Interruption of Homologous Desensitization in Cyclic Guanosine 3',5'-Monophosphate Signaling Restores Colon Cancer Cytostasis by Bacterial Enterotoxins

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Abstract

Bacterial diarrheagenic heat-stable enterotoxins induce colon cancer cell cytostasis by targeting guanylyl cyclase C (GCC) signaling. Anticancer actions of these toxins are mediated by cyclic guanosine 3',5'-monophosphate (cGMP)–dependent influx of Ca²⁺ through cyclic nucleotide-gated channels. However, prolonged stimulation of GCC produces resistance in tumor cells to heat-stable enterotoxin–induced cytostasis. Resistance reflects rapid (tachyphylaxis) and slow (bradyphylaxis) mechanisms of desensitization induced by cGMP. Tachyphylaxis is mediated by cGMP-dependent protein kinase, which limits the conductance of cyclic nucleotide-gated channels, reducing the influx of Ca²⁺ propagating the antiproliferative signal from the membrane to the nucleus. In contrast, bradyphylaxis is mediated by cGMP-dependent allosteric activation of phosphodiesterase 5, which shapes the amplitude and duration of heat-stable enterotoxin–dependent cyclic nucleotide accumulation required for cytostasis. Importantly, interruption of tachyphylaxis and bradyphylaxis restores cancer cell cytostasis induced by heat-stable enterotoxins. Thus, regimens that incorporate cytostatic bacterial enterotoxins and inhibitors of cGMP-mediated desensitization offer a previously unrecognized therapeutic paradigm for treatment and prevention of colorectal cancer. (Cancer Res 2005; 65(23): 11129-35)

Introduction

Colorectal cancer is the third most common, and the second most deadly, cancer in the developed world (1, 2). The mortality rate for colon cancer, 50%, reflects metastatic disease progression (1) and the lack of efficacious adjuvant chemotherapy (3). Indeed, ~20% of patients have unresectable disease at presentation and the majority of patients (>90%) that develop metastases (~33%) do not benefit from current pharmacotherapeutic interventions (1, 3).

Major obstacles to the development of effective therapeutic regimens include the genetic and phenotypic heterogeneity of colorectal tumors (4) and the emergence of drug-induced adaptive escape mechanisms in cancer cells (5). Thus, individualized therapy, more effective molecular targets, and strategies to circumvent drug resistance are paramount for future therapeutic paradigms for colon cancer (6, 7).

Bacterial heat-stable enterotoxins induce secretory diarrhea in endemic populations, travelers, and animal herds (8) by serving as superagonists for the intestine-specific receptor guanylyl cyclase C (GCC; ref. 9). Heat-stable enterotoxins, which have evolved to facilitate bacterial dissemination and propagation, exemplify molecular mimicry of the endogenous hormones guanylin and uroguanylin, which mediate autocrine/paracrine control of intestinal fluid and electrolyte homeostasis by activating GCC and inducing cyclic guanosine 3',5'-monophosphate (cGMP)–dependent chloride efflux through the cystic fibrosis regulator channel (10–12).

Intriguingly, longitudinal exposure to heat-stable enterotoxin–producing bacteria seems to protect endemic populations against colon cancer by reducing rates of enterocyte proliferation (13) and intestinal tumorigenesis (14). In contrast to fluid and electrolyte secretion, regulation of intestinal cell proliferation by heat-stable enterotoxin–induced activation of GCC consists of cGMP-dependent stimulation of Ca²⁺ entry through cyclic nucleotide-gated (CNG) channels (13).

Heat-stable enterotoxin–induced Ca²⁺ currents, in turn, are coupled to suppression of DNA synthesis (13) and tumor cell cytostasis (15).

Normally, GCC is selectively expressed in apical membranes of enterocytes, “outside” mucosal cell tight junctions, and inaccessible to the systemic vascular compartment (10, 16, 17). Intestinal epithelial cells that have undergone neoplastic transformation overexpress functionally competent GCC (18) displayed on their surface during metastatic dissemination to extraintestinal tissues, making it paradoxically accessible to the systemic vascular compartment (19, 20).

Indeed, GCC represents a unique target for selectively delivering imaging and therapeutic agents to metastatic colorectal tumors in vivo (16, 17). Moreover, GCC agonists have been proposed as novel cytostatic agents for targeted therapy for colorectal cancer metastases (13). However, receptor desensitization (21–23) and activation of phosphodiesterases (21, 24) represent mechanisms by which colorectal cancer cells could develop resistance to cGMP-dependent cytostasis, limiting the therapeutic potential of GCC ligands.

The present study reveals the previously unrecognized emergence of homologous desensitization of GCC-mediated cell cycle regulation in human colon cancer cells. Thus, elevations in intracellular cGMP ([cGMP]ₖ) induce rapid (tachyphylaxis) and slow (bradyphylaxis) mechanisms of desensitization, imposed by the integrated regulation of discreet cGMP-dependent effectors, which prevent GCC-mediated cytostasis. Importantly, interruption of the molecular mechanisms underlying tachyphylaxis and bradyphylaxis permits sustained inhibition of cancer cell proliferation by GCC ligands without the development of escape or resistance.
Materials and Methods

Reagents. Eagle’s MEM (EMEM), Ca\(^{2+}\)-free minimal essential medium (S-MEM), t-glutamine, and other reagents for cell culture were obtained from Life Technologies, Inc. (Rockville, MD). Fetal bovine serum (FBS) and the DMEM/F12 were from Mediatech, Inc. (Herndon, VA). Native heat-stable enterotoxin was prepared as described (15). \(^{45}\)Ca\(^{2+}\) (24 mCi/mL) was purchased from ICN Biochemicals, Inc. (Costa Mesa, CA), [methyl-\(^{3}H\)] Thymidine (1 mCi/mL) was obtained from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). RP8P-CPT-cGMPs was from Biolog Biochemicals (San Diego, CA), whereas Zaprinast, 8-br-cGMP, milrinone, propidium iodide, and all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell culture. T84 (passages 40-60) human colon carcinoma cells (American Type Culture Collection, Manassas, VA) were maintained at 37°C (5% CO\(_2\)) in DMEM/F12 containing 2.5 mmol/L t-glutamine, 100 IU/mL penicillin, 100 \(\mu\)g/mL streptomycin, and 10% FBS. Cells were fed every third day and split when subconfluent.

Cell proliferation. Proliferation of cancer cells was quantified in 96-well plates by [methyl-\(^{3}H\)] thymidine (0.2 \(\mu\)Ci/well) incorporation into DNA (15). Cells were pulse-labeled (3 hours) with [\(^{3}H\)] thymidine at the end of respective checkpoints. For studies examining cancer cell cytostasis, ~50,000 cells per well were plated, permitted to recover for 6 hours, and synchronized for 18 hours with FBS-free DMEM. Then, cells were stimulated to proliferate (up to 34 hours) by adding 10% FBS, in the presence of the indicated treatments. For studies examining acute regulation of DNA synthesis, exponentially growing cancer cells (~60% confluent) were synchronized by FBS starvation in EMEM for 48 hours followed by stimulation of DNA synthesis with 10 mmol/L t-glutamine (in EMEM, for ~24 hours). Where appropriate, pretreatments with heat-stable enterotoxins and/or other agents were done during the stimulation period and terminated by washing cells with EMEM (37°C, thrice) at the 20th hour following glutamine addition. Then, treatments in t-glutamine-containing EMEM were added to cells ~15 minutes before [methyl-\(^{3}H\)] thymidine for quantification of effects on DNA synthesis (13, 15). Unless otherwise indicated, effects on DNA synthesis were expressed as the percentage of parallel control cultures employing the corresponding vehicle.

Flow cytometry. T84 cells (~10\(^6\) per well) were plated in six-well plates, permitted to recover for 6 hours, and synchronized for 18 hours by serum starvation. Then, cell proliferation was induced with 10% FBS for 24 hours, in the presence of the indicated treatments. Following incubations, cells were collected by trypsinization, washed with PBS (three times with ice-cold ethanol (75%), and stained with a propidium iodide–containing solution (50 \(\mu\)g/mL propidium iodide, 100 \(\mu\)g/mL RNase A, 1 mmol/L EDTA, and 0.1% Triton X-100). Quantification of the percentage of cells in each phase of the cell cycle was done using a Coulter EPICS XL-MCL flow cytometer (15).

Cyclic guanosine 3',5'-monophosphate. Studies were done in exponentially growing T84 cells (~60% confluent), and cGMP was determined by RIA (19). Cells were synchronized, stimulated with 10 mmol/L t-glutamine in EMEM, and pretreated where appropriate as described for studies examining DNA synthesis. Then, for total cellular cGMP accumulation, cells in 96-well plates were washed ( thrice) and incubated for 15 minutes in EMEM at 37°C, in the presence of the indicated treatment. Reactions were terminated with ice-cold 100% ethanol, and supernatants separated from pellets by centrifugation and processed for cGMP determinations. In contrast, for guanylyl cyclase activity, cells in T-75 flasks were collected by scraping into 1 mL of TEED buffer [50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 1 mmol/L DTT, and 1 mmol/L phenylmethylsulfonyl fluoride], homogenized on ice by aspiration through a series of narrow gauge (20-25 gauge) needles, and centrifuged at 1,000 \(\times\) g (4°C) for 5 minutes. Supernatants were further centrifuged at 100,000 \(\times\) g (4°C) for 60 minutes to produce a pellet, which was resuspended in TEED at 1 mg protein/mL. Membrane proteins (20 \(\mu\)g) were incubated for 5 minutes (37°C) in 100 \(\mu\)L of 50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L isobutylmethylyxanthine, 15 mmol/L creatine phosphate, 2.7 units of creatine phosphokinase, 4 mmol/L MgCl\(_2\), 1 mmol/L GTP in the presence of the indicated treatment. Enzyme reactions were terminated by addition of 50 mmol/L sodium acetate (pH 4.0) followed by boiling for 3 minutes. cGMP production was linear with respect to time and protein concentration.

Reverse transcription-PCR. Total RNA (1 \(\mu\)g) from human retina (positive control, purchased from BD Biosciences, Franklin Lakes, NJ) and T84 cells (obtained with the Quagen RNA Easy kit, Quagen, Valencia, CA) underwent reverse transcription with Superscript II (Life Technologies, Gaithersburg, MD), and resultant oligo(dcthytymidine)\(_{18}\)-primed cDNAs were subjected to PCR for 35 cycles (94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds) using Taq DNA polymerase (Applied Biosystems, Foster City, CA). CGN11 channel mRNA was detected with sense (5'-TCTGGAGGATGATGACAGTGCC-3') and antisense (5'-CAGG-TACTGCTCCTCTGTGAT-3') primers designed to amplify a product of ~123 bp. PCR products were subjected to electrophoresis on 2% agarose gels in Tris-borate EDTA buffer (90 mmol/mL Tris, 2 mmol/mL EDTA (pH 8.3)) containing ethidium bromide and visualized by transillumination. Template-negative controls were run in each PCR experiment.

Calcium transport. Exponentially growing T84 cells (~60% confluent in 24-well plates) were incubated in S-MEM containing low (300 \(\mu\)mol/L) CaCl\(_2\) (13). Cells were pretreated with heat-stable enterotoxins for the indicated times followed by washing (37°C, thrice) with incubation medium. Then, unidirectional \(^{45}\)Ca\(^{2+}\) fluxes induced by heat-stable enterotoxins (1 mmol/L, 20 minutes) were quantified (13).

Statistics. Reverse transcription-PCR experiments were done in duplicate and repeated twice. Studies of calcium transport were done in duplicate and expressed as mean ± SE of four separate experiments. All other determinations were done in triplicate and repeated at least thrice. Data are expressed as the mean ± SE of a representative experiment done in triplicate. Statistical analysis was done employing the unpaired two-tailed Student’s t test, and significance was assumed for \(P < 0.05\).

Results

Human colon carcinoma cells develop resistance to heat-stable enterotoxin–induced cytostasis. Heat-stable enterotoxins delayed cell cycle progression ~40% (quantified by double reciprocal analysis) in human colon cancer cells (Fig. 1A), in the absence of checkpoint arrest or apoptosis (Fig. 1B; ref. 15). Cell cycle delay reflected an acute effect that underwent desensitization, because suppression of DNA synthesis was lost following chronic exposure to heat-stable enterotoxins (Fig. 1C). Desensitization of the antiproliferative effects of heat-stable enterotoxins was associated with a reduced ability of that enterotoxin to elevate [cGMP], (Fig. 1D), which mediates cytostasis produced by GCC (13, 15). Moreover, pharmacologic inhibition of phosphodiesterase 5 (PDE5), but not PDE3, potentiated heat-stable enterotoxin effects on proliferation by further elevating [cGMP], (Fig. 1D), and restored maximal heat-stable enterotoxin–induced [cGMP], accumulation in desensitized cells (Fig. 1D, arrows). However, suppression of DNA synthesis by heat-stable enterotoxins was only partially rescued by inhibiting PDE5 in desensitized tumor cells (Fig. 1D). Thus, colon cancer cell resistance to the antiproliferative effects of heat-stable enterotoxins reflects a complex mechanism mediated, in part, by PDE5, which shapes GCC signaling by limiting the amplitude of [cGMP], accumulation.

Enterotoxin-induced resistance to cytostasis is mediated by tachyphylaxis and bradyphylaxis. Colon cancer cells developed resistance to the antiproliferative effects of heat-stable enterotoxins as early as 30 minutes following exposure to that ligand (Fig. 2, left). Following complete desensitization, heat-stable enterotoxins induced a paradoxical stimulation of DNA synthesis, which peaked after 3 hours of exposure to the enterotoxin (Fig. 2, left). Moreover, mechanisms of cancer cell adaptation were completely reversible within 9 hours following withdrawal of heat-stable enterotoxins (Fig. 2, left). These temporal kinetics of...
desensitization and recovery of the antiproliferative effects of heat-
stable enterotoxins are nearly identical to those of induction and
decline of PDE5 activity mediated by GCC and cGMP signaling
(21, 25). Of importance, linear regression analysis of the onset
of cellular adaptation revealed two principle components character-
ized by rapid (tachyphylaxis) and slow (bradyphylaxis) rates of
desensitization (Fig. 2, right).

**Bradyphylaxis to heat-stable enterotoxin–induced cytostasis
is mediated by PDE5.** Bradyphylaxis was characterized by a
paradoxical stimulation of DNA synthesis (Fig. 2) associated with a
similarly paradoxical decrease in [cGMP], accumulation induced by
heat-stable enterotoxins (Fig. 3A). Inhibition of PDE5, but not
PDE3, prevented paradoxical stimulation of DNA synthesis
(Fig. 3B) by restoring maximal heat-stable enterotoxin–induced
[cGMP], accumulation (Fig. 1D, arrows). In that context, cGMP
induces durable allosteric activation of PDE5 (26, 27), which
could mediate paradoxical enterotoxin-dependent stimulation of DNA
synthesis (Fig. 3B) and decreases in [cGMP], accumulation (Fig. 3A)
following chronic stimulation of GCC by heat-stable enterotoxins.
Indeed, a membrane-permeant phosphodiesterase-resistant ana-
logue of cGMP, 8-br-cGMP (28), which mimicked heat-stable
enterotoxin–induced cytostasis and desensitization in colon cancer
cells (Fig. 3C), failed to induce paradoxical stimulation of DNA
synthesis in heat-stable enterotoxin– or 8-br-cGMP-desensitized
cells (Fig. 3C, compare with the respective PBS condition). In
addition, 8-br-cGMP was equiefficacious with the combination of
heat-stable enterotoxins and a PDE5 inhibitor in partially reversing
heat-stable enterotoxin–induced desensitization (Fig. 3C). Impor-
tantly, cancer cells desensitized with 8-br-cGMP, a poor allosteric
activator of PDE5 (29), did not exhibit paradoxical stimulation of
DNA synthesis upon heat-stable enterotoxin exposure and were
insensitive to the combination of heat-stable enterotoxin and a
PDE5 inhibitor (Fig. 3C). It is noteworthy that, although heat-stable
enterotoxins failed to maximally stimulate GCC activity over
long durations (Fig. 4A), receptor down-regulation (30) and/or
decreased cGMP synthetic rates (21) did not substantially
contribute to heat-stable enterotoxin resistance in intact cells,
because inhibition of PDE5 activity fully prevented bradyphylaxis
(Fig. 3B). Thus, PDE5 largely mediates bradyphylaxis to heat-stable
enterotoxin–dependent cytostasis in colon cancer cells by up-
regulation of its catalytic activity induced by chronic cGMP
stimulation (25–27).

**Tachyphylaxis to heat-stable enterotoxin–induced cytostasis
is mediated by PKG.** Tachyphylaxis contributed ~50% of the
desensitization to heat-stable enterotoxin–induced cytostasis
(y intercepts of extrapolated fitted curves, Fig. 2, right). Rapid
desensitization also was mediated by cGMP (Fig. 3C) but reflected
a mechanism other than synthesis or degradation of cGMP by GCC
(Fig. 4A) or PDE5 (Fig. 1D), respectively. Thus, tachyphylaxis was
induced by the phosphodiesterase-resistant 8-br-cGMP (28), which
increased [cGMP], independently of GCC without inducing
bradyphylaxis by PDE5 (Fig. 3C). Ca++ entry through CNG channels
expressed in colorectal cancer cells (ref. 31; Fig. 4B, top) mediated
heat-stable enterotoxin–induced cytostasis (Fig. 4B, bottom; ref. 13).
Although [cGMP], remained stable, this Ca++ current was
attenuated over the time course of tachyphylaxis (Fig. 4C) and
could be fully restored by inhibiting PKG but not PDE5 (Fig. 4D).
Diminished Ca++ entry through CNG channels mediated tachy-
phylaxis, because protection of the cytostatic Ca++ current by
inhibiting PKG, but not PDE5, prevented acute desensitization to
the antiproliferative effects of heat-stable enterotoxins (Fig. 4D).
Importantly, tachyphylaxis and the associated attenuation of Ca++
entry were not influenced by removal of extracellular Ca++
(Fig. 4D), indicating that PKG-induced desensitization is not
dependent on mechanisms downstream of the tertiary messenger

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**Figure 1.** A, synchronized T84 cells were stimulated to proliferate with
10% FBS for the indicated times in the presence of vehicle (PBS, □) or
heat-stable enterotoxins (ST, 1 μmol/L, inducing maximal inhibition
of cancer cell proliferation; ref. 15). Cells were pulse-labeled (3 hours) with
[methyl-3H]thymidine at the end of incubations. Percentage of the maximal
thymidine incorporation induced by serum (PBS, 24 hours). B, cell cycle
distribution by flow cytometry (see Materials and Methods) of T84 cells treated
for 24 hours with PBS or heat-stable enterotoxins (1 μmol/L). C, DNA synthesis
of synchronized T84 cells was stimulated for 20 hours by L-glutamine in the
presence of PBS or heat-stable enterotoxins (1 μmol/L). Cells were then
washed and exposed to heat-stable enterotoxins (1 μmol/L) in L-glutamine-containing
medium. After 15 minutes, [methyl-3H]thymidine was added and incubations
were continued for an additional 3 hours. D, PBS or heat-stable enterotoxins
(1 μmol/L) pretreatments and inhibition of DNA synthesis by 1 μmol/L heat-stable
enterotoxins was assessed as in C. In parallel incubations, total cellular cGMP
accumulation was determined by stimulating cells for 15 minutes with heat-stable
enterotoxins (1 μmol/L) following pretreatments. Inhibitors of cGMP-regulated,
cAMP-specific PDE3 [milrinone (MLR), 10 μmol/L], and cGMP-regulated,
cGMP-specific PDE5 [zaprinast (ZAP), 10 μmol/L] were added 15 minutes
before the final application of heat-stable enterotoxins to completely inhibit the
respective targeted phosphodiesterase (25, 49).

**Figure 2.** Heat-stable enterotoxins (ST, 1 μmol/L) effects on DNA synthesis
were determined in T84 cells following pretreatment (1 μmol/L heat-stable
enterotoxins) and washing as indicated (left). [3H]Thymidine incorporation into
DNA was done and measured as described in Materials and Methods. Linear
regression analyses were generated to fit acute (0-1 hours) and chronic
(1-3 hours) onset of desensitization (right). Rates of desensitization (Kdes) were
calculated from the slope of respective lines.
minutes before the final application of heat-stable enterotoxins. Experiments were done as described in Materials and Methods. The PBS control of cells pretreated with 1 μmol/L heat-stable enterotoxins for 20 hours. *, P < 0.05; **, P < 0.01, compared with the PBS control. C, following pretreatment for 20 hours with PBS, 1 μmol/L heat-stable enterotoxins or 5 mmol/L 8-br-cGMP, T84 cells were reexposed to the same reagents 15 minutes before being pulse labeled (3 hours) with [methyl-3H]thymidine to assess DNA synthesis. Where indicated, zaprinast (10 μmol/L) was added 15 minutes before the final application of heat-stable enterotoxins.

(Ca2+) mediating cytostasis by heat-stable enterotoxins (13). Hence, in colon cancer cells, tachyphaxia to heat-stable enterotoxin--induced cytostasis is a negative feedback mechanism mediated by PKG and targeting CNG channels.

Inhibition of tachyphaxia and bradyphaxia restores heat-stable enterotoxin--induced cytostasis in human colon carcinoma cells. Cancer cell resistance to cytostasis induced by heat-stable enterotoxins or 8-br-cGMP was prevented by the combination of two selective pharmacologic inhibitors of PDE5 and PKG, respectively (Fig. 5A). Prevention of enterotoxin-induced desensitization, mediated by tachyphaxia and bradyphaxia, by these agents was additive, reflecting the contribution of both PKG and PDE5 to these mechanisms (Fig. 5A, left). In contrast, the PDE5 inhibitor zaprinast had little effect on, whereas the PKG inhibitor RP8pCPT-cGMPs completely reversed, 8-br-cGMP-induced desensitization, reflecting the dominant contribution of tachyphaxia to this mechanism (Fig. 5A, right). Moreover, combining these inhibitors with heat-stable enterotoxins unmasked durable suppression of tumor cell proliferation (Fig. 5B). These observations show that the cell cycle delay induced by heat-stable enterotoxins (Fig. 1A) reflects the integration of cancer cell adaptation and escape superimposed on cytostasis rather than enduring cell cycle inhibition. Finally, the combination of heat-stable enterotoxins and inhibitors of PDE5 and PKG induced cytostasis but not cell cycle arrest or apoptosis (Fig. 5C), underscoring their specific action in preventing desensitization to the antiproliferative effects of GCC and cGMP (Fig. 1B; ref. 15).

Cytostasis induced by bacterial diarrheagenic heat-stable enterotoxins represents a novel therapeutic approach against colon

Discussion

Current pharmacotherapy for colorectal cancer has limited clinical effect (3), and ~50% of patients die within 5 years from diagnosis as a result of metastatic disease (1). Adjuvant chemotherapy, the best available intervention for colon cancer metastasis, increases median survival only ~14 months (32). One principle mechanism underlying the minimal efficacy of current therapeutic regimens is the acquisition of tumor cell resistance to anticancer drugs (5). For example, the drug of choice in colon cancer, 5-fluorouracil, is ineffective in tumors expressing high levels of dihydroorotid dehydrogenase (33) or thymidylate synthase (34), which increases drug catabolism and decreases drug-induced inhibition of DNA synthesis, respectively. Moreover, tumors frequently adopt opportunistic and nonspecific but highly effective escape mechanisms against anticancer drugs, such as multidrug resistance associated with increased drug efflux following induction of ATP-dependent transporters (35). The varied molecular mechanisms underlying drug resistance underscore the importance of designing strategies to circumvent tumor cell adaptation, which affects even the most recently introduced therapeutically efficacious agents (5, 7).

A, guanylyl cyclase activity was quantified as described in Materials and Methods. Membrane preparations were obtained from T84 cells following incubations with PBS or 1 μmol/L heat-stable enterotoxins (ST). *, P < 0.05, compared with the effect of heat-stable enterotoxins in membranes from cells not preincubated with heat-stable enterotoxins. B, RTPCR for CNG channel (top), DNA synthesis (bottom left), and 45Ca2+ influx (bottom right) were examined as described in Materials and Methods. NTC, nontemplate control. Heat-stable enterotoxins (1 μmol/L) and the specific inhibitor of CNG channels -cis-diltiazem (L-DLT, 200 μmol/L) were employed to examine heat-stable enterotoxin--mediated regulation of antiproliferative 45Ca2+ entry through CNG channels (13). Measurements of 45Ca2+ influx are expressed as % increase over respective controls. Acute desensitization was studied in T84 cells following preincubations with 1 μmol/L heat-stable enterotoxins for 0-60 minutes in C) or 1 μmol/L heat-stable enterotoxins in the presence of the indicated reagents (for 60 minutes in D). DNA synthesis, 45Ca2+ influx, and cGMP accumulation induced by heat-stable enterotoxins (1 μmol/L) were examined in parallel incubations and measured as described in Materials and Methods. cGMP is expressed as the relative fold of respective PBS controls (baselines). Ca2+-free, Ca2+-deficient cell culture media (S-MEM); ZAP, the PDE5 inhibitor zaprinast (10 μmol/L); RP8pCPT, the PKG inhibitor RP8pCPT-cGMPs, employed at 100 μmol/L to completely inhibit PKG (60).
PKG and PDE5 Mediate Resistance to GCC-Induced Cytostasis

enterotoxin-induced cytostasis in tumor cells exposed to heat-stable enterotoxins. In agreement with the present findings, cGMP-dependent PDE5 activation reduced maximum stimulation of GCC by heat-stable enterotoxins in human colon cancer cells (21, 25). Similarly, enhanced activation of PDE5-dependent cGMP hydrolysis was observed in the heart (40) and kidney (41) upon persistent stimulation of cGMP synthesis by natriuretic peptide receptors, particulate guanylyl cyclases homologous to GCC. Moreover, cGMP-stimulated PDE5 opposed chronic nitric oxide stimulation of soluble guanylyl cyclase in platelets and vascular smooth muscle (42, 43). Together, these observations support the suggestion that desensitization, in the form of bradyphylaxis, shapes the amplitude and duration of cGMP responses in all cells coexpressing guanylyl cyclases and PDE5 (27, 43).

In contrast to PDE5 and bradyphylaxis, PKG-mediated tachyphylaxis represents a previously unrecognized mechanism of cellular adaptation to GCC and cGMP signaling. PKG regulated rapid desensitization to heat-stable enterotoxin–induced cytostasis by reducing the amplitude of entry through CNG channels of Ca^{2+} propagating the antiproliferative signal from the membrane to the nucleus. Tachyphylaxis induced by heat-stable enterotoxins was not altered by inhibition of PDE5 or application of 8-br-cGMP, suggesting that cGMP hydrolysis is not central to this process. Rather, 8-br-cGMP, a potent activator of PKG (44), induced tachyphylaxis to acute cGMP signaling. In addition, removal of extracellular Ca^{2+} from the medium did not affect the development of tachyphylaxis to heat-stable enterotoxins, indicating that Ca^{2+} entering through CNG channels and the associated downstream intracellular effectors did not mediate acute desensitization. These observations are particularly relevant, because the influx of extracellular Ca^{2+} through store-operated Ca^{2+} channels, but not through CNG channels, opposes heat-stable enterotoxin–dependent cytostasis by allosterically inhibiting GCC-induced accumulation of [cGMP], in human colon cancer cells (45).

GCC activation by heat-stable enterotoxins engages a previously unrecognized intracellular signaling network regulating cell cycle progression of colon carcinoma cells (Fig. 6). Cyclic GMP-dependent activation of PKG and PDE5 represents one gating mechanism for this network, shaping the duration and amplitude of heat-stable enterotoxin–induced cytostasis. In this model, elevated [cGMP], generated by GCC activation initiates the tumor cell cycle by stimulating Ca^{2+} entry through CNG channels (13). Acutely, cGMP signaling induces PKG-dependent reduction of Ca^{2+} entry through CNG channels, uncoupling cytostasis from [cGMP]. Subsequently, prolonged GCC stimulation induces cGMP-dependent activation of PDE5 (25, 27), which lowers [cGMP], further desensitizing colon cancer cells to heat-stable enterotoxin actions. PKG likely plays a role in tachyphylaxis and bradyphylaxis by regulating Ca^{2+} entry through CNG channels and sensitizing PDE5 to allosteric cGMP stimulation (26, 39). Notably, elimination of negative feedback mechanisms in cGMP signaling with specific pharmacologic inhibitors of PDE5 and PKG completely rescues heat-stable enterotoxin–induced cytostasis.

Mechanisms underlying desensitization to GCC activation described herein may have important implications for intestinal homeostasis. Indeed, the endogenous GCC ligands, guanylin and uroguanylin, are novel regulators of intestinal epithelial cell dynamics. Uroguanylin mimics the effects of heat-stable enterotoxins on intestinal epithelial cell proliferation (15) and inhibits...
References

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Figure 6. Proposed mechanisms underlying negative feedback regulation of heat-stable enterotoxin–induced cytostasis in human colon cancer cells.

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