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Modulatory effect of adenosine receptors on the ascending and descending neural reflex responses of rat ileum

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Abstract

Background: Adenosine is known to act as a neuromodulator by suppressing synaptic transmission in the central and peripheral nervous system. Both the release of adenosine within the small intestine and the presence of adenosine receptors on enteric neurons have been demonstrated. The aim of the present study was to characterize a possible involvement of adenosine receptors in the modulation of the myenteric reflex. The experiments were carried out on ileum segments 10 cm in length incubated in a single chambered organ bath, and the reflex response was initiated by electrical stimulation (ES).

Results: ES caused an ascending contraction and a descending relaxation followed by a contraction. All motility responses to ES were completely blocked by tetrodotoxin, indicating that they are mediated by neural mechanisms. Atropine blocked the contractile effects, whereas the descending relaxation was significantly increased. The A1 receptor agonist N6-cyclopentyladenosine increased the ascending contraction, whereas the ascending contraction was reduced by the A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine. Activation of the A1 receptor further reduced the descending relaxation and the latency of the peristaltic reflex. The A2B receptor antagonist alloxazine increased ascending contraction, whereas descending relaxation remained unchanged. For A2A and A3 receptors, we found contradictory effects of the agonists and antagonists, thus there is no clear physiological role for these receptors at this time.

Conclusions: This study suggests that the myenteric ascending and descending reflex response of the rat small intestine is modulated by release of endogenous adenosine via A1 receptors.

Background

Purines such as adenosine triphosphate (ATP) and adenosine are known to act as neurotransmitters as well as neuromodulators in the central, peripheral, and enteric nervous system [1,2].

The pharmacological actions of adenosine on smooth-muscle preparations are well established, and the effects are mediated via receptors that have been classified as P1-purinoceptors (adenosine receptors) [3]. The adenosine receptors may be subdivided into A1 and A2 adenosine receptors, based on their functional coupling to adenylyl cy-

class activity [4]. Thus, A1 receptors are generally linked to inhibition of cyclic adenosine monophosphate (cAMP) generation, while A2 receptors stimulate the formation of cAMP [5]. In addition, an A3 adenosine receptor with inhibitory effect on cAMP formation has been cloned [6], and the A2 receptor has been further subdivided into the A2A and A2B receptor subtypes on a molecular basis [4]. Using either ligand binding, functional studies, or molecular techniques, A1, A2A, A2B, and A3 receptors have been identified within the rat small intestine [7–10].

A1 receptors in the rat ileum are known to be situated on cholinergic nerve endings innervating the smooth muscle, and activation of these A1 receptors reduces smooth-muscle contraction following electrical stimulation, by reducing acetylcholine (ACH) release [9–12]. However, there is also strong evidence that in the circular layer of the rat ileum, the smooth muscle can be relaxed by A1 receptor-mediated mechanisms [9]. In contrast, the A2A receptor increases electrically induced twitch contractions in the guinea-pig ileum, which is attributed to facilitation of ACH release [13].

Within the peristaltic reflex, ACH and tachykinins (substance P, neurokinin A) are responsible for the ascending excitatory motor response of the peristaltic reflex activated by cholinergic interneurons (nicotinic receptors) [14–16], whereas vasoactive intestinal peptide, pituitary adenylate cyclase-activating peptide, and nitric oxide are responsible for the descending relaxant pathway [17,18].

Various models of the enteric reflex have been described, such as cut-open gut segments that are stimulated by stretching oral and aboral to the recording site [15,19], or intact gut segments stimulated either by increasing intraluminal pressure or by electrical stimulation, either with intraluminal pressure being recorded or with force transducers being connected to the serosal surface [20,21]. In our laboratory, a myenteric reflex model has been developed that uses whole segments of rat ileum. Intrinsic nerves are stimulated by electrical stimulation, and motility changes are detected by force transducers attached to record the activity of the circular muscle [22–24]. The pharmacology of the reflex response induced by electrical stimulation as used in our experiments has been shown to be similar to that activated by intraluminal balloon dilatation [25].

With various experimental set-ups designed for investigation of the peristaltic reflex, there have been reports of involvement of adenosine receptors in small-intestinal peristalsis. However, due to the different experimental set-ups used, the differing outcome variables, and the variety of agonists and antagonists used, a number of contradictory results have been published. Whereas the peristaltic

reflex model concentrates on the initiation of the peristaltic reflex, measuring propulsion and contraction of the longitudinal smooth muscle, the model investigating the myenteric reflex focuses on ascending and descending neural pathways, measuring contraction of the circular smooth muscle.

Since most investigations have described possible influences of single adenosine receptor subtypes, the aim of the present study was to characterize a possible role of adenosine A1, A2A, A2B and A3 receptors in the ascending and descending myenteric reflex in the rat ileum in vitro.

Results

Characterization of the set-up used to investigate the ascending excitatory and descending inhibitory reflexes

Electrical field stimulation caused an ascending contraction and a descending relaxation, which was followed by a subsequent contractile response (Fig. 1). All motility responses caused by electrical field stimulation were abolished by TTX (3×10^{-7} M: oral contraction: $-3.9 \pm 4.5\%^*$; anal relaxation: $-6.4 \pm 4.5\%^*$; anal contractile response: $-0 \pm 0.0\%^*$; $* = P < 0.05$; $n = 5$) as well as hexamethonium (10^{-4} M: oral contraction: $-0 \pm 0.0\%^*$; anal relaxation: $-9.8 \pm 4.7\%^*$; anal contractile response: $-0 \pm 0.0\%^*$; $* = P < 0.05$; $n = 5$). Blockade of muscarinic receptors with atropine (10^{-6} M) decreased the oral and anal contraction (atropine 10^{-6} M: oral: $-6.2 \pm 4.7\%^*$, $n = 8$; anal: $-13.6 \pm 10.9\%^*$, $n = 5$), whereas the anal relaxation was significantly increased by cholinergic blockade (10^{-6} M: $+221.2 \pm 82.4\%^*$, $n = 6$).

None of the adenosine receptor agonists or antagonists used had any influence on the basal activity of the ileum preparation – either on basal tone or on the occurrence of spontaneous contractions (data not shown).

Effect of the A1 agonist CPA and the A1 antagonist DPCPX

On the ascending contraction CPA (10^{-14} – 10^{-8} M) caused an increase in the contraction force at lower concentrations and a decrease at the highest concentration applied ($n = 17$) (Figs. 1, 2). On the descending relaxation, CPA caused a concentration-dependent reduction ($n = 17$) (Figs 1,3). The latency of the ascending contraction was significantly increased by CPA (Table 1).

DPCPX (10^{-10} – 10^{-6} M) also caused a concentration-dependent reduction in the ascending contraction ($n = 11$) (Figs. 1, 2). The descending relaxation was significantly reduced by DPCPX ($n = 11$) (Figs 1,3). The latency of the ascending contraction was significantly increased by DPCPX (Table 1).

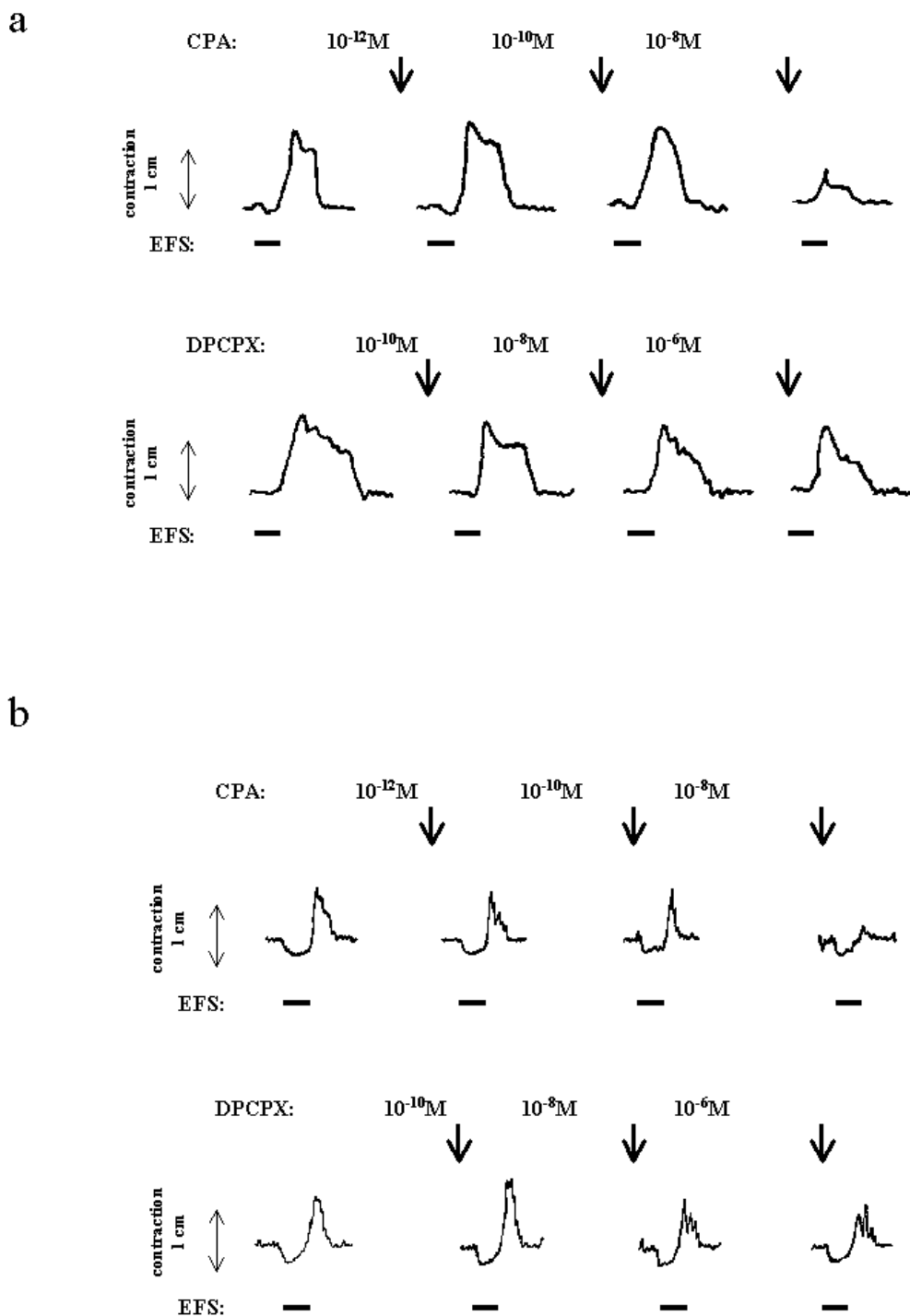


Figure 1
 Representative tracings showing the effect of the A1-receptor agonist CPA and the A1-receptor antagonist DPCPX on the (a) ascending excitatory reflex response and (b) descending relaxant reflex response in the rat ileum. The reflex response was induced by electrical stimulation (EFS; 20 V, 2 ms, 3 pps, 15 s), and the response was recorded by contractility recording 2 cm oral from the stimulation site. (1 cm pen deflection distance = 6 mN)

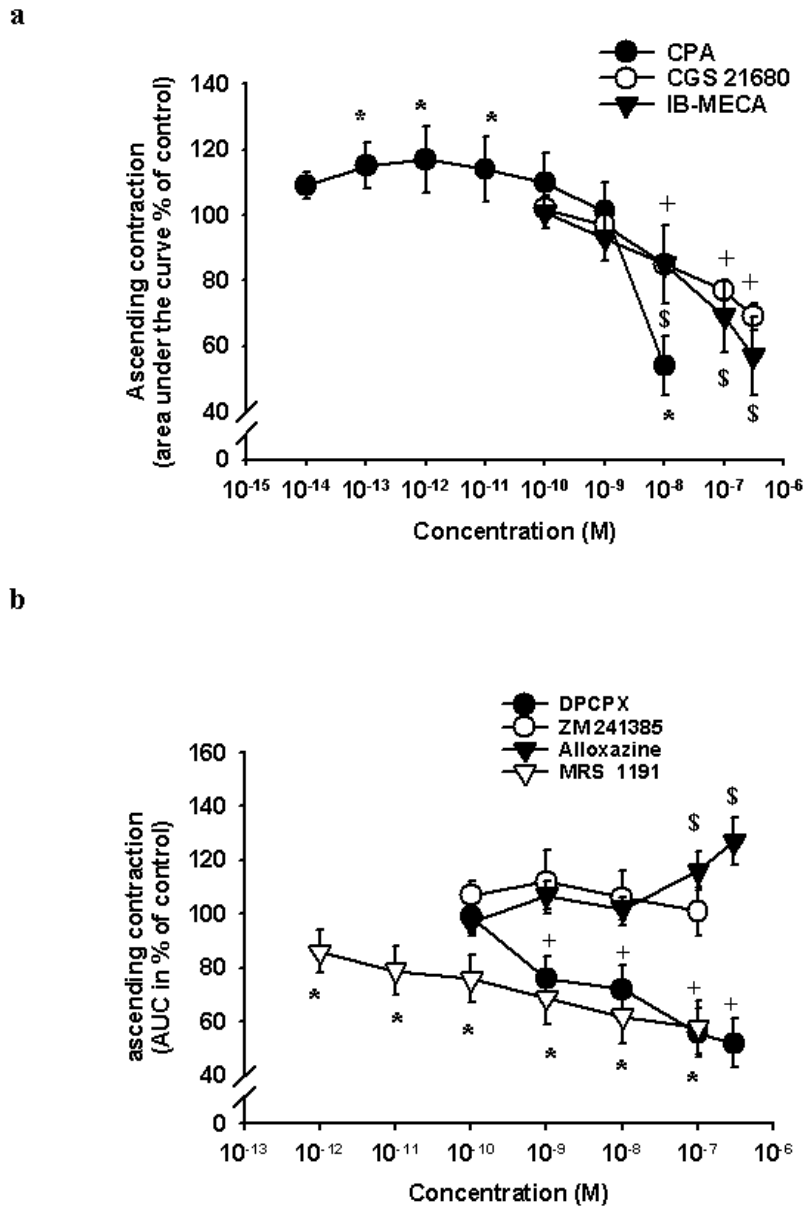
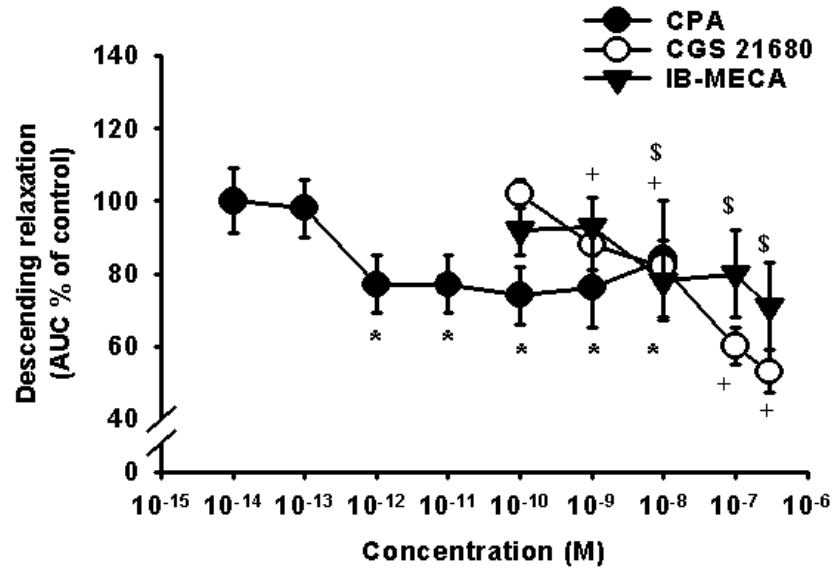


Figure 2

Concentration-response curves of the different adenosine receptor agonists (a) and antagonists (b) on the excitatory ascending reflex response. The reflex response was induced by electrical stimulation (EFS; 20 V, 2 ms, 3 pps, 15 s), and the response was recorded by contractility recording 2 cm oral from the stimulation site (* = $P < 0.05$ for CPA/MRS 1191; + = $P < 0.05$ for CGS 21680/DPCPX; \$ = $P < 0.05$ for IB-MECA/Alloxazine).

a



b

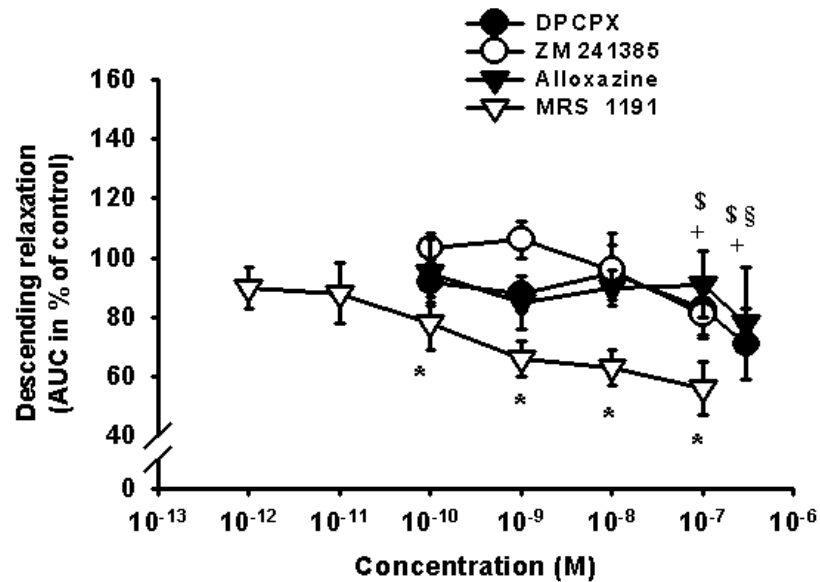


Figure 3

Concentration-response curves of the different adenosine receptor agonists (a) and antagonists (b) on the inhibitory descending reflex response. The reflex response was induced by electrical stimulation (EFS; 20 V, 2 ms, 3 pps, 15 s), and the response was recorded by contractility recording 2 cm aboral from the stimulation site (* = $P < 0.05$ for CPA/MRS 1191; + = $P < 0.05$ for CGS 21680/DPCPX; § = $P < 0.05$ for IB-MECA/Alloxazine; § = $P < 0.05$ for ZM 341285).

Table 1: An overview of the influence of the different adenosine receptor agonists/antagonists used on the latency of the ascending contraction. The influence on latency is given for the highest concentration applied

Drug	Experiments (n)	Mean \pm SEM	Significance
DPCPX 10^{-6} M	10	134 \pm 14%	$P < 0.05$
CPA 10^{-8} M	16	171 \pm 22%	$P < 0.05$
ZM 241385 10^{-6} M	16	106 \pm 6%	n.s.
CGS 21680 3×10^{-7} M	11	104 \pm 9%	n.s.
Alloxazine 3×10^{-7} M	9	104 \pm 7%	n.s.
MRS 1191 10^{-7} M	11	113 \pm 15%	n.s.
IB-MECA 3×10^{-7} M	8	111 \pm 14%	n.s.

Effect of the A2A agonist CGS 21680 and the A2A antagonist ZM 241385

CGS 21680 (10^{-10} - 3×10^{-7} M) reduced the ascending contraction and the descending relaxation in a concentration-dependent fashion ($n = 11$) (Figs. 2, 3), whereas the latency of the ascending contraction remained unchanged (Table 1).

The A2A receptor antagonist ZM 241385 (10^{-10} - 10^{-7} M) did not significantly change the ascending contraction ($n = 17$) (Fig. 2), descending relaxation (Fig. 3), or the latency of the ascending contraction (Table 1).

Effect of the A2B antagonist alloxazine

Alloxazine (10^{-10} - 3×10^{-7} M) caused a small but significant increase in the ascending contractile response ($n = 8$) (Fig. 2) and a significant decrease in the descending relaxation ($n = 8$) (Fig. 3), whereas the latency of the ascending relaxation was not significantly changed (Table 1).

Effect of the A3 agonist IB-MECA and the A3 antagonist MRS 1191

IB-MECA (10^{-10} - 3×10^{-7} M) reduced the ascending contraction in a concentration-dependent fashion ($n = 8$) (Fig. 2) and reduced the descending relaxation (Fig. 3), whereas the latency of the ascending relaxation was not significantly changed (Table 1).

MRS 1191 (10^{-14} - 10^{-7} M) caused a significant decrease in the ascending contraction ($n = 12$) (Fig. 2) and descending relaxation (Fig. 3), whereas the latency of the ascending relaxation was not significantly changed (Table 1).

Discussion

Since the release of adenosine 5'-triphosphate from isolated myenteric nerve varicosities was first demonstrated in the guinea-pig small intestine in 1982 [29], it has been postulated that endogenous purines are involved in the neural enteric reflex pathways.

The data presented in this study demonstrate involvement of the A1 adenosine receptor in the myenteric reflex of the rat small intestine. The ascending contractile response is increased by the A1 agonist CPA at lower concentrations, whereas it is attenuated at higher concentrations. This finding is different compared to studies focused on the peristaltic reflex and peristalsis, in which activation of A1 receptors causes an attenuation of the ascending contraction in guinea-pig ileum [30] or a decrease in peristalsis in rat jejunum [31] but direct comparison in this case is hampered by the different setups, different species and the different and higher agonist concentration used. The experimental set-up used in the present study also showed an attenuation of the ascending contractions, but only at high concentrations – an effect that may be nonspecific, whereas at lower concentrations the effect of CPA has to be regarded as a specific A1 receptor-mediated effect.

Interestingly, the A1 receptor antagonist DPCPX also attenuated the ascending contractile response in our experiments, an observation that is in agreement with the finding that CPA increases the ascending contractile response in this set-up at the more specific lower concentrations. Since in the present experimental set-up, the A1 receptor antagonist DPCPX when given alone also attenuates the myenteric reflex responses, these data suggest that activation of A1 receptors by endogenously released adenosine under our experimental conditions stimulates the myenteric reflex responses. The fact that the specific A1 antagonist DPCPX was able to attenuate the reflex response in the preparations used contrasts with the findings of Hancock and Coupar [31]. Using a different preparation, with reflex stimulation by fluid inflation and recording of longitudinal muscle contraction and of volume expulsion, they were unable to find any influence of the A1 antagonist DPCPX on the peristaltic reflex response [31]. This difference might be due to different experimental set-ups (electrical vs. fluid inflation), to the use of different muscle layers to record contractile activity (longitudinal vs. circular smooth muscle), or possibly to the fact that different regions were investigated (ileum vs.

jejunum). These differences limit a comparison on the few published attempts investigating components of the peristaltic reflex.

Both the A1 receptor agonist and the antagonist caused significant decreases in descending relaxation. In addition, the latency of the ascending contraction appears to be modulated by the A1 receptor, since activation by CPA causes an increase in the timing of the occurrence of the ascending contraction after electrical stimulation. Interestingly, the latency of the ascending contraction was also increased in the presence of DPCPX. A possible explanation for this might be that exogenously applied agonists cause activation of mechanisms that may not be activated by endogenous adenosine receptor agonists under physiological conditions.

Alloxazine, the selective A2B receptor antagonist, provides further evidence for a modulatory involvement of endogenous adenosines on the myenteric reflex. In the presence of alloxazine, the ascending contractile response and the descending relaxant response following initiation of the myenteric reflex are both reduced. In contrast to the A1 receptor, which appears to modulate the myenteric reflex permanently after being activated through endogenous adenosines, the A2B receptor antagonist does not influence the latency of the ascending contraction. The timing of the ascending contractions is also not influenced by agonists or antagonists of either the A2A or A3 receptors, making a physiological role for these receptors in the timing of the myenteric reflex unlikely, but giving further strength to the specificity of A1 receptor involvement in the timing of the myenteric reflex.

The A2A receptor agonist caused attenuation of both ascending contraction and descending relaxation within the myenteric reflex response. However, since the A2A receptor antagonist did not exert any effects on the ileum preparation, this finding may not reflect a physiological pathway within the myenteric reflex under the experimental conditions used here and furthermore an effect of CGS 21680 on other than A2A receptors (e.g. A1 receptors) can not be ruled out.

The effects observed with the receptor agonist and antagonist for the A3 receptor might only be pharmacological ones, although the effect of the A3 receptor antagonist might reflect the fact that endogenous adenosines within the myenteric reflex also act via the A3 receptor. Since both agonists and antagonists show attenuation of either the ascending contraction or the descending relaxation, these findings may reflect involvement of these receptor subtypes, but nonspecific actions cannot be completely ruled out.

All of the above considerations are reflected by a number of observations reported in the literature, but there are many limitations to the interpretation of adenosine receptor-mediated effects in a complex physiological system such as the myenteric reflex. Especially the fact that the selectivity of the different agonists and antagonists used is limited might limit the conclusions drawn when the effects at higher concentrations are discussed. Since the agonist and antagonist selectivity is based on rank orders, effects on other receptors can not be ruled out for the higher concentrations and therefore the conclusions were drawn very carefully and effects at the lower concentrations were regarded as more specific.

Adenosine A1 and A2 receptors within the gastrointestinal tract are known to reduce ACH release, as well as tachykinin (substance P) release, from neuronal endings [32,33]. However, adenosine receptors not only influence neurotransmitter release, but are also potent in modulating neuron excitability [34], and adenosine released from neuronal endings have been thought to represent relaxant neurotransmitters in the gastrointestinal tract, with direct actions on smooth muscle [35–37].

With regard to rat ileum, A1 receptor agonists in whole segments of rat ileum cause relaxation of the longitudinal muscle in which NECA and CPA are equipotent, although it is known that NECA is a weak A1 receptor agonist that also has a high agonist potency on A2 receptors [9]. Isolated longitudinal ileal muscle is also relaxed by A1 receptor activation, but in contrast to whole segments, CPA is more potent than NECA [9]. In contrast, isolated circular muscle is contracted, and the potency order again suggests that an A1 receptor is involved [9]. This difference in A1 receptor-mediated effects on circular and longitudinal smooth muscle is very important, and may explain the differences in motility changes reported in studies investigating peristaltic or myenteric reflex pathways.

At present, most of the available agonists and antagonists are characterized by a rank order of receptor activity that makes interpretation of the findings in a complex in-vitro experiment difficult, and implies that effects at lower concentrations may be regarded as more specific than effects obtained at higher concentrations. Antagonist-on-agonist application experiments were not carried out in the present set-up, since results obtained from such experiments would be hard to interpret in a complex arrangement such as that used here, in which adenosine receptors are located at more than one site. Interpretation is made even more difficult by the ability of adenosine receptors to interact with other receptors for neurotransmitters/neuromodulators. This extends the range of opportunities for adenosine receptor agonists to interfere with neuronal function and communication [8].

Comparisons with reports by other investigators focusing on the peristaltic reflex are hampered not only by the fact that each investigator uses a different set-up and evaluates different patterns of the peristaltic reflex (contraction force vs. contraction occurrence), but also by the fact that either agonists or antagonists of the adenosine receptors are used, and that most of the investigations focus only on a single adenosine receptor subtype, instead of the broad spectrum of all receptor subtypes [10,21,38–40]. An even greater problem in interpreting the effects of adenosine agonists and antagonists in a complex experimental set-up such as the peristaltic reflex is the fact that fairly specific agonists and antagonists, acting on a variety of adenosine receptor subtypes in a large number of possible involved locations (neuronal and muscular), can only be interpreted as the sum of actions that might result from a large number of activating and inactivating effects at different sites. Though all of the above mentioned limitations also account for the here presented study, this is the first attempt to completely work out all adenosine receptor subtypes in one and the same setup.

Conclusion

In conclusion, the results presented here provide evidence that the adenosine receptors participate in the peristaltic reflex in a complex manner. A1 receptors appear to modulate the timing physiologically, whereas for the A2A, A2B, and A3 receptors, no evidence was found that they are involved in the timing of the peristaltic reflex. The study also demonstrates that A1 receptors are physiologically involved in the regulation of the force of ascending contraction in the myenteric part of the peristaltic reflex, with A1 receptor activation causing an increase in the ascending contraction and an attenuation of the descending relaxation. A2B receptors appear to be physiologically involved in the regulation of the ascending contraction of the myenteric reflex pathways, with A2B receptor blockade causing an increase in the ascending contraction and an attenuation of the descending relaxation.

Methods

Male Wistar rats (300–400 g) were killed by intraperitoneal injection of pentobarbital sodium (100 mg/kg). The ileum was removed immediately and kept in oxygenated Krebs-Ringer bicarbonate solution (KRS: NaCl 115.5 mM, MgSO₄ 1.16 mM, NaH₂PO₄ 1.16 mM, glucose 11.1 mM, NaHCO₃ 21.9 mM, CaCl₂ 2.5 mM, KCl 4.16 mM). All experiments lasted less than 3 h, and each muscle strip was used for a single concentration-response curve only. Experiments were carried out in accordance with institutional animal ethics committee guidelines.

Myenteric reflex

The experiments investigating the myenteric reflex were carried out in an in-vitro organ bath model, as described

previously [24], modified from similar models used before [20,25,26]. A segment 10 cm in length was carefully dissected, and the mesenteric arcade was removed. The segment was placed in an organ bath filled with 35 ml oxygenated KRS and maintained at 37°C. A glass rod 2 mm in diameter was passed through the lumen, and fixed the gut in the organ bath. Mechanical activity of the circular muscle was recorded using force-displacement transducers attached with tips to the serosa 2 cm orally and 2 cm anally from the pair of stimulation electrodes and a tension of 1 g was applied. Contractile changes were recorded using Grass FTO3C force transducers (Grass, Quincy, MA, USA) and recorded on a Sormedics R611 chart recorder (Sormedics, Anaheim, CA, USA). Field stimulation impulses for neuronal responses were applied using a Grass S11 stimulator, stimulating for 15 s at intervals of 2 min at standard parameters of 20 V pulse strength and 3 Hz, 2 ms pulse duration. The electrical stimulatory signal was connected to an AC/DC coupler and recorded simultaneously with the motility signal.

The gut segment was stimulated every 2 min, and this periodic stimulation was maintained throughout the experiment. A stable response to the electrical stimulation (identical contractions to at least three consecutive stimuli) was established after 10–20 minutes. When the response stabilized, a stable response was achieved for a minimum of 3 hours and control experiments to a maximum period of 5 hours were performed as described formerly. The experiment was started after an equilibration period of 30 min. Drugs were added 60 s after the last stimulation and before the next electrical stimulation in the organ bath so that all of the tissue was equilibrated with each drug. For each concentration, at least three reflex responses were elicited before the next concentration was tested. Concentration-response curves were recorded in a cumulative manner. At the end of the experimental protocol, the buffer was exchanged several times, and after a period of 15 min a control recording was performed after the wash-out.

Drugs

The drugs used were N⁶-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), alloxazine, 4-[2-[[[6-amino-9-(N-ethyl-b-D-ribofuranuroamidoyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid (CGS 21680), ZM 241385, MRS 1191, IB-MECA (RBI, Natick, MA, USA), N^G-nitro-L-arginine-methyl-ester (L-NAME), atropine, hexamethonium, tetrodotoxin (TTX) (Sigma, Irvine, UK). Drugs were freshly dissolved and further diluted in KRS on the day of use. DPCPX, alloxazine, CGS 21680, ZM 241385, MRS 1191 and IB-MECA were soluble in dimethyl sulfoxide (DMSO) and further diluted in KRS. Control experiments investigating DMSO at the highest concentration applied and performed in a time course

comparable to the other experiments had no effect on the recorded motility. References for the pharmacological profiles of the used drugs can be found at Fredholm et al., 2001 and Liang et al. 1995 [27,28].

Data evaluation and analysis

As a measure of the contractile activity, the area under the curve (mm²) of the contraction induced at the oral recording sites and the relaxation at the aboral recording sites were determined using a digitizing tablet (Sigma Scan, Jandel, CA, USA) and changes in the area under the curve in presence of drugs are expressed in % of pre-drug controls. The latency of the reflex response (seconds) was determined as the time interval between the start of stimulation and the onset of contraction, and expressed as a percentage change in comparison with the pre-drug control.

Data are given as mean plus or minus standard error (m ± SEM); n indicates the number of independent observations in ileal segments from different animals. Using a commercial statistical package (SigmaStat, Jandel Scientific, San Rafael, CA, USA), analysis of variance for repeated measures was used to test for a statistical difference, and the Dunnett post-hoc test was used to establish the significance against the control value. A null hypothesis with a probability of less than 5% was considered significant.

Authors contributions

JT and MS carried out all experimental work, participated in the design of the study and carried out statistical data analysis. MS additionally finalized the manuscript for publication. RD participated in the design and coordination of the study and in the data preparation. VS and HA conceived of the study, gave intellectual input, participated in the design and coordination of the study.

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