A novel role of intestine epithelial GABAergic signaling in regulating intestinal fluid secretion

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1Department of Physiology, Shandong University School of Medicine, Jinan, People’s Republic of China; 2Key Laboratory of Medical Neuroiology of Shandong University, Jinan, People’s Republic of China; and 3Department of Physiology & Pharmacology and Robarts Research Institute, University of Western Ontario, London, Ontario, Canada

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Li Y, Xiang Y, Lu W, Liu C, Li J. A novel role of intestine epithelial GABAergic signaling in regulating intestinal fluid secretion. Am J Physiol Gastrointest Liver Physiol 303: G453–G460, 2012. First published June 14, 2012; doi:10.1152/ajpgi.00497.2011.—γ-Aminobutyric acid (GABA) is one of the main neurotransmitters in the central nervous system, and it is produced via the enzymatic activity of glutamic acid decarboxylase (GAD). GABA generates fast biological signaling through type A receptors (GABA_ARs), an anionic channel. Intriguingly, GABA is found in the jejunal epithelium of rats. The present study intended to determine whether a functional GABA signaling system exists in the intestinal epithelium and if so whether the GABA signaling regulates intestinal epithelial functions. RT-PCR, Western blot, and immunohistochemical assays of small intestinal tissues of various species were performed to determine the expression of GABA-signaling proteins in intestinal epithelial cells. Perforated patch-clamp recording was used to measure GABA-induced transmembrane current in the small intestine epithelium cell line IEC-18. The fluid weight-to-intestine length ratio was measured in mice that were treated with GABA_Ar agonist and antagonist. The effect of GABA_Ar antagonist on allergic diarrhea was examined using a mouse model. GABA, GAD, and GABA_Ar subunits were identified in small intestine epithelial cells of mice, rats, pigs, and humans. GABA_Ar agonist induced an inward current and depolarized IEC-18. Both GABA and the GABA_Ar agonist muscimol increased intestinal fluid secretion of rats. The increased intestinal secretion was largely decreased by the GABA_Ar antagonist picrotoxin or gabazine, but not by tetrodotoxin. The expression levels of GABA-signaling proteins were increased in the intestinal epithelium of mice that were sensitized and challenged with ovalbumin (OVA). The OVA-treated mice exhibited diarrhea, which was alleviated by oral administration of gabazine or picrotoxin. An endogenous autocrine GABAergic signaling exists in the mammalian intestinal epithelium, which up-regulates intestinal fluid secretion. The intestinal GABAergic signaling becomes intensified in allergic diarrhea, and inhibition of this GABA signaling system alleviates the allergic diarrhea.

γ-Aminobutyric acid; type A γ-aminobutyric acid receptor; intestinal epithelial cell; electrolyte transport; allergic diarrhea

γ-AMINOBUTYRIC ACID (GABA) is one of the main neurotransmitters in the central nervous system. GABA is produced from glutamate by decarboxylation via the enzymatic activity of glutamic acid decarboxylase (GAD). GABA is a transmitter of enteric interneurons and that GABA signaling regulates the function of the gastrointestinal tract (22). Interestingly, GABA is also found in the cytoplasm and the brush border of epithelial cells in rat jejunum (33). However, the issues as to whether intestinal epithelial cells (IECs) express GABA receptors and if so whether GABA signaling plays a role in the regulation of IEC functions remain to be addressed. In relation to these notions, previous studies, including ours, show that GAD and GABA_Ar subunits are expressed in lung epithelial cells (38). This GABA signaling system in the lung epithelium is involved in the regulation of mucus production (38) and fluid transport through luminal secretion of Cl− (20). Cl− secretion from IECs is also the major driving force for fluid secretion, which is especially evident during diarrhea (35). Although cystic fibrosis transmembrane conductance regulator (CFTR) is one of the key players of transepithelial Cl− movement, the mechanisms of Cl− transport in IECs are not fully understood.

In the present study, we determined whether IECs are endowed with a GABAergic signal system, and if so whether this system contributes to the intestinal Cl−-fluid secretion, hence the pathogenesis of diarrhea. Our results showed that GAD, GABA, and GABA_Ars were localized in mammalian IECs, and activation of GABA_Ars depolarized the cells, indicating an autocrine GABAergic signal system in the intestinal epithelium. In addition, our studies showed that stimulation of GABA_Ars increased intestinal fluid secretion. Moreover, the GABAergic signaling was upregulated in IECs of mice with ovalbumin (OVA)-induced diarrhea. Importantly, blocking this GABA signaling reduced the occurrence of allergic diarrhea.

MATERIALS AND METHODS

What is already known about this subject? 1) The GABA_Ar-mediated autocrine signaling system in the lung epithelia involves the regulation of bronchial mucus production in a mouse model of allergic asthma and alveolar fluid transport by upregulating luminal secretion of chloride. 2) Both GABA and GAD65 are found in the cytoplasm epithelial cells in rat jejunum.

What are the new findings? 1) GABA and the GABA-signaling molecules GAD and GABA_Ar subunits were identified in the small intestinal epithelium, indicating the existence of an endogenous autocrine GABAergic system in the tissue. 2) GABA_Ars in intestinal epithelial cells were functional, and they mediate efflux of anionic ions in the cell. 3) Stimulation of GABA_Ars increases intestinal fluid...
secretion in intact rats. 4) The intestinal GABAergic system is upregulated in a mouse model of allergic diarrhea. 5) Inhibiting this GABAergic signal system alleviates allergic diarrhea.

How might it impact on clinical practice in the foreseeable future? Targeting the intestinal epithelium GABAergic system may be new therapeutic strategies for the management of allergic diarrhea.

Ethics statement. The experimental procedures in rats, mice, and pigs were approved by the Committee on Animal Research and Ethics of Shandong University School of Medicine (approval nos.: 2008-012 and 2010-17). Human small intestine samples were obtained from four patients (23–56 yr) who were undergoing surgery for intestinal trauma. The collection of human intestine samples was approval by the Ethics Committee of Qilu Hospital, Shandong University, and a written informed consent was obtained from each participant.

Cell culture. The intestinal epithelial cell line IEC-18 (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 1.5 g/l sodium bicarbonate, 10% FBS, and 0.1 U/ml insulin (Sigma, St. Louis, MO). The cells were incubated with 10% CO2 at 37°C. Cells were grown on 35-mm culture dishes for patch-clamp recordings and on glass coverslip slips in six-well culture plates for immunofluorescent staining.

RT-PCR. IEC-18 cells and the cortex of Wistar rats (6–8 wk old) were homogenized in Trizol (Invitrogen, Carlsbad, CA), and the total RNA was extracted from the homogenates. The genomic DNA was removed with DNase (Promega, Madison, WI). RNA samples (2 μg each) were reverse-transcribed into cDNA using a cDNA reverse-transcription kit (TOYOBO, Osaka, Japan). The primers were shown in Table 1. PCR was conducted at 94°C for 4 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 80 s, and 72°C for 5 min. Quantity of mRNA of the GABAAR subunits was normalized to 18S rRNA.

Immunohistochemistry. A segment of the ileum removed from mice was soaked in 4% paraformaldehyde for 12 h. The fixed tissue was rinsed for 100 min and then cleaned, dehydrated, and immersed in wax. The tissue was sectioned into slices with 4 μm thickness. After being removed from the patients, the human ileum slices were prepared using the same procedure described above. The pig fetus ileum was obtained by cesarean section of pregnant sow at 105 days of gestation. Next, the ileum was soaked in 4% paraformaldehyde for 24 h. On the next day, the fixed tissues were dehydrated, and then the tissues were sectioned into 10 μm slices. After deparaffinization, hydration, and two rinses with PBS (pH 7.3), sections were boiled in citrate acid buffer for 15 min. After three rinses in PBS, sections were incubated with PBS that consisted of 10% normal goat serum for 1 h. The sections were incubated with rabbit anti-GAD65/67 (Sigma), rabbit polyclonal anti-GABAAR β2/3-subunit (Abcam, Cambridge, UK), or anti-GABAAR β2/3-subunit (Upstate, Lake Placid, NY) diluted with the blocking buffer overnight in a humid chamber at 4°C. The final concentrations of anti-GAD65/67 and anti-GABAAR β2/3-subunits were 1:2,000, 8 μg/ml, and 1:1,000, respectively. After three rinses, the sections were incubated with Alexa Fluor 568-conjugated goat anti-rabbit (1:600; Invitrogen) and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:600) at room temperature for 1 h, followed by washes with PBS. The dye 4’,6-diamidino-2-phenylindole (1:5,000) was used to stain the nucleus. The cover slips were mounted with 75% glycerol. Negative controls were stained without primary antibodies.

Western blot. The mouse, rat ileum, or IEC-18 cell homogenates were electrophoresed (10% SDS-PAGE) and transferred to polyvinylidene fluoride (Millipore, Bedford, MA) membranes. The membranes were blocked in TTBS buffer that contained of 5% nonfat milk (Difco, Detroit, MI) in 0.1% Tween 20, 50 mmol/l Tris, and 150 mmol/l NaCl. The membranes were blocked in TTBS buffer overnight in a humid chamber at 4°C. After washes with TTBS, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (Beyotime, Shanghai, China) or goat anti-mouse (Solarbio, Beijing, China) secondary antibodies at room temperature for 1 h. The concentrations of goat anti-rabbit and goat anti-mouse were 1:3,000 and 1:1,000, respectively. Immunoreactivities were detected by enhanced chemiluminescence (Millipore).

Table 1. Primer used in RT-PCR for GABA receptor subunits and GAD65/67

<table>
<thead>
<tr>
<th>Subunits Direction</th>
<th>Primer Sequence</th>
</tr>
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<tbody>
<tr>
<td>α1 Forward</td>
<td>5'-AGAATGTCAGAAGTGGTGAAGTTCAAGTTTAT-3'</td>
</tr>
<tr>
<td>α2 Reverse</td>
<td>5'-CAATTTGCCGACCGTGTTTACATG-3'</td>
</tr>
<tr>
<td>α3 Reverse</td>
<td>5'-ATGTATACGTTTCGAGCTTATG-3'</td>
</tr>
<tr>
<td>α4 Reverse</td>
<td>5'-GCACCCAAATCTTTGAAATATC-3'</td>
</tr>
<tr>
<td>α5 Reverse</td>
<td>5'-ATTATTCCTGGCAGAATGTTG-3'</td>
</tr>
<tr>
<td>α6 Forward</td>
<td>5'-CTTACCACCAATGTCAGACAT-3'</td>
</tr>
<tr>
<td>β1 Forward</td>
<td>5'-ATATCCCTCCTGAAGAGAAAACTG-3'</td>
</tr>
<tr>
<td>β2 Forward</td>
<td>5'-TTTACCTGGTACCTCTCCTCACTG-3'</td>
</tr>
<tr>
<td>β3 Forward</td>
<td>5'-TCAACACTGCACTTTTGAATCAATC-3'</td>
</tr>
<tr>
<td>δ Forward</td>
<td>5'-ATGGTCTGACACAGAGAAAGCAGG-3'</td>
</tr>
<tr>
<td>θ Forward</td>
<td>5'-GTCCCTGTAACATGCTACAGAC-3'</td>
</tr>
<tr>
<td>ε Forward</td>
<td>5'-TTGAGTTTGAAATTCTCCCACTTG-3'</td>
</tr>
<tr>
<td>π Forward</td>
<td>5'-AAAATTCTTGGGAGAAATGCTAAT-3'</td>
</tr>
<tr>
<td>ρ1 Forward</td>
<td>5'-TGTCATATTGAAAAGATTTGAG-3'</td>
</tr>
<tr>
<td>ρ2 Forward</td>
<td>5'-GACACCGTCTGGTGATATTAC-3'</td>
</tr>
<tr>
<td>ρ3 Forward</td>
<td>5'-GACCTCTTGCTGCTGCAGAGATA-3'</td>
</tr>
<tr>
<td>GAD65 Forward</td>
<td>5'-GTCATCTCACAACCTTGCA-3'</td>
</tr>
<tr>
<td>GAD67 Forward</td>
<td>5'-CTTCGACACTCTGCTGCAGAGATA-3'</td>
</tr>
<tr>
<td>18S rRNA Forward</td>
<td>5'-CGACCCGCTACCTAACCAGCT-3'</td>
</tr>
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</table>

GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase.
**Electrophysiology.** The cultured IEC-18 cells were bathed in a solution containing (in mmol/l): 155 NaCl, 1.3 CaCl2, 5.4 KCl, 25 HEPES, and 33 glucose (pH 7.4, osmolarity ~315 mosmol/kgH2O). Perforated patch-clamp recordings were performed in IEC-18 cells at room temperature (22°C) using a multiclamp 700B amplifier (Axon Instruments). The patch electrodes were filled with a solution containing (in mmol/l) 155 KCl, 15 KOH, 10 HEPES, 2 MgCl2, 1 CaCl2, and 2 tetraethylammonium (pH 7.35, osmolarity 315 mosmol/kgH2O). Gramicidin (30 mg/ml; Sigma) was included in the electrode solution for membrane perforation. GABA and/or the GABA AR agonist muscimol was applied to the test cell using a computer-controlled multibarrel perfusion system (SF-77B; Warner Instruments). Electrical signals were digitized and filtered (1–2 kHz). The electrical signals were acquired on-line by means of Clampex (Axon Instruments) and analyzed off-line using Clampfit (Axon Instruments).

**In vivo measurement of intestinal fluid secretion.** After 24 h fasting, male BALB/c mice (8–10 wk, body wt 25–30 g) were anesthetized with 2% pentobarbital sodium (45–50 mg/kg ip injection). Body temperature of mice was maintained between 36 and 38°C during surgery using a heating pad. An abdominal incision (1.5 cm) was made to expose the small intestine, and a closed loop of empty ileum (20 mm of length) proximal to the cecum was isolated by sutures. Ileal loops were injected with 100 μl normal saline (NS) alone or NS containing GABA (1–100 μM, A-2129; Sigma), tetrodotoxin (TTX) (1 μM; Sigma), gabazine (100 μM, SR-95531; TOCRIS, Ellisville, MO), or muscimol (10 μM; TOCRIS). The effects of genistein (50 μM; Sigma), forskolin (10 μM; Sigma), and bumetanide (10 μM; Sigma) were also tested. The abdominal incision was closed with sutures, and the animal was allowed to recover from anesthesia. Five hours later, the mice were killed, and ileal loops were isolated. The ileal loops were weighted after removal of mesentery and connective tissues. After the luminal fluid was released through a longitudinal incision, the weight and length of ileal loops were measured. The ratio of intestinal fluid weight to intestinal length was calculated.

**Induction of allergic diarrhea.** A mouse model of allergic diarrhea was conducted by following a previously reported protocol (8, 18). Briefly, male BALB/C mice (20–25 g) were divided into four groups randomly. On the first day, an individual mouse was immune sensitized by OVA (Sigma) [1 mg in 50 μl NS mixed with 50 μl aluminum potassium sulfate adjuvant [alum: AIK (SO₄)₂-12H₂O (Sigma)] by intraperitoneal injection. At the 7th day after sensitization, mice were fasted for 4 h. Mice in different groups were given, via intragastric administration, NS (control), OVA (50 mg), OVA mixed with gabazine (1.2 mg/kg), OVA mixed with picrotoxin (1.2 mg/kg; TOCRIS), and CFTR(inh)-172 (250 μg/kg ip; dissolved in DMSO for a stock solution; Sigma) every other day for 3 wk. OVA and drugs were dissolved in 250 μl NS. Mice were housed individually in cages that were sterilized by ultraviolet exposure for 20 min before and after the administration. The properties of mouse stool were observed 1 h after intragastric administration of OVA. The occurrence of diarrhea in mice was determined by comparing the property of stools before and after OVA. Mice were killed by cervical dislocation 25 days after OVA administration. The ileum (0.5 cm from cecum, 2–3 cm length) was excised from each mouse and was prepared for immunohistochemistry assay or Western blot.

**Statistics.** The data were presented as means ± SE. Data were analyzed with SigmaStat 3.5 software (SPSS, Chicago, IL). Significant differences between two groups were determined by one-way ANOVA. *P* < 0.05 was considered as significant difference.

**RESULTS**

**Endogenous GABA signaling exists in small intestinal epithelium.** To demonstrate the expression of GABA-signaling molecules in the intestinal epithelial cells, we carried out RT-PCR assays of IEC-18 cells. The assay detected the mRNAs encoding GAD65, GAD67, and the subunits of GABAARs in IEC cells (Fig. 1A). In line with the RT-PCR results, immunoblot assays revealed that GAD65 and GAD67, as well as several types of GABAAR subunits, were expressed in the small intestine of rat, mouse, and IEC-18 cells (Fig. 1B). Immunofluorescent staining results also confirmed the existence of GABAergic molecules in rat ileum epithelial cell line IEC-18.

**Fig. 1.** γ-Aminobutyric acid (GABA) signaling proteins were expressed in small intestinal epithelial cells (IECs). A: RT-PCR for glutamic acid decarboxylase (GAD) 65/67 and type A GABA receptor (GABAAR) subunits in IEC-18 cells (M, marker; rat brain cortex, positive control). B: Immunoblot of IEC-18 cells, rat and mouse small intestine for GAD65/67 and some GABAAR subunits. Rat/p1, tissue was obtained from a postnatal day 1 rat. C: Immunohistochemistry showing GABAergic molecules in rat ileum epithelial cell line IEC-18.
tence of GABA and GAD, as well as several GABA<sub>A</sub>R subunits, including \( \beta_{2/3} \)- and \( \pi \)-subunits in IEC-18 cells (Fig. 1C). These results suggest that small intestinal epithelial cells are endowed with an autocrine GABA-signaling system.

To investigate the subcellular localization of the GABA-signaling molecules in the small intestine, we carried out immunofluorescent staining of small intestine slices of mice, fetal pig, and human for GAD65/67 and some GABA<sub>A</sub>R subunits. The immunofluorescent staining results showed that GAD65/67 was mainly expressed in the cytosol of IECs (Fig. 2A, top). Given the \( \beta_2 \)-subunits are required for the formation of functional GABA<sub>A</sub>Rs in neurons, and a larger amount of the \( \beta_3 \)-subunit protein was expressed in the mouse and rat intestines, we stained the mouse intestine for GABA<sub>A</sub>Rs using an antibody against both \( \beta_2 \) and \( \beta_3 \) subunits. Our result showed that the \( \beta_{2/3} \)-subunits were expressed on the apical membrane of IECs (Fig. 2A, bottom). We also made immunofluorescent staining of pig and human intestinal tissues for the \( \pi \)-subunit, a GABA<sub>A</sub>R subunit that is widely expressed in nonneuronal cells. Our

Fig. 2. Auto/paracrine GABAergic system in small intestine epithelium. Immunohistochemistry showed cellular distribution of GABAergic molecules in the mouse (A), fetal pig small intestine (B), and human ileum (C). Epithelial cells were identified by immunostaining with an antibody to pan-cytokeratin (green). Immunostaining of GAD65/67 and GABA<sub>A</sub>R \( \beta_2 \) and \( \beta_3 \) (\( \beta_{2/3} \))-subunits (red). D: representative trace of GABA-evoked currents. E: muscimol-evoked currents. F: muscimol-induced depolarization in IEC-18.

Fig. 3. GABAergic system regulates intestinal fluid secretion. A: representative mouse ileal loops 6 h after luminal injection with GABA or 0.9% saline solution [normal saline (NS)]. B: the bar graphs show the normalized volume of intestinal fluid induced by GABA. C: bar graphs show averaged loop fluid accumulation. Tetrodotoxin (TTX) cannot totally block the effect induced by GABA. D: muscimol, a selective GABA<sub>A</sub>R agonist, mimics the effect induced by GABA. E: gabazine, a selective GABA<sub>A</sub>R antagonist, largely blocks the effect induced by GABA.

G456 GABA<sub>A</sub> RECEPTORS INVOLVED IN INTERSTITIAL FLUID SECRETION

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results showed that GABA_\text{AR} \pi-\text{subunit was expressed in IECs of pig fetus (Fig. 2B) and human ileum (Fig. 2C).}

\textit{GABA_\text{AR subunits in IECs form functional ion channels.} To investigate whether the GABA_\text{AR} subunits form functional ion channels in IECs, we performed perforated patch recordings in IEC-18 cells. Under voltage-clamp mode at a holding potential of \(-60\) mV, application of GABA (1 mmol/l) evoked inward currents in 8 out of 22 cells (Fig. 2D), and the amplitude of GABA-induced currents was relatively small (6.4 \pm 0.5 pA, \(n = 8\)). Current was also evoked in IEC-18 cells by application of muscimol, a selective GABA_\text{AR} agonist (Fig. 2E). In addition, our results revealed that, under current-clamp mode, the endogenous membrane potential of these cells was \(-37 \pm 8.6\) mV (\(n = 8\)). Moreover, muscimol induced a membrane depolarization in these cells (Fig. 2F). Together, these results indicate that GABA_\text{AR}s in IECs are functional and activation of the receptor induces an efflux of Cl\(^-\).

\textit{IEC GABAergic signaling is involved in intestinal fluid secretion.} Fluid secretion to the intestinal lumen critically relies on activation of Cl\(^-\) channels in the apical membrane of IECs. Given GABA_\text{AR}s are high-conductance Cl\(^-\) channels, we examined whether these Cl\(^-\) channels in IECs are involved in electrolyte transport and fluid secretion. Specifically, we measured in vivo intestinal fluid secretion in adult mice. Our results showed that intraintestinal application of GABA increased intraluminal fluid secretion in a dose-dependent manner (Fig. 3A and B). GABA receptors are expressed in enteric nervous cells (22), and fluid secretion is regulated by the nervous system. To avoid a potential action of intraluminal GABA on nervous cells, we used the Na\(^+\) channel inhibitor TTX (1 \(\mu\)M) to block the nerve activity when administering GABA. Notably, the GABA-induced increase of intestinal fluid secretion persisted in the presence of TTX (Fig. 3C). Intraluminal administration of the GABA_\text{AR} agonist muscimol also enhanced the fluid secretion (Fig. 3D). In contrast, the GABA_\text{AR} antagonist gabazine blocked the GABA effect on fluid secretion (Fig. 3E).

Both CFTR, a cAMP-dependent Cl\(^-\) channel (3), and calcium-activated Cl\(^-\) channels (4, 19, 27) mediate Cl\(^-\) efflux from IECs. Indeed, stimulation of CFTR with genistein (50 \(\mu\)M) and forskolin (10 \(\mu\)M) increased intestinal fluid secretion (data not shown). Under our experimental conditions, the efficacy of GABA on enhancing intestinal fluid secretion was significant although it was less effective than that of CFTR stimulants (data not shown). Furthermore, the GABA-induced fluid secretion was abolished by bumetanide, an inhibitor of Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (data not shown), confirming that the effect of GABA on intestinal fluid secretion is associated with transepithelial Cl\(^-\) transport. The combined data indicate that an endogenous GABAergic signal system does exist in the small intestine epithelia, where it critically regulates electrolyte transport and fluid secretion.

\textit{Inhibiting GABAergic signaling alleviates allergen-induced diarrhea.} We then examined whether the intestinal epithelial GABAergic signaling is involved in pathogenesis of diarrhea. To this end, we created a mouse model of allergic intestinal anaphylaxis by sensitizing and challenging the animals with OVA. Our immunohistochemical staining results showed that, 25 days after OVA challenge, the expression of GAD65/67 and GABA_\text{AR} \pi-subunit in the intestinal epithelia increased significantly compared with that of control animals (Fig. 4A). Notably, the increased expression of GAD65/67 and GABA_\text{AR} \pi-subunit was reversed in mice that were treated with GABA_\text{AR} antagonists (Fig. 4A). Our immunoblot assays also

\begin{figure}[h]
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\caption{Upregulation of the GABAergic system in the small intestinal epithelium of ovalbumin (OVA)-induced allergic diarrhea mice. A: immunofluorescence staining of GAD65/67 and GABA_\text{AR} \pi-subunit (red). B: immunoblot shows that GAD65/67 and GABA_\text{AR} are expressed in intestinal mucosa and are upregulated in small intestinal epithelium of OVA-induced allergic diarrhea mice, whereas blockers of GABA_\text{AR}, gabazine and picrotoxin, inhibit the upregulation induced by OVA.}
\end{figure}
after 10 treatments with the GABAAR antagonist gabazine or ated the OVA-induced allergic diarrhea. As shown in Fig. 5

Control (n = 10), OVA + gabazine (n = 10) and OVA + picrotoxin (n = 7).

showed that treatment with GABAAR antagonists suppressed the increase of GABAAR β2/3-subunits in the intestinal tissues (Fig. 4B).

We further analyzed whether GABAAR antagonists alleviated the OVA-induced allergic diarrhea. As shown in Fig. 5B, after 10 treatments with the GABAAR antagonist gabazine or picrotoxin, almost 20% of the animals escaped from the OVA-induced allergic diarrhea while the progress of OVA-induced diarrhea was slowed down in other animals. Importantly, the efficacy of picrotoxin on the occurrence of diarrhea was comparable to that of the treatment with the CFTR inhibitor Cfr(inh)-172 (250 µg/kg ip) (data not shown). This result indicated that the intestinal epithelial GABAergic signal system is indeed involved in the process of allergic diarrhea.

DISCUSSION

The present study features two major findings: 1) the intestinal epithelial cells are endowed with a functional GABAergic signal system, and 2) this system is critically involved in the regulation of intestinal electrolyte transport and targeting the pathogenesis of the allergic diarrhea. Besides CFTR, the ionotropic GABA receptor is another identified Cl− channel that contributes to the formation of the intestinal fluid (Fig. 6). Importantly, this study may have clinical potential. Because the GABAAR antagonists alleviated the allergic diarrhea, the GABAergic signal system could become a new candidate for the resolution of diarrhea.

Data from previous studies have hinted that an autocrine or a paracrine GABA signaling mechanism exists in rodent gastrointestinal epithelial cells. For example, Erdo et al. (12, 13) find that GABAARs are expressed in rat stomach mucosal, whereas Wang et al. (33) demonstrate that GABA and GAD65 are mainly expressed in crypt and villi epithelial cells of rat jejunum. Consistent with previous findings, we found that GABA and GAD65/67 are expressed in rat, mouse, pig, and human ileum epithelium, as well as IEC-18 cells. GABA is also synthesized, stored, and secreted by mucosal endocrine-like cells throughout the rat antrum and intestine (9, 23). The mucosa, therefore, may well be under the influence of GABA released from epithelial cells and endocrine cells. Importantly, we first discovered that some ionotropic GABA receptor subunits also existed in small intestinal epithelium, confirming that the intestinal epithelium of mammals contains an endogenous GABA signaling system. Moreover, our perforated patch-clamp recording showed that application of GABA and/or muscimol, a selective GABAAR agonist, to IEC-18 cells induced a membrane depolarization. It is well known that GABAARs are ligand-gated Cl− channels. This result not only proved that the GABAARs in the intestinal epithelial cells were functional but also established that activation of GABAARs in the intestinal epithelial cells induced efflux of Cl−. Fluid secretion from the IECs to the intestinal lumen mainly depends on the activation of Cl− channels in the apical membrane of IECs. Our in vivo experiments showed that administration of GABA increased intestinal fluid secretion in a dose-dependent manner, making evident that the endogenous GABA signaling system in the intestine contributed to the intestinal fluid secretion although it was less effective than that of CFTR stimulants.

It has been reported that the enteric nervous system contains GABAergic neurons (22), and activation of GABAARs in submucosal secretomotor neurones stimulates Cl− secretion by intestinal epithelial cells in guinea pig ileum in vitro (25). In

Fig. 6. A working model of the ionotropic GABA receptor, a ligand-gated Cl− channel, and cystic fibrosis transmembrane conductance regulator (CFTR), another known Cl− channel in small IECs. GABA, which was synthesized, stored, and secreted by small intestinal epithelial cells, binds its type A receptors (GABAARs) in the apical membrane. GABAARs are opened, which evoke Cl− efflux. Fluid secretion to the intestinal lumen critically relies on activation of Cl− channels in the apical membrane, so fluid formation was increased. GABA-induced intestinal fluid secretion is associated with transepithelial Cl− transport. However, it is unknown whether both Cl− channels interact directly or indirectly.
addition, GABA receptors are also expressed in intestinal neuroendocrine cells (21) that regulate intestinal secretion and motility. However, our in vivo experiments showed that the stimulatory effect of GABA on intestinal secretion is still there in the presence of TTX, a bioactive peptide that eliminates nervous and endocrine control by blocking voltage-gated Na+ channels. Our result indicates that the GABA-evoked fluid secretion largely relies on activation of the intestinal epithelial cells.

GABA Rs can be selectively activated by muscimol (1) and preferentially blocked by gabazine (26). In our intestinal fluid secretion (in vivo) experiments, muscimol mimicked the GABA-induced excitatory effect on intestinal fluid secretion. On the contrary, gabazine antagonized that effect, indicating that GABA Rs play a critical role in intestinal fluid secretion.

Allergic diarrhea is characterized by imbalanced ion exchange and excessive production of fluid (30). A substantial body of evidence suggests that, in the process of allergic diarrhea, the activated Th2 immune cells release a wide range of cytokines, such as IL-13 and IL-4, which in turn strongly stimulate intestinal epithelial fluid and electrolyte transport by increasing Cl− secretion (40). However, the underlying mechanism remains unclear. In this study, we sensitized and then challenged mice by OVA, a widely used approach for inducing allergic diarrhea in animals (7), and investigated the role of the GABAergic signal system in allergic diarrhea. Our experiments revealed that the expression of GAD65/67 and GABA AR subunits was increased significantly after allergen challenge (Fig. 4, A and B), which was consistent with our previous study in asthma (38). Intragastric administration of picrotoxin and gabazine, two different GABA AR antagonists, reversed the upregulated expression of GABA AR subunits after allergen challenge (Fig. 4B). Importantly, the diarrhea progression slowed down after administration of the GABA AR antagonists picrotoxin and gabazine (Fig. 5B). The efficacy of picrotoxin on the occurrence of diarrhea was similar to that of the treatment with the CFTR inhibitor Cftr(inh)-172 (data not shown). These results demonstrated that this GABAergic system in intestinal epithelium is critically associated with the pathogenesis of allergic diarrhea.

We believed bicuculline to be a selective antagonist of GABA ARs (37). We did not use bicuculline in our in vivo studies because bicuculline is metabolized rapidly to the less active form bicucine under physiological pH and temperature (28). In addition, bicuculline also antagonizes glycine, 5-hydroxytryptamine, nicotinic aceetylcholine, and N-methyl-D-aspartate receptor (17, 32). In contrast, gabazine is a more selective GABA AR antagonist than bicuculline (39). Most importantly, in a range of lower concentrations, gabazine rarely crosses the blood-brain-barrier (36), thus excluding potential influence on the central nervous system. Indeed, in our experiments, the dose of gabazine was effective in treatment of allergic diarrhea but did not induce any sign of convulsion.

Taken together, the present study has demonstrated a novel GABAergic signal system is expressed in small intestinal epithelium, which is involved in electrolyte transport and fluid secretion. In addition, the GABAergic signal system in the intestinal epithelial cells is upregulated during allergic diarrhea. Intragastric administration of GABA AR antagonists retards the progress of allergic diarrhea and reduces the occurrence of allergic diarrhea. Our data not only demonstrate the essential role of a previously unknown epithelial GABAergic signal system in intestinal fluid secretion but may also lead to new therapeutic strategies for the management of allergic diarrhea.

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DISCLOSURES
The authors state no conflict of interest.

AUTHOR CONTRIBUTIONS
Author contributions: Y.L. and Y.-Y.X. performed experiments; Y.L., Y.-Y.X., and J.L. analyzed data; Y.L., Y.-Y.X., and J.L. prepared figures; Y.L., C.L., and J.L. drafted manuscript; Y.L. and J.L. approved final version of manuscript; W.-Y.L. and J.L. conception and design of research; J.L. interpreted results of experiments; J.L. edited and revised manuscript.

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