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RESEARCH PAPER

Purinergic P2X receptor activation induces emetic responses in ferrets and *Suncus murinus* (house musk shrews)

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Background and purpose: Despite the rapid progress made in understanding the significant role played by signalling via extracellular ATP in physiology and pathology, there has been no clear information generated on its involvement in the emetic response.
Experimental approach: In the present study, the emetogenic potential of extracellular ATP signalling in mammalian species was examined using ferrets and *Suncus murinus* (house musk shrews). A slowly degradable ATP analogue, α,β-methyleneATP (α,β-meATP), was used to activate the P2X receptors, and either the non-selective P2 receptor antagonist, pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), or the specific $P2X_3$ homomer and $P2X_{2/3}$ heteromer antagonist, A-317491, were tested against the agonist-induced response.
Key results: Intraperitoneal injection of α,β-meATP produced significant emetic responses in ferrets (1 - 30 mg kg^{-1}) and in *Suncus murinus* (5 - 50 mg kg^{-1}). The responses occurred frequently within the first 10 min after administration, much less frequently from 11 to 60 min and no responses occurred later than 60 min. The emetic responses were completely inhibited by intraperitoneal pre-treatment with PPADS (100 mg kg^{-1}) or A-317491 (100 mg kg^{-1}). Abdominal surgical vagotomy did not reduce the emetic response in *Suncus murinus* significantly.
Conclusions and implications: These results for the first time indicate that the activation of P2X receptors evokes emetic responses in mammalian species. The $P2X_3$ homomer and · or $P2X_{2/3}$ heteromer in the area postrema could be responsible for the emetic response. This finding contributes to the elucidation of the roles played by extracellular ATP signalling in various emetic symptoms.
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Keywords: ATP; emesis; purinergic P2X receptor; A-317491; area postrema; vagotomy; $P2X_3$ homomer; $P2X_{2/3}$ heteromer; vomit

Abbreviations: α,β-meATP, α,β-methylene ATP; PBS, phosphate-buffered saline; PPADS, pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid; NTS, nucleus of the solitary tract

Introduction

A large number of patients suffer from different conditions where nausea and vomiting are major symptoms, including functional dyspepsia, following surgery, chemotherapy and radiation therapy, and motion sickness (Sanger and Andrews, 2006). Emesis is a reflex that integrates the visceral afferent pathway, the vomiting centre (medulla oblongata of the brainstem), and the efferent pathway (Wang and Borison, 1952). Some endogenous transmitters are known to modulate the emetic reflex system. 5-Hydroxytryptamine (5-HT) is considered to be one of causal molecules in chemotherapy-induced emesis and nausea. For instance, 5-HT from the enterochromaffin cells of the gastrointestinal tract stimulates $5\text{-}HT_3$ receptors located on the vagus afferents, which transmit the impulses to the vomiting centre (Naylor and Rudd 1996). However, cases of emesis and nausea resistant to treatment with currently available anti-emetic drugs are still a clinical problem and new, more effective treatments are needed to improve the management of nausea and vomiting.

ATP, an important extracellular signalling molecule, exerts physiological and pathological responses by acting on two families of purinergic P2 receptors, P2X ionotropic ligand-gated ion channel receptors and P2Y metabotropic G protein-coupled receptors. Seven P2X receptor subunits ($P2X_1$–$P2X_7$) have been identified so far. It has been shown that native functional P2X receptors have either homomeric

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or heteromeric trimeric subunit organisation (Jiang *et al*., 2003). Recent rapid progress in P2 receptor research has resulted in the elucidation of the kinetics and pharmacological profiles of some P2X receptors, such as the $P2X_3$ homomer and $P2X_{2/3}$ heteromer (North, 2002). Purinergic signalling is involved in a wide range of physiological and pathological events, including synaptic neurotransmission, neuromuscular transmission that leads to the contraction or relaxation of smooth muscle, and exocrine or endocrine secretion (Burnstock, 2006).

It has also been shown that extracellular ATP signalling plays an important role in multiple activities in the gastrointestinal tract. It is active in synaptic transmission in the myenteric and submucosal ganglia, and is involved in controlling peristalsis in the gut, vascular activity in the gastrointestinal tract, and mucosal secretion (Burnstock, 2006). An immunohistochemical staining study has revealed that $P2X_1$–$P2X_6$ subunits are present in the brainstem where the vomiting centre (a complex formed by the area postrema, the nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus) is located (Yao *et al*., 2000). In addition, it has been shown that the activation of P2X receptors located on neurons within the NTS facilitates presynaptic glutamate release and this may imply their possible contribution to the autonomic control (Jin *et al*., 2004). However, there has been no clear information on the involvement of extracellular ATP signalling in the emetic response.

This study was undertaken to examine the emetogenic potential of P2X receptor activation in mammalian species. A slowly degradable ATP analogue α,β-methylene ATP (α,β-meATP) was systemically (i.p.) administered to activate P2X receptors. In addition, the effects of a non-selective P2 receptor antagonist, pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), as well as a $P2X_3$ homomer- and $P2X_{2/3}$ heteromer-specific antagonist, A-317491 (Jarvis *et al*, 2002), on agonist-induced emetic response were investigated to identify the P2X receptor subtypes. The doses of the agonist (Gyires *et al*., 1985) and antagonists (Honore *et al*., 2002; Jarvis *et al*., 2002; Wu *et al*., 2004; Sharp *et al*., 2006; Kindig *et al*., 2007) were chosen based on the previous studies where they were assessed in P2X receptor-related animal models *in vivo* and in our preliminary dose-finding study. Abdominal vagotomy was also performed in an attempt to determine the site of action of the P2X receptor agonist in the emetic reflex pathway. The goal of this study was to elucidate the key molecules involved in emetogenesis so that the mechanism of refractory emesis and nausea symptoms could be better understood.

Materials and methods

Animals

All experimental procedures performed on animals were approved by the Astellas Pharma Inc. Animal Experiment Committee. Nine male ferrets (*Mustela putorius furo*; Marshall Farms Inc., NY, USA) weighing 0.9–1.3 kg and 80 male house musk shrews (*Suncus murinus*; Sun-Her, CLEA Japan Inc., Tokyo, Japan) weighing 55–80 g were used for the study. They were housed in animal rooms maintained at constant temperature ($24\pm2^{\circ}C$ for ferrets and $23\pm2^{\circ}C$ for *Suncus murinus*) and humidity ($55\pm10\%$ for both species) levels, with a 13 h day (7:30–20:30)–night cycle. The animals had free access to tap water and pellet chow (5L14 (Toyoda Tusho, Japan) for ferrets and CLEA-311 (CLEA Japan Inc.) for *Suncus murinus*).

Measurement of emesis in ferrets

The ferrets were fasted overnight before the experiments, but were allowed free access to water at all times. Each animal was placed unconstrained in an observation box (40 cm wide, 40 cm long and 40 cm high) and acclimated for at least 30 min before drug administration. The animals were observed continuously for 120 min following the administration of α,β-meATP and the number of episodes were recorded and displayed in 5 min time bins. An emetic episode was characterised by rhythmic abdominal contractions that either resulted in the oral expulsion of solid or liquid material (that is, vomiting) or those producing no material (that is, retching). The investigation of the emetogenic effect of α,β-meATP was conducted using a five-ferret cross-over design, that is, each animal received all doses (vehicle, 1, 3, 10 and 30 mg kg^{-1} i.p.) of α,β-meATP, with intervals of 7 days or more between each. On each day of the experiment, each animal received a dose of α,β-meATP that was different from all the other animals. The effect of morphine was also examined as a reference emetogenic compound. The dose of 0.5 mg kg^{-1} s.c. was used so that a comparison could be made with the results of previous reports (Gardner *et al*., 1996; Rudd *et al*., 1996). The antagonistic effect of PPADS on the α,β-meATP-induced emetic response was evaluated using a four-animal cross-over design, that is, each animal received all doses (vehicle, 10, 30 and 100 mg kg^{-1}) of PPADS with intervals of 7 days or more between each. On each day of the experiment, 30 min before α,β-meATP (30 mg kg^{-1} i.p.) injection, each animal received a dose of PPADS that was different from all the other animals. All observations were recorded by the same author to avoid interobserver differences.

Measurement of emesis in Suncus murinus

The measurement of emetic response was conducted using a modified version of the previously reported method (Kakimoto *et al*., 1997; Andrews *et al*., 2000). On the day of the experiment, each animal was transferred to a transparent observation chamber, and the number of emetic episodes was recorded and displayed in 1 min time bins for 60 min after the administration of α,β-meATP. The latency period between the drug administration and the first response was also recorded. An emetic episode was defined as described in 'Measurement of emesis in ferrets,' above. The emetogenic effect of α,β-meATP was investigated using a 25-animal parallel design (five groups of five animals each). The inhibitory effects of the P2 receptor antagonists were also evaluated using a 35-animal parallel design (seven groups of five animals each). PPADS (30 or 100 mg) or A-317491 (3, 10, 30 or 100 mg kg^{-1}) was intraperitoneally administered

30 min before α,β-meATP (50 mg kg^{-1} i.p.) injection. All emetic episode observations were recorded by the same author to avoid interobserver differences.

Abdominal vagotomy in Suncus murinus

Abdominal surgical vagotomy in *Suncus murinus* was performed according to the previously reported procedure (Andrews *et al*., 2000). The abdomen was opened via a midline incision under anaesthesia (sodium pentobarbital, 50 mg kg^{-1}, i.p.). The dorsal and ventral abdominal vagal trunks running over the oesophagus were identified, mobilised, ligated and sectioned. The abdomen was closed in layers. Ten animals underwent vagotomy, and 10 underwent sham surgery. Sham surgery was performed using similar procedures except the vagal trunks were not either ligated or sectioned. A recovery period of at least 1 week was allowed between the surgery and emetic testing. Either phosphate-buffered saline (PBS) or α,β-meATP (50 mg kg^{-1} i.p.) was administered to the vagotomised or sham-operated animals ($n=5$ per group). The number of emetic episodes was recorded for 60 min after administration. After the observation of the emetic responses to α,β-MeATP, the success of the vagotomy was confirmed by the absence of emetic responses caused by oral copper sulphate administration (40 mg kg^{-1} 10 ml^{-1}).

Data analysis

The total number of emetic responses and the latency to the first response during the observation period after the administration of the emetogenic agents were tallied for each animal. Data for the number of emetic responses and the latency were expressed as the mean ± s.e. mean and the median (min—max.), respectively, for each treatment group. The statistical differences of the number of emetic responses between the drug-treated groups and the vehicle-treated group were analysed using Dunnett's multiple comparison test for *Suncus murinus*, or Dunnett's test using within-subject error for ferrets. The statistical differences of the latency between the drug-treated groups and the vehicle-treated group were analysed using Steel multiple comparison test for *Suncus murinus*, or Friedman test for ferrets. The Student's *t*-test or Wilcoxon's rank-sum test was used for the comparison of PBS vs α,β-meATP or sham vs vagotomy in the vagotomy study. $P<0.05$ was considered statistically significant. The number of emetic responses with A-317491 pretreatment was converted to percentage (the number with vehicle pretreatment was set as 100% response). Then, the dose that yielded 50% inhibitory effect (ED_{50}) was determined by linear regression analysis, using three doses (3, 10 and 30 mg kg^{-1} i.p.) of A-317491.

Drugs

α,β-meATP lithium and pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) tetrasodium and A-317491 sodium were obtained from Sigma Chemical Co. (St Louis, MO, USA). Morphine hydrochloride was purchased from Takeda Chemical Industries (Osaka, Japan). α,β-MeATP, PPADS and A-317491 were dissolved in PBS and administered intraperitoneally in a volume of 1 ml kg^{-1} for ferrets or 10 ml kg^{-1} for *Suncus murinus*. The morphine was dissolved in saline and administered subcutaneously to ferrets in a volume of 0.5 ml kg^{-1}. Drug concentrations were calculated as the base form.

Results

Emetogenic effect of α,β-meATP in ferrets

Intraperitoneal administration of α,β-meATP with the dose range of 1–30 mg kg^{-1} produced emetic responses (vomiting and retching) in ferrets during the observation period (2 h), but treatment with the vehicle (PBS) did not. After the administration of α,β-meATP, the emetic responses occurred most frequently during the first 10 min and much less frequently from 10 to 60 min. No emetic response was observed between 60 and 120 min (Figure 1). The number of emetic responses during the first 60 min after α,β-meATP administration at the doses of 10 and 30 mg kg^{-1} was significantly larger than that after PBS administration. The number of emetic responses caused by 30 mg kg^{-1} of α,β-meATP was almost as many as that caused by the positive reference emetogenic compound, morphine (0.5 mg kg^{-1} s.c.) (Table 1). α,β-meATP did not cause any other behavioural changes such as writhing during the observation period.

Effect of PPADS on agonist-induced emesis in ferrets

The emetic response induced by α,β-meATP (30 mg kg^{-1} i.p.) was inhibited by intraperitoneal pretreatment with PPADS 30 min before administration of α,β-meATP in ferrets. The antagonistic effect of PPADS at the doses 10–100 mg kg^{-1} was statistically significant compared with that of the vehicle (PBS). The emetic responses were completely inhibited at the dose of 100 mg kg^{-1} (Figure 2). The effect of PPADS on responder rate and latency of emetic responses was summarised in Table 2.

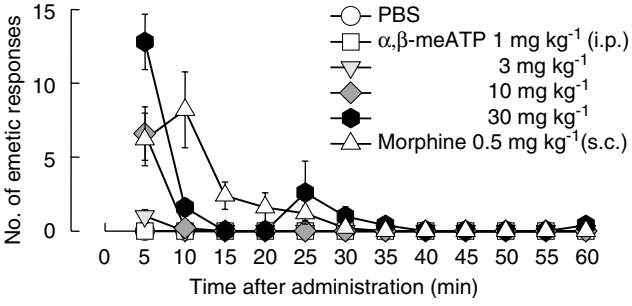

Figure 1 Time course of emetic responses after intraperitoneal administration of α,β-meATP or the subcutaneous administration of morphine (a reference drug) in ferrets. The animals were observed continuously for 120 min after the administration of α,β-meATP and the number of emetic responses (vomiting and retching) was recorded and displayed in 5 min time bins. Data are presented as the mean ± s.e. mean for five animals. α,β-meATP, α,β-methylene ATP.

Table 1 Relationship between doses of α,β-meATP and emetic responses in ferrets

Drug	*Dose (mg kg^{-1})*	*No. of animals (responder/tested)*	*No. of emetic responses*	*Latency (s)*
PBS (i.p.)	—	0/5	0.0±0.0	∞ [∞–∞]
α,β-meATP (i.p.)	1	0/5	0.0±0.0	∞ [∞–∞]
	3	3/5	1.4±0.6	∞ [52–∞]
	10	5/5	8.0±2.5***	130 [79–145]
	30	5/5	22.8±1.0***	51 [47–78]
Morphine (s.c.)	0.5	5/5	19.8±1.9	192 [138–271]

Abbreviations: α,β-meATP, α,β-methylene ATP; PBS, phosphate-buffered saline.
Emetic responses were measured during 60 min after administration of α,β-meATP. The number of emetic responses was expressed as the mean±s.e. mean. ***$P<0.001$, significantly different from the vehicle-treated group (Dunnett's test using within-subject error). The latency (s) to the first emetic responses was expressed as the median (min—max). If an animal did not show emetic responses, the latency was taken to be equal to 3600 s (60 min) and expressed as ∞. Friedman test applied for latency data showed a significant variance ($P=0.002$) among five groups (α,β-meATP at 0, 1, 3 10 and 30 mg kg^{-1}).

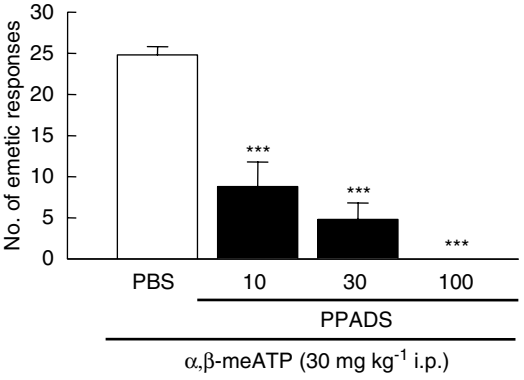

Figure 2 Inhibitory effect of PPADS on the α,β-meATP-induced emetic response in ferrets. PPADS was intraperitoneally administered 30 min before α,β-meATP administration (30 mg kg^{-1} i.p.). The total number of emetic responses (vomiting and retching) during the first 60 min after administration of α,β-methylene ATP was tallied. The open and closed columns represent the means, and the vertical bar represents + s.e. mean. ***$P<0.001$, significantly different from the vehicle-treated group (Dunnett's test using within-subject error). α,β-meATP, α,β-methylene ATP; PPADS, pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid.

Table 2 Effect of PPADS on responder rate and latency of emetic responses in ferrets

Drug	*Dose (mg kg^{-1} i.p.)*	*No. of animals (responder/tested)*	*Latency (s)*
PBS	—	4/4	87 [55–99]
PPADS	10	3/4	54 [31–∞]
	30	3/4	70 [35–∞]
	100	0/4	∞ [∞–∞]

Abbreviations: α,β-meATP, α,β-methylene ATP; PBS, phosphate-buffered saline; PPADS, pyridoxal phosphate-6-azophenyl-2′.
Emetic responses were observed during the first 60 min after administration of α,β-meATP. PPADS or PBS was intraperitoneally administered 30 min before α,β-meATP (30 mg kg^{-1}, i.p.) injection. The latency (s) to the first emetic responses was expressed as the median (min—max). If an animal did not show emetic responses, the latency was taken to be equal to 3600 s (60 min) and expressed as ∞. Friedman test applied for latency data showed no significant variance ($P=0.14$) among four groups (PPADS at 0, 10, 30 and 100 mg kg^{-1}).

Emetogenic effect of α,β-meATP in Suncus murinus

Intraperitoneal administration of α,β-meATP with the dose range of 5–50 mg kg^{-1} produced emetic responses (vomiting and retching) in *Suncus murinus* during the observation period (1 h), although treatment with the vehicle (PBS) did not. The emetic responses occurred frequently during the first 10 min, but no emetic response was observed from 10 to 60 min after administration (Figure 3). At a dose of 20 or 50 mg kg^{-1}, the emetogenic effect was statistically significant compared to that of vehicle (Table 3). α,β-meATP did not cause any other behavioural changes such as writhing during the observation period.

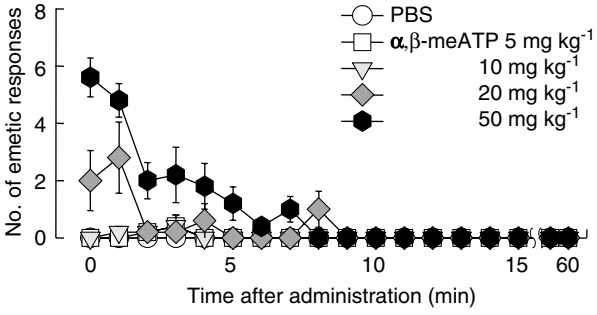

Figure 3 Time course of emetic responses after intraperitoneal administration of α,β-meATP in *Suncus murinus*. The number of emetic responses (vomiting and retching) was recorded and displayed in 1 min time bins during the observation period. No response was observed from 11 to 60 min. Data are presented as the mean±s.e. mean for five animals. α,β-meATP, α,β-methylene ATP.

Effect of PPADS and A-317491 on agonist-induced emesis in Suncus murinus

α,β-meATP (50 mg kg^{-1} i.p.)-induced emetic responses were significantly inhibited by intraperitoneal pretreatment with both 30 and 100 mg kg^{-1} doses of PPADS 30 min before α,β-meATP administration in *Suncus murinus*. The emetic response was completely inhibited by a dose of 100 mg kg^{-1}. Intraperitoneal pretreatment with A-317491 with the dose range of 3–100 mg kg^{-1} significantly inhibited the α,β-meATP (50 mg kg^{-1} i.p.)-induced emetic responses. The emetic response was completely inhibited by the dose of 100 mg kg^{-1} (Figure 4). The ED_{50} value with 95% confidence limit of A-317491 was 4.3 (1.4–7.3) mg kg^{-1}. The effect of PPADS and A-317491 on responder rate and latency of emetic responses was summarised in Table 4.

Effect of vagotomy on agonist-induced emesis in Suncus murinus
α,β-meATP (50 mg kg^{-1} i.p.) induced emesis in both sham and vagotomised *Suncus murinus*. The number of agonist-induced emetic responses did not differ significantly between the sham and vagotomy groups (Table 5).

Table 3 Relationship between doses of α,β-meATP and emetic responses in *Suncus murinus*

Drug	*Dose (mg kg^{-1} i.p.)*	*No. of animals (responder/tested)*	*No. of emetic responses*	*Latency (s)*
PBS	—	0/5	0.0±0.0	∞ [∞–∞]
α,β-meATP	5	1/5	0.6±0.6	∞ [173–∞]
	10	2/5	0.8±0.5	∞ [113–∞]
	20	4/5	6.8±2.4*	38 [19–∞]
	50	5/5	19.0±2.9***	21 [15–38]$^{\#}$

Abbreviations:α,β-meATP, α,β-methylene ATP; PBS, phosphate-buffered saline; PPADS, pyridoxal phosphate-6-azophenyl-2′.
Emetic responses were observed during 60 min after administration of α,β-meATP. The number of emetic responses was expressed as the mean±s.e. mean. *$P<0.05$, ***$P<0.001$, significantly different from the vehicle-treated group (Dunnett's multiple comparison test). The latency (s) to the first emetic responses was expressed as the median (min—max). If an animal did not show emetic responses, the latency was taken to be equal to 3600 s (60 min) and expressed as ∞. $^{\#}P<0.05$, significantly different from the vehicle-treated group (Steel multiple comparison test).

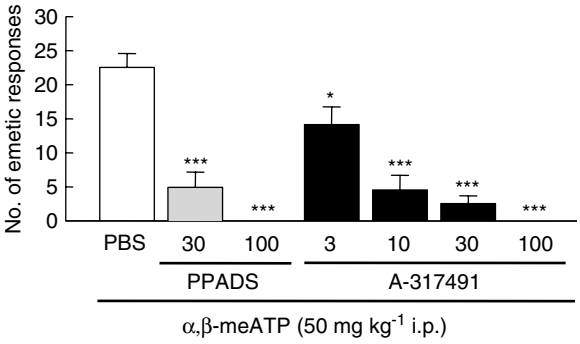

Figure 4 Inhibitory effect of PPADS and A-317491 on α,β-meATP-induced emetic response in *Suncus murinus*. Antagonists were given intraperitoneally 30 min before α,β-meATP (50 mg kg^{-1} i.p.) injection. The total number of emetic responses in 60 min after α,β-meATP administration was tallied. Data were presented as the mean + s.e. mean for five animals per group. *$P<0.05$, ***$P<0.001$, significantly different from the vehicle-treated group (Dunnett's multiple comparison test). α,β-meATP, α,β-methylene ATP; PPADS, pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid.

Discussion and conclusions

Ferrets and Suncus murinus *for the study of emesis*
Ferrets and *Suncus murinus* (the house musk shrew) were used in this study because they were species commonly used for the research of emesis. It has been reported that a P2X receptor agonist α,β-meATP induces contractile responses with a potency similar to that of ATP ($pD_2=4.75$) in the isolated ileum of *Suncus murinus* (Nagata *et al*., 1993). In addition, there is a significant increase in responses to von Frey hairs stimulation during the application of α,β-meATP (1 μM) in the *in vitro* preparation of ferret oesophagus with oesophagitis (Page *et al*., 2000). These studies suggested that the ferret and *Suncus murinus* could provide suitable models for the analysis of P2 receptor-mediated gastrointestinal functions, although the potency of the agonist to cause responses is relatively low.

P2X receptor activation induces emetic responses
In this study, the activation of P2X receptors by systemic administration of a slowly degradable ATP analogue α,β-meATP (a P2X receptor agonist) induced significant and consistent emetic responses both in ferrets and *Suncus*

Table 4 Effect of P2X receptor antagonists on responder rate and latency of emetic responses in *Suncus murinus*

Drug	*Dose (mg kg^{-1} i.p.)*	*No. of animals (responder/tested)*	*Latency (s)*
PBS	—	5/5	32 [19–45]
PPADS	30	4/5	472 [29–∞]
	100	0/5	∞ [∞–∞]$^{\#\#}$
A-317491	3	5/5	35 [31–115]
	10	3/5	166 [28–∞]
	30	4/5	252 [61–∞]$^{\#}$
	100	0/5	∞ [∞–∞]$^{\#}$

Abbreviations: α,β-meATP, α,β-methylene ATP; PBS, phosphate-buffered saline; PPADS, pyridoxal phosphate-6-azophenyl-2′.
Emetic responses were observed during 60 min after administration of α,β-meATP. PPADS, A-317491 or PBS was intraperitoneally administered 30 min before α,β-meATP (50 mg kg^{-1} i.p.) injection. The latency (s) to the first emetic responses was expressed as the median (min—max). If an animal did not show emetic responses, the latency was taken to be equal to 3600 s (60 min) and expressed as ∞. $^{\#}P<0.05$, $^{\#\#}P<0.01$, significantly different from the vehicle-treated group (Steel multiple comparison test).

Table 5 Effect of abdominal surgical vagotomy on α,β-meATP-induced emesis

Treatment	*Drug*	*No. of animals (responder/tested)*	*No. of emetic responses*	*Latency (s)*
Sham operation	PBS	0/5	0.0±0.0	∞ [∞–∞]
	α,β-meATP (50 mg kg^{-1} i.p.)	5/5	18.0±1.8***	21 [17–58]$^{\#\#}$
Vagotomy	PBS	0/5	0.0±0.0	∞ [∞–∞]
	α,β-meATP (50 mg kg^{-1} i.p.)	5/5	14.4±1.2***	41 [9–51]$^{\#\#}$

Abbreviations: α,β-meATP, α,β-methylene ATP; PBS, phosphate-buffered saline.
Emetic responses were observed during 60 min after i.p. administration of α,β-meATP (50 mg kg^{-1}). The number of emetic responses was expressed as the mean±s.e. mean. ***$P<0.001$, significantly different from the corresponding vehicle-treated group (Student's *t*-test). There was no significant difference in the effect of α,β-meATP between sham and vagotomised groups (Student's *t*-test). The latency (s) to the first emetic responses was expressed as the median (min—max). If an animal did not show emetic responses, the latency was taken to be equal to 3600 s (60 min) and expressed as ∞. $^{\#\#}P<0.01$, significantly different from the corresponding vehicle-treated group (Wilcoxon's rank-sum test). There was no significant difference in the effect of α,β-meATP between sham and vagotomized groups (Wilcoxon's rank-sum test).

murinus. The response was completely inhibited by pretreatment with either of the P2 receptor antagonists PPADS or A317491. These results are the first to suggest that ATP signalling is involved in the induction of emetic symptoms in mammalian species.

The P2X receptor subtype involved in the emetic response

Purinergic P2 receptors consist of two families, ionotropic P2X and metabotropic P2Y subtypes. Currently, seven subtypes of P2X receptors ($P2X_{1-7}$) and eight subtypes of P2Y receptors have been identified (Burnstock, 2006). Because α,β-meATP is a selective agonist for P2X receptor subtypes, especially $P2X_1$, $P2X_3$ homomer, and $P2X_{2/3}$ heteromer (North and Surprenant, 2000), it is likely that the P2X receptor was responsible for the emetic response we observed. Furthermore, A-317491 is an antagonist that is highly selective for the $P2X_3$ homomer and $P2X_{2/3}$ heteromer over other P2X subtypes (Jarvis *et al*. 2002). The selectivity of the compounds, α,β-meATP and A-317491, suggests that $P2X_3$ homomer and|or $P2X_{2/3}$ heteromer were responsible for the emetic response. The potency of A-317491 and PPADS in inhibiting the emetic responses seems consistent with those obtained in the previous studies where the antagonists were assessed in other *in vivo* animal models involving $P2X_3$ homomer and/or $P2X_{2/3}$ heteromer receptors, although direct comparison seems difficult due to the difference of protocol such as administration route, species and end point responses, and so on. A-317491 has an analgesic effect (ED_{50} value of 2.3 mg kg^{-1} s.c.) in Freund's complete adjuvant-induced hyperalgesia, where the $P2X_3$ homomer and/or $P2X_{2/3}$ heteromer are considered to play an important role (Wu *et al*., 2004). It produces analgesia with the ED_{50} value of 10–15 μmol kg^{-1} (6–8 mg kg^{-1}) s.c. for thermal hyperalgesia and mechanical allodynia after chronic nerve constriction injury in rats (Jarvis *et al*., 2002). Furthermore, it significantly inhibits C-fiber- and Aδ-fiber-evoked electrophysiological responses at the dose of 3 and 10 mg kg^{-1} i.v., respectively, in anesthetised rats (Sharp *et al*., 2006). In the present studies, PPADS significantly inhibited emetic responses at 10 and 30 mg kg^{-1} i.p. in the ferret and *Suncus murinus*, respectively, and this potency seems a little lower than that of A-317419. This rank order of potency (A-317491 > PPADS) is consistent with that in the cell line expressing human $P2X_{2/3}$ receptor, that is, A-317491 and PPADS inhibit α,β-meATP (10 μM)-induced calcium influx with pIC_{50} of 7.37 and 5.28, respectively (Jarvis *et al*., 2004). Studies i*n vivo* have shown that PPADS dose-dependently reduces acetic acid-induced abdominal constrictions with an ED_{50} of 70 μmol kg^{-1} (42 mg kg^{-1}) i.p. in mice (Honore *et al*., 2002). Furthermore, intra-arterial administration of PPADS at 10 mg kg^{-1} significantly attenuates afferent nerve activity in anesthetised cats (Kindig *et al*., 2007).

The contribution of the vagal afferent nerve ending to the response is not large

The $P2X_3$ homomer and $P2X_{2/3}$ heteromer were initially identified as a new P2X receptor family predominantly localised on the peripheral sensory neurons (Chen *et al*., 1995; Lewis *et al*., 1995). It has now become clear that P2X receptors are present on the vagal afferent nerves in the gastrointestinal tract and are involved in the control of gut functions in mammalian species; specifically, the P2X receptors are localised presynaptically on the vagal afferent terminals in rats (Atkinson *et al*., 2000). The $P2X_3$ subunits are expressed on the intraganglionic laminar nerve endings (mechanosensory endings of vagal afferent nerves) in the rat stomach, where they are probably involved in physiological reflex activity (Xiang and Burnstock, 2004). It is also known that ATP sensitises the vagal afferent nerve activity in the ferret oesophagitis model (Page *et al*., 2000). The $P2X_3$ homomer and/or $P2X_{2/3}$ heteromer on the terminals of the vagal afferent nerve in the gut region may have become activated by α,β-meATP in our model. However, the result of the surgical vagotomy in this study suggests that the activation of the P2X receptors on the ending of afferent vagal nerve does not play a prominent role in the α,β-meATP-induced emetic response.

The area postrema may be responsible for the response

The area postrema is located on the dorsal surface of the medulla oblongata and has been known as a chemoreceptor trigger zone for emesis. Since this region lacks the blood–brain barrier, it is anatomically positioned to detect emetic signals mediated by substances in the blood. In addition, the area postrema along with the NTS and the dorsal motor nucleus of the vagus comprise the dorsal vagal complex, which is the site of emetic reflex integration (vomiting centre) (Miller and Leslie, 1994). Interestingly, an immunohistochemical study using antibody has revealed that $P2X_2$ and $P2X_3$ subunits are expressed in the area postrema (Yao *et al*., 2000). The co-localisation of the $P2X_2$ and $P2X_3$ subunits was observed in sensory neurons and their central terminals, although the co-localisation in the area postrema was not investigated in that study (Vulchanova *et al*., 1997). Furthermore, ATP (injected into the vertebral artery) enhanced the electrical unit activity of loci in the area postrema in anesthetised cats (Borison *et al*., 1975). It is, therefore, possible that the $P2X_3$ homomer and/or the $P2X_{2/3}$ heteromer in the area postrema play an important role in the emetic response observed in this study. On the other hand, $P2X_3$ homomer and/or $P2X_{2/3}$ heteromer in the CNS (the area protected by the blood–brain barrier) are unlikely involved in the emetic response, because A-317491 is a peripherally acting blocker that penetrates poorly into the CNS (brain–plasma ratio is 0.008) (Wu *et al*., 2004).

Possible influence of local irritation by intraperitoneal administration

An important consideration in this study is the possibility that the emetic response could be caused by non-specific local irritation in the peritoneal cavity by intraperitoneal injections. This, however, would not account for the emetic response observed in this study due to following reasons. (1) The drug was dissolved in the buffer (PBS) and the pH of the α,β-meATP solution was between 4 and 7. It was confirmed that the intraperitoneal injection of PBS solution with pH 4 did not induce any emetic responses. (2) The receptor antagonist completely blocked the emetic response and the

intraperitoneal administration of antagonists themselves did not show any behavioural changes in animals. (3) The subcutaneous administration of α,β-meATP at doses of 10 or 50 mg kg^{-1} similarly caused emetic responses in *Suncus murinus* in our preliminary study (data not shown). It is therefore reasonable to consider that the emetic response was caused by the specific receptor activation rather than simple irritation of the peritoneal cavity by intraperitoneal injections.

Absence of pain-related behaviour by α,β-meATP injection

The ATP signal is well known to be involved in the nociception in mammalian species. Intraperitoneal injection of ATP (4 mg kg^{-1}) induces abdominal writhing responses (a nociception-related behaviour) in mice (Gyires and Torma, 1984; Gyires *et al*., 1985). On the other hand, the intravenous injection of ATP induces analgesia in the hot plate and phenylquinone-induced writhing assays in mice (Gomaa, 1987). In this study, α,β-meATP did not cause any other behavioural change such as writhing during the observation period. The absence of nocifensive behaviour may be due to differences of animal species (mice vs ferrets and *Suncus murinus*) and/or agonists (ATP vs α,β-meATP) used. The involvement of exogenous purinergic signals in the control of nociception in ferrets and *Suncus murinus* remains to be investigated.

To summarise, the results of this study provide compelling evidence suggesting that the activation of the P2X receptor has emetogenic potential in mammalian species. The $P2X_3$ homomer and/or $P2X_{2/3}$ heteromer in the area postrema could be responsible for the emetic response. The findings provided by this study should be viewed as steps towards clarifying the significance of the roles extracellular ATP signalling plays in various emetic symptoms.

Conflict of interest

The authors state no conflict of interest.

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