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A_{2A} and A_3 receptors mediate the adenosine-induced relaxation in spontaneously active possum duodenum *in vitro*

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1 The aim of this study was to define the P1 purinergic receptors that regulate spontaneous or adenosine-induced duodenal motor activity.

2 Spontaneous contractile activity was recorded isometrically from possum longitudinal duodenal muscle strips. Adenosine (0.5 μM – 1 mM) was administered noncumulatively and repeated after pretreatment with a P1 antagonist or tetrodotoxin (TTX, 1 μM), ($n = 4 – 7$). Antagonists used were: A_1, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 10 nM); A_{2A}, 8-(3-chlorostyryl)caffeine (CSC, 10 μM); A_{2B}, 3-isobutyl-8-pyrrolidinoxanthine (IPDX, 10 μM); A_3, 9-chloro-2-(2-furanyl)-5-[(phenylacetyl) amino][1,2,4]-triazolo(1,5-c)quinazoline (MRS1220, 10 μM). Changes in activity are expressed as percentage of baseline. Statistical analysis utilised nonparametric tests.

3 Adenosine ($n = 34$) induced a long-lasting, concentration-dependent decrease in activity by $55.6 \pm 3.2\%$ area under curve (AUC), $47.3 \pm 4.0\%$ contraction amplitude, $31.6 \pm 3.6\%$ basal tension and $10.4 \pm 1.7\%$ contraction frequency (all $P < 0.001$). The adenosine-induced decrease in contraction amplitude was blocked by CSC ($P < 0.01$) or inhibited by MRS1220 ($P < 0.03$) pretreatment, but not modified by TTX, DPCPX or IPDX pretreatment.

4 Adenosine antagonists modified spontaneous contractile activity. Pretreatment with DPCPX or CSC increased basal tension, whereas IPDX or MRS1220 pretreatment decreased contractile activity.

5 In conclusion, exogenous adenosine reduced duodenal longitudinal motor activity via A_{2A} and A_3 receptors. Our findings suggest that endogenous purines may modulate spontaneous duodenal motor activity.

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Abbreviations: ATP, adenosine 5′-triphosphate; CSC, 8-(3-chlorostyryl)caffeine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; IPDX, 3-isobutyl-8-pyrrolidinoxanthine; MRS1220, 9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino][1,2,4]-triazolo(1,5-c)quinazoline; TTX, tetrodotoxin

Introduction

Extracellular purines (ATP, ADP, adenosine) and pyrimidines (UTP, UDP) are known modulators of gastrointestinal function, affecting motility, secretion and absorption. Purinergic receptors are classified into two classes. Adenosine acts via the P1 receptors of which there are four recognised receptor subtypes: A_1, A_{2A}, A_{2B} and A_3. ATP, ADP, UTP and UDP act through P2 receptors, which are further classified into P2X and P2Y classes.

There are several reports of adenosine causing relaxation in the duodenum, however, the magnitude of the response appears to differ between species and the preparations studied. In the rabbit, adenosine causes a decrease in the contraction amplitude of spontaneous contractions (Small & Weston, 1979), whereas in the rat a rapid and short-lasting decrease in basal tension occurs followed by a marked decrease in the contraction amplitude (Gaion *et al*., 1988). Furthermore, studies using an isometric/isovolumic preparation of rat duodenum found that exogenous adenosine caused a fall in pressure and tension (Mule *et al*., 1989; Serio *et al*., 1990). The neurotoxin, tetrodotoxin (TTX), failed to modify this response suggesting that the P1 receptors were present on the duodenal muscle (Mule *et al*., 1989; Serio *et al*., 1990).

Subsequent studies with specific agonists/antagonists using precontracted rat duodenal muscle strips revealed the presence of A_1 receptor (R) (Nicholls *et al*., 1992). However, non-selective concentrations of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (1 μM) were required to inhibit the effect of exogenous adenosine thus raising the possibility that A_2 receptors may be involved. Furthermore, the A_{2A}R agonist CGS21680 had no effect, thereby suggesting that A_{2B}R could be involved (Nicholls *et al*., 1992).

The location of various receptors within the duodenum was examined in studies using longitudinal muscle strips and muscularis mucosae strips (Nicholls *et al*., 1996). An inhibitory response was observed in the longitudinal muscle strip mediated by A_1R and possibly A_{2B}R. In contrast, a contractile response was identified in the muscularis mucosae, possibly mediated by A_{2B}R because of the low potency of the A_{2A}R agonist CGS21680. However, the responses of the longitudinal

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muscle were found to dominate when full thickness muscle strips were used (Nicholls *et al*., 1992).

In summary, these studies in the rat suggested that A_1R are present in the duodenum; however the response to adenosine may be mediated by A_2 receptors, probably $A_{2B}R$. Conclusive data showing $A_{2B}R$ to be involved in the adenosine response however is lacking. Furthermore, the possible involvement of A_3R has not been addressed.

Our previous studies relating to biliary motility (i.e. gallbladder, common bile duct and sphincter of Oddi) have used the Australian Brush-tailed possum (Baker *et al*., 1993; Cox *et al*., 1998; Chen *et al*., 2000; Al-Jiffry *et al*., 2001). The possum is an excellent model to study biliary motility, because unlike man, and most laboratory species, its sphincter of Oddi is predominantly extra-duodenal and therefore allows interpretation of sphincter of Oddi motility as a separate entity to duodenal motility. Furthermore, we have demonstrated that modulation of duodenal motility can influence sphincter of Oddi motility via a neural reflex (Saccone *et al*., 1994; Simula *et al*., 1997; Woods *et al*., 2000; Konomi *et al*., 2002). An extension of these studies relating to the possible modulatory role of purines/pyrimidines on biliary motility firstly requires the characterisation of purinergic receptors in the duodenum near the sphincter of Oddi. Therefore, the aim of this study was to identify the purinergic receptors involved in mediating the response to exogenous adenosine. We used specific antagonists to each of the A_1, A_{2A}, A_{2B} and A_3 receptors.

Methods

Tissue collection

A total of 46 Australian Brush-tailed possums (*Trichosurus vulpecula*) of either sex were fasted overnight and anaesthetised by intramuscular injection of ketamine (20 mg kg^{-1}, Ketamil Injection, Troy Laboratories Pty. Ltd, NSW, Australia) and xylazine (5 mg kg^{-1}, Rompun, Bayer Australia Ltd, NSW, Australia). Following an abdominal incision, the proximal duodenum, between the pyloric sphincter and the sphincter of Oddi, was removed. After surgery each animal was euthanised with a lethal dose of pentobarbitone sodium (Lethabarb, Virbac Pty. Ltd, NSW, Australia).

In vitro *organ bath studies*

The isolated duodenum was immediately placed in oxygenated modified Krebs solution containing: NaCl 133.4 mM, KCl 0.6 mM, NaH_2PO_4 1.3 mM, $NaHCO_3$ 16.3 mM, D-glucose 7.7 mM, $CaCl_2$ 2.12 mM at room temperature (Baker *et al*., 1992; Woods *et al*., 2000). The duodenum was pinned to a Sylgard (Dow Corning Corporation, MI, U.S.A.)-lined Petri dish and covered with oxygenated modified Krebs solution. The mesenteric vessels and connective tissue were removed from the duodenum and eight full thickness longitudinal muscle strips (10 mm × 2 mm) were prepared from the tissue immediately adjacent to the sphincter of Oddi.

Muscle strips were secured in double-jacketed 7 ml organ baths, containing continuously oxygenated modified Krebs solution at 37°C. Each strip was attached to an isometric force – displacement transducer (FT03, Grass Instrument Co., Quincy, MA, U.S.A.), which was then connected to a MacLab recording system (ADInstruments, Castle Hill, NSW, Australia), which utilised the software Chart v3.5.6/s (ADInstruments).

Strips were equilibrated at a basal tension of 5 mN for 60 min, with freshly modified Krebs solution exchanged every 10 min. Spontaneous contractile activity was recorded. A paired protocol was utilised where adenosine (0.5 μM – 1 mM; RBI, MA, U.S.A.) was added (5 min) to each strip. After washout, strips were pretreated with a receptor antagonist (30 min) or TTX (10 min; 1 μM; Alomone Laboratory, Jerusalem, Israel) before the same concentration of adenosine was reapplied (5 min) to the same muscle strip. The antagonists used were: A_1R antagonist DPCPX (10 nM, RBI); $A_{2A}R$ antagonist 8-(3-chlorostyryl) caffeine (CSC, 10 μM, RBI); $A_{2B}R$ antagonist 3-isobutyl-8-pyrrolidinoxanthine (IPDX, 10 μM, gift from Dr Italo Biaggioni, Vanderbilt University, U.S.A.; (Feoktistov *et al*., 2001); A_3R antagonist 9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino][1,2,4]-triazolo[1,5-c]quinazoline (MRS1220, 10 μM, RBI). Dimethyl sulphoxide (DMSO, BDH Merck Pty Ltd, Victoria, Australia) was used in some of these studies as a solvent. The addition of DMSO alone (0.1%; $n=4$), or modified Krebs solution, failed to show any significant effect on spontaneous activity over a 30 min period, or the adenosine-induced response. Electrical field stimulation (70 V, 30 Hz, 0.1 ms duration for 10 s) was performed to ensure nerve blockade was obtained for experiments with TTX (Huang *et al*., 1998; Woods *et al*., 2000).

Analysis

Changes in contractile activity were quantified for area under curve (AUC, mN.s; AUC is a combined measure of contraction amplitude and frequency, and is used as an indicator of motility (Saccone *et al*., 1994; Konomi *et al*., 2002)) basal tension (mN), contraction amplitude (mN) and frequency of contractions. To normalise muscle strip activity, i.e. take into consideration the variation of the magnitude of spontaneous activity between individual muscle strips, the response was calculated as the change from baseline activity for each adenosine/antagonist application for each muscle strip. The determination of the initial adenosine response was calculated by taking a 1 min period immediately prior to adenosine application, and a 1 min period representing the peak response to adenosine, and determining the change in activity. The determination of the effect of an antagonist on basal activity was calculated by taking a 1 min period immediately prior to antagonist application and a 1 min period 30 min after antagonist application and determining the change in activity. The determination of the adenosine response in the presence of the antagonist was calculated by taking a 1 min period immediately prior to adenosine application (i.e. 30 min after antagonist application) and a 1 min period representing the peak response to adenosine in the presence of the antagonist and determining the change in activity. The two adenosine responses were then compared to determine if the antagonist had an effect on the adenosine-induced response. Therefore, the change in basal activity due to the antagonist application is accounted for and the actual change in the adenosine-induced response is determined.

Group data is expressed as the change from baseline activity, or as percentage of baseline activity, mean ± s.e.m. For each group, n represents the number of muscle strips at

each concentration of adenosine (note: for a single adenosine concentration, each muscle strip was from a different animal). Minor changes in contraction frequency were observed in the overall group data for adenosine, however, dose – response relationships were not observed in the smaller groups for each antagonist and are therefore not presented.

Graphs were prepared using GraphPad Prism (GraphPad Software Inc., San Diego, CA, U.S.A.) using nonlinear regression to generate curves of best fit and EC_{50} values. Statistical analyses were performed using the statistical package SPSS (SPSS Inc., Chicago, IL, U.S.A.) and utilised nonparametric paired or unpaired tests as appropriate: Kruskal – Wallis, Mann – Whitney, Wilcoxon and Friedman tests.

Results

Adenosine

Exogenous adenosine ($n \geqslant 34$ muscle strips/concentration) decreased spontaneous longitudinal duodenal smooth muscle activity (Figure 1). The response consisted of long-lasting, concentration-dependent decreases in AUC (to $44.4 \pm 3.2\%$ of baseline, $P < 0.001$), contraction amplitude (to $52.8 \pm 4.0\%$ of baseline, $P < 0.001$) and basal tension (to $68.4 \pm 3.6\%$ of baseline, $P < 0.001$). A minor decrease in contraction frequency (to $89.6 \pm 1.7\%$ of baseline, $P < 0.001$) was also observed. Estimates of the EC_{50} values were 89, 105, 86 and 383 μM for AUC, contraction amplitude, basal tension and contraction frequency, respectively. The magnitude and duration of the adenosine response varied between individual muscle strips, with some responses persisting until the adenosine was washed out (5 min).

A_1R antagonist: DPCPX

Pretreatment with DPCPX (10 nM; $n = 56$ muscle strips) produced a small increase in the basal tension but did not influence the contraction amplitude or contraction frequency of spontaneous contractions ($P < 0.01$) (Table 1). However, DPCPX pretreatment (10 nM; $n \geqslant 7$ muscle strips/concentration; from seven animals) had no effect on the response to adenosine for any parameter measured (Figure 2). A higher concentration of DPCPX (1 μM) also failed to have a significant effect on the response to adenosine (data not shown).

A_{2A}R antagonist: CSC

Pretreatment with CSC (10 μM; $n = 48$ muscle strips) increased the basal tension ($P < 0.01$) but did not significantly alter the contraction amplitude or contraction frequency of spontaneous contractions (Table 1). In contrast, pretreatment with CSC (10 μM; $n \geqslant 6$ muscle strips/concentration; from six animals) completely blocked the adenosine-induced decrease in contraction amplitude ($P < 0.01$) and decreased its effect on AUC ($P < 0.02$), but had no effect on the adenosine-induced decrease in basal tension (Figure 3).

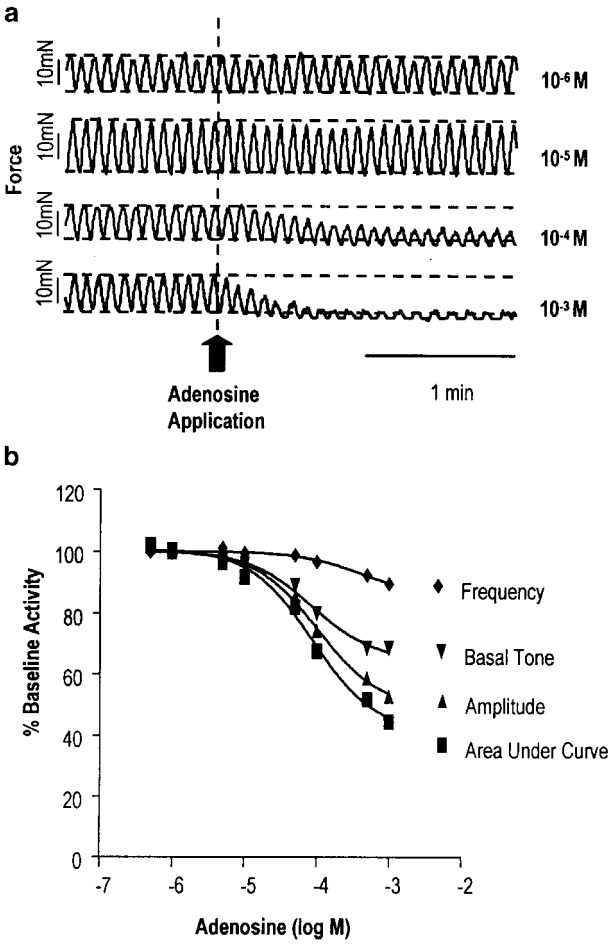

Figure 1 (a) Representative chart recordings from four different muscle strips displaying responses to various concentrations of adenosine. Horizontal dashed lines indicate baseline activity for contraction amplitude and basal tension in each strip. Vertical dashed line with arrow indicates the addition of adenosine. (b) Group data, as a percentage of baseline activity, illustrating the concentration-dependent effect of adenosine on area under curve, contraction amplitude, basal tension and contraction frequency. Each point is the mean of 34 muscle strips, where each muscle strip is from a different animal. Error bars (s.e.m.) are within the size of the symbols.

Table 1 Effect of purinergic antagonists and TTX on spontaneous duodenal activity

Receptor	*Antagonist*	*AUC*	*Contraction amplitude*	*Basal tension*	*Contraction frequency*
A_1R	DPCPX (10 nM, $n = 56$)	$98.3 \pm 1.0\%$	$98.1 \pm 1.0\%$	$105.7 \pm 1.8\%$*	$101.1 \pm 0.8\%$
A_{2A}R	CSC (10 μM, $n = 48$)	$97.7 \pm 1.5\%$	$97.1 \pm 1.2\%$	$108.5 \pm 1.6\%$*	$100.9 \pm 0.7\%$
A_{2B}R	IPDX (10 μM, $n = 41$)	$77.9 \pm 2.4\%$*	$86.0 \pm 2.4\%$*	$79.1 \pm 3.8\%$*	$97.7 \pm 1.3\%$
A_3R	MRS1220 (10 μM, $n = 40$)	$90.4 \pm 2.4\%$*	$92.4 \pm 1.0\%$*	$104.4 \pm 2.2\%$	$101.7 \pm 0.9\%$
Nerve blockade	TTX (1 μM, $n = 60$)	$96.9 \pm 1.9\%$	$98.8 \pm 2.2\%$	$94.5 \pm 1.3\%$*	$97.8 \pm 1.2\%$

Data expressed as percentage of baseline activity and is presented as mean $\pm$ s.e.m. *$P < 0.05$ as determined by Wilcoxon matched pairs test. n = number of muscle strips (from at least four animals). See text for complete antagonist names.

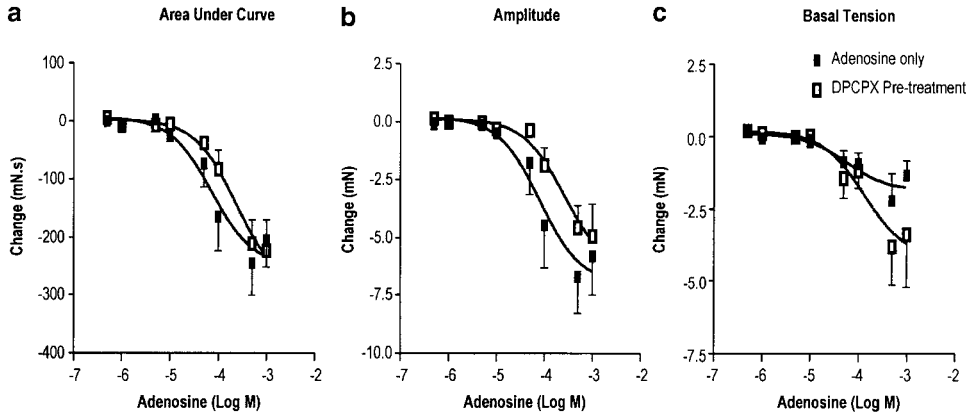

Figure 2 Group data, as a change from baseline activity, illustrating the lack of effect of pretreatment of longitudinal duodenal muscle strips with the A_1R antagonist DPCPX (10 nM) on the adenosine-induced relaxation for AUC (a), contraction amplitude (b) and basal tension (c) ($P>0.05$ for all). Each point is the mean ± s.e.m. of at least seven muscle strips, where each muscle strip is from a different animal.

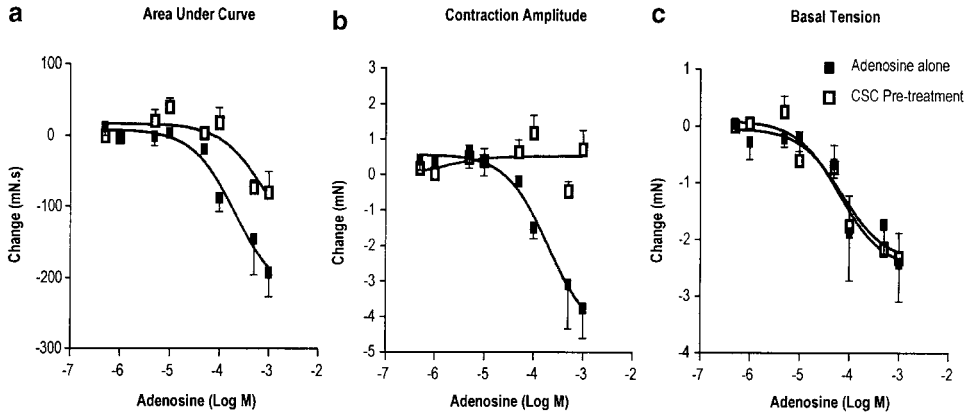

Figure 3 Group data, as a change from baseline activity, illustrating the effect of pretreatment of longitudinal duodenal muscle strips with the A_{2A}R antagonist CSC (10 μM) on the adenosine-induced relaxation. CSC pretreatment antagonised the response for AUC (a; $P<0.02$), blocked the decrease in contraction amplitude (b; $P<0.01$), but did not affect basal tension (c; $P>0.05$). Each point is the mean ± s.e.m. of at least six muscle strips, where each muscle strip is from a different animal.

A_{2B} antagonist: IPDX

Pretreatment with IPDX (10 μM; $n=41$ muscle strips) produced a significant decrease in spontaneous contractile activity for AUC ($P<0.01$), contraction amplitude ($P<0.01$) and basal tension ($P<0.01$) (Table 1). However, IPDX pretreatment (10 μM; $n\geqslant 4$ muscle strips/concentration; from six animals) had no effect on the response to adenosine for any parameter measured (Figure 4).

A3 antagonist: MRS1220

Pretreatment with MRS1220 (10 μM; $n=40$ muscle strips) modified spontaneous contractile activity by reducing AUC ($P<0.03$) and the contraction amplitude ($P<0.01$) (Table 1). MRS1220 pretreatment (10 μM; $n\geqslant 5$ muscle strips/concentration; from five animals) also reduced the adenosine-induced decrease in contraction amplitude ($P<0.03$) and AUC ($P<0.05$), and furthermore caused a slight enhancement of the adenosine-induced decrease in basal tension ($P<0.05$) at higher adenosine concentrations (Figure 5).

Neural blockade: TTX

Pretreatment with the neural toxin TTX (1 μM; $n=60$ muscle strip) produced a small decrease in basal tension ($P<0.001$) but did not modify other parameters (Table 1). However, TTX pretreatment (1 μM; $n\geqslant 5$ muscle strips/concentration; from five animals) had no significant effect on the response to adenosine for any parameter measured (Figure 6).

Discussion

This study reports for the first time that exogenous adenosine acts via receptor subtypes A_{2A} and A_3 to reduce duodenal longitudinal muscle activity, predominantly affecting contraction amplitude. The adenosine response was not affected by neural blockade. Furthermore, this study suggests the involvement of endogenous adenosine in the maintenance of spontaneous contractile activity.

Our findings were somewhat unexpected, however they support previous studies in rabbit and rat duodenum where exogenous adenosine also induced a relaxation (Small &

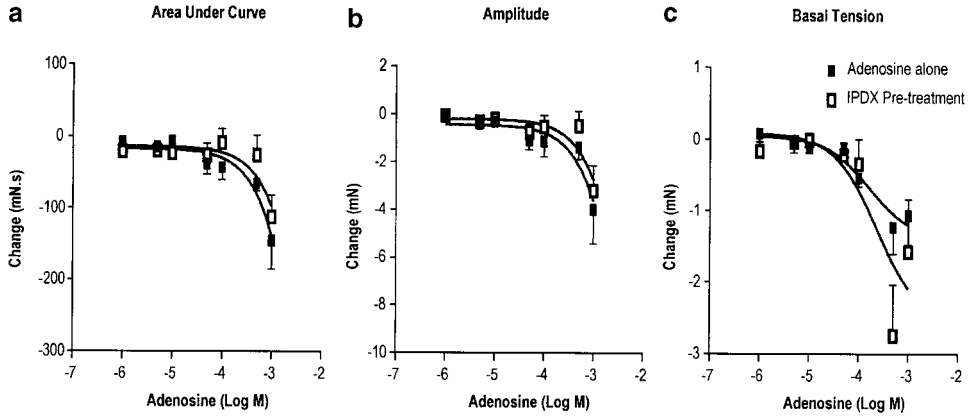

Figure 4 Group data, as a change from baseline activity, illustrating the lack of effect of pretreatment of longitudinal duodenal muscle strips with the $A_{2B}R$ antagonist IPDX (10 μM) on the adenosine-induced relaxation. IPDX pretreatment did not effect the response to adenosine for AUC (a), contraction amplitude (b) or basal tension (c) ($P>0.05$ for all). Each point is the mean $\pm$ s.e.m. of four to six muscle strips, where each muscle strip is from a different animal.

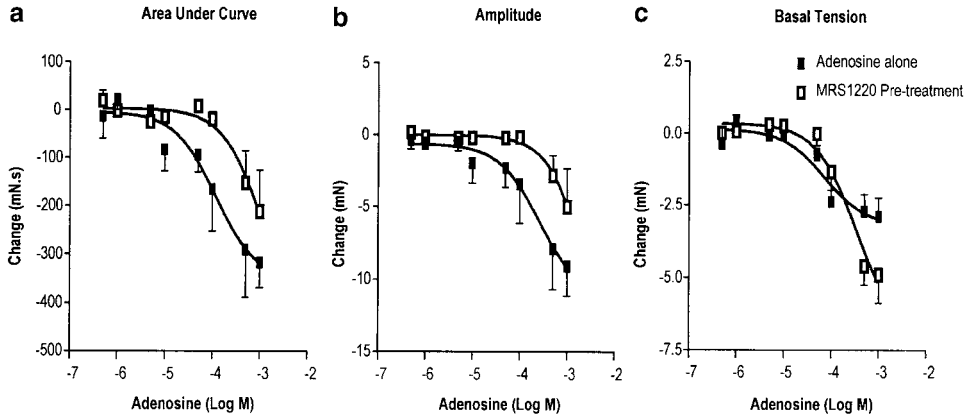

Figure 5 Group data, as a change from baseline activity, illustrating the effect of pretreatment of longitudinal duodenal muscle strips with the A_3R antagonist MRS1220 (10 μM) on the adenosine-induced relaxation. MRS1220 pretreatment antagonised the response to adenosine for AUC (a; $P<0.05$), contraction amplitude (b; $P<0.03$) but caused an enhancement of the adenosine response for basal tension (c; $P<0.05$) at the higher concentrations. Each point is the mean $\pm$ s.e.m. of at least five muscle strips, where each muscle strip is from a different animal.

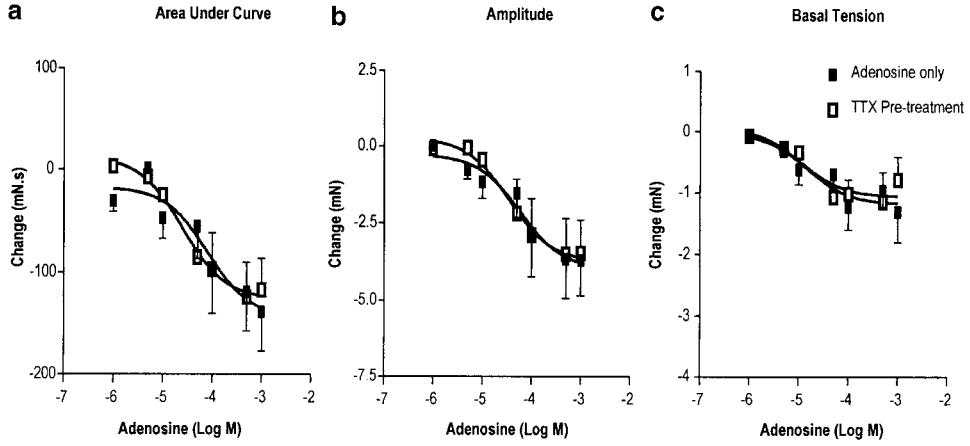

Figure 6 Group data, as a change from baseline activity, illustrating the lack of effect of pretreatment of longitudinal duodenal muscle strips with the nerve blocker TTX (1 μM) on the adenosine-induced relaxation for AUC (a), contraction amplitude (b) and basal tension (c) ($P>0.05$ for all). Each point is the mean $\pm$ s.e.m. of at least five muscle strips, where each muscle strip is from a different animal.

Weston, 1979; Gaion *et al*., 1988; Mule *et al*., 1989). Our data extends the more recent studies in the rat duodenum, which identified A_1R and predicted $A_{2B}R$ to be present (Nicholls *et al*., 1992, 1996). In agreement with the rat studies, we also found that nanomolar concentrations of DPCPX did not affect the adenosine-induced response, thereby suggesting that the A_1R may not be involved in mediating the relaxation to exogenous adenosine. In the possum duodenum, we have provided evidence implicating the $A_{2A}R$ and the A_3R as mediators for the response to exogenous adenosine. Nicholls *et al*. (1992) found that nonselective concentrations of DPCPX were required to have an effect on exogenous adenosine, thereby suggesting the presence of A_2 receptors. Due to the lack of specific antagonists, it was suggested that $A_{2B}R$ were involved based on the lack of response to an $A_{2A}R$ agonist CGS12680, which has low potency for the $A_{2B}R$ (Nicholls *et al*., 1992, 1996). However, the possible role of A_3R was not examined, and our findings would support the role of A_3R. We also found the $A_{2A}R$ antagonist blocked the adenosine-induced decrease in contraction amplitude, but had no effect on basal tension, whereas Nicholls *et al*. (1992) found an $A_{2A}R$ agonist to have little effect. This anomaly could be due to the technical differences, as precontracted rat duodenal strips were used to demonstrate a decrease only in basal tension and changes in contraction amplitude would not be observable. Our findings in spontaneously active possum duodenum are comparable to those found in the rat duodenum studies.

This study used the possum as a model rather than the more common rodent models and as a consequence it has been assumed that the adenosine antagonists behave in the possum in a manner similar to that described for rat and human. In an effort to determine if these antagonists did indeed act in a manner similar to that already described, we attempted to estimate and compare the antagonists' dissociation constant (K_B). The Schild equation can be used to estimate K_B values for simple competitive antagonism (one-receptor systems), however, this study implicates both the A_{2A} and the A_3 receptors in the response to adenosine. When two receptors are involved, the agonist will encounter the second receptor, which is not blocked by the antagonist, and results in the generation of a greater response. This response is greater than that predicted by a competitively antagonised single-receptor population resulting in a deviation from the Schild regression and an erroneous estimation of K_B (Kenakin, 1992). Furthermore, it should be noted that the A_3 antagonist MRS1220 is active in human tissue and does not bind rat A_3 receptors, suggesting that the possum model may be more similar to human than rat models.

Studies have been performed using immunohistochemical techniques to indicate which adenosine receptors are present in tissues. Immunohistochemical studies have characterised the distribution of neural P1 receptors in the human enteric system (Christofi *et al*., 2001). Recently, $P2X_2$ and $P2X_5$ receptor proteins have been localised to the interstitial cells of Cajal in guinea-pig ileum (Burnstock & Lavin, 2002), and it was speculated that the release of ATP from enteric nerves, glial cells or contracting smooth muscle may provide a feedback mechanism for pacemaker activity in the intestine. The minor decrease in contraction frequency observed with adenosine in our study may be consistent with activation of purinergic receptors on interstitial cells of Cajal.

We have also provided some evidence for the role of endogenous purines in the maintenance of spontaneous duodenal activity. The regulation of spontaneous activity is complex, involving myogenic, neural and non-neural mechanisms. Application of the A_1 and A_{2A} antagonists increased basal tension, suggesting that endogenous adenosine has a modulatory role in spontaneous contractile activity. In contrast, we found that antagonism of the $A_{2B}R$ and A_3R decreased spontaneous activity. This response suggests that blockade of these receptors in various sites within the tissue (i.e. muscle, myenteric neurons, epithelial cells, etc.) could produce complex interactions, which are expressed as a net reduction in spontaneous activity. Furthermore, in support of our findings, a previous study showed that the addition of nucleotide pyrophosphatase to rat duodenum, which would reduce the level of endogenous purines, reduced the tone, contraction amplitude and contraction frequency of spontaneous activity (Manzini *et al*., 1985), also suggesting a role of endogenous purines in maintaining spontaneous activity. These observations raise the question as to the source of these endogenous purines. ATP may be released from nerves in the myenteric plexus or the submucous plexus, or perhaps directly from the smooth muscle cells during contraction (Katsuragi *et al*., 1996). Thus, the potential complex interactions with purinergic receptors within the tissue makes it difficult to reconcile the actions of antagonists on spontaneous activity.

In conclusion, we have found in the possum duodenum that exogenous adenosine mediates its inhibitory actions acting via the $A_{2A}R$ and A_3R, and that endogenous adenosine may play a role in modulating spontaneous activity.

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