Desensitization of the P₂-purinoceptors on the rat colon muscularis mucosae

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1 Adenosine 5'-triphosphate (ATP) and adenosine have been shown to contract the rat colon muscularis mucosae, and the receptors at which they act have been classified as P_{2Y} and A₁ respectively. Uridine 5'-triphosphate (UTP) also contracts this tissue, and desensitization was used to investigate the receptors by which it acts, in the light of recent suggestions that specific pyrimidinoceptors may exist for UTP, or that nucleotide receptors may exist which are responsive to both ATP and UTP but not to some ATP analogues such as 2-methylthioadenosine 5'-triphosphate (2-MeSATP).

2 ATP, UTP and adenosine each contracted the rat colon muscularis mucosae in a concentration-dependent manner over the concentration range 0.3–300 µM, although maximal responses to ATP and UTP were not obtained. ATP was approximately 4 times as potent as UTP and approximately equipotent with adenosine although the maximal response to adenosine appeared to be less than that to ATP or UTP.

3 Desensitization of the tissue with ATP (200 µM) given immediately before each concentration of the agonists reduced subsequent contractions induced by ATP itself and also by UTP, but did not reduce contractions induced by adenosine. Desensitization of the tissues with UTP (200 µM) also reduced contractions induced by ATP and UTP but not by adenosine, whereas desensitization with adenosine (200 µM) reduced contractions induced by adenosine itself but not by ATP or UTP.

4 Desensitization of the tissue with 2-MeSATP (200 µM), which is a more potent agonist than ATP at P_{2Y}-purinoceptors, greatly reduced the responses to ATP and to UTP, but had no effect on responses induced by adenosine. Attempts to desensitize the tissue with adenosine 5'-(α,β-methylene)triphosphonate (AMPCPP), which is a more potent agonist than ATP at P_{2X}-purinoceptors but is less potent at P_{2Y}-purinoceptors, were unsuccessful.

5 These results show that cross desensitization to ATP and UTP occurred and was specific for these agonists rather than being due to a general decrease in the ability of the muscle to contract. This implies that ATP and UTP act at the same receptor, which does not support the existence of specific pyrimidinoceptors but which could be taken as evidence for the existence of a nucleotide receptor on this tissue. However, the ability of 2-MeSATP, which is inactive at the proposed nucleotide receptors, also selectively to desensitize this receptor indicates instead that ATP and UTP are both acting at a purinoceptor of the P_{2Y} type in this tissue.

Keywords: Rat colon muscularis mucosae; purinoceptors; pyrimidinoceptors; ATP; UTP; nucleotide receptors; desensitization

Introduction

The classification of P₂-purinoceptors on smooth muscle into P_{2X}, at which adenosine 5'-triphosphate (ATP) acts to cause contraction, and P_{2Y}, at which ATP generally acts to cause relaxation, is now well accepted. These receptors are defined by the potency order of agonists, with 2-methylthioadenosine 5'-triphosphate (2-MeSATP) being more potent than ATP which is more potent than adenosine 5'-(α,β-methylene)triphosphonate (AMPCPP) at P_{2Y}-purinoceptors, while on P_{2X}-purinoceptors AMPCPP is more potent than ATP which is equipotent with 2-MeSATP (Burnstock & Kennedy, 1985; Kennedy, 1990). No selective competitive antagonists are currently available to consolidate this receptor classification, and the best available ATP antagonist, suramin, does not discriminate between P_{2X} and P_{2Y} receptors (Hoyle *et al.*, 1990). The pharmacological actions of adenine nucleotides may be complicated by their breakdown to adenosine which acts via its own receptors of which two major subclasses exist, A₁ which generally mediates contraction of smooth muscle and A₂ which mediates relaxation. The breakdown of ATP and its analogues to adenosine may reduce its potency in some tissues (Welford *et al.*, 1987), and in others may result in ATP acting largely via adenosine receptors rather than P₂-purinoceptors (Bailey & Hourani, 1992). Use of more stable analogues of ATP such as AMPCPP can help to avoid this problem, although they are not as potent as ATP at P_{2Y}-purinoceptors and may not be completely resistant to

degradation in some tissues (e.g. Bailey & Hourani, 1992). An additional complication in interpreting results is that the related analogue adenylyl 5'-(β,γ-methylene)diphosphonate (AMPPCP) has been shown to act apparently directly via A₁ adenosine receptors in some tissues, showing that one cannot make assumptions about the receptor selectivity of a compound on the basis of its structure (Bailey & Hourani, 1990; Hourani *et al.*, 1991; Von Kügelgen *et al.*, 1992).

Although most research has centred on the actions of adenine nucleotides, there is increasing interest in the effects of other nucleoside triphosphates, in particular the pyrimidine nucleotide uridine 5'-triphosphate (UTP). In many tissues these nucleotides are also active, but there is debate as to whether these actions are via P_{2X} and P_{2Y}-purinoceptors or some other receptor subtype. Pyrimidinoceptors, analogous to purinoceptors but responding to uracil nucleotides, have been proposed to exist largely on the basis of differences in the responses of some tissues to ATP and UTP (reviewed by Seifert & Schultz, 1989). In the absence of selective antagonists, desensitization has been used in an attempt to discriminate between receptors for ATP and for UTP, and in a number of blood vessels which contract to ATP studies of this type have been interpreted as providing some support for the existence of separate UTP receptors (Von Kügelgen *et al.*, 1987; Sääg *et al.*, 1987; 1990; Von Kügelgen & Starke, 1990). However, in other tissues, such as the mouse vas deferens,

there is cross desensitization between ATP and UTP and their contractile responses therefore appear to be mediated by a common receptor (Von Kügelgen *et al.*, 1990). In these tissues the P_2 -purinoceptor is of the P_{2X} subtype and may therefore be a receptor-operated cation channel (Benham, 1990; Kennedy, 1990), but recently there has been more interest in the effects of ATP and UTP on cells in which the receptor is linked to phospholipase C activation. These include vascular endothelial cells, which respond to ATP by releasing factors such as nitric oxide which are responsible for the relaxation by ATP of many blood vessels. Because of differences in the structure-activity relationships observed on different cells and in particular the effects of 2-MeSATP, it has been proposed that two types of receptor recognising ATP may be involved. As well as P_{2Y} purinoceptors at which the potency order is 2-MeSATP > ATP > UTP, there may be nucleotide receptors at which the potency order is ATP = UTP >> 2-MeSATP, and some cells may have a mixed population of receptors, which could result in a potency order of 2-MeSATP > ATP = UTP, with a lower maximal response being achieved by 2-MeSATP than by ATP due to the activation of both receptor types by ATP (O'Connor *et al.*, 1991). Phospholipase C-coupled receptors at which UTP and ATP are equipotent, and which are therefore equivalent to nucleotide receptors, have also been called P_{2U} -receptors (Dubyak, 1991).

Although UTP is much less potent than ATP in the guinea-pig taenia caeci (Brown & Burnstock, 1981), which relaxes to ATP via the archetypal P_{2Y} -purinoceptors (Burnstock & Kennedy, 1985; O'Connor *et al.*, 1991), little is known about the effects of UTP and the receptors at which it acts in other non-vascular smooth muscle preparations which contain P_{2Y} -like purinoceptors. P_{2Y} -purinoceptors mediating relaxation of smooth muscle are often resistant to desensitization (Burnstock & Kennedy, 1985), and there are also technical difficulties and problems of interpretation in working with tissues in which the tone has to be raised before addition of nucleotides for detection of relaxation responses. We have recently shown that the rat colon muscularis mucosae contracts to ATP via a P_2 -purinoceptor at which the potency order of analogues was 2-MeSATP > ATP > AMPCPP, and which we therefore characterized as P_{2Y} (Bailey & Hourani, 1990). However the maximal response to 2-MeSATP was less than that to ATP, and preliminary studies indicated that UTP was also active here, so it seemed likely that this would be a suitable tissue in which to investigate the possible existence of pyrimidinoceptors or nucleotide receptors using desensitization. This tissue also contracts to adenosine via A_1 receptors (Bailey *et al.*, 1992), so this compound was used as a control agonist to ensure that any desensitization was selective.

Methods

Normally fed Wistar Albino rats (200–250 g), University of Surrey strain, were killed by cervical dislocation. A 40 mm length of the distal colon was removed and placed in warm (32°C) Tyrode buffer of the following composition (mM): NaCl 136.9, KCl 2.8, CaCl₂ 1.8, MgCl₂ 2.1, NaHCO₃ 11.9, NaH₂PO₄ 0.3, glucose 5.6, pregassed with 95% O₂/5% CO₂. The tissue was cleared of faecal matter and a glass pipette, external diameter 5 mm, was placed inside the colon. The outer muscularis propria was removed by gently rubbing with moist cotton wool and discarded. From the remaining thick walled tube of mucosal tissue, consisting of epithelial tissue and muscularis mucosae, a 20 mm length was suspended in Tyrode buffer in a 4 ml organ bath bubbled with 95% O₂/5% CO₂ at 32°C. Contractions were recorded isometrically at a resting tension of 1 g with a Grass FT03 transducer and displayed on a Grass 79C polygraph.

The tissues were allowed to equilibrate for 90 min with regular washes at approximately 20 min intervals, following

Figure 1 Contraction of the rat colon muscularis mucosae by ATP (■), UTP (●) or adenosine (▲). Each point is the mean $\pm$ s.e.mean of four determinations.

Figure 2 Contraction of the rat colon muscularis mucosae by (a) ATP, (b) UTP or (c) adenosine in the absence (closed symbols) or presence (open symbols) of desensitization by ATP (200 μ M). Each point is the mean $\pm$ s.e.mean of four determinations.

which concentration-response curves to ATP, UTP or adenosine were determined with or without desensitization with ATP, UTP, adenosine, 2-MeSATP or AMPCPP. For the control concentration-response curves increasing doses of agonist were given non-cumulatively, with a contact time of approximately 1 min, a washout period of 30 s and a recovery period of 10 min for ATP and adenosine and 15 min for UTP. These recovery periods were shown in preliminary experiments to allow repeated administration of the agonists

Figure 3 Contraction of the rat colon muscularis mucosae by (a) ATP, (b) UTP or (c) adenosine in the absence (closed symbols) or presence (open symbols) of desensitization by UTP (200 μM). Each point is the mean $\pm$ s.e.mean of four determinations.

(10 μM) to be given without a decrease in the observed responses. Concentration-response curves to ATP and UTP were determined on the same tissue by applying doses of each agonist alternately, and concentration-response curves to adenosine were determined on separate tissues. Desensitization was achieved in separate experiments by applying the agonists at a concentration of 200 μM with a contact time of 5 min (4 min the case of 2-MeSATP) during which time the contraction reached a peak and declined, followed by a 30 s washout to allow the tissue to relax completely before challenging with ATP, UTP or adenosine. The desensitizing agonist was applied before each dose of the test agonists, and a recovery period of 15 min was allowed between doses. All contractions were expressed as a percentage of the contraction induced by carbachol (50 μM) applied at the end of the experiments, and results were expressed graphically as the mean and standard error of the mean (s.e.mean) of the responses obtained. EC₅₀ values referred to each agonist's maximal responses could not be obtained because the concentration-response curves did not achieve a plateau. The potency of the agonists was therefore calculated as the pEC₃₀ value (the negative log of the concentration required to give 30% of the maximal response to carbachol), calculated by linear regression analysis of the linear portions of the individual log concentration-response curves. The pEC₃₀ values were calculated and compared in the presence and absence of desensitization using analysis of variance followed by Dunnett's one-tailed test, and EC₃₀ values given in Table 1 were obtained from the mean pEC₃₀ values.

Adenosine, ATP, UTP, AMPCPP and carbachol were obtained from Sigma, Poole, Dorset, 2-MeSATP from Research

Figure 4 Contraction of the rat colon muscularis mucosae by (a) ATP, (b) UTP or (c) adenosine in the absence (closed symbols) or presence (open symbols) of desensitization by adenosine (200 μM). Each point is the mean $\pm$ s.e.mean of four determinations.

Biochemicals, Natick, MA (U.S.A.), and buffer salts (analytical grade) from BDH, Poole, Dorset.

Results

ATP, UTP and adenosine each contracted the rat colon muscularis mucosae in a dose-dependent manner, although maximal responses were not obtained for ATP and UTP even at a concentration of 300 μM. ATP was approximately 4 times more potent than UTP, and adenosine was of similar potency to ATP (Figure 1). Pretreatment with ATP (200 μM) significantly reduced responses to ATP and to UTP, increasing the EC₃₀ values for these agonists approximately 10 fold and 3 fold respectively (Figure 2a,b). Pretreatment with UTP (200 μM) also reduced responses to both ATP and UTP, increasing the EC₃₀ values for these agonists 3 fold and 11 fold respectively, although this increase failed to achieve statistical significance for ATP (Figure 3a,b). Pretreatment with ATP (200 μM) caused a very small (< 2 fold) increase in the EC₃₀ value to adenosine, which was not statistically significant ($P > 0.05$) (Figure 2c). Pretreatment with UTP (200 μM) did not affect the concentration-response curve to adenosine (Figure 3c). Pretreatment with adenosine (200 μM) significantly reduced responses to adenosine, increasing the EC₃₀ value approximately 4 fold, but did not affect the concentration-response curve to ATP or UTP (Figure 4). Pretreatment with 2-MeSATP (200 μM) greatly reduced the response to both ATP and UTP, increasing the EC₃₀ values for these agonists 38 fold and 21 fold respectively

Figure 5 Contraction of the rat colon muscularis mucosae by (a) ATP, (b) UTP or (c) adenosine in the absence (closed symbols) or presence (open symbols) of desensitization by 2-methylthioadenosine 5'-triphosphate (200 μM). Each point is the mean $\pm$ s.e.mean of four determinations.

(Figure 5a,b). The responses to adenosine were not affected by pretreatment with 2-MeSATP (200 μM) (Figure 5c). Pretreatment of the tissues with AMPCPP (200 μM) did not significantly affect the concentration-response curves to any of the agonists (results not shown). EC_{50} values for ATP, UTP and adenosine in the presence and absence of desensitization are given in Table 1.

Discussion

These results show that UTP, like ATP, contracts the rat colon muscularis mucosae and is approximately 4 times less potent than ATP itself. We have previously shown that 2-MeSATP is much more potent than ATP on this tissue and

had therefore characterized the receptor as a P_{2Y} -purinoceptor (Bailey & Hourani, 1990). The present study, however, indicates that UTP is somewhat more potent here than in other tissues, such as the guinea-pig taenia caeci (Brown & Burnstock, 1981) which have been classified as containing P_{2Y} -purinoceptors. In addition, 2-MeSATP does not achieve the same maximal response as ATP in this tissue (Bailey & Hourani, 1990), in contrast to the guinea-pig taenia caeci (Satchell & Maguire, 1975). In the classification of O'Connor *et al.* (1991), the rat colon muscularis mucosae could therefore contain a mixture of P_{2Y} -purinoceptors and nucleotide receptors, with 2-MeSATP acting on the former, UTP on the latter and ATP on both. Alternatively, this tissue could contain pyrimidinoceptors (see Seifert & Schultz, 1989, for review) on which only UTP acts, together with P_{2Y} -purinoceptors which respond to ATP and 2-MeSATP. In the absence of any antagonist known to be selective for any of these putative receptors we attempted to use desensitization to investigate these possibilities, in the hope that a contractile response might prove easier to manipulate in this way than the relaxations observed in most smooth muscle preparations containing receptors of this type.

Desensitization of the tissue was achieved with ATP, UTP, 2-MeSATP and adenosine, but not with AMPCPP. The failure of this agonist to cause any desensitization probably reflects its relatively low potency in this tissue (Bailey & Hourani, 1990), and is further evidence that ATP does not contract via P_{2X} -purinoceptors here as these receptors are very readily desensitized by AMPCPP (Burnstock & Kennedy, 1985). 2-MeSATP was the most effective desensitizing agonist, again probably reflecting its high potency in this tissue (Bailey & Hourani, 1990). In each case the contractile effect of the desensitizing dose of agonist used was similar except for 2-MeSATP which caused a smaller contraction than the other agonists, so the ability to cause desensitization was not simply related to the size of the contraction and therefore fatigue of the tissue. The desensitization also demonstrated some degree of receptor selectivity, as responses to ATP and UTP were not reduced by desensitization with adenosine and responses to adenosine were not decreased by desensitization with UTP or 2-MeSATP and were only slightly decreased, at low adenosine concentrations, by desensitization with ATP. As part of the desensitization protocol, ATP was left in contact with the tissues for 5 min and we have previously shown that during this time and under these conditions ATP is significantly degraded and adenosine is produced (Bailey & Hourani, 1990). Indeed use of the antagonist, suramin, revealed that contractions induced by high concentrations of ATP had a minor component which was due to interaction with A_1 receptors (Bailey *et al.*, 1992). The effect of desensitization with ATP in reducing the effects of low concentrations of adenosine is therefore most likely to be due to desensitization of the A_1 receptor by the adenosine produced.

The lack of cross-desensitization between adenosine and any of the nucleotides therefore suggested that any reduction in responses was not due to tissue fatigue, but was occurring at some process closer to the receptor interaction. However,

Table 1 Effect of desensitization by ATP, UTP, adenosine (Ado), 2-methylthioadenosine 5'-triphosphate (2-MeSATP) or adenosine 5'-(α,β -methylene)triphosphonate (AMPCPP) (200 μM) on EC_{50} values (μM) for contraction of the rat colon muscularis mucosae induced by ATP, UTP and adenosine

	Control	Desensitizing agonist				
		ATP	UTP	Ado	2-MeSATP	AMPCPP
ATP	7.5	77**	20	9.5	280**	10
UTP	34	110*	380**	49	710**	57
Adenosine	22	36	20	89*	20	32

Agonist EC_{50} values are calculated from pEC_{50} values which were the mean of 4 determinations.

Values significantly different from control values are indicated.

* $P < 0.05$; ** $P < 0.01$ (Dunnett's one-tailed test).

there was cross-desensitization between ATP and UTP, which suggests that in this tissue ATP and UTP are interacting with the same receptor, and there is therefore no evidence for a separate pyrimidinoceptor. These results are comparable to those found previously in the mouse vas deferens (Von Kügelgen *et al.*, 1990) but not in a number of blood vessels (Von Kügelgen *et al.*, 1987; Saiag *et al.*, 1987; 1990; Von Kügelgen & Starke, 1990), although as mentioned in the introduction, the P₂-purinoceptor in these tissues is of the P_{2X} subtype rather than P_{2Y}. To investigate the possibility that the effects of ATP and UTP may be mediated via nucleotide receptors as proposed by O'Connor *et al.* (1991), we used 2-MeSATP which is a potent agonist at P_{2Y}-purinoceptors but is inactive at the putative nucleotide receptor. 2-MeSATP proved however to be the most effective desensitizing agonist, inhibiting not only responses to ATP but also those to UTP, which does not support the existence of nucleotide receptors.

It is of course possible that the cross-desensitization observed between the nucleotides but not with adenosine may be due to all the nucleotides causing contraction via the same

second messenger pathways (e.g. phospholipase C activation), which may be different from that used by the A₁ receptor. In this case the desensitization may not be a true effect at the receptors, but may instead reflect an alteration in the function of this pathway. However, we do not know how adenosine or the nucleotides cause contraction of this tissue, or how the process of desensitization occurs and so these questions remain unanswered. With this reservation however, the results presented here do not support the existence of either pyrimidinoceptors or nucleotide receptors on the rat colon muscularis mucosae, but instead suggest that ATP, UTP and 2-MeSATP all act at the same receptor. There seems no reason at the present time to call this receptor anything other than a P_{2Y}-purinoceptor, although it may not be identical to P_{2Y}-purinoceptors found on other tissues, such as the guinea-pig taenia caeci, at which UTP is considerably less potent.

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