Mechanical stimulation activates Gαq signaling pathways and 5-hydroxytryptamine release from human carcinoid BON cells

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5-Hydroxytryptamine (5-HT) released from enterochromaffin cells activates secretory and peristaltic reflexes necessary for lubrication and propulsion of intestinal luminal contents. The aim of this study was to identify mechanosensitive intracellular signaling pathways that regulate 5-HT release. Human carcinoid BON cells displayed 5-HT immunoreactivity associated with granules dispersed throughout the cells or at the borders. Mechanical stimulation by rotational shaking released 5-HT from BON cells or from guinea pig jejunum during neural blockade with tetrodotoxin. In streptolysin O–permeabilized cells, guanosine 5′-*O*- (2-thiodiphosphate) (GDP-β-S) and a synthetic peptide derived from the COOH terminus of Gαq abolished mechanically evoked 5-HT release, while the NH_2-terminal peptide did not. An antisense phosphorothioated oligonucleotide targeted to a unique sequence of Gαq abolished mechanically evoked 5-HT release and reduced Gαq protein levels without affecting the expression of $G\alpha_{11}$. Depletion and chelation of extracellular calcium did not alter mechanically evoked 5-HT release, whereas depletion of intracellular calcium stores by thapsigargin and chelation of intracellular calcium by 1,2-bis (*o*-Aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM) reduced 5-HT release. Mechanically evoked 5-HT release was inhibited by somatostatin-14 in a concentration-dependent manner. The results suggest that mechanical stimulation of enterochromaffin-derived BON cells directly or indirectly stimulates a G protein–coupled receptor that activates Gαq, mobilizes intracellular calcium, and causes 5-HT release.

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Introduction

Enterochromaffin cells that store 5-hydroxytryptamine (5-HT) have been linked to several bowel disorders including malignant carcinoid, dumping, and irritable bowel syndromes (IBSs) (1–3). Carcinoid syndrome represents a spectrum of heterogeneous symptoms from vague abdominal complaints to flushing, bronchoconstriction, and diarrhea that result from release of bioactive mediators from carcinoid tumors (4). Although the incidence of carcinoid tumors is relatively low (1.5 per 100,000 population), they comprise about 25% of all small intestinal tumors (4). 5-HT, the major secretory product of carcinoid tumors, is one of the putative mediators of carcinoid diarrhea, which is often relieved by 5-HT receptor antagonists or treatment with somatostatin derivatives (5, 6). The disadvantages to the latter treatment are the broad spectrum of actions and failure of some patients to respond to somatostatin.

BON cells are a human carcinoid cell line derived from a metastasis of a pancreatic carcinoid tumor of enterochromaffin cell origin (7, 8). BON cells have characteristics of foregut and midgut tumors and contain a variety of mediators, including neurotensin and pancreastatin in addition to 5-HT (8, 9). Understanding the mechanisms triggering release of 5-HT from BON cells may provide new insights into the biology of carcinoid tumors and into the development of therapeutic interventions for tumors not responding to conventional therapies.

Alteration in 5-HT release is not confined to neoplastic enterochromaffin cells. In a subset of IBS patients, an increased number of nontransformed enterochromaffin cells was associated with an increased sensitivity to transmucosal pressure (1, 9). Selective $5\text{-}HT_3$ receptor antagonists and $5\text{-}HT_4$ agonists have proven to be beneficial in the treatment of diarrhea- and constipation-predominant symptoms, respectively (10–12). Identifying the signaling mechanisms that activate enterochromaffin cells is necessary in understanding the symptoms of several disease states of the gut, as well as its normal physiology. Therefore, the BON cell line provides a unique model to study the mechanosensitive signaling pathways regulating 5-HT release in cells with similarities to nontransformed, intestinal enterochromaffin cells.

Since processes of enteric neurons do not penetrate into the lumen, 5-HT from enterochromaffin cells plays a critical role in activating intrinsic primary afferents that are in reflex pathways regulating secretion and motility (13–18). Although mechanical stimuli are important modulators of cellular function, little is known about mechanisms of mechanotransduction in enterochromaffin cells. In other cell systems, mechanical stimulation such as pressure, strain, or shear force is associated with activation of quanine nucleotide-binding proteins (G proteins) and an increase in intracellular calcium concentration (19–23).

G proteins are heterotrimeric consisting of α, β, and γ subunits. They are classified by the amino acid sequences of the α subunit into subfamilies (Gαs, Gαi, Gαq, and $G\alpha_{12/13}$) (24). Upon activation, guanosine 5′-diphosphate–bound (GDP-bound) α subunit releases GDP and binds to GTP, causing dissociation of the heterotrimer into the active α subunit and the βγ dimer. The Gαq family consists of Gαq, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, and $G\alpha_{16}$. They are all regulators of the phospholipase C-β (PLCβ), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes Ca^{2+} from intracellular stores whereas DAG activates protein kinase C (PKC) (24). Activation of several G protein–coupled receptors, such as muscarinic receptors (M_1, M_3), adrenoreceptors (β), and purinoceptors (A_2), is positively or negatively associated with 5-HT release from enterochromaffin cells (ref. 25 and unpublished data).

The overall aim of this study was to identify mechanosensitive intracellular signaling pathways that regulate 5-HT release. To this end, it was necessary to determine if mechanical stimulation could release 5-HT from the human carcinoid cell line, BON, which has characteristics of enterochromaffin cells and to identify whether G proteins were involved. The results showed that mechanical stimulation activates the Gαq subunit, mobilizes intracellular Ca^{2a}, and releases 5-HT.

Methods

Cell culture. BON cells were kindly provided by C.M. Townsend, Jr. (University of Texas, Galveston, Texas, USA). BON is a noncloned cell line that is a mixed population of large round cells and small cells with dendritic-like extensions. BON cells were subcloned by serial dilution, and clone 7 was chosen on the basis of having the highest content of 5-HT released by the calcium ionophore, A23187 (10 μM). Monolayers of BON cells were maintained in DMEM: nutrient mixture F-12 (DMEM/F12) (1:1) media, supplemented with 10%FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C (8). Cells were trypsinized and seeded at a density of 10^5 or 10^6 cells per well in 12-well culture plates (Corning-Costar Corp., Corning, New York, USA). Cells were grown for 48 hours to achieve confluence before experiments, with the exception of the antisense experiments in which cells were grown for 6 days. Passage numbers were 10–30.

Materials. Concentrations of drugs were chosen based on published values and from preliminary experiments. Alaproclate, pargyline, and thapsigargin were purchased from RBI (Natick, Massachusetts, USA). Tetrodotoxin was obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). BAPTA-AM [1,2-bis (*o*-Aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra (acetoxymethyl) ester] was purchased from Calbiochem-Novabiochem Corp. (San Diego, California, USA). Somatostatin-14 (lot number ZO146) was obtained from Bachem California (Torrance, California, USA). Phosphorothioated oligonucleotides were synthesized and purified by high-performance liquid chromatography by Integrated DNA Technologies Inc. (Coralville, Iowa, USA). Synthetic COOH-terminal peptide (amino acid residues 340 to 359) and NH_2-terminal peptide (amino acid residues 13 to 29) of Gαq were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA).

Antiserum. The primary Ab was a monoclonal mouse anti–5-HT Ab, clone number M0758, used at a dilution of 1:25 (DAKO Corp., Carpinteria, California, USA). The secondary Ab was a horse anti-mouse IgG conjugated to FITC (Vector Laboratories, Burlingame, California, USA) used at a dilution of 1:40. The specificity of the 5-HT Ab was determined by preadsorbing with 0.5 mM 5-HT for 1 hour at 37°C. The primary rabbit polyclonal Gαq Ab (E-17; Santa Cruz Biotechnology) was raised against a peptide epitope within the amino terminal domain of Gαq of mouse origin. Rabbit polyclonal $G\alpha_{11}$ Ab (D-17; Santa Cruz Biotechnology) was raised against a peptide mapped to the amino terminal domain of $G\alpha_{11}$ of mouse origin. Both Ab's are specific and do not cross-react with each other or to other Gα subunit proteins. The primary Ab's were used in a dilution of 1:2,000. The secondary Ab was a peroxidase-conjugated anti-rabbit IgG used at 1:3,000. Immunopositive bands were visualized with the enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

Immunofluorescent detection of 5-HT. Bon cells (10^5–10^6) were plated on number 0 coverslips and maintained in DMEM/F12 at 37°C in a 5% CO_2 incubator for 3 days. After fixation for 1 hour at 4°C in modified Zamboni's fixative, the cells were washed three times at 10-minute intervals with PBS. The cells were incubated in normal horse serum (3%) in 0.5% Triton-X/PBS for 30 minutes at room temperature before an overnight incubation with the 5-HT Ab. The secondary Ab was conjugated to FITC at 4°C. The cells were washed with PBS three times at 10-minute intervals and incubated for 2 hours at room temperature with the secondary Ab, a horse anti-mouse IgG. A third and final PBS wash was performed. The specificity of the 5-HT Ab was determined by preadsorbing with 0.5 mM 5-HT for 1 hour at 37°C. Cells were visualized by laser-confocal imaging using the Zeiss LSM 410 microscope (Carl Zeiss Inc., Thornwood, New York, USA). The cells were excited with an

Ar-Kr laser at 488 nm. Emissions were captured by a photomultiplier tube through a LP 590 filter and saved on an IBM computer as Tagged Image File Format (TIFF) images. The thickness of each optical slice was 0.5–1.5 μm. Images represent averages of four scans.

Electron microscopy. BON cells were fixed onto 35-mm dishes (Corning-Costar Corp.) using 1.6% glutaraldehyde, 0.4 mM $CaCl_2$, and 0.05 M sucrose in 0.1 M PBS, pH 7.4, for 30 minutes at 4°C. The cells were rinsed in 0.1 M PBS, pH 7.4, containing 0.1 M sucrose, postfixed in 1% osmium tetroxide, and dehydrated through an ethanol series (50%, 70%, 80%, 95%, 100%, 100%). Cells were rinsed in hydroxypropylmethacrylate for 30 minutes followed by rinsing in three changes of Polybed 812 resin overnight at 60°C. Approximately 800-nm sections were cut using a Reichert Ultracut E Ultramicrotome, placed on copper grids, and stained with 2% uranyl acetate and Reynold's lead citrate. Specimens were observed in a Philips CM 12 transmission electron microscope at 60 kV.

Mechanical stimulation. The medium was removed before washing the cells with Earle's balanced buffer solution (EBBS) (pH 7.3) containing 0.1% BSA. After preincubation for 30 minutes at 37°C, the medium was removed and replaced with 0.5 ml EBBS that contained 10^{-5} M of alaproclate (5-HT uptake inhibitor) and pargyline (monoamine oxidase inhibitor). The cells were exposed to static conditions or to a mechanical stimulus generated by a shaker (Lab-Line, Melrose Park, Illinois, USA), which rotated the plates to generate movement of the assay buffer. After mechanical stimulation for 20 minutes, the assay buffer was collected and centrifuged for 20 seconds to remove any detached cells. The supernatants were frozen at –80°C until the 5-HT assay was performed. The cells in the culture plate were washed two times with cold PBS solution and stored at –80°C for protein assay.

For tissue experiments, male albino Hartley guinea pigs (Sprague-Dawley Harlan, Indianapolis, Indiana, USA) were stunned and exsanguinated, a method approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. A 10- to 15-cm segment of the small intestine (jejunum) was removed and flushed with cold Krebs-Ringer solution (pH 7.3), and cut along the mesenteric border. Whole-thickness small intestinal segments were mounted in modified circular chambers (8 mm diameter) with the mucosal side upward. The mucosal compartment contained 0.3 ml Krebs-Ringer solution with 5×10^{-7} M tetrodotoxin and 1×10^{-5} M of alaproclate and pargyline. Mechanical stimulation was the same as above.

Cell permeabilization with streptolysin O. Confluent BON cells in 12-well culture plates were preincubated in EBBS at 37°C for 15 minutes. Cells were washed two times with 0.5 ml of permeabilizing buffer (pH 7.3) containing 120 mM KCl, 25 mM $NaHCO_3$, 5 mM HEPES, 10 mM $MgCl_2$, 1 mM KH_2PO_2, 1 mM EGTA, 300 mM $CaCl_2$, and 100 μM MgATP. Cell membrane permeabilization was carried out by incubation with 20 U/ml streptolysin O (Sigma Chemical Co.) in 0.5 ml permeabilizing buffer for 5 minutes. After the incubation, the cells were washed two times with permeabilizing buffer and preincubated with 200 μM guanosine 5′-*O*- (2-thiodiphosphate) (GDP-β-S) (Calbiochem-Novabiochem Corp.) or 25 μg/ml of synthetic peptide in permeabilizing buffer containing 10^{-5} M alaproclate and pargyline for 5 minutes before they were exposed to mechanical stimulation. For peptide treatment, buffers contained 1× protease inhibitor cocktail (Calbiochem-Novabiochem Corp.).

Treatment of cells with oligonucleotides. BON cells (10^5) were plated in 12-well culture plates and incubated for 48 hours before treatment. Preliminary data showed that high concentrations of phosphorothioated oligonucleotides (5 μM and 10 μM) were toxic to cells. Therefore, 1 μM of phosphorothioated oligonucleotide was added daily for 6 days. The sequence of the antisense oligonucleotide was 5′-GCT TGA GCT CCC GGC GGG CG-3′ (105-125 bp from translation start codon) for Gαq (26) and was 5′-GGG CGC GCC GAC TCC GTG TG-3′ for the scrambled oligonucleotide.

Cell toxicity assay for lactate dehydrogenase. Lactate dehydrogenase activity released from the cytosol was measured by colorimetric assay using a Cytotoxicity Detection kit (Roche Molecular Biochemicals, Indianapolis, Indiana, USA) according to the manufacturer's instructions. The sensitivity of the assay was confirmed by the manufacturer. About 0.2×10^4 to 2×10^4 cells/well were sufficient for most experimental setups.

5-HT enzyme immunoassay. 5-HT release was measured by enzyme immunoassay using an ELISA kit (Coulter Corp., Miami, Florida, USA) according to the manufacturer's instructions. The absorbance was measured at 405 nm and 5-HT concentration was determined from a standard curve. The sensitivity of the assay is approximately 0.5 nM (1.8 pg/well). With a sample range between 4.5 and 5.5 nM (n = 30), the interassay standard deviation and coefficient of variation were 0.4 nM and 8.9%, respectively.

Immunoblotting. The frozen cells were thawed and gently scraped from culture plates with a cell scraper. After centrifugation (3,000 *g* for 5 minutes at 4°C), pellets were resuspended in lysis buffer. It contained 10 mM Tris-HCl, pH 8.0, the detergents 1% Triton X-100 and 60 mM octyl glucose, 150 mM NaCl, 5 mM EDTA, 500 μM 4-(2-Aminoethyl) benzenesulfonylfluoride-HCl, 1 μM E-64, 150 nM aprotinin, which are serine, cysteine, and trypsin inhibitors, respectively, and in addition the trypsin-like and cysteine protease inhibitor 1 μM leupeptin. The resuspended pellets were incubated for 30 minutes on ice after sonication and insoluble material was removed by centrifugation (10,000 *g* for 3 minutes at 4°C). Protein concentration of each well was determined (Bio-Rad, Hercules, California, USA). Solubilized proteins (29 μg) were separated by 10% denaturing SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The α subunits of G proteins were detected by immunostaining with rabbit

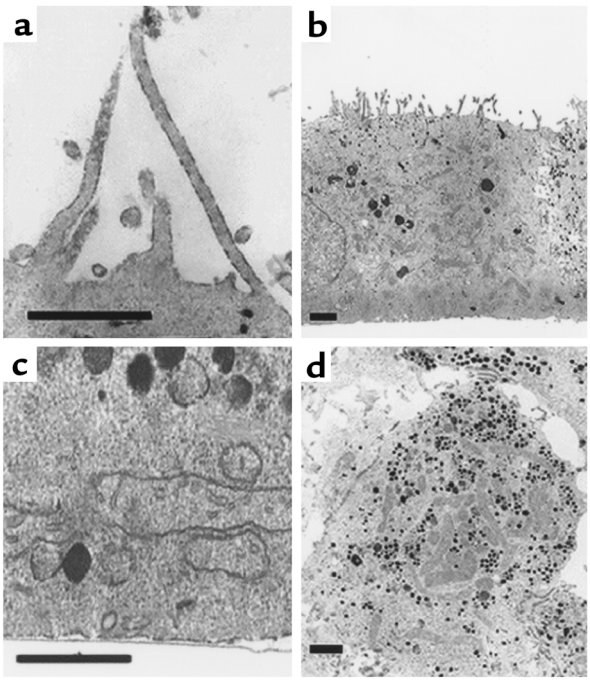

Figure 1
Transmission electron micrographs of BON cells. Sections (80 nm) were examined with a Philips CM 12 transmission electron microscope at 60 kV. (**a** and **b**) Electron micrographs showing microvilli with varying shapes and sizes on the plasma membrane of BON cells. Note abundance of granules adjacent to microvilli (**b**). (**c**) Bottom surface has no microvilli-like protrusions. (**d**) The cytoplasm is rich in secretory granules. Scale bar, 1 μm. ×7,500 (**b**); ×12,500 (**d**); ×70,000 (**a** and **c**).

polyclonal Ab's against Gαq or $G\alpha_{11}$. Nitrocellulose membranes were blocked with 5% nonfat milk for 2 hours at 37°C and probed with primary Ab's (1:2,000) for 1 hour at 37°C. Membranes were rinsed with PBS/Tween-20 (80 mM Na_2HPO_4 Æ7H_2O, 20 mM KH_2PO_4, 100 mM NaCl, 1% [vol/vol] Tween 20, pH 7.5) and incubated with peroxidase-conjugated anti-rabbit IgG at 1:3,000 dilution for 30 minutes at room temperature. Immunopositive bands were visualized with the ECL kit (Amersham Pharmacia Biotech). The protein bands visualized on Hyperfilm ECL (Amersham Pharmacia Biotech) were scanned, and the intensity of each band was measured by ScanWizard 5 software (Microtek Lab Inc., Redondo Beach, California, USA).

Statistics. All data are expressed as means plus or minus standard errors of the means (SEM). Statistical significance was evaluated by paired or unpaired Student's *t* test or ANOVA with Bonferroni's multiple comparison post hoc test, depending on experimental design.

Results

Electron microscopy. BON cells were subcloned by serial dilution and clone 7 was chosen on the basis of having the highest releasable content of 5-HT. BON cells were characterized by membrane protrusions reminiscent of microvilli in enterochromaffin cells (27). (Figure 1, a and b). On the side attached to the culture plate, membrane protrusions were very rarely seen (Figure 1, b and c). BON cells contained a large number of dense-core secretory granules distributed throughout the cell or localized at one pole of the cell often associated with microvilli (Figure 1, b and d).

5-HT immunoreactivity in BON cells. 5-HT immunoreactivity indicated by the white color was evident in nearly all BON cells (>98%) (Figure 2, a, b, d, and e). 5-HT immunoreactivity was present in granules (Figure 2, d and e). Preadsorption of the 5-HT Ab with 5-HT abolished any positive reaction, indicating that the Ab is specific for 5-HT (Figure 2, c and f).

5-HT release from BON cells in response to secretagogues. Ca^{2a} ionophore A23187, which allows entry of Ca^{2a} into cells from extracellular sources, and forskolin, which stimulates adenylyl cyclase to increase cAMP production, caused a very large increase in 5-HT release compared with basal 5-HT release (control: 0.9 ± 0.1 pmol/well/20 min) (Table 1). In contrast, receptor-mediated 5-HT release was less, as illustrated by isoproterenol, which activates β-adrenergic receptors, Gαs, adenylyl cyclase, and cAMP, and bethanechol, which activates muscarinic receptors, Gαq, IP_3, and calcium or a Gαi pathway (Table 1).

Effect of mechanical stimulation on 5-HT release. To determine whether mechanical stimulation by rotational shaking would enhance 5-HT release from enterochromaffin cells in vitro, whole-thickness preparations of the guinea pig small intestine (jejunum) were exposed to increasing speeds of shaking. The assay buffer contained 500 nM of tetrodotoxin to prevent neuronally evoked 5-HT release. Increasing the shaking speed from 150 to 250 rpm caused a graded increase in 5-HT release (Figure 3a).

BON cells exposed to mechanical stimulation by shaking the culture plates on an orbital shaker at

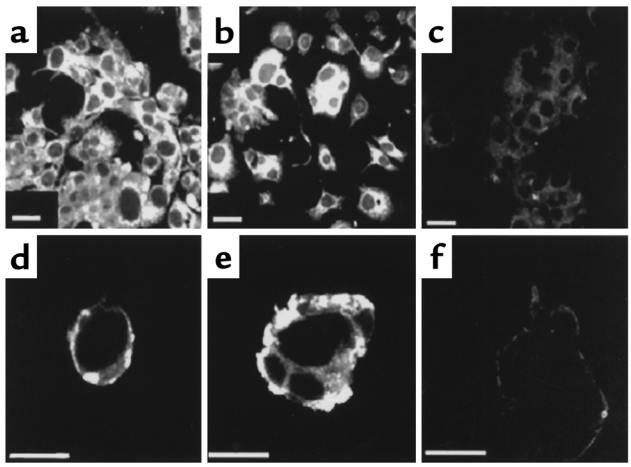

Figure 2
5-HT immunoreactivity in BON cells. (**a**, **b**, **d**, and **e**) 5-HT immunoreactivity, indicated by white, was detected in most BON cells. (**c** and **f**) Preadsorption with 0.5 mM 5-HT abolished any positive reaction. Optical slices were 0.5–1.5 μm thick. (**a**–**c**) Bars, 40 μm; (**d**–**f**) bars, 20 μm.

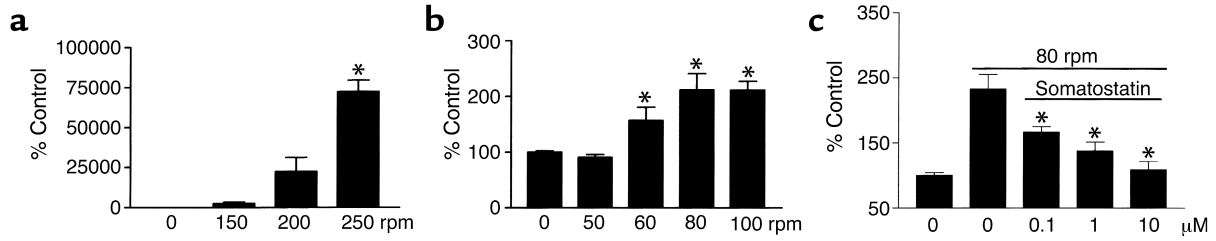

Figure 3
Effect of mechanical stimulation on 5-HT release from guinea pig small intestine (**a**) and human BON cells without (**b**) and with somatostatin-14 (**c**). Tissues and cells were maintained in a static condition (0 rpm; control) or exposed to mechanical stimulation ranging from 50 to 100 or 150 to 250 rpm for 20 minutes at 37°C. Values represent 5-HT release relative to controls in picomoles per well per 20 minutes. (**b**) Cells 0.8 ± 0.08. (**c**) Cells 1.3 ± 0.1. (**a**) Tissue 9.8 ± 4. Increasing rpm resulted in rpm-dependent increase in 5-HT release (n = 32, 0 rpm; n = 4, 50 rpm; n = 10, 60 rpm; n = 6, 80 rpm; n = 16, 100 rpm). *P < 0.05 versus 0 rpm.

increasing rpm from 50 to 100 for 20 minutes showed an rpm-dependent increase in 5-HT release (Figure 3b). Significant increases in 5-HT release above controls (0 rpm) occurred at 60 rpm (157% ± 24%) with a maximum at 80 rpm (211% ± 29%). Cell viability after shaking, assessed by trypan blue exclusion, total protein assay, cell numbers, and lactate dehydrogenase (LDH) release, were not significantly different from the control group (Table 1).

Effects of streptolysin O on LDH activity and 5-HT release. There was a sharp increase in LDH activity, a marker of plasma membrane integrity, within the first 5 minutes after exposure to streptolysin O (SLO), followed by a more gradual rise with time after SLO wash out (Figure 4a). 5-HT release in static controls and in mechanically stimulated cells gradually declined in parallel as a function of time after SLO treatment (Figure 4b). Despite the high activity of LDH 115 minutes after permeabilization with SLO and the gradual decrease in basal and mechanically stimulated 5-HT release, the cells were still able to respond to mechanical stimulation by an increase in 5-HT release similar to nonpermeabilized cells. The decrease in 5-HT release in the basal state paralleled that in the stimulated states (Figure 4b). Linear regression analysis showed that the slopes are not significantly different (–0.018 ± 0.0050, 0 rpm, and –0.032 ± 0.0098, 80 rpm; P = 0.21) under static or stimulated conditions.

Role for G proteins in mechanically evoked 5-HT release. There was no detectable change in basal 5-HT release from permeabilized cells compared with nonpermeabilized cells in the static or stimulated condition. Pretreatment of permeabilized BON cells with GDP-β-S (200 μM) abolished mechanically evoked 5-HT release without affecting basal release (Figure 5a). To investigate the role of Gαq in mechanically evoked release of 5-HT, BON cells were treated with a phosphorothioated antisense oligonucleotide derived from a unique sequence of Gαq. Controls consisted of untreated cells or cells exposed to an oligonucleotide with a scrambled sequence. The latter had the same guanine/cytosine content as the antisense oligonucleotide and the nucleotides were arranged in random order. Treatment of the cells with 1 μM antisense oligonucleotide for 6 days did not alter basal 5-HT release. Mechanical stimulation increased 5-HT release in both control groups (Figure 6a). However, in antisense oligonucleotide-treated cells, mechanical stimulation did not increase 5-HT release (Figure 6a). Inhibition of mechanically evoked 5-HT release occurred without compromising functional integrity of the 5-HT exocytosis/release mechanisms. Ability to release 5-HT was assessed by treatment of the cells with a calcium ionophore (10 μM A23187). A23187 evoked a similar release of 5-HT in pmol/μg protein/well/20 min for scrambled oligonucleotide-treated cells (0 M, 0.3 ± 0.1 [n = 5]; 10 μM, 9.8 ± 0.2 [n = 5]) and antisense oligonucleotide-treated cells (0 M, 0.5 ± 0.1; 10 μM, 8.3 ± 0.5).

The results of Western blot analysis indicated that the antisense oligonucleotide targeting Gαq reduced expression of Gαq protein compared with control and scrambled oligonucleotide-treated cells (Figure 6, b and

Table 1
Secretagogue-evoked 5-HT release and cell viability after shaking

10 μM secretagogues/5-HT release			Cell viability	
Drug	Signaling path	5-HT release	Assay	80 rpm/0 rpm
Calcium ionophore (%)	Ca^2 influx	1,910 ± 645 (3)	LDH (% total)	0.9 ± 0.1/0.7 ± 0.1 (4)
Forskolin (%)	AC/cAMP	2,104 ± 453 (3)	Trypan blue (%)	8.7 ± 1/8.4 ± 1 (7–8)
Isoproterenol β-R (%)	Gαs, AC/cAMP	357 ± 99 (3)	Protein (μg/μl)	3.1 ± 0.1/3.5 ± 0.1 (4–10)
Bethanechol M_1/M_3-R (%)	Gαq, IP_3, Ca^2	179 ± 21 (3)	Cell no. × 10^6	1.1 ± 0.03/1.0 ± 0.04 (7–8)

Means ± SEM are shown. 5-HT release is percent control value, 0.9 ± 0.1 pmol/well/20 min. Numbers in parentheses are n values. AC, adenylyl cyclase.

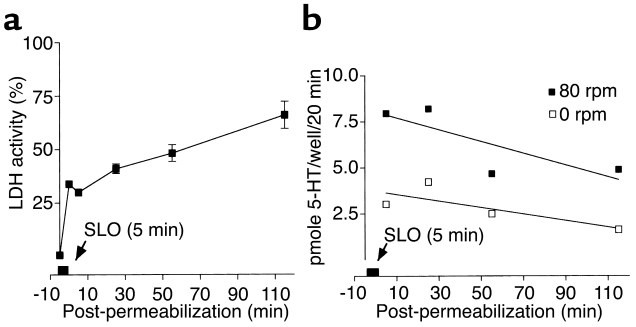

Figure 4
Effects of SLO on LDH activity and 5-HT release. BON cells were permeabilized by 20 U/ml SLO for 5 minutes at 37°C in permeabilizing buffer. After permeabilization, SLO was washed out, and BON cells were incubated at 37°C for different time periods from 5 minutes to 115 minutes. (**a**) Buffers were collected after each time period and the amount of LDH activity was measured. Values represent percentage of total LDH activity (n = 3). (**b**) After incubations, BON cells were stimulated with/without rotational shaking (80 rpm) and 5-HT release was measured after the stimulation (20 minutes). Values represent concentrations of 5-HT in wells. Linear regression analysis of 5-HT release showed that the slopes were not significantly different (–0.018 ± 0.0050 and –0.032 ± 0.0098; P = 0.21) under static or stimulated conditions (80 rpm; n = 3).

c). Studies were done to ensure that the amount of protein loaded in each lane was the same and the antisense treatment affected only Gαq rather than $G\alpha_{11}$, which has high amino acid sequence homology (88%) with Gαq. The immunoblot was stripped and reprobed with polyclonal Ab's specific for $G\alpha_{11}$. The results indicate that $G\alpha_{11}$ is still expressed at the same levels in control and after Gαq antisense oligonucleotide treatment (Figure 6, b and c).

Studies were done to investigate whether the synthetic COOH-terminal peptide (residues 340–359, VFAAVKDTILQLNLKEYNLV) of Gαq would affect shaking-evoked release of 5-HT. In SLO-permeabilized BON cells, pretreatment of 25 μg/ml of COOH-terminal peptide for 5 minutes abolished shaking-evoked 5-HT release (Figure 5b). On the other hand, the NH_2-terminal peptide (residues 13–29, EEAKEARRINDEIERQL) of Gαq had no significant effect on 5-HT release (Figure 5b).

Effect of Ca^{2+}-free buffer, Ca^{2+} chelators, or depletion of intracellular Ca^{2+} stores on basal and mechanically evoked 5-HT release. The next series of studies were designed to assess the involvement of Ca^{2+} in mediating mechanically evoked release of 5-HT. Ca^{2+} ionophore A23187 (Table 1) and thapsigargin, which releases intracellular Ca^{2+}, significantly increased 5-HT release compared with controls (control; 0.9 ± 0.1 pmol/well/20 min; 1 μM thapsigargin, 2.1 ± 0.6 pmol/well/20 min; P < 0.05, n = 3-6).

Since these results suggested that 5-HT release by BON cells is Ca^{2+} dependent, studies were done to determine the role of extracellular and intracellular Ca^{2+} pools in mechanically evoked 5-HT release. In Ca^{2+}-free EBBS containing the extracellular chelator 1 mM EGTA, basal 5-HT release was not different from control (calcium-containing buffer: 0.8 ± 0.08 pmol/well/20 min; Ca^{2+}-free buffer: 0.9 ± 0.1 pmol/well/20 min) (Figure 7a). At this concentration of EGTA there was no apparent cell detachment. In the absence of extracellular Ca^{2+}, mechanical stimulation (80 rpm) significantly increased 5-HT release. The relative enhanced rate, 176% ± 10% of static control, was not significantly different from the rate in Ca^{2+}-containing buffer (211% ± 29%) (Figure 7a).

Because mechanical stimulation can induce 5-HT release in the absence of extracellular Ca^{2+}, additional experiments were performed to investigate the possibility that mechanical stimulation mobilizes Ca^{2+} from internal Ca^{2+} stores. To investigate this possibility, the cells were pretreated with high concentrations of thapsigargin (10 μM), an inhibitor of the endoplasmic reticular Ca^{2+}-ATPase, to deplete intracellular Ca^{2+} stores. Thapsigargin pretreatment inhibited shaking-evoked 5-HT release by 75% (Figure 7b). Second, BON cells were pretreated with cell-permeable Ca^{2+} chelator, BAPTA-AM (50 μM), for 1 hour to buffer internal Ca^{2+} levels before being tested in Ca^{2+}-free EBBS. Mechanically evoked (80 rpm) 5-HT release was completely abolished by BAPTA-AM pretreatment (111% ± 5% of the static value) (Figure 7c). Taken together, these data strongly indicate that shaking mobilizes Ca^{2+} from intracellular stores.

Effect of somatostatin on 5-HT release. Because somatostatin derivatives are often used therapeutically in patients with carcinoid syndrome, we examined the effect off somatostatin-14 on 5-HT release. Somatostatin-14 had no effect on basal release (0 rpm: 1.3 ± 0.1 pmol/well/20 min; 0 rpm + 10 μM somatostatin-14: 1.0 ± 0.04 pmol/well/20 min). However, this peptide significantly inhibited mechanically stimulated 5-HT release in a concentration-dependent manner (Figure

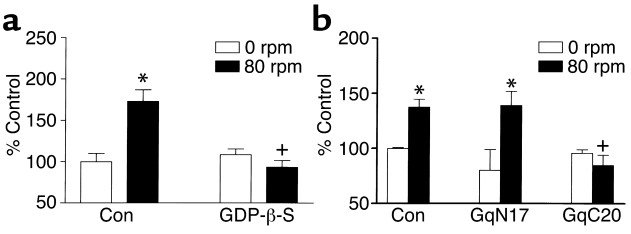

Figure 5
(**a**) SLO-permeabilized BON cells. Cells were permeabilized by 20 U/ml SLO for 5 minutes in permeabilizing buffer and preincubated with 200 μM of GDP-β-S for 5 minutes before stimulation (shaking at 80 rpm): 0 rpm, 2.9 ± 0.4 pmol/well/20 min; 0 rpm + GDP-β-S, 3.1 ± 0.3 pmol/well/20 min. *P < 0.05 versus permeabilized static control, ^{+}P < 0.05 versus shaking at 80 rpm of permeabilized cells (n = 9). (**b**) BON cells were permeabilized by 20 U/ml SLO for 5 minutes in permeabilizing buffer and preincubated with 25 μg/ml of NH_2-terminal peptide (GqN17) or COOH-terminal peptide (GqC20) for 5 minutes before mechanical stimulation at 80 rpm. Experiments were performed in the presence of 1× protease inhibitor cocktail. 5-HT release in pmol/well/20 min was 0 rpm, control: 2.2 ± 0.02; GqN17: 1.8 ± 0.4; GqC20: 2.1 ± 0.07. *P < 0.05 versus permeabilized static control, ^{+}P < 0.05 versus shaking at 80 rpm of permeabilized cells (n = 3).

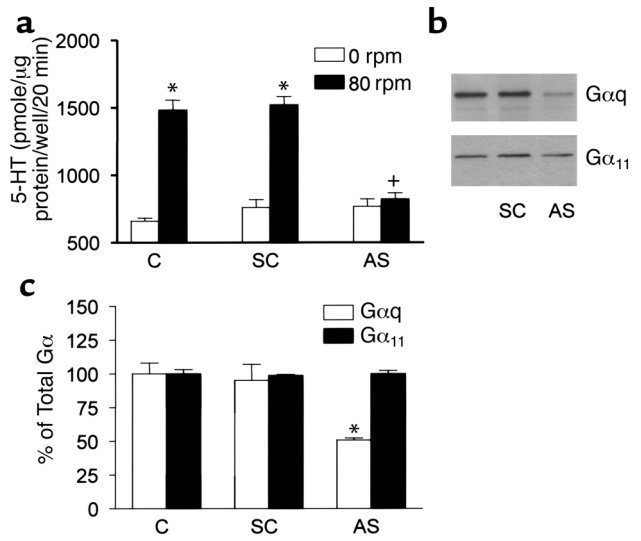

Figure 6
Selective suppression of Gαq protein with antisense oligonucleotide. (**a**) BON cells were untreated (C) or treated with 1 µM of antisense (AS) or scrambled (SC) phosphorothioated oligonucleotide for 6 days. 5-HT release was measured after stimulation (filled bar; shaking at 80 rpm for 20 minutes) or from static controls (open bar). 5-HT release in pmol/µg protein/well/20 min at 0 rpm: C, 0.7 ± 0.02; SC, 0.8 ± 0.06; AS, 0.8 ± 0.06. $*P < 0.0001$ versus 0 rpm, $^{+}P > 0.05$ versus 0 rpm: $n = 4$, 0 rpm, C, SC, and AS; $n = 6$, 80 rpm, AS; $n = 7$, 80 rpm, SC; $n = 10$, 80 rpm, C. (**b**) Membrane extracts (29 µg of protein) from each group were separated by 10% SDS gel electrophoresis and then analyzed for the expression levels of Gαq protein using specific polyclonal Ab's against Gαq. After exposure of the immunoblot to film, the membrane was stripped of the anti-Gαq primary-secondary Ab complexes with 0.2 M NaOH for 5 minutes and reprobed with specific polyclonal Ab's against $G\alpha_{11}$. The first lane is control; the second and third lanes are SC and AS, respectively. (**c**) The density of visualized bands was analyzed and expressed as percentage of untreated control. $*P < 0.05$ versus control ($n = 3$).

3c). At a concentration of 10 µM, somatostatin-14 abolished mechanically evoked 5-HT (Figure 3c).

Discussion

Our results indicate that mechanical stimulation by rotational shaking causes 5-HT release from the small intestine of the guinea pig even when neural conduction was blocked. While mechanical stimulation releases 5-HT independent of any neural input, it is unclear if this is a direct effect on enterochromaffin cells or whether it is due to release of other mediators from a variety of cell types found in tissue preparations. This possibility could explain why 5-HT release was considerably greater in tissues compared with BON cells. The number of cell types found in tissues was minimized by using BON cells that displayed similarities to nontransformed enterochromaffin cells. Clone BON7 was relatively homogenous in that nearly all the cells (> 98%) contained 5-HT immunoreactivity localized to granules (Figure 2). Granules were often associated with one pole of the cell ,as has been reported for enterochromaffin cells in vivo (27).

The factor that transduces a mechanical force into a biological response in BON cells is unknown. However, the protrusion of microvilli into the buffer where they can "taste" the contents suggests that they may contain mechanotransducing elements analogous to hair cells (28). BON cells also have other sensory modalities by virtue of their chemosensitivity to hexoses (29).

Rotational movement of assay buffer in culture plates generates several mechanical forces, including shear stress or continuously changing hydrostatic pressure gradients, or both (30). In vivo, shear stress is likely to occur in the small intestine and proximal colon where the luminal contents are fluid, or in the distal colon when they become fluid such as in diarrheal states. Mechanical stimulation generated by direct contact of the enterochromaffin cells with the luminal contents occurs frequently throughout the intestinal tract. Others have reported that balloon distention, puffs of nitrogen gas ejected from a pipette, and stroking with a brush evoke 5-HT release (31, 32). Many of these stimuli are composed of several different forces acting simultaneously. Thus in the continuously changing

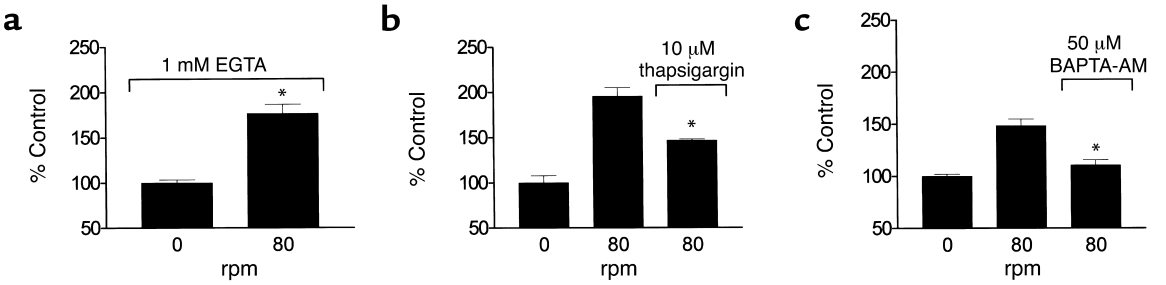

Figure 7
Effects of chelating or depleting extracellular and intracellular Ca^{2+} on mechanically evoked 5-HT release. (**a**) For extracellular Ca^{2+} free conditions, experiments were performed in Ca^{2}-free EBBS containing 1 mM EGTA. Mechanical stimulation was shaking at 80 rpm. $*P < 0.05$ versus static controls (0 rpm; $n = 6$). (**b**) Pretreated with 10 µM thapsigargin for 30 minutes. $*P < 0.05$ versus shaking of untreated cells at 80 rpm ($n = 3$). (**c**) Pretreated with 50 µM BAPTA-AM for 1 hour. $*P < 0.05$ versus shaking of untreated cells at 80 rpm ($n = 6$). Controls at 0 rpm are (**a**) 0.9 ± 0.1 pmol/well/20 min; (**b**) 1.2 ± 0.09 pmol/well/20 min; (**c**) 0.9 ± 0.3 pmol/well/20 min.

environment of the bowel, composite stimuli may more readily reflect the in vivo condition.

Our results suggest that rotational shaking of cultured BON cells activates heterotrimeric G proteins causing 5-HT release. Based on published reports, it seemed likely that the Gαq family, which consists of Gαq, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, and $G\alpha_{16}$, might be involved. $G\alpha_{14}$, $G\alpha_{15}$, and $G\alpha_{16}$ are unlikely candidates because they are found in epithelial, stromal, or hematopoietic cells. Evidence for Gαq in BON cells comes from several different experimental regimens in which stimulated 5-HT release was reduced or abolished: (a) GDP-β-S, which prevents activation of heterotrimeric G proteins inhibited 5-HT release; (b) an antisense oligonucleotide directed against a unique sequence of human Gαq cDNA reduced expression of Gαq protein and abolished mechanically evoked 5-HT release without decreasing cell density, total cell protein, cellular responsiveness to a calcium ionophore; and (c) the synthetic COOH-terminal peptide of Gαq abolished 5-HT release.

One puzzle is the observation that the antisense treatment reduced Gαq protein levels by 50%, whereas it abolished mechanically evoked 5-HT. Our results are compatible with the possibility that there is more than one pool of Gαq in BON cells. In endothelial cells, most of the Gαq associates with caveolin in membrane invaginations called caveolae (33).

The effect of the antisense oligonucleotides in abolishing 5-HT release was unlikely to be due to impairment of the vesicular monoamine transporters, VMAT1 and VMAT2, and a reduction of 5-HT content in secretory vesicles. Whereas these transporters in carcinoid tumors are regulated by pertussis toxin-sensitive Go_2, mechanically stimulated 5-HT release is mediated by Gαq that is resistant to pertussis toxin.

The COOH-terminus of Gαq encodes an intracellular domain that interacts with the agonist-occupied, G protein–coupled receptors (34). Because the C termini of Gαq and $G\alpha_{11}$ have considerable sequence homology, the synthetic COOH-terminal peptide does not discriminate between the two. This is not problematic, because the antisense experiments described above targeted a sequence that was unique to Gαq, and therefore they provide strong evidence that the Gαq pathway is involved in mechanically evoked 5-HT release. On the other hand, the purpose of the experiments with the synthetic peptide was to test whether it could disrupt the coupling of the G protein with its receptor by competing with the endogenous domain of Gαq as described for Gαq-coupled α_{1B} adrenergic receptor-mediated signaling (35). The finding that antisense oligonucleotides abolished mechanically evoked 5-HT release coupled with the observation that the COOH-terminal peptide of Gαq also abolished 5-HT release suggests that a ligand-occupied receptor is coupled to Gαq during mechanotransduction in BON cells. Consistent with the conclusion that mechanical stimulation activates a G protein–coupled receptor is the observation in endothelial cells that fluid flow increased mRNA levels of FEB-1 and edg-1, two G protein–coupled receptor genes (36).

All Gα subunits are modified at or near their NH_2-termini by covalent attachment of the fatty acids myristate and/or palmitate that support membrane association and signaling of G proteins (24). Therefore, mechanical stimulation of the phospholipid bilayer could activate G proteins directly without the necessity of an agonist-occupied receptor (23). Indeed, shear stress applied to reconstituted phospholipid liposomes increased GTPase activity of Gαq and $G\alpha i_3$ under conditions when neither receptor proteins nor cytoskeletal proteins were present. While our results cannot completely rule out the possibility that the mechanical stimulus activates the phospholipid bilayer/G protein complex directly, studies with the COOH-terminal peptide suggest that this is unlikely. Since the synthetic peptide that interferes with the coupling of the G protein with the receptor completely abolished 5-HT release in BON cells, there was no residual 5-HT release to attribute to another mechanism.

Although our results rule out the possibility that paracrine substances from other cell types mediate mechanically evoked 5-HT release; they do not eliminate autocrine mediators. Indeed, mechanical stimuli such as shear, tensile, and osmotic stress were shown to increase ATP release, which then can activate P_2 receptors (37–39). It is unknown whether P_2 receptors are present on BON cells, although they have been reported to inhibit 5-HT release from enterochromaffin cells (25).

Regardless of the mechanism, activation of Gαq leads to elevation of intracellular Ca^{2+}, which triggers 5-HT release (25). An increase in intracellular Ca^{2+} due to influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels on the plasma membrane is not essential for mechanically evoked 5-HT release in BON cells, whereas Ca^{2+} mobilization from intracellular stores is a necessity. This conclusion is supported by the finding that 5-HT is still released under Ca^{2+}-free conditions. Furthermore, this conclusion is reinforced by the findings that 5-HT release was inhibited by depletion of intracellular Ca^{2+} stores and buffering free Ca^{2+} in the cytosol. Elevation of intracellular Ca^{2+} concentration has been shown to be due to release from Ca^{2+} stores by an activation of IP_3 receptors (40). In endothelial cells, shear stress generated by fluid flow activated PLCβ, which hydrolyses PIP_2 into IP_3 (41). Phosphoinositide hydrolysis is considered to be mediated by either protein tyrosine phosphorylation of PLCγ or G protein activation of PLCβ (42).

In summary, we have devised a noncytotoxic, mechanical stimulus for releasing 5-HT from BON cells. The results indicate that detection of mechanical stimuli by BON cells involves receptor-Gαq coupling and mobilization of intracellular Ca^{2+}. These novel findings in BON cells as a model for studying mechanotransduction and 5-HT release begin to uncover the cellular and molecular events that transform mechan-

ical forces into biological responses. Drugs targeting the Gαq-coupled signaling pathway to facilitate or inhibit 5-HT release would be important therapeutic interventions in disease states where improper sensation and function of enterochromaffin cells cause symptoms of diarrhea or constipation.

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