Guanosine negatively modulates the gastric motor function in mouse

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Abstract The aim of the present study was to evaluate if guanine-based purines may affect the gastric motor function in mouse. Thus, the influence of guanosine on the gastric emptying rate in vivo was determined and its effects on spontaneous gastric mechanical activity, detected as changes of the intraluminal pressure, were analyzed in vitro before and after different treatments. Gastric gavage of guanosine (1.75–10 mg/kg) delayed the gastric emptying. Guanosine (30 μ M–1 mM) induced a concentration-dependent relaxation of isolated stomach, which was not affected by the inhibition of the purine nucleoside phosphorylase enzyme by 4'-deaza-1'-aza-2'-deoxy-1'-(9-methylene)-immucillin-H. The inhibitory response was antagonized by *S*-(4-nitrobenzyl)-6-thioinosine, a membrane nucleoside transporter inhibitor, but not affected by 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine, a nonselective adenosine receptor antagonist, or by tetrodotoxin, a blocker of neuronal voltage-dependent Na⁺ channels. Moreover, guanosine-induced effects persisted in the presence of 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one, an inhibitor of soluble guanylyl cyclase or tetraethylammonium, a nonselective potassium channel blocker, but they were progressively reduced by increasing concentrations of 2'5'dideoxyadenosine, an adenylyl cyclase inhibitor. Lastly, the levels of cyclic adenosine monophosphate (cAMP), measured by ELISA, in gastric full thickness preparations were increased by guanosine. In conclusion, our data indicate that, in mouse, guanosine is able to modulate negatively the gastric motor function, reducing

gastric emptying and inducing muscular relaxation. The latter is dependent by its cellular uptake and involves adenylyl cyclase activation and increase in cAMP intracellular levels, while it is independent on neural action potentials, adenosine receptors, and K⁺ channel activation.

Keywords Stomach · Guanosine · Endoluminal pressure · Muscular relaxation · Gastric emptying · Mouse

Introduction

Guanine-based purines have been shown to be released from neurons and/or glia both under basal conditions and after various types of stimulation, as stress conditions [1–3]. Extracellular guanosine exerts many trophic effects [4, 5], including stimulation of astrocyte proliferation [6, 7], synthesis and release of various trophic factors [8, 9], and promotion of neurite outgrowth from neurons and neuron-like cells [10, 11]. In addition, guanine-based purines, including guanosine, have been shown to exert inhibitory effects on the glutamatergic system activity in physiological and pathological conditions [4, 12] and to modulate memory processes [13].

Although some of the actions of guanosine may be intracellularly mediated after its uptake, many trophic effects are not substantially affected by the nucleoside uptake inhibitors suggesting that they are independent of intracellular mechanism [14]. In support of this hypothesis, binding and functional studies have shown the presence of specific binding sites for guanosine in rat brain and primary cultured rat astrocytes [15, 16]. Exogenous guanosine 5'-triphosphate and guanosine have been suggested to participate in the regulation of arterial tone since they relax both endothelium-intact- and endothelium-denuded arterial preparations, likely via activation of a guanine nucleotide-specific receptor [17, 18].

Recent research studies provided evidence that guanine-based purines may modulate the functionality also of the

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enteric nervous system, interfering negatively with the cholinergic neurotransmission in mouse colon [19]. Gastrointestinal functions are under control of the well-characterized adenine-based purinergic system but, so far, data are too scarce to establish a definitive modulatory role for the guanine-based purinergic system.

Since numerous experimental evidences indicate that adenine-based purinergic system is able to regulate the contractility of mouse stomach, inducing relaxant effects acting both directly on the smooth muscle cells and indirectly on the enteric nerves [20, 21], the purpose of the present study was to evaluate whether guanosine may affect gastric motor function too.

In particular, we evaluated the responses induced by guanosine *in vivo* and *in vitro*, analyzing the effects of guanosine on gastric emptying and verifying the influence of guanosine on the mouse gastric spontaneous mechanical activity, using isolated gastric preparations.

Methods

Experiments were performed using adult male mice (C57BL/6) weighing about 25 g obtained from Harlan Laboratories (San Pietro al Natisone Udine, Italy). Animals were kept under environmentally controlled condition, ambient temperature 24 °C, humidity 40 %, and 12 h light/dark cycle with food and water *ad libitum*. Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national (D. L. n. 116, G. U., suppl. 40, 18 February 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, 1 December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication no. 80-23, 1985 and Guidelines for the Use of Animals in Biomedical Research, *Thromb. Haemost.* 58, 1078-1084, 1987). Mice were euthanized using isoflurane anesthesia followed by cervical dislocation.

Gastric emptying

Gastric emptying to a non-nutrient meal was assessed in overnight-fasted mice, habituated to intragastric gavage before starting the experiments. Mice received by gavage 0.5 ml of 2 % methylcellulose solution and were sacrificed 30 min after the gavage. After laparotomy, stomach was dissected by quickly cutting the lower esophageal sphincter and pyloric region. The stomach was opened and the remaining methylcellulose was collected and the weight annotated. The rate of gastric emptying was assessed by subtracting the weight of methylcellulose remaining in the stomach to the weight of methylcellulose administered [22]. Different doses of guanosine, ranging from 0.875 to

10 mg/kg, dissolved in 0.1 ml of distilled water, were gavaged, using a catheter, 15 min before receiving methylcellulose. Control mice received an equal volume of distilled water. Gastric emptying was measured in comparison with that of control mice ($n=5$ for each group) which received an equal volume of 2 % methylcellulose solution.

Functional studies *in vitro*

After animal sacrifice, the abdomen was immediately opened, the esophagus was tied proximal to the lower esophageal sphincter, and the entire stomach was excised. Preparations were mounted in a custom-designed organ bath, which was continuously perfused with oxygenated (95 % O₂ and 5 % CO₂) and heated (37 °C) Krebs solution with the following composition (in millimolar): NaCl 119, KCl 4.5, MgSO₄ 2.5, NaHCO₃ 25, KH₂PO₄ 1.2, CaCl₂ 2.5, and glucose 11.1. The pyloric end was tied around the mouth of a J-tube, which was connected to a standard pressure transducer (Statham Mod. P23XL; Grass Medical Instruments, Quincy, MA, USA), and the changes of endoluminal pressure were recorded on ink-writer polygraph (Grass model 7D). Preparations were allowed to equilibrate for about 60 min before starting the experiment. Under these conditions, mouse stomach exhibits spontaneous small rhythmic contractions and basal tone, allowing testing the relaxant activity directly without the use of a contractile agent.

At the beginning of each experiment, the preparation was challenged with isoproterenol (1 μM) until reproducible responses were obtained, to ensure that a stable and acceptable level of sensitivity had been reached before the experimental procedure was begun. The responses to noncumulative concentrations of guanosine (30 μM–1 mM) were examined. Guanosine was added into the bath at increasing concentrations in volumes of 50 μl after switching off the perfusion. Each concentration was left in contact with the tissue for 4 min at 20-min intervals.

To establish the contribution of guanosine breakdown products in the observed effects, concentration–response curve for guanosine was constructed in the presence of 4'-deaza-1'-aza-2'-deoxy-1'-(9-methylene)-immucillin-H (DADMe-ImmH, 1 μM), a recently synthesized potent and selective inhibitor of purine nucleoside phosphorylase enzymes [23, 24].

In order to evaluate the mechanism of action, the responses to guanosine were also tested in presence of the following: tetrodotoxin (TTX, 1 μM), a voltage-dependent Na⁺ channel blocker; *S*-(4-nitrobenzyl)-6-thioinosine (NBTL, 10 μM), a nucleoside transporter inhibitor; 2'5' dideoxyadenosine (DDA, 10 μM), an inhibitor of adenylate cyclase; 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 10 μM), a selective inhibitor of nitric oxide (NO)-stimulated soluble guanylate cyclase; tetraethylammonium (TEA, 20 mM), a nonselective potassium channel blocker;

and 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS 15943, 10 μ M), a nonselective adenosine receptor antagonist. The different inhibitor agents were added to the perfusing solution at least 20 min before testing guanosine. A single inhibitor was tested in one preparation and its concentration was chosen on the basis of our previous studies in mouse gastrointestinal tract [19, 25–27]. TTX was proven to be effective at blocking response to electrical field stimulation [28].

Measurement of intracellular levels of cAMP

The cyclic adenosine monophosphate (cAMP) content of gastric full thickness preparation was measured by enzyme immunoassay. Pre-weighted muscular strips were incubated with vehicle (Krebs solution), guanosine (0.3–1 mM), or forskolin (10 μ M) for 5 min, both dissolved in Krebs solution. The tissue was rapidly frozen in liquid nitrogen and homogenized in 5 % trichloroacetic acid (TCA). The precipitate was removed by centrifugation at 1500 $\times$ *g* for 10 min and the supernatant was transferred to a clean tube and the TCA was extracted using water-saturated ether. The residual of ether was removed by heating the sample to 70 °C for 5 min. Levels of cAMP were measured using a cAMP enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI). The detection limit of the assay was 0.1 pmol/ml. The cAMP content was expressed as picomoles per gram of tissue wet weight.

Solution and drugs

The following drugs were used: DDA, DADMe-ImmH, guanosine, isoproterenol hydrochloride, NBTI, TEA, TTX (all purchased from Sigma-Aldrich, Inc., St Louis, USA), CGS 15943 and ODQ (Tocris-Bioscience Bristol, UK), and methylcellulose (Sigma 274429-5G). DADMe-ImmH was a generous *gift* from Dr. Vern Schramm (Albert Einstein Medical College, New York, NY). DDA, NBTI, and ODQ were dissolved in dimethyl sulfoxide and further diluted in Krebs. The maximal final concentration of dimethyl sulfoxide in the organ bath did not affect the contractility of the stomach. Guanosine was dissolved in 10 % solution of 1 N NaOH to prepare 1 M stock solution. At the highest concentration tested, the final concentration of NaOH in the organ bath was 0.3 %, concentration that did not affect gastric tone. However, concentrations of guanosine higher than 3 mM could not be tested due to the effects of the solvent on the mechanical activity. All the other drugs were dissolved in distilled water. The working solutions were prepared fresh on the day of the experiment by diluting the stock solutions in Krebs. Methylcellulose solution was prepared the day of the experiments, dispersing methylcellulose in hot water (80 °C) under continuous stirring. The solution was then allowed to cool to 35 °C.

Statistical analysis

Gastric emptying in guanosine-gavaged animals was expressed as a percentage of the gastric empty of the vehicle-treated animals, taken as 100 %. Gastric relaxation induced by guanosine *in vitro* was expressed as a percentage of the response to isoproterenol (Iso, 1 μ M). All data are expressed as mean values $\pm$ SEM. The letter *n* indicates the number of experiments and it is equivalent to the number of experimental animals. Statistical analysis was performed by means of paired Student's *t* test or analysis of variance followed by Bonferroni test when appropriate. A probability value of less than 0.05 was regarded as significant.

Results

Gastric emptying

Gastric gavage of guanosine (from 1.75 to 10 mg/kg) significantly delayed gastric emptying in a dose-related manner compared with control group (Fig. 1). The maximum inhibitory effect of guanosine was induced by a dose of 7.5 mg/kg being the gastric emptying about 53 % than that observed in vehicle-treated animals ($P<0.05$, $n=5$).

Functional studies *in vitro*

Exogenously administered guanosine (30 μ M–1 mM) induced a muscular relaxation of isolated stomach (Fig. 2a), which developed slowly, persisted throughout the contact time, and was reversible after washing out. The effect enhanced by increasing the concentration and the relaxation to the maximal concentration tested (1 mM) was 2.4 ± 0.8 cm

Fig. 1 Effects of guanosine on gastric emptying. Different doses of guanosine (from 0.875 to 10 mg/kg) were administered by gavage 15 min before receiving methylcellulose. Data are means $\pm$ SEM and are expressed as a percentage of the gastric emptying measured in vehicle-treated animals, taken as 100 % ($n=5$ each). * $P<0.05$ when compared to vehicle-treated animals

Fig. 2 **a** Typical tracings showing the relaxation induced by increasing concentrations of guanosine on isolated mouse gastric preparations. **b** Concentration–response curves for the relaxation induced by guanosine (30 μM–1 mM) alone ($n=26$) and in the presence of 1 μM DADMe-

ImmH, a potent and selective inhibitor of PNP ($n=8$). Data are means $\pm$ SEM and are expressed as a percentage of the response induced by 0.1 μM Iso in the same tissue

H₂O ($n=26$) (about 60 % of the relaxation to 1 μM isoproterenol) ($EC_{50}=0.22$ mM, 95 % CLs 0.10–0.52 mM, $n=26$) (Fig. 2b). DADMe-ImmH (1 μM), a potent and selective inhibitor of purine nucleoside phosphorylases [23, 24], only slightly affected the concentration–response curve for guanosine, indicating that the effect we observed was due to guanosine itself and not to some of its breakdown products. The response to guanosine was deeply antagonized by pretreatment of the preparation with NBTI (10 μM), a membrane nucleoside transporter inhibitor (Fig. 3a), suggesting that it depends by guanosine intracellular uptake.

Instead, relaxation induced by guanosine was not affected by the blockade of adenosine receptors with CGS 15943 (10 μM), a nonselective adenosine receptor antagonist (Fig. 3b), which antagonized the gastric relaxation induced by adenosine in the same preparation (data not shown).

To investigate if a neuronal mechanism was responsible for the response observed, we tested the effect induced by the purine in the presence of TTX, blocker of neuronal voltage-

dependent Na⁺ channels. TTX (1 μM) failed to affect significantly the responses to guanosine at all concentration tested (Fig. 4a).

Since relaxation of gastrointestinal smooth muscle involves several intracellular mechanisms, including increase of cyclic guanosine monophosphate (cGMP), increase of cAMP, and hyperpolarization of the cell membrane via direct or indirect activation of K⁺ channels, we tested the effects induced by guanosine in the presence of ODQ, a selective inhibitor of soluble guanylate cyclase; DDA, an adenylate cyclase inhibitor; or TEA, a nonselective potassium channel blocker. The relaxation induced by guanosine (30 μM–1 mM) was not affected by ODQ (10 μM), but it was significantly reduced by DDA (10–30 μM) in a concentration-dependent manner (Fig. 4b, c) Lastly, pretreatment with TEA (20 mM), which per se induced a marked increase of the gastric tone (about 2 cm H₂O), had no effect on the guanosine responses (Fig. 4d). None of the other antagonists per se had any effect on the gastric tone.

Fig. 3 Concentration–response curves for the relaxation induced by guanosine (30 μM–1 mM) before and after **a** 10 μM NBTI, a membrane nucleoside transporter inhibitor, or **b** 3 μM CGS 15943, a nonselective adenosine receptor antagonist. Data are means $\pm$ SEM of at least five experiments and are expressed as a percentage of the response induced by 0.1 μM Iso in the same tissue. * $P<0.05$ when compared to the control

Fig. 4 Concentration–response curves for the relaxation induced by guanosine (30 μ M–1 mM) before and after **a** 1 μ M TTX, blocker of neuronal voltage-dependent Na^+ channels, or **b** 10 μ M ODQ, an inhibitor of soluble guanylyl cyclase, or **c** 10–30 μ M DDA, an inhibitor of adenylyl cyclase, or **d** 20 mM TEA, a nonselective potassium channel blocker. Data are means $\pm$ SEM of at least five experiments and are expressed as a percentage of the response induced by 0.1 μ M Iso in the same tissue. * P <0.05 when compared to the control

Effects of guanosine on cAMP content

In gastric full thickness preparations, cAMP levels were concentration-dependent increased by guanosine (0.3–1 mM) vs vehicle, although to a lesser extent than cAMP increase induced by forskolin (10 μ M) (Fig. 5).

Discussion

Our results provide evidence for the ability of guanosine to influence gastric motor activity in mouse. In fact, exogenous guanosine reduces the gastric emptying rate and the gastric tone, the latter effect is dependent upon its cellular uptake and involves adenylyl cyclase activation.

Several lines of evidence stress the influence exerted by the adenine-based purinergic system on motor functions in the gastrointestinal tract. The extracellular purine nucleoside, adenosine, and nucleotide, ATP, have roles as neurotransmitters and neuromodulators in the enteric nervous systems [29]. Their effects are transduced through specific purinergic (P1 and P2) receptor subtypes, widely distributed in various regions of the gut wall, including myenteric neurons, smooth muscle cells, glial cells, and interstitial cells of Cajal [30, 31].

Guanine-based purines can be considered as a part of the purinergic system. However, although increasing evidences indicate that guanine-based purines may exert extracellular effects in the central nervous systems [4–11], so far, little attention has been given to the possibility that these purinergic compounds also may affect enteric nervous

Fig. 5 Effects of guanosine and forskolin on the cAMP content of mouse gastric preparation. Each value is mean $\pm$ SEM of four separate experiments. * P <0.05 when compared to the vehicle. \$ P <0.05 when compared to 0.3 mM guanosine. # P <0.05 when compared to 10 μ M forskolin

system and, thereby, gastrointestinal functions. Recently, it has been shown that in mouse colon guanine-based purines are able to modulate negatively cholinergic transmission, without affecting spontaneous mechanical activity [19].

Data from the present study demonstrated that guanine-based purines also are involved in the control of gastric motility. In fact, we observed that intragastric gavage of guanosine *in vivo* delays gastric emptying and that exogenous guanosine *in vitro* relaxes murine gastric preparations.

Since guanosine could be rapidly converted into guanine by purine nucleotide phosphorylase enzymes [5] and then to other active metabolites, we tested guanosine in the presence of a potent purine nucleotide phosphorylase inhibitor [23, 24]. The observation that inhibition of the ectonucleotidases did not alter the response to guanosine implies that guanosine itself is responsible for the observed effects. Guanosine could activate specific membrane binding site [15, 16] or exert its effects after uptake into cells. In our preparations, NBTI, a nucleoside uptake inhibitor [32], markedly reduced the guanosine-induced relaxation, suggesting that guanosine intracellular uptake is an essential step for the effects to be mediated. Myenteric neurons, smooth muscle cells, glial cells, and interstitial cells of Cajal are all potential target of guanosine.

TTX, a blocker of neuronal voltage-dependent Na^+ channels, failed to affect the relaxation induced by guanosine suggesting that the response is independent from neural action potentials. Thus, in mouse stomach, we have found a pharmacological profile similar to that previously reported in mouse colon, where guanosine after its cellular uptake acts via a mechanism not dependent on neuronal action potential [19].

In addition, in cultured rat astrocytes [7], guanosine has been reported to stimulate the release of adenine-based purines from the cells, inducing extracellular accumulation of adenosine, which interacting with purinergic P1 receptors might be responsible for guanosine-induced effects. The observation that, in our preparations, guanosine effects were not modified in the presence of a nonselective adenosine receptor antagonist discards such a possibility.

A further step of our research was to clarify the intracellular mechanism responsible for the observed muscular relaxation. Cyclic nucleotides as cAMP and cGMP are important second messengers associated with gastrointestinal smooth muscle relaxation [33, 34]. Thus, we tested the possibility that guanosine could induce either activation of adenylyl cyclase or of guanylyl cyclase, leading to an increase in the intracellular cyclic nucleotide levels. Inhibitory responses to guanosine were resistant to pretreatment with guanylyl cyclase inhibitor but were strongly antagonized in a concentration-dependent manner by a blocker of adenylyl cyclase, DDA. These observations suggest that increase of the intracellular cAMP levels is involved in the transduction pathway responsible for the evoked purinergic relaxation.

Moreover, the conclusion that guanine-based purines can lead to an increase of intracellular concentration of cyclic nucleotides [14, 16, 35, 36] is also supported by the observations that incubation with guanosine increased in a concentration-dependent manner the amount of cAMP in the gastric preparation. We are aware that a second, independent source of cAMP in mammalian cells is, the more recently discovered, soluble adenylyl cyclase [37]. However, the observation that guanosine effects are antagonized by the adenylyl cyclase inhibitors, DDA, directed to the P-site of conventional transmembrane cyclases [38], supports the hypothesis of an involvement of transmembrane adenylyl cyclases. Thus, it remains to be determined the metabolic steps that after the intracellular uptake of guanosine are activated to affect adenylyl cyclase activity. The release of some enteric modulator (which is not adenosine) acting on receptors coupled via G proteins to conventional transmembrane adenylyl cyclases has to be taken into account. In addition, it has been demonstrated that some effects of guanosine could occur via activation of K^+ channels [39] and increase of the K^+ conductance plays a central role in the muscular relaxation, being target also of cAMP [40]. However in our preparations, pretreatment with TEA, a widely used nonselective K^+ channel blockers, was ineffective on the guanosine-induced relaxation, indicating that, in contrast to the effects of other purinergic compound in mouse gastrointestinal smooth muscle as ATP or adenosine [41–43], guanosine in gastric preparations did not enhance the K^+ channel open probability. Therefore, there will be required further investigations to define the action mechanism by which guanosine induces muscular relaxation.

In conclusion, our data indicate that, in mouse, guanosine is able to modulate negatively the gastric motor function, reducing gastric empty and inducing muscular relaxation. The latter is dependent by its cellular uptake and involves adenylyl cyclase activation, which leads to an increase in the cAMP intracellular levels. Moreover, neural action potentials, adenosine receptors, and K^+ channel activation are not involved in the developing muscular relaxation.

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