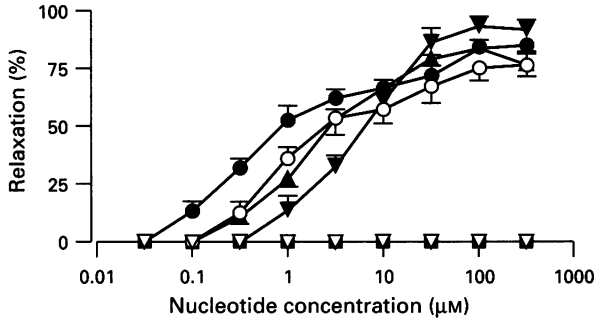

Figure 1 Relaxation of the rat duodenum longitudinal muscle by 2-MeSATP (●), ATP (○), ATP-γ-S (▲), AMPCPP (▼), UTP (▽) or UDP (■), expressed as % reversal of the contraction induced by carbachol (0.3 μM). Each point is the mean with s.e. mean of at least four determinations. See text for abbreviations.

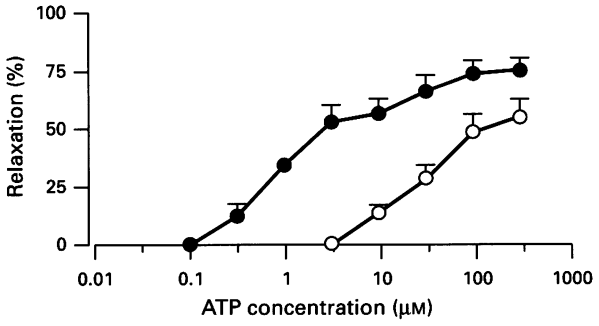

Figure 2 Relaxation by ATP of the rat duodenum longitudinal muscle in the absence (●) or presence (○) of suramin (1 mM), expressed as % reversal of the contraction induced by carbachol (0.3 μM). Each point is the mean with s.e. mean of at least four determinations.

and 300 μM), achieving approximately 10 and 25% respectively of the response to carbachol. These contractile responses were qualitatively similar to those seen in the whole duodenum (see representative traces in Johnson & Hourani, 1994). The other agonists (2-MeSATP, AMPCP, AMPCPP, ATP, UTP, ADP and UDP) at concentrations of 300 μM did not contract the longitudinal muscle (data not shown).

Rat duodenum muscularis mucosae

ATP-γ-S, AMPCP, AMPCPP, ATP, UTP, ADP and UDP each contracted the muscularis mucosae in a concentration-dependent manner with mean pEC_{50} values of 5.11 ± 0.12 (EC_{50} = 7.8 μM), 5.00 ± 0.11 (EC_{50} = 10 μM), 4.84 ± 0.12 (EC_{50} = 15 μM), 4.67 ± 0.16 (EC_{50} = 21 μM), 4.59 ± 0.13 (EC_{50} = 26 μM), 4.70 ± 0.17 (EC_{50} = 20 μM) and 4.76 ± 0.35 (EC_{50} = 17 μM) respectively. However, the curves were not sigmoidal in shape being incomplete at 300 μM. 2-MeSATP also weakly contracted the muscularis mucosae, but at 300 μM achieved only 35% and so no EC_{50} value could be otained. The agonist potency order for contraction was ATP-γ-S ≥ AMPCP ≥ AMPCPP = ATP = UTP = ADP = UDP > > 2-MeSATP (Figure 3). Contractile responses to all agonists were sustained and declined slowly, reaching a maximum after approximately 30–45 s. The muscularis mucosae did not relax to any of the agonists at concentrations of 300 μM (data not shown).

Pretreatment with suramin (1 mM) shifted the contraction concentration-response curve for AMPCPP to the right and this shift was statistically significant ($P<0.001$), resulting in a mean pEC_{50} value of 3.31 ± 0.12 (EC_{50} = 490 μM) and a dose ratio of 33 (Figure 4a). In contrast, pretreatment with suramin (1 mM) did not significantly shift the contraction concentration-response curves to ATP, UTP or ATP-γ-S, resulting in mean pEC_{50} values of 4.90 ± 0.18 (EC_{50} = 13 μM), 4.25 ± 0.13 (EC_{50} = 56 μM) and 4.74 ± 0.20 (EC_{50} = 18 μM) respectively in the presence of suramin (Figures 4b–d).

Desensitization was observed to AMPCPP (100 μM), as subsequent contractions were significantly different from the response to the first dose of agonist (Figure 5a and 5b). In contrast no statistically significant desensitization was observed to UTP or ATP-γ-S (100 μM) when repeatedly administered over 90 min (Figure 5a). Cross-desensitization between AMPCPP and UTP or ATP-γ-S (100 μM) was not observed, as pretreatment with AMPCPP did not significantly reduce contractions induced by UTP or ATP-γ-S (Figure 5b).

Degradation studies on the rat duodenum muscularis mucosae

ATP (100 μM) was rapidly degraded by the muscularis mucosae to ADP, adenosine 5'-monophosphate (AMP) and inosine with no adenosine being detected, and around 25% of the ATP remained after 5 min (Figure 6a). A similar rate of degradation was seen for UTP (100 μM), with UDP, uridine 5'-monophosphate (UMP) and uridine being formed (Figure 6b), and for 2-MeSATP (100 μM), with 2-methylthioadenosine 5'-diphosphate (2-MeSADP), 2-methylthioadenosine 5'-monophosphate (2-MeSAMP) and 2-methylthioadenosine being formed (Figure 7a). In contrast, ATP-γ-S (100 μM) was degraded much more slowly than ATP, around 60% of ATP-γ-S remaining after 5 min, with ADP, AMP and inosine being formed (Figure 7b). AMPCPP was also degraded at a slower rate than ATP, around 55% remaining after 5 min, with AMPCP being formed (Figure 7c). ATP, UTP and 2-MeSATP were almost completely degraded after 20 min, whereas for ATP-γ-S and AMPCPP around 40% and 45% respectively remained (Figures 6 and 7). Suramin (1 mM) did not significantly affect the rate or pattern of degradation of the nucleotides compared at 5 min, apart from AMPCPP which was degraded more slowly in the presence of suramin ($P<0.01$) (Figures 6 and 7).

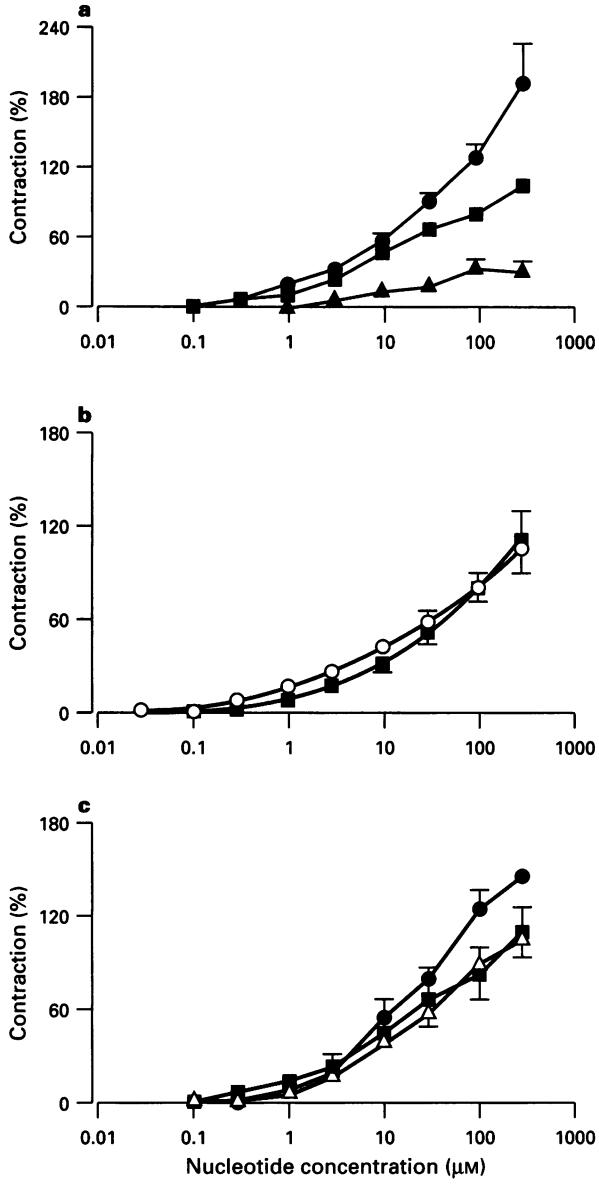

Figure 3 Contraction of the rat duodenum muscularis mucosae by (a) ATP-γ-S (●), AMPCPP (■) or 2-MeSATP (▲); (b) ATP (○) or UTP (■); and (c) AMPCP (●), ADP (△) or UDP (■), expressed as % of contraction induced by carbachol (100 μM). The results are shown as three separate figures for clarity. Each point is the mean with s.e. mean of at least four determinations. See text for abbreviations.

Discussion

The whole rat duodenum has been shown to contain P_{2Y}-purinoceptors via which it relaxes to a series of nucleotides with agonist potency orders of 2-MeSATP > ATP = ADP > ATP-γ-S = AMPCPP > > GTP > > UTP = UDP (Nicholls *et al.*, 1990; Johnson & Hourani, 1994). When examined for a relaxant response to series of nucleotides, the rat duodenum muscularis mucosae was unresponsive. In contrast, the rat duodenum longitudinal muscle relaxed with an agonist potency order of 2-MeSATP > ATP = ATP-γ-S > AMPCPP, with UTP and UDP being inactive. In addition, suramin antagonized the relaxant response to ATP on the longitudinal muscle, significantly shifting its relaxation concentration-response curve to the right. Our data thus clearly shows that it is the longitudinal muscle which contains the P_{2Y}-purinoceptor previously demonstrated in the whole duodenum as the potency order and the qualitative nature of the response are similar (Nicholls *et al.*, 1990; Johnson & Hourani, 1994;

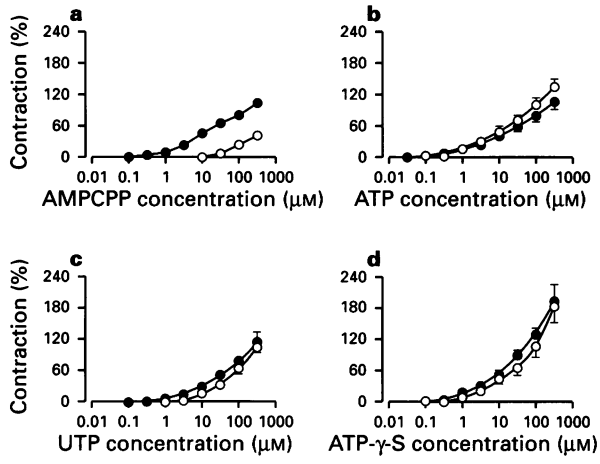

Figure 4 Contraction of the rat duodenum muscularis mucosae by (a) AMPCPP, (b) ATP, (c) UTP and (d) ATP-γ-S in the absence (●) or presence (○) of suramin (1 mM). Contraction was expressed as % of contraction induced by carbachol (100 μM). Each point is the mean with s.e. mean of at least four determinations. See text for abbreviations.

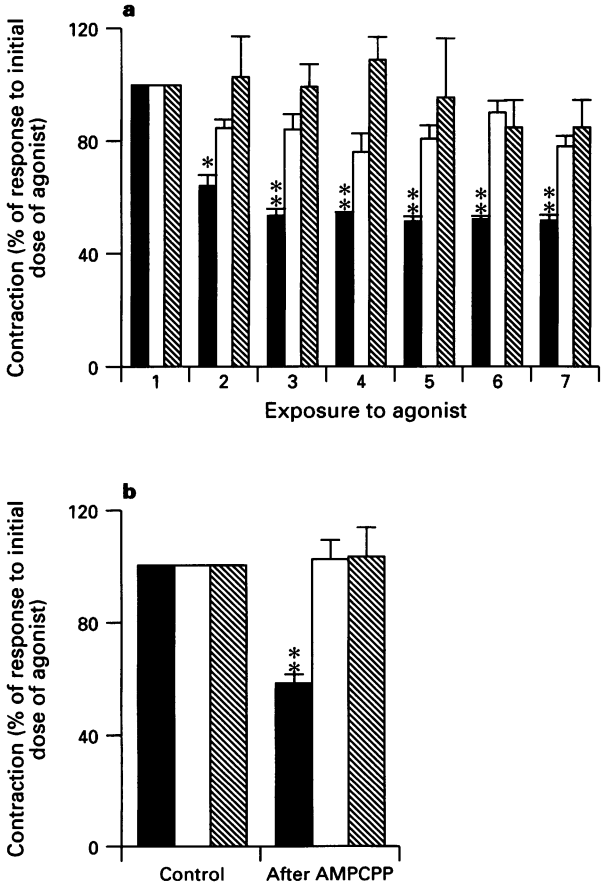

Figure 5 Histograms showing contraction of the rat duodenum muscularis mucosae in response to repeated doses of agonist (100 μM), and expressed as the % of response to the initial dose of the agonist administered. (a) Shows contraction to repeated administration of the same agonist and (b) shows contraction following exposure to a desensitizing dose of AMPCPP. AMPCPP is shown as solid columns, ATP-γ-S as open columns and UTP as hatched columns. Each column is the mean with s.e. mean of four determinations. Values significantly different from control values are indicated by * $P<0.05$ and ** $P<0.01$ (Student's t test). See text for abbreviations.

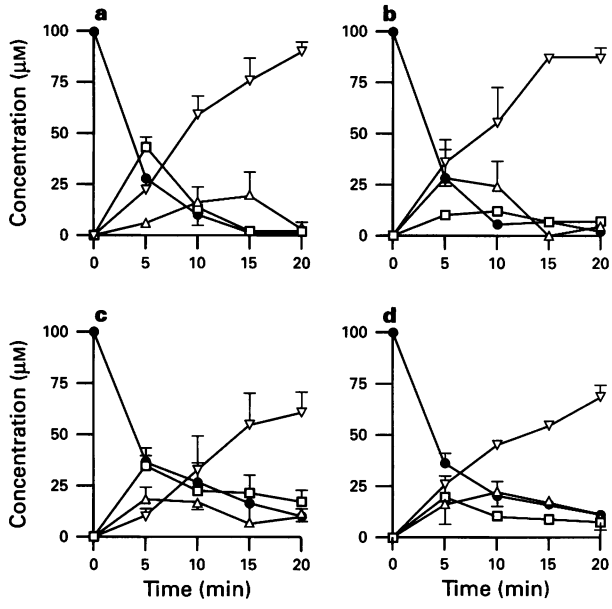

Figure 6 Degradation by the rat duodenum muscularis mucosae of (a and c) ATP (100 μM) (●), to ADP (□), AMP (△) and inosine (▽) or (b and d) UTP (100 μM) (●), to UDP (□), UMP (△) and uridine (▽). (a) and (b) are in the absence and (c) and (d) are in the presence of suramin (1 mM). Each point is the mean with s.e. mean of three determinations. See text for abbreviations.

Windscheif *et al.*, 1995). AMPCPP is rather more potent here than might be expected from its reported lack of activity at P_{2Y} purinoceptors cloned from chick or turkey brain, rat or mouse insulinoma cells and bovine endothelium (Webb *et al.*, 1993; Filtz *et al.*, 1994; Henderson *et al.*, 1995; Tokuyama *et al.*, 1995). This could be a consequence of its resistance to breakdown, as previously discussed for P_{2X}-purinoceptors (Kennedy & Leff, 1995), or could indicate that the P_{2Y}-purinoceptor on smooth muscle is of a different subtype.

The relaxation concentration-response curves to 2-MeSATP and ATP appeared to be biphasic whereas those to AMPCPP and ATP-γ-S were not. This could imply that ATP and 2-MeSATP were acting via two receptors to cause relaxation, and the fact that the more stable compounds AMPCPP and ATP-γ-S were monophasic suggests that this second receptor could be a P_1-purinoceptor. The longitudinal muscle relaxes to adenosine via A_1 and A_{2b} receptors (Nicholls *et al.*, 1996). However, 2-methylthioadenosine is only a weak agonist at P_1-purinoceptors (Satchell & Maguire, 1975), and the effect of ATP was largely inhibited by suramin, with no hint that this antagonism was blocking only one component of the action of ATP. In addition, in the whole duodenum, the relaxant responses which appear to be due to relaxations of the longitudinal muscle, responses to ATP and 2-MeSATP as well as those to AMPCPP were not inhibited by 8-SPT (Hourani *et al.*, 1993). It therefore seems unlikely that ATP and 2-MeSATP are acting at a P_1-purinoceptor, but the nature of this second site is unknown.

The whole rat duodenum has also been shown to contract to UTP, UDP and ATP-γ-S, but not to ATP, ADP, AMPCPP or 2-MeSATP (Johnson & Hourani, 1994). As suramin inhibited contractions to ATP-γ-S, but not those to UTP, it was suggested that there may be two contraction mediating receptors, one a suramin-insensitive receptor at which UTP acts and the other a suramin-sensitive receptor at which ATP-γ-S acts. The longitudinal muscle only contracted weakly to high concentrations of ATP-γ-S which may reflect the presence of a small amount of the muscularis mucosae left attached to the longitudinal muscle during the dissection. In contrast the

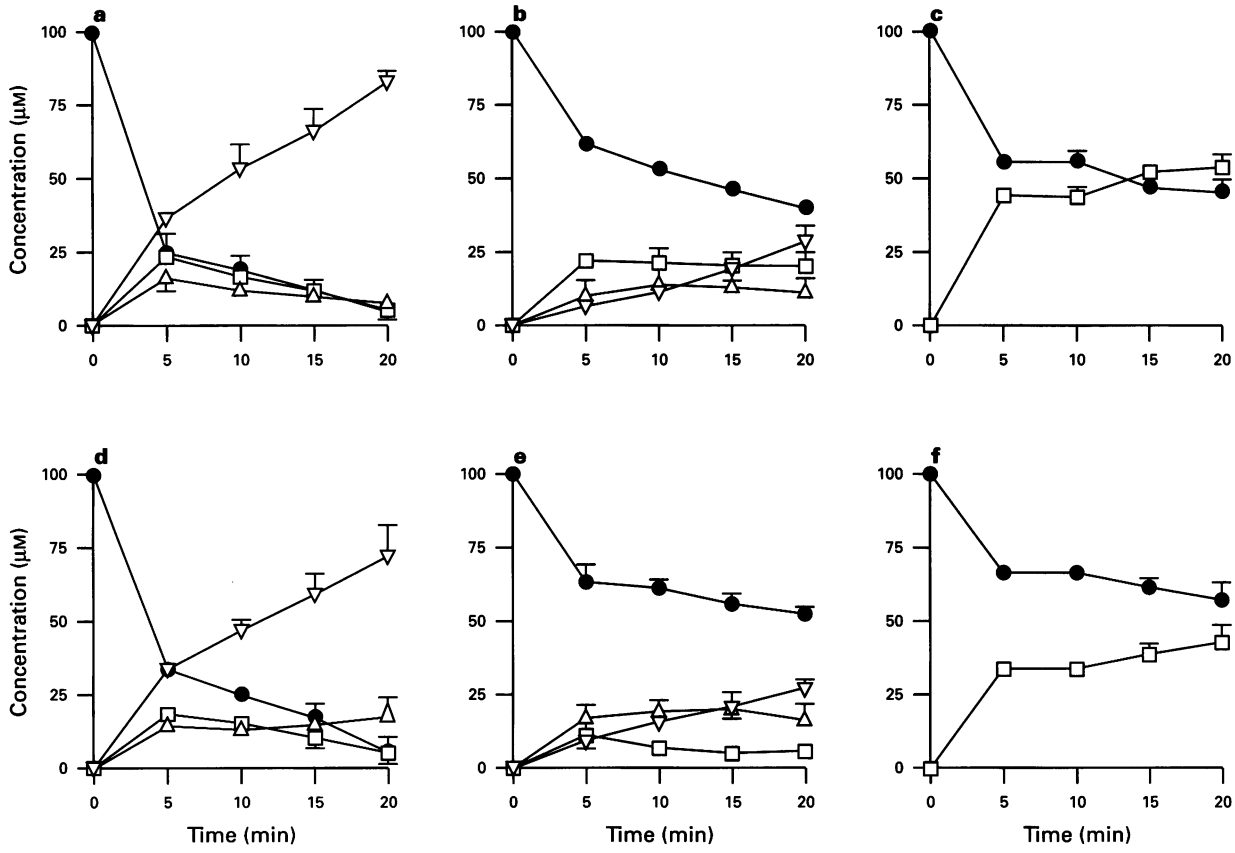

Figure 7 Degradation by the rat duodenum muscularis mucosae of (a and d) 2-MeSATP (100 μM) (●), to 2-MeSADP (□), 2-MeSAMP (△) and 2-MeSadenosine (▽), (b and e) ATP-γ-S (100 μM) (●), to ADP (□), AMP (△) and inosine (▽) or (c and f) AMPCPP (100 μM) (●), to AMPCP (□). (a), (b) and (c) are in the absence and (d), (e) and (f) are in the presence of suramin (1 mM). Each point is the mean with s.e. mean of three determinations. See text for abbreviations.

muscularis mucosae contracted with an agonist potency order of ATP-γ-S ⩾ AMPCP ⩾ AMPCPP = ATP = UTP = ADP = UDP > > 2-MeSATP, which is not characteristic of any one P_2-purinoceptor subtype. The fact that ATP contracted the muscularis mucosae whereas no contractions were observed in the whole duodenum is presumably because in the unseparated tissue these contractions are counteracted by the strong relaxations of the longitudinal muscle. Contractile responses to all agonists were slow and sustained in contrast to tissues such as the rat colon muscularis mucosae (P_{2Y}) in which the contractile response to purines is rapid and reversible (Bailey & Hourani, 1990).

The P_{2X} subtype is characterized by a contractile response and an agonist potency order in smooth muscles of AMPCPP > ATP = 2-MeSATP (Burnstock & Kennedy, 1985), although as discussed in the introduction this does not reflect the true potency but is distorted by degradation of the agonists (Kennedy & Leff, 1995). Suramin is also an antagonist (Hoyle *et al.*, 1990; Leff *et al.*, 1990), and rapid desensitization is observed (Kasakov & Burnstock, 1983; Meldrum & Burnstock, 1983). In the rat duodenum muscularis mucosae AMPCPP was much more potent than 2-MeSATP in eliciting contraction suggesting the presence of a P_{2X}-purinoceptor. In addition, suramin significantly shifted the contraction concentration-response curve of AMPCPP to the right, and desensitization to AMPCPP was observed. Taken together, this would suggest that there is a P_{2X}-purinoceptor in the muscularis mucosae at which AMPCPP is able to elicit contraction. However, our data also indicate the presence of an additional contraction-mediating receptor in the muscularis mucosae, probably of the P_{2U} subtype.

The P_{2U} receptor has been characterized as a receptor linked to phospholipase C and at which the agonist potency order is UTP = ATP = ATP-γ-S > > 2-MeSATP = AMPCPP (O'Connor *et al.*, 1991; O'Connor, 1992; Dubyak & El-Moatassim, 1993; Fredholm *et al.*, 1994). The agonist potency order for contractions of the muscularis mucosae suggests an action at a P_{2U} receptor, as ATP was equipotent with UTP, and 2-MeSATP is only weakly active. The high potency of AMPCPP may be due to the presence of the P_{2X} receptor, and this may also explain why ATP-γ-S has a higher potency here than would be expected for a P_{2U} receptor, as it is also a potent P_{2X} agonist. The lack of antagonism by suramin is also consistent with this suggestion, as there appear to be subclasses of the P_{2U} subtype based on sensitivity to suramin. At the P_{2U} receptor in PC12 rat phaechromocytoma cells for example, suramin is a competitive antagonist (Murrin & Boarder, 1991), whereas in bovine aortic endothelial cells and bovine aortic collateral artery rings, which contain a mixed population of P_{2Y} and P_{2U} receptors, suramin is a competitive antagonist of the P_{2Y}-selective agonists, but has little effect on the response to UTP (Wilkinson *et al.*, 1993; 1994a). Suramin shifts the concentration-response curves for ATP, UDP and ATP-γ-S in the rat aorta, but not in canine tracheal epithelial cells, which both contain P_{2U} receptors (Dainty *et al.*, 1994). In the muscularis mucosae we found that suramin although it inhibited responses to AMPCPP did not antagonize the responses to ATP, UTP or ATP-γ-S, which indicates that contraction to all three of these agonists is mediated via a suramin-insensitive P_{2U} receptor. In the muscularis mucosae responses to AMPCPP were readily desensitized unlike those to UTP and ATP-γ-S, and no cross-desensitization between AMPCPP and UTP or ATP-γ-S was observed. This further supports our proposal that UTP and ATP-γ-S are acting via a receptor different from that at which AMPCPP acts.

In the whole rat duodenum, contractions to ATP-γ-S but not those to UTP were abolished in the presence of suramin, suggesting that UTP and ATP-γ-S act via different receptors

(Johnson & Hourani, 1994). However, the results presented here suggest that in the muscularis mucosae UTP and ATP-γ-S act via a common receptor, and as the use of suramin as a P_2 antagonist can also be complicated by its ability to inhibit ectonucleotidases (Hourani & Chown, 1989), it is possible that this differential antagonism in the whole duodenum was instead due to concomitant inhibition by suramin of the breakdown of UTP but not of ATP-γ-S. This mechanism has been suggested to explain differential effects of suramin on ATP analogues in the rabbit ear artery (Crack *et al.*, 1994), in which suramin has been shown to cause a large shift to the right in the concentration-response curve for AMPCPP (an ATP analogue resistant to degradation by ectonucleotidases; Welford *et al.*, 1986; 1987) (Leff *et al.*, 1990), whereas the concentration-response curve to ATP is only slightly shifted to the right (Crack *et al.*, 1994). This has been taken to indicate a 'self cancelling' of P_2-purinoceptor antagonism and inhibition of ectonucleotidases by suramin. For this reason we decided to investigate the rate and pattern of degradation of some nucleotides and the effect of suramin to see if this could account for the differential antagonism observed in the muscularis mucosae.

The whole rat duodenum has previously been shown to dephosphorylate ATP rapidly via ectonucleotidases, with inosine rather than adenosine being formed as the ultimate product due to the presence of adenosine deaminase extracellulary on the duodenum (Franco *et al.*, 1988; Nicholls *et al.*, 1992b). In the present study we also found that inosine rather than adenosine was the major breakdown product of ATP. ATP, UTP and 2-MeSATP were all rapidly degraded at similar rates ultimately to inosine, uridine and 2-methylthioadenosine respectively, whilst ATP-γ-S and AMPCPP were both degraded more slowly, ultimately to inosine and AMPCP respectively. A similar rate and pattern of degradation was seen in the absence and presence of suramin for all of these agonists apart from AMPCPP which was dephosphorylated significantly slower at 5 min in the presence of suramin. It has been shown in other tissues that the lack of antagonist action of suramin can be explained by its action as an ectonucleotidase inhibitor. In the muscularis mucosae however this does not appear to be the case. The contraction concentration-response curve to AMPCPP was shifted by suramin whereas the contraction concentration-response curve to ATP-γ-S was not, while suramin inhibited the breakdown of AMPCPP but not that of ATP-γ-S. It is therefore hard to explain the lack of effects of suramin on the response to ATP-γ-S simply in terms of suramin slowing removal of the agonist by inhibiting ectonucleotidases. It is possible that the nature of the product formed as a result of the degradation of AMPCPP and ATP-γ-S may complicate this interpretation. The product of degradation of AMPCPP, AMPCP, was also a potent contractile agonist, whereas the ultimate degradation product of ATP-γ-S was the inactive inosine so dephosphorylation may not affect the observed action of AMPCPP as greatly as that of ATP-γ-S. However, as ATP-γ-S was degraded significantly more slowly than ATP, the fact that suramin had exactly the same effect on ATP-γ-S and ATP argues against a 'self-cancelling' effect of suramin as an antagonist and an ectonucleotidase inhibitor. In this context it is worth noting that as 2-MeSATP was degraded at a similar rate to ATP and UTP, its low potency in the muscularis mucosae cannot be due to the action of ectonucleotidases. In this study it was only possible to detect the concentration of nucleotide remaining and its breakdown products in the bulk phase (the organ bath buffer), so the rate of degradation measured was certainly an underestimate of the rate of degradation occurring in the biophase (i.e. in the vicinity of the receptors), due to lack of equilibration between the bulk phase and the biophase (Kennedy & Leff, 1995). However, the relative rates of degradation of the various nucleotides measured here should be a true reflection of their relative rates of degradation in the biophase, although it is possible that diffusion into the biophase may be different for different agonists.

In conclusion, our data indicate that the rat duodenum longitudinal muscle contains a P_{2Y}-purinoceptor which mediates relaxation, and that the rat duodenum muscularis mucosae may contain two receptors which both mediate contraction. One of these is suramin-sensitive at which AMPCPP is a potent agonist readily causing desensitization, suggesting it is probably a P_{2X}-purinoceptor. The other is suramin-insensitive at which ATP and UTP show equal potency, and at which ATP-γ-S also acts, suggesting it is probably a P_{2U}-purinoceptor. In addition, the rat duodenum muscularis mucosae is able to dephosphorylate rapidly adenine and uracil nucleotides, via ectonucleotidases and adenosine deaminase, and this may complicate interpretation of the results.

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