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Original Article

P2Y receptor-mediated transient relaxation of rat longitudinal ileum preparations involves phospholipase C activation, intracellular Ca^{2+} release and SK channel activation

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Aim: Purinergic signaling plays a major role in the enteric nervous system, where it governs gut motility through a number of P2X and P2Y receptors. The aim of this study was to investigate the P2Y receptor-mediated motility in rat longitudinal ileum preparations.
Methods: Ileum smooth muscle strips were prepared from rats, and fixed in an organ bath. Isometric contraction and relaxation responses of the muscle strips were measured with force transducers. Drugs were applied by adding of stock solutions to the organ bath to yield the individual final concentrations.
Results: Application of the non-hydrolyzable P2 receptor agonists α,β-Me-ATP or 2-Me-S-ADP (10, 100 μmol/L) dose-dependently elicited a transient relaxation response followed by a sustained contraction. The relaxation response was largely blocked by SK channel blockers apamin (500 nmol/L) and UCL1684 (10 μmol/L), PLC inhibitor U73122 (100 μmol/L), IP_3 receptor blocker 2-APB (100 μmol/L) or sarcoendoplasmic Ca^{2+} ATPase inhibitor thapsigargin (1 μmol/L), but not affected by atropine, NO synthase blocker *L*-NAME or tetrodotoxin. Furthermore, α,β-Me-ATP-induced relaxation was suppressed by $P2Y_1$ receptor antagonist MRS2179 (50 μmol/L) or $P2Y_{13}$ receptor antagonist MRS2211 (100 μmol/L), and was abolished by co-application of the two antagonists, whereas 2-Me-S-ADP-induced relaxation was abolished by $P2Y_6$ receptor antagonist MRS2578 (50 μmol/L). In addition, $P2Y_1$ receptor antagonist MRS2500 (1 μmol/L) not only abolished α,β-Me-ATP-induced relaxation, but also suppressed 2-Me-S-ADP-induced relaxation.
Conclusion: P2Y receptor agonist-induced transient relaxation of rat ileum smooth muscle strips is mediated predominantly by $P2Y_1$ receptor, but also by $P2Y_6$ and $P2Y_{13}$ receptors, and involves PLC, IP_3, Ca^{2+} release and SK channel activation, but is independent of acetylcholine and NO release.

Keywords: ileum smooth muscle strips; transient relaxation; purinergic signaling; α,β-methylene-ATP; 2-Me-S-ADP, SK channel; PLC; IP_3; Ca^{2+} release

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Introduction

A balance of contraction and relaxation of gastrointestinal (GI) smooth muscle cells is the physiological prerequisite for digestive and non-digestive motility. Numerous sympathetic fibers and particularly parasympathetic fibers innervate the smooth muscle and are known to be involved in coordinating the motility of large portions of the GI tract. However, there is an increasing interest in non-adrenergic non-cholinergic (NANC) neurotransmission, and the enteric nervous system (ENC) has been acknowledged to play a major role in governing smooth muscle motility[1]. Generally, nitric oxide (NO) and adenosine 5′ triphosphate (ATP) are thought to be key molecules in inhibitory NANC signaling, giving rise to hyperpolarization and relaxation of GI smooth muscle[2–4]. While NO leads to a long-lasting inhibition of smooth muscle motility, the available data on purinergic responses are less consistent, as both excitatory and inhibitory effects have been observed and seem to depend on the subtype and cellular location of P2 purinoceptors. In addition, species differences should also be considered[2–10]. Seven cloned ionotropic P2X receptors that combine

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heteromerically, as well as eight cloned metabotropic P2Y receptors, account for the large heterogeneity of purinoceptor-mediated effects[11]. Hence, P2 purinoceptors are intriguing target candidates for future therapies in conditions associated with GI dysmotility. However, development of these therapies is currently hampered by the lack of specific inhibitors for the majority of P2 receptors. Immunohistochemical data have demonstrated that ionotropic $P2X_2/P2X_3$ and metabotropic $P2Y_1/P2Y_2$ receptors are expressed on pre-junctional enteric neurons[7, 12], while smooth muscle cells are predominantly immunoreactive for $P2Y_1$ receptors[7]. Moreover, there is a great consensus that the purinergic inhibitory junction potential and the corresponding relaxation are highly sensitive to MRS2179 and MRS2500, showing that the $P2Y_1$ is the receptor type involved in purinergic nerve-mediated relaxation[13–15]. This finding has also been confirmed in $P2Y_1$ knock-out (KO) mice[16–18]. Consistent with this differential expression, the ATP release and smooth muscle contraction from cholinergic fibers that are induced by the P2 agonist α,β-methylene ATP (α,β-Me-ATP) are mediated by pre-junctional P2Y and P2X receptor activation, respectively[8]. Post-junctional P2Y receptors, however, activate apamin-sensitive SK channels as a consequence of Ca^{2+} release from thapsigargin-sensitive stores and thus promote relaxation[4, 9, 19, 20]. Recently, an elegant study tested the hypothesis that spontaneous hydrolysis products of ATP, such as ADP and adenosine, mediate the biphasic effects observed following ATP administration and found that ADP and adenosine were agonists of P2Y and adenosine A_1 receptor, respectively, thereby having the opposite effects from ATP[6].

In the present study, we tested the hypothesis that purinergic signaling is involved in the control of ileum motility in the rat and found that P2 receptor activation led to both transient relaxation and slow-onset sustained contraction of longitudinal segments of the rat ileum.

Materials and methods

Preparation of ileum smooth muscle strips

Male Sprague–Dawley rats (50–120 days old, 200–350 g, Charles River, Sulzfeld, Germany) were deeply anesthetized with diethyl ether and decapitated. All procedures conformed to the national and international guidelines on the ethical use of animals (European Council Directive 86/609/EEC) and were approved by local authorities (LALLF Mecklenburg-Vorpommern). The abdomen was incised, and the full length of the small intestine was removed. Ileum preparation was obtained by cutting a piece 10−15 cm in length from the ileocecal junction in the oral direction. This preparation was subsequently submerged into a dissection solution containing (in mmol/L) 120 NaCl, 4.5 KCl, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 1.6 $CaCl_2$, 1.0 $MgSO_4$, 0.025 Na_2-EDTA, 5.5 glucose, and 5 HEPES (pH=7.4) at 4 °C and was further cut while submerged in the solution into 4–8 specimens of approximately 2 cm in length. Thin nylon threads were sutured to either end of these specimens to enable longitudinal fixation in the organ bath (Panlab ML0146/C, ADInstruments, Spechbach, Germany). The organ bath was filled with a buffer that contained (in mmol/L) 120 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 30 $NaHCO_3$, 0.5 Na_2-EDTA, 5.5 glucose, 2 sodium pyruvate (pH=7.4) and was gassed with carbogen (95% O_2 and 5% CO_2).

Isometric contractions *in vitro*

After fixation in the organ bath, the ileum specimens were allowed to recover for at least 1 h before the experiments started. During this time, the organ bath temperature was raised from room temperature to 37 °C. The initial tension of the gut specimens within the organ bath was adjusted to yield a stable baseline tone. Briefly, the specimens were inserted and fixed with a mean force. Occasionally, the tissue displayed an increase or a decrease in baseline tone, which was abolished by re-adjustment of the baseline force. Before the start of the experiment, a stable baseline tone was achieved for at least 50 min. Under these conditions, both contractions and relaxations have been found to be very reproducible and to show low variance between different tissue specimens in previous studies[21, 22]. Isometric contractions and relaxations of the smooth muscle strips induced by KCl or purinergic ligands were measured by force transducers (MLT0201, ADInstruments, Oxford, UK) and recorded with a bridge amplifier (ML224, ADInstruments) connected to an analog-to-digital converter (Powerlab 4/30, ADInstruments) and then analyzed by the Chart 5 software (ADInstruments).

α,β-Me-ATP, 2-methylthioadenosine-5'-diphosphate (2-Me-S-ADP), UCL1684, 2-aminoethoxydiphenylborane (2-APB), thapsigargin, U73122, pertussis toxin, MRS2211, MRS2179, MRS2578, MRS2500 and suramin were purchased from Tocris Bioscience (Bristol, UK). Apamin was obtained from Alomone Labs (Jerusalem, Israel). All other chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany). The application of drugs was performed by adding 100 μL of stock solution (α,β-Me-ATP, 2-Me-S-ADP, apamin, UCL1684, 2-APB, thapsigargin, U73122, pertussis toxin, MRS2211, MRS2179, MRS2578, MRS2500, suramin, tetrodotoxin) or 1000 μL of stock solution (KCl) to the organ bath (volume 25 mL) to yield the individual final concentration. The organ bath fluid was not corrected for osmolarity after the addition of KCl. However, the resulting increase in osmolarity was well tolerated by the preparations, and hyperosmotic control experiments with addition of the same amount of NaCl revealed relaxation of the smooth muscle rather than contraction[21]. Each gut preparation was tested with KCl for depolarization-induced contractions at the beginning and at the end of the experiment and was included in the study when the KCl-induced contraction at the end of the experiment was at least 50% of the initial response.

Statistical analysis

All data are expressed as mean±SEM. Statistical comparison of drug effects was performed using ANOVA followed by Holm-Sidak *post-hoc* test for pairwise comparisons (Figure 2–6). If only two groups were being compared (Figure 1), the unpaired two-tailed Student's *t*-test was applied. Significant differences were assumed when the *P* value was less than 0.05.

Results

P2 receptor activation causes a transient relaxation and a sustained contraction

The aim of this study was to analyze the effect of P2 receptor signaling on the motility of the ileum longitudinal smooth muscle. To this end, we used α,β-Me-ATP as a non-hydrolyzable specific P2 receptor agonist. Figure 1A illustrates the experimental paradigm. At the beginning of the experiment, each gut preparation was challenged with a high concentration of KCl (60 mmol/L) for 10 min, and a strong depolarization-induced contraction was obtained in all cases (Figure 1A, leftmost traces). After the washout of KCl for at least 30 min, a single dose of α,β-Me-ATP was applied for 20 min using a randomly chosen concentration of 10 or 100 μmol/L (n=8 and 5; Figure 1A, middle traces). This agonist elicited a dose-dependent transient relaxation (100 μmol/L α,β-Me-ATP: -11.0±2.0 mN, n=5, Figure 1B) and a slow-onset, but sustained contraction (100 μmol/L α,β-Me-ATP: 13.8±5.7 mN, n=5), which was also dose-dependent (Figure 1C). We confined our experiment to a single dose of α,β-Me-ATP for each gut preparation, because preliminary experiments have shown that repeated applications of this agonist produce significant desensitization, as has also been reported previously[23]. Hence, after the washout of α,β-Me-ATP, we tested the effect of a high concentration of KCl solution again. This final depolarization-induced contraction (Figure 1A, rightmost traces) was as strong as that at the beginning of the experiment (100%±7% of initial KCl-response, n=25).

To demonstrate the P2 receptor-dependent nature of the α,β-Me-ATP-induced effects, we pre-applied the specific P2 receptor antagonist suramin (100 μmol/L; Figure 1Ab, d). This blocker was added to the organ bath 10 min prior to ATP application and almost abolished both relaxation (100 μmol/L α,β-Me-ATP: -1.2±0.6 mN, n=6, P<0.01, unpaired t-test) and contraction (100 μmol/L α,β-Me-ATP: 2.8±0.7 mN, n=6, P<0.05, unpaired t-test), hence providing evidence that P2 receptor-activation is the predominant mechanism of action of α,β-Me-ATP (Figure 1B, C). While α,β-Me-ATP has been reported to act primarily on P2X receptors[24], we aimed to determine whether the P2 receptor-induced transient relaxation could also be achieved by 2-Me-S-ADP, a non-hydrolyzable agonist with preferential action on P2Y receptors. At a concentration of 10 μmol/L, this compound also produced transient relaxation (-6.7±0.9 mN, n=20) but less pronounced contraction (3.7±1.3 mN, n=20).

Intracellular cascade leading to P2 receptor-mediated relaxation and contraction

Because purinoceptors are localized to pre-junctional enteric neurons[7, 10, 12], the effects of P2 receptor-mediated relaxation could be due to the inhibition of acetylcholine release or to the production of NO. However, atropine (1 μmol/L) had no effect on relaxation induced by α,β-Me-ATP (-10.1±0.6 mN, n=4) or by 2-Me-S-ADP (-6.8±2.0 mN, n=4, Table 1). The NO synthase blocker L-NAME (100 μmol/L) was also ineffective at reducing the relaxation induced by α,β-Me-ATP (-9.0±1.1 mN, n=15) or by 2-Me-S-ADP (-5.2±0.9 mN, n=8, Table 1). To inhibit the activity of all enteric nerve fibers, we pre-applied tetrodotoxin (TTX, 500 nmol/L) to the organ bath. Similarly to atropine and L-NAME, the relaxation induced by α,β-Me-ATP (-11.4±1.5 mN, n=9) or by 2-Me-S-ADP (-7.0±1.4 mN, n=10, Table 1) was not altered by TTX. In contrast, as previously published[4, 9, 19, 20], SK channel inhibition by apamin (0.5 μmol/L) or UCL1684 (10 μmol/L) abolished the relaxation induced by α,β-Me-ATP and by 2-Me-S-ADP (see asterisks in Figure 2Aa and 2Ab). Thus, P2 receptor-mediated relaxation was entirely due to SK channel-activation (0±0 mN for all, Figure 2B), while the contraction following purinergic stimulation did not involve these channels (Figure 2C).

We next tested whether Ca^{2+} release from internal stores triggers SK channel activation. To this end, we used the IP_3 receptor blocker 2-APB (100 μmol/L) and the sarcoendoplasmic reticulum ATPase (SERCA) inhibitor thapsigargin (1 μmol/L). Pre-treatment of ileum segment preparations with 2-APB caused a transient contraction followed by a persistent relaxation (not shown), which could be due to a brief activation of store-operated Ca^{2+} entry that is activated by 2-APB at concentrations below 10 μmol/L, but inhibited by higher concentrations[25]. Application of thapsigargin led to a pronounced but transient contraction (not shown), which has been shown to be correlated with a transient rise in cytosolic Ca^{2+} concentration following SERCA inhibition[26]. Therefore,

Table 1. Control experiments.

Pre-treatment	α,β-Me-ATP		2-Me-S-ADP	
	Relaxation	Contraction	Relaxation	Contraction
None	−11.0±2.0 mN (n=5)	13.8±5.7 mN (n=5)	−6.7±0.9 mN (n=20)	3.7±1.3 mN (n=20)
Atropine (1 μmol/L)	−10.1±0.6 mN (n=4)	11.7±3.3 mN (n=4)	−6.8±2.0 mN (n=4)	5.8±1.8 mN (n=4)
L-NAME (100 μmol/L)	−9.0±1.1 mN (n=15)	14.2±2.2 mN (n=15)	−5.2±0.9 mN (n=8)	0.9±0.6 mN (n=8)
TTX (500 nmol/L)	−11.4±1.5 mN (n=9)	7.1±2.6 mN (n=9)	−7.0±1.4 mN (n=10)	2.9±0.4 mN (n=10)

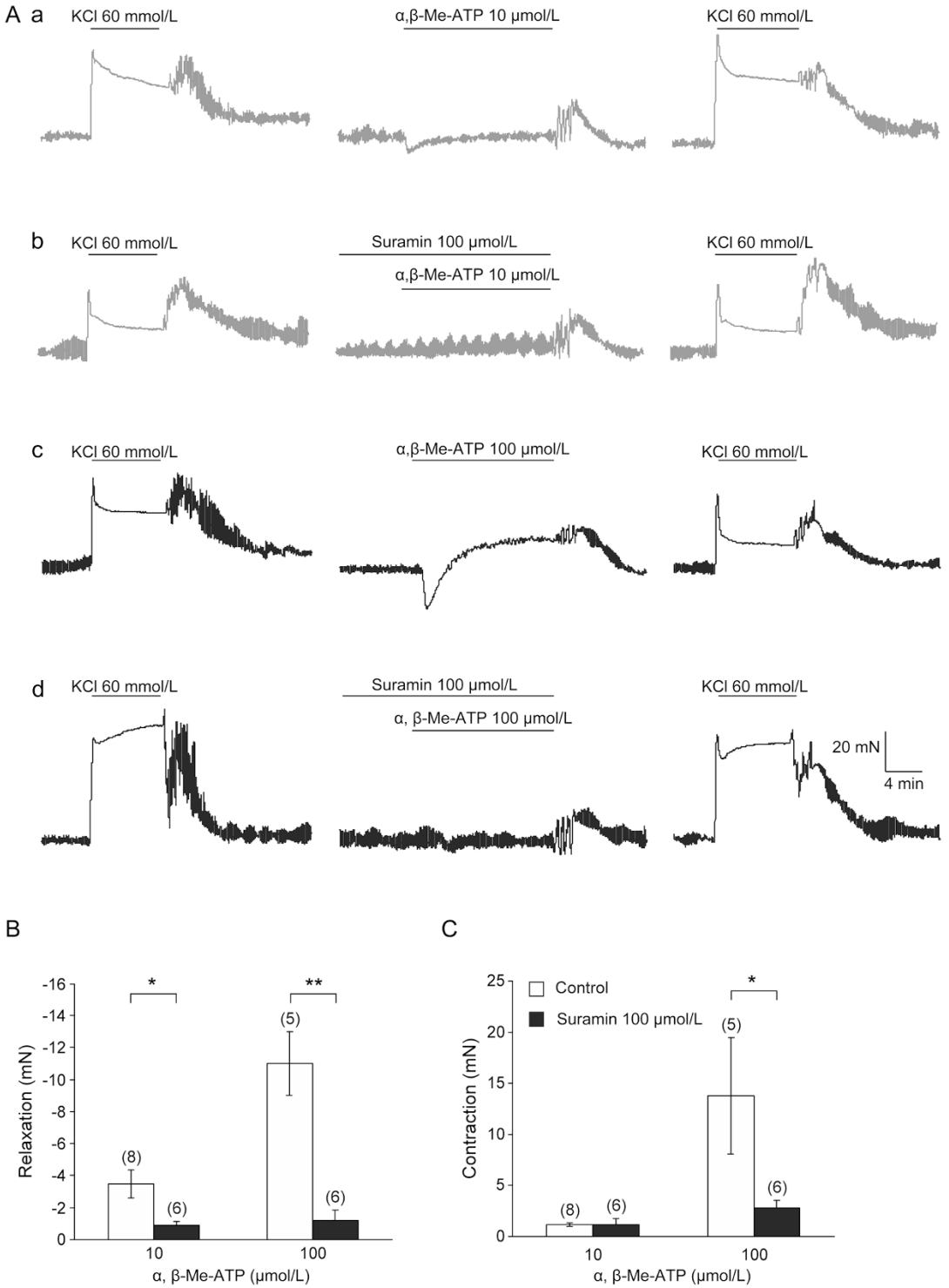

Figure 1. P2 receptor activation causes a transient relaxation and a sustained contraction. (A) At the beginning of each experiment, the ileum longitudinal smooth muscle strip was challenged by application of KCl to the organ bath at concentrations that reached 60 mmol/L, which produced a strong isometric peak contraction followed by a less pronounced sustained contraction, indicating stable recording conditions. After washout and recovery for 30 min, α,β-Me-ATP induced a transient relaxation and a sustained contraction (Aa and Ac). When the P2 receptor blocker suramin was present in the organ bath, both relaxation and contraction were abolished (Ab and Ad). At the end of the experiment, application of KCl again induced a contraction, which was similar to that at the beginning of the experiment. (B) The transient relaxation elicited by α,β-Me-ATP was dose-dependent and significantly inhibited by suramin (n=5–8 as indicated by numbers in brackets). (C) The sustained contraction by α,β-Me-ATP was also dose-dependent and significantly inhibited by suramin (n=5–8 as indicated by numbers in brackets). Mean±SEM. $^{*}P$<0.05, $^{**}P$<0.01.

smooth muscle preparations were allowed to equilibrate with these compounds before the agonists α,β-Me-ATP or 2-Me-S-ADP was added to the organ bath. As shown in representative traces in Figure 3A, both 2-APB and thapsigargin, prevented the purinergic agonist-induced transient relaxation (see asterisks in Figure 3Aa and Figure 3Ab). A series of independent experiments confirmed the significant reduction of the transient relaxation induced by α,β-Me-ATP (control: -9.0±1.2 mN, n=13; 2-APB: -0.2±0.1 mN, n=22, P<0.01; thapsigargin: -3.9±0.8 mN, n=15, P<0.01; Figure 3B) and by 2-Me-S-ADP (control: -9.0±0.7 mN, n=20; 2-APB: -0.2±0.2 mN, n=14, P<0.01; thapsigargin: -0.2±0.1 mN, n=17, P<0.01; Figure 3B). Interestingly, the purinergic agonist-induced contraction was not affected by these compounds (Figure 3C), indicating that Ca^{2+} influx through voltage-gated Ca^{2+} channels might be sufficient to trigger smooth muscle contraction. Indeed, co-application of thapsigargin and the L-type Ca^{2+} channel blocker verapamil (100 μmol/L) abolished the contraction (α,β-Me-ATP: 2.7±0.2 mN, n=2, P<0.05 versus control; 2-Me-S-ADP: 0.8±0.3 mN, n=9, P<0.01 versus control, data not shown). Collectively, our results so far indicate that Ca^{2+} release from internal stores and subsequent activation of SK channels are downstream mechanisms of purinergic agonist-induced relaxation.

Because IP_3 receptor-mediated Ca^{2+} release is a consequence of phospholipase C (PLC) activation, we next tested the effect of the PLC inhibitor U73122 (100 μmol/L) on the relaxation induced by the P2 agonists α,β-Me-ATP and 2-Me-S-ADP (Figure 4). Both α,β-Me-ATP (control: -10.1±0.7 mN, n=9; U73122: -2.7±0.4 mN, n=16, P<0.01) and 2-Me-S-ADP (control: −8.3±0.8 mN, n=8; U73122: -1.0±0.1 mN, n=10, P<0.01) no longer stimulated relaxation of the ileum smooth muscle when PLC was inhibited prior to agonist administration (Figure 4B). Hence, PLC activation plays a crucial role in P2 receptor-mediated relaxation. With respect to purinergic contractions, U73122 did not affect α,β-Me-ATP-induced contraction, but significantly blocked the contraction by the P2Y-preferring agonist 2-Me-S-ADP (control: 5.6±1.3 mN, n=6; U73122: 1.4±0.6 mN, n=10, P<0.01; Figure 4C). These results indicate that P2Y

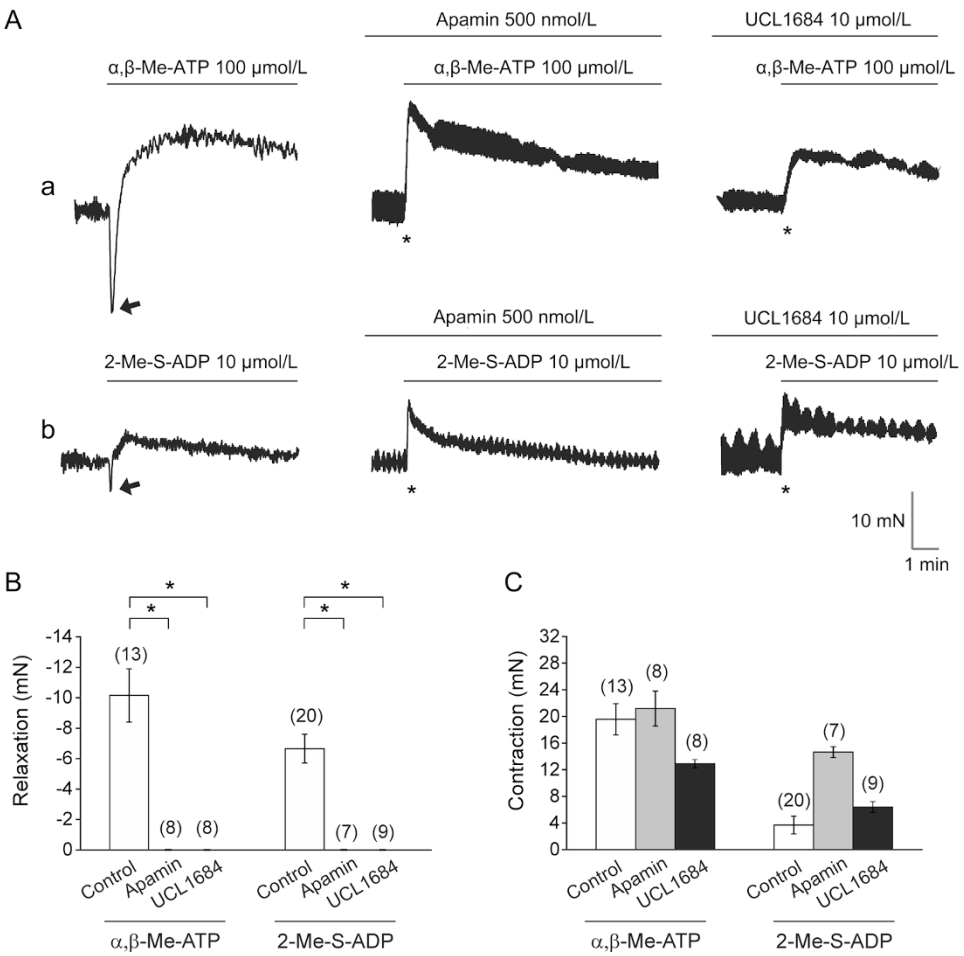

Figure 2. P2 receptor-mediated relaxation relies on SK channel activation. (A) The transient relaxation induced by α,β-Me-ATP and by 2-Me-S-ADP (see arrows in Aa and Ab) was prevented by pre-application of 500 nmol/L apamin or 10 μmol/L UCL1684 (see asterisks in Aa and Ab). All traces in A are derived from individual smooth muscle specimens. (B) The transient relaxation induced by α,β-Me-ATP or by 2-Me-S-ADP was abolished by apamin and by UCL1684, indicating SK channel dependence (n=7–20 as indicated by numbers in brackets). (C) The sustained contraction elicited by both P2 receptor agonists was unaffected by apamin or UCL1684 (n=7–20 as indicated by numbers in brackets). Mean±SEM. $^*P$<0.05.

receptor-mediated contraction requires PLC activation, while P2X receptor-mediated contraction may also be triggered by depolarization-induced Ca^{2+} influx via voltage-gated L-type Ca^{2+} channels. We therefore tested the effect of verapamil (100 μmol/L) on α,β-Me-ATP-induced contractions and observed a significant reduction (8.8±1.5 mN, n=6, P<0.05 versus control), but the contraction induced by the P2Y-preferring agonist 2-Me-S-ADP was not affected by inhibition of L-type Ca^{2+} channels (5.8±0.2 mN, n=4). These results suggest that P2Y receptor activation under normal circumstances does not substantially activate depolarization-induced Ca^{2+} influx.

P2Y receptors involved in purinergic relaxation and contraction

Heterotrimeric G proteins play a major role in PLC activation. In particular, the $G\alpha_{q/11}$ and βγ-subunits of $G_{i/o}$ proteins have been shown to recruit PLC and thereby lead to IP_3 production[27]. Pertussis toxin (PTX), however, specifically interferes with $G_{i/o}$ protein signaling and is thus a powerful tool to study this pathway[28] and to discriminate between the $G\alpha_{q/11}$ and βγ-subunit of $G_{i/o}$. Importantly, PTX (40 ng/mL) did not affect baseline motility, but had differential effects on purinergic relaxation. While this compound failed to block the α,β-Me-ATP-induced relaxation (see arrow in Figure 4Aa; -12.1±1.8 mN, n=5, Figure 4B), it significantly reduced the relaxing effect of 2-Me-S-ADP (see asterisk in Figure 4Ab; -3.1±0.8 mN, n=5, P<0.01, Figure 4B). In contrast, contractions induced by either agonist were largely unaffected by the PTX (Figure 4C). Together with our findings on PLC inhibition, these results suggest that α,β-Me-ATP-induced relaxation predominantly involves $G\alpha_{q/11}$ activation, whereas PTX-sensitive $G_{i/o}$ coupling is involved in 2-Me-S-ADP-induced relaxation.

Having ascertained that PLC activation is instrumental in relaxation induced by α,β-Me-ATP, as well as by 2-Me-S-ADP, we sought to identify the P2Y receptor(s) involved in this process. As shown in Figure 5, the $P2Y_1$ receptor antagonists MRS2179 and MRS2500, as well as the $P2Y_{13}$ receptor

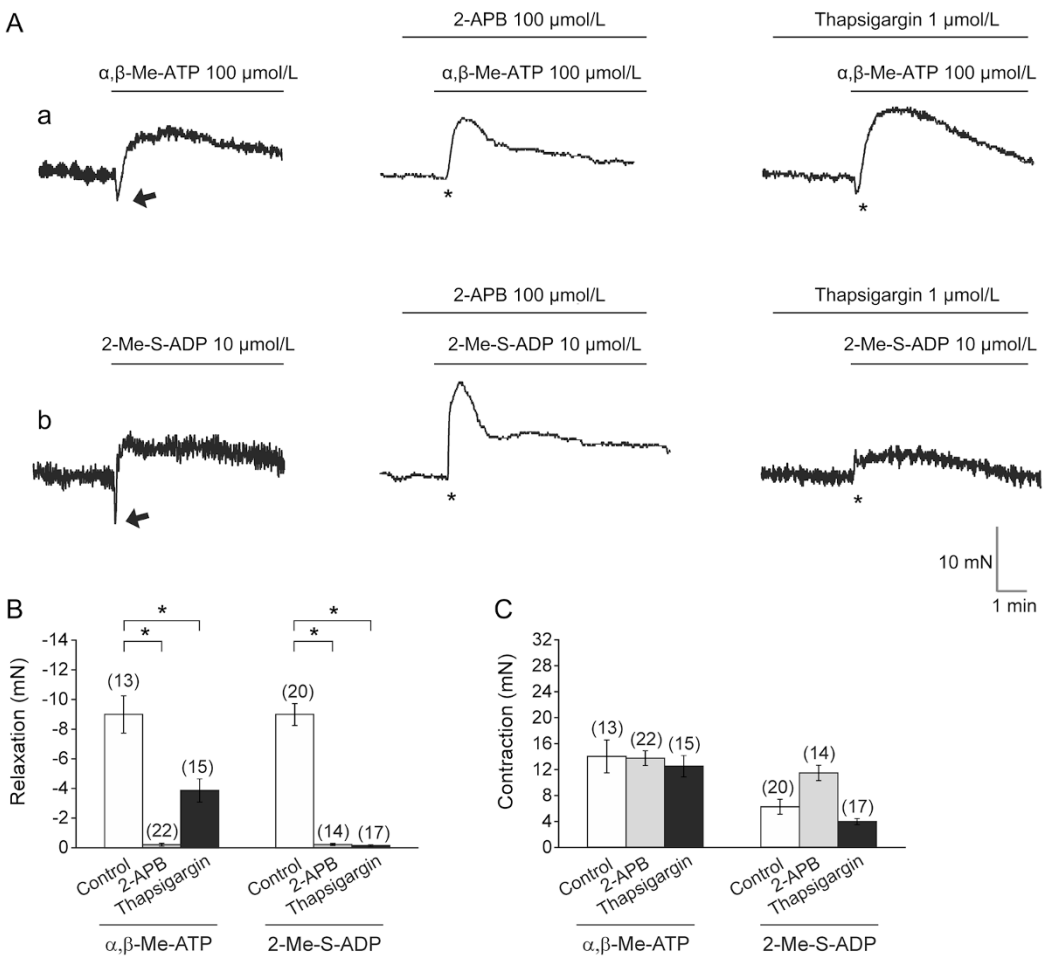

Figure 3. P2 receptor-mediated relaxation involves Ca^{2+} release from internal stores. (A) The transient relaxation induced by α,β-Me-ATP and by 2-Me-S-ADP (see arrows in Aa and Ab) was prevented by pre-application of 100 μmol/L 2-APB or 1 μmol/L thapsigargin (see asterisks in Aa and Ab). All traces in A are derived from individual smooth muscle specimens. (B) The transient relaxation induced by α,β-Me-ATP and by 2-Me-S-ADP was significantly reduced by both 2-APB and thapsigargin, indicating the involvement of IP_3 receptor-mediated Ca^{2+} release from internal stores (n=13–22 as indicated by numbers in brackets). (C) The sustained contraction induced by either P2 receptor agonist was unaffected by 2-APB and by thapsigargin (n=13–22 as indicated by numbers in brackets). *P<0.05.

antagonist MRS2211, significantly reduced the α,β-Me-ATP-induced relaxation (see asterisks in Figure 5Aa; -2.2±0.5 mN, *n*=12 for MRS2179, 0±0 mN, *n*=6 for MRS2500, and -1.0±0.3 mN, *n*=11 for MRS2211; Figure 5B), but the 2-Me-S-ADP-induced relaxation was only reduced by MRS2500 (-2.0±1.0 mN, *n*=12). Moreover, the sustained contraction induced by α,β-Me-ATP was also significantly reduced by all three P2Y receptor antagonists (Figure 5C). These data demonstrate that α,β-Me-ATP not only activates P2X receptors but also is a potent $P2Y_1$ and $P2Y_{13}$ agonist. We therefore expected that co-application of MRS2179 and MRS2211 would almost abolish the α,β-Me-ATP-induced effects. This was indeed the case for both the relaxation (0±0 mN, *n*=8, Figure 6B; see asterisk in Figure 6Aa) and the contraction (2.2±0.2 mN, *n*=8, Figure 6C). However, the 2-Me-S-ADP-induced relaxation was not affected by co-application of MRS2179 and MRS2211 (-5.0±1.7 mN, *n*=8, Figure 6B), indicating that 2-Me-S-ADP probably involves receptors other than $P2Y_1$ and $P2Y_{13}$.

In contrast to the $P2Y_1$ and $P2Y_{13}$ receptor antagonists, the $P2Y_6$ receptor antagonist MRS2578 completely blocked 2-Me-S-ADP-induced relaxation (−0.8±0.3 mN, *n*=8, Figure 6B; see asterisk in Figure 6Ab), but enhanced the contraction (10.7±1.1 mN, *n*=8). Furthermore, MRS2578 partially but significantly reduced the α,β-Me-ATP-induced relaxation. These data suggest that the α,β-Me-ATP-induced relaxation is preferentially mediated by the $P2Y_1$ and $P2Y_{13}$ receptors but may also involve other receptors, while the 2-Me-S-ADP-induced relaxation is mediated by $P2Y_6$ receptor activation (Figure 6B).

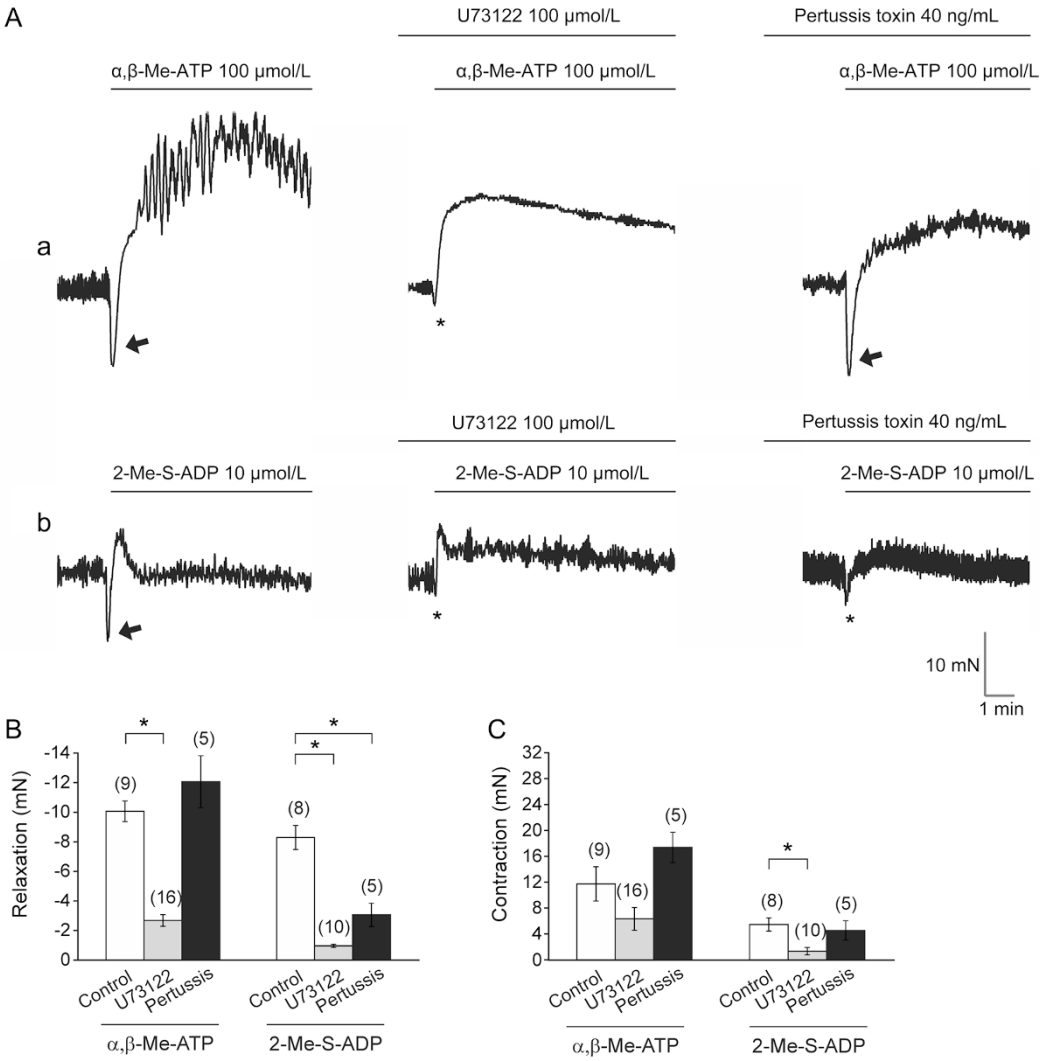

Figure 4. P2 receptor-mediated relaxation is mediated by phospholipase C (PLC), but only partially involves inhibitory G proteins. (A) The transient relaxation by α,β-Me-ATP and by 2-Me-S-ADP (see arrows in Aa and Ab) was prevented by pre-application of 100 μmol/L U73122, but 40 ng/mL pertussis toxin blocked only the relaxation induced by 2-Me-S-ADP, but not that induced by α,β-Me-ATP (see asterisks in Aa and Ab). All traces in A are derived from individual smooth muscle specimens. (B) The transient relaxation induced by α,β-Me-ATP and by 2-Me-S-ADP was significantly reduced by U73122, indicating the involvement of PLC. Pertussis toxin significantly reduced the relaxation induced by 2-Me-S-ADP, indicating the involvement of inhibitory G proteins (*n*=5–16 as indicated by numbers in brackets). (C) The sustained contraction induced by 2-Me-S-ADP was significantly reduced by U73122, but contraction by α,β-Me-ATP was unaffected by either inhibitor (*n*=5–16 as indicated by numbers in brackets). Mean±SEM. $^*P<0.05$.

Discussion

The aim of this study was to investigate the molecular mechanisms of P2Y receptor-mediated motility in longitudinal ileum preparations. The non-hydrolyzable P2 receptor agonists α,β-Me-ATP and 2-Me-S-ADP caused a transient relaxation and a more sustained contraction of rat ileum smooth muscle. Moreover, we found that the transient relaxation was mediated by P2Y receptor activation and involved PLC, IP_3-dependent Ca^{2+} release from internal stores and subsequently SK-type Ca^{2+}-activated K^+ channels. In contrast, the P2 receptor-induced contraction was abolished by the combined inhibition of sarcoendoplasmic reticulum ATPase and L-type voltage-dependent Ca^{2+} channels.

P2 receptors are involved in purinergic motility of the rat ileum

The purinergic agonist α,β-Me-ATP was introduced as an inhibitor of ionotropic P2X receptors[24, 29]. However, Kitajima and co-workers have demonstrated that the α,β-Me-ATP-induced rise in the intracellular Ca^{2+} in aortic smooth muscle cells is not entirely prevented by verapamil, which indicates the involvement of non-L-type voltage-dependent Ca^{2+} channels and/or intracellular Ca^{2+} release following P2Y receptor activation[30]. More recently, α,β-Me-ATP was demonstrated to activate the $P2Y_1$ receptor in the murine and human colon[31]. Indeed, in our study, both α,β-Me-ATP-induced relaxation and contraction were significantly reduced by the P2Y receptor blockers MRS2179, MRS2211 and MRS2500 and were

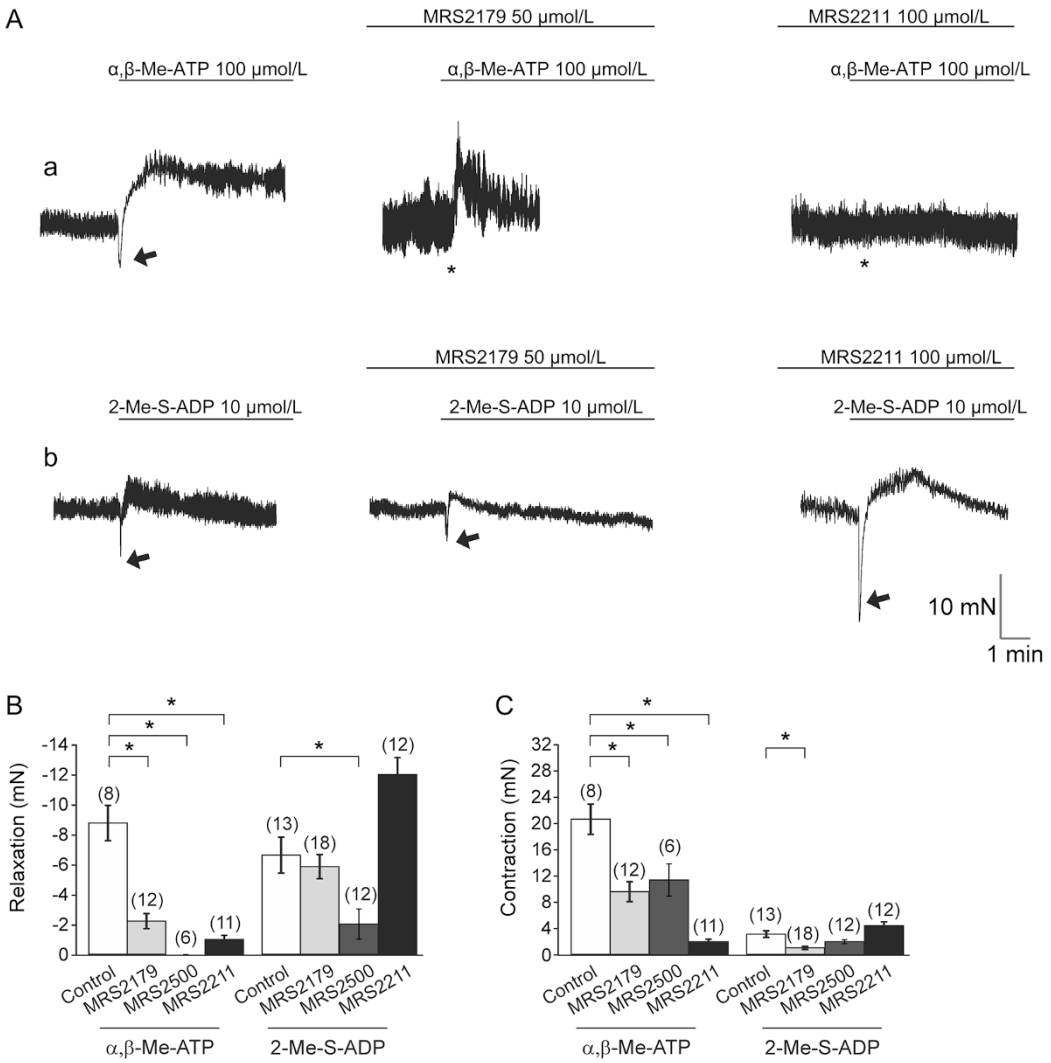

Figure 5. Differential effects of MRS2179, MRS2500 and MRS2211 on P2 receptor-mediated relaxation. (A) While 50 μmol/L MRS2179 and 100 μmol/L MRS2211 prevented the transient relaxation induced by α,β-Me-ATP (see arrow and asterisks in Aa), these inhibitors had no effect on the 2-Me-S-ADP-induced relaxation (see arrows in Ab). All traces in A are derived from individual smooth muscle specimens. (B) The transient relaxation induced by α,β-Me-ATP was significantly reduced by MRS2179, MRS2500 or MRS2211. MRS2500 (1 μmol/L) also blocked the transient relaxation induced by 2-Me-S-ADP, indicating the involvement of $P2Y_1$ receptors (n=6–18 as indicated by numbers in brackets). (C) The sustained contraction induced by α,β-Me-ATP was significantly reduced by MRS2179, MRS2500 or MRS2211. The contraction induced by 2-Me-S-ADP was significantly reduced only by MRS2179 (n=6–18 as indicated by numbers in brackets). Mean±SEM. $^*P$<0.05.

abolished by co-application of both antagonists, suggesting the involvement of P2Y rather than P2X receptors in the rat ileum. Moreover, MRS2578 application led to a significant reduction in α,β-Me-ATP-induced relaxation. This observation might indicate that either $P2Y_6$ is involved in α,β-Me-ATP-induced relaxation or that $P2Y_1$ is also inhibited by MRS2578. Importantly, α,β-Me-ATP-induced contraction was also significantly reduced by verapamil, indicating the involvement of L-type Ca^{2+} channels presumably activated via P2X-mediated depolarization.

Eight subtypes of P2Y receptors have been cloned so far, five of which ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) are coupled to the activation of PLC via $G_{q/11}$ proteins and the remainder of which ($P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) negatively regulate adenylate cyclase via $G_{i/o}$ proteins but may also activate PLC via βγ-subunits[11]. While MRS2179 and MRS2500 are quite selective for $P2Y_1$[32], MRS2211 has a high affinity for the $P2Y_{13}$ receptor but a lower affinity for $P2Y_1$ and $P2Y_{12}$ receptors[33, 34]. Thus, we conclude that α,β-Me-ATP-induced relaxation and contraction were mediated by the activation of the $P2Y_1$ and $P2Y_{13}$ receptors, but the experiments using MRS2500 point to $P2Y_1$ receptors having the predominant role.

The other purinoceptor agonist used in this study, 2-Me-S-ADP, has been developed as a P2Y receptor-preferring agonist[35]. While 2-Me-S-ADP-induced relaxation was not affected by co-application of MRS2179 and MRS2211, it was abolished by MRS2578, a $P2Y_6$ receptor-antagonist, indicating that this receptor subtype was sufficient to generate the 2-Me-S-ADP-induced relaxation. Intriguingly, MRS2211 applied alone led to the induction of increased relaxation by

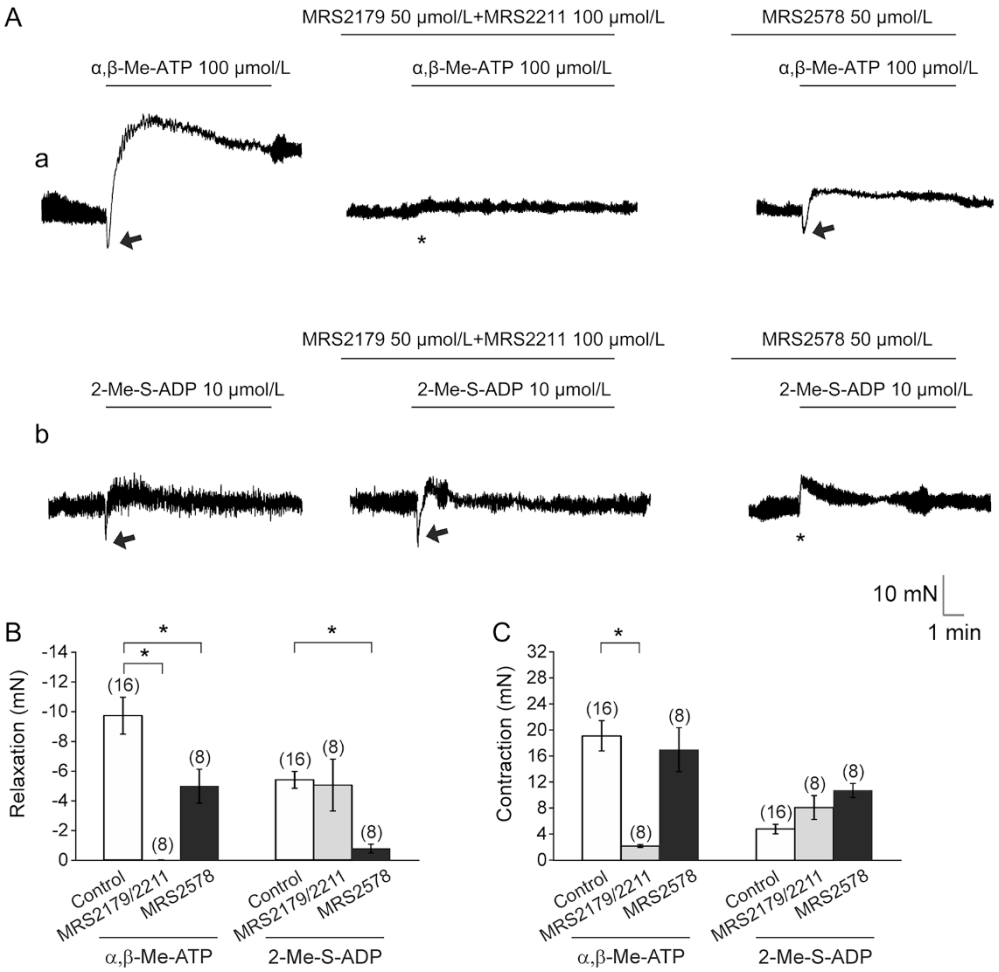

Figure 6. Differential effects of MRS2179, MRS2211 and MRS2578 on P2 receptor-mediated relaxation. (A) While the transient relaxation induced by α,β-Me-ATP was abolished by co-application of 50 μmol/L MRS2179 and 100 μmol/L MRS221, it was unaffected by application of MRS2578 (see arrows and asterisk in Aa). In contrast, the transient relaxation induced by 2-Me-S-ADP was unaffected by co-application of MRS2179 and MRS2211, but was abolished by application of MRS2578 (see arrows and asterisk in Ab). All traces in A are derived from individual smooth muscle specimens. (B) The transient relaxation induced by α,β-Me-ATP, but not by 2-Me-S-ADP, was significantly reduced by co-application of MRS2179 and MRS2211, indicating the involvement of the $P2Y_1$ and $P2Y_{13}$ receptors. The transient relaxation induced by 2-Me-S-ADP was abolished by MRS2578, indicating the involvement of the $P2Y_6$ receptor (n=8–16 as indicated by numbers in brackets). (C) The sustained contraction induced by α,β-Me-ATP was significantly reduced by co-application of MRS2179 and MRS2211, but the contraction induced by 2-Me-S-ADP was unaffected by any of the inhibitors (n=8–16 as indicated by numbers in brackets). Mean±SEM. $^*P<0.05$.

2-Me-S-ADP, for as yet unknown reasons. In contrast, the contraction induced by 2-Me-S-ADP was not affected by any of the P2Y receptor antagonists used in this study and was thus mediated by purinergic receptors other than $P2Y_1$, $P2Y_6$, $P2Y_{12}$ or $P2Y_{13}$.

Morphological data on P2 receptor distribution in the gut are scarce. At least in the mouse ileum, smooth muscle cells were strongly immunoreactive for $P2Y_1$ receptors[7], and myenteric plexus neurons in the guinea-pig ileum were shown to express the $P2Y_6$ and $P2Y_{12}$ receptor[36]. However, interspecies differences are very likely, because α,β-Me-ATP-induced contractions of guinea-pig ileum were sensitive to the $P2X_1$ receptor antagonist NF279 and the P2X receptor-preferring blocker PPADS[37]. This finding is in contrast to the present results using rat ileum, which point to a predominant P2Y receptor-mediated contraction following exposure to α,β-Me-ATP.

Another important issue is pre-junctional P2 receptor expression. In particular, it is known that α,β-Me-ATP application leads to neural fiber-mediated cholinergic contractions[5], and altered ATP release from cholinergic fibers in response to pre-junctional P2Y receptor activation may have occurred in our preparations[8]. Therefore, we examined the P2Y receptor-induced transient relaxation following pre-incubation with atropine, L-NAME or TTX and found that at least the P2Y receptor-mediated relaxation was independent of acetylcholine or nitric oxide release from pre-junctional nerve fibers.

Intracellular signaling cascade following purinergic receptor activation

The pharmacological experiments in the present study showed that the transient relaxation induced by either α,β-Me-ATP or 2-Me-S-ADP was abolished by apamin or UCL1684, indicating that SK channel activation was instrumental in purinergic relaxation. Hence, we suggest that purinergic relaxation might be due to membrane hyperpolarization following SK channel activation. Consistent with this idea, the sustained contraction following application of the P2 receptor agonists was not sensitive to SK channel inhibition. Moreover, the transient relaxation induced by either agonist was significantly reduced by 2-APB, thapsigargin and U73122. These results indicate that activation of PLC and subsequent IP_3-dependent Ca^{2+} release from internal stores were important steps in the intracellular pathway leading to purinergic relaxation, and these steps are upstream of SK channel activation. In agreement with previous reports[4, 9, 19, 20], we propose that a major pathway leading to purinergic relaxation involves $P2Y_1$ receptor and PLC activation, followed by IP_3-mediated Ca^{2+} release from internal stores and subsequent SK channel activation.

It is important to note that smooth muscle cells may not be the only cell types involved in $P2Y_1$ and SK channel-mediated purinergic relaxation. Recently, a new class of fibroblast-like cells expressing platelet derived growth factor receptor α (PDGFRα) have been described in the subepithelial layer of the gastrointestinal tract[38]. As has been demonstrated in both the murine detrusor muscle and colon, these cells express $P2Y_1$ receptors coupled to SK channels, leading to purinergic inhibition[39-41]. Moreover, the effect of ATP was significantly reduced in PDGFRα-expressing cells from $P2Y_1$-knockout mice, indicating a major role of $P2Y_1$ in these cells[40]. It is therefore conceivable that these cells are also important mediators of purinergic relaxation in the rat ileum, which harbors SK3-expressing fibroblast-like cells that form gap junctions with smooth muscle cells[42], as has recently been shown in the murine colon[43]. Another limitation of our study might be that purinergic receptors are also expressed in epithelial cells and hence are involved in secretory processes[44-46]. Therefore, we cannot exclude the possibility that purinoceptor activation-mediated secretion could have affected the relaxation and contraction in our preparations.

To further explore the subtype of the purinoceptor involved, we used pertussis toxin, which specifically and irreversibly inactivates $G_{i/o}$ proteins. The G proteins not only inhibit adenylate cyclase but also activate PLC through their βγ-subunits[47, 48]. Pertussis toxin can thus both stimulate adenylate cyclase and inhibit PLC[28, 49]. In our study, pertussis toxin blocked 2-Me-S-ADP-induced relaxation, but had no effect on α,β-Me-ATP-induced relaxation. Because the effects of both agonists involved PLC signaling, the pertussis toxin data suggest that 2-Me-S-ADP involved the βγ-subunits of $G_{i/o}$ proteins, whereas α,β-Me-ATP predominantly coupled to $G\alpha_{q/11}$ proteins. As discussed above, α,β-Me-ATP-induced relaxation and contraction were mediated by $P2Y_1$ and $P2Y_{13}$ receptor activation. Because, according to the current knowledge, only $P2Y_1$ is coupled to $G\alpha_{q/11}$[11], $P2Y_1$ receptor-mediated motility fully compensated for the effects of both receptors under normal conditions. Thus, $P2Y_1$ is a major purinoceptor on smooth muscle cells that governs rat ileum motility. In contrast, $P2Y_6$ receptor inhibition abolished the relaxation induced by 2-Me-S-ADP, which was also largely pertussis toxin-sensitive. These data suggest that $P2Y_6$ is not only coupled to $G_{q/11}$ as currently thought[11] but also leads to $G_{i/o}$ activation. Activation of different G protein species has previously been described for adenosine receptors[50].

Purinergic contraction, however, was not affected by 2-APB or thapsigargin, suggesting that Ca^{2+} release from internal stores may not be involved in this process. Because 2-APB has been observed to interfere with store-operated Ca^{2+} entry[25, 51], this mechanism is not required. However, 2-Me-S-ADP-induced contraction, but not α,β-Me-ATP-induced contraction, was sensitive to U73122, indicating a potential role for PLC and subsequent intracellular Ca^{2+} signaling in mediating P2Y purinergic contraction for both motility responses, confirming previous reports[52, 53]. In contrast, contraction induced by either agonist was abolished by co-application of the SERCA inhibitor thapsigargin and the L-type Ca^{2+} channel blocker verapamil. Thus, purinergic contraction might partially involve Ca^{2+} influx through voltage-gated Ca^{2+} channels, Ca^{2+} release from internal stores and other mechanisms downstream of PLC activation such as Rho kinase signaling[54].

One potential caveat of our study might be that contractile activity measured in the longitudinal direction could be

affected by epithelium-derived factors and/or by modulation via the electrically coupled circular layer of the smooth muscle. Although this possibility cannot be excluded, several observations argue against it. The ileum segments were closed on both ends by sutures to fix them in the organ bath. Thus, when the drug diffuses, it should first reach the outer longitudinal layer, while the inner circular layer and the epithelium should be reached much later. The latency between the administration of purinergic agonists (injection of 100 μL into 25 mL volume) and transient relaxation was approximately 8–10 s. Hence, we think that it is unlikely, albeit possible, that purinergic agonists exerted their effects indirectly by acting on the circular layer and/or the epithelium.

Another limitation of the present study is that pharmacological manipulation may activate multiple receptors, including extra-junctional ones, unlike electrical field stimulation (EFS) (which acts only on junctional receptors). EFS studies are required to confirm the findings made here.

Conclusion

In summary, our data provide evidence that P2Y receptor activation by α,β-Me-ATP or 2-Me-S-ADP leads to both $G_{q/11}$- and $G_{i/o}$-dependent and PLC-mediated IP_3 production. While α,β-Me-ATP acts via P2X, $P2Y_1$ and $P2Y_{13}$ receptors, 2-Me-S-ADP activates both the $P2Y_1$ and $P2Y_6$ receptors. Overall, the $P2Y_1$ receptor appears to be the major purinoceptor subtype involved in purinergic relaxation. Following PLC activation, IP_3 is produced and activates the release of Ca^{2+} from internal thapsigargin-sensitive stores, which leads to the activation of SK channels. Because these channels sense the intracellular Ca^{2+} concentration, they give rise to a K^+ outward current, leading to hyperpolarization and thus relaxation.

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Author contribution

Felix MADER, Ludwig KRAUSE, and Tursonjan TOKAY performed the experiments; Timo KIRSCHSTEIN designed the study, performed the statistical analysis, and prepared the manuscript; Oliver W HAKENBERG and Rüdiger KÖHLING participated in the design of the study and prepared the manuscript. All authors read and approved the final manuscript.

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