

# Enterotoxin preconditioning restores calcium-sensing receptor-mediated cytostasis in colon cancer cells

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**Guanylyl cyclase C (GCC), the receptor for diarrheagenic bacterial heat-stable enterotoxins (STs), inhibits colorectal cancer cell proliferation by co-opting  $Ca^{2+}$  as the intracellular messenger. Similarly, extracellular  $Ca^{2+}$  ( $Ca^{2+}_o$ ) opposes proliferation and induces terminal differentiation in intestinal epithelial cells. In that context, human colon cancer cells develop a phenotype characterized by insensitivity to cytostasis imposed by  $Ca^{2+}_o$ . Here, preconditioning with ST, mediated by GCC signaling through cyclic nucleotide-gated channels, restored  $Ca^{2+}_o$ -dependent cytostasis, reflecting posttranscriptional regulation of calcium-sensing receptors (CaRs). ST-induced GCC signaling deployed CaRs to the surface of human colon cancer cells, whereas elimination of GCC signaling in mice nearly abolished CaR expression in enterocytes. Moreover, ST-induced  $Ca^{2+}_o$ -dependent cytostasis was abrogated by CaR-specific antisense oligonucleotides. Importantly, following ST preconditioning, newly expressed CaRs at the cell surface represented tumor cell receptor targets for antiproliferative signaling by CaR agonists. Since expression of the endogenous paracrine hormones for GCC is uniformly lost early in carcinogenesis, these observations offer a mechanistic explanation for the  $Ca^{2+}_o$ -resistant phenotype of colon cancer cells. Restoration of antitumorigenic CaR signaling by GCC ligand replacement therapy represents a previously unrecognized paradigm for the prevention and treatment of human colorectal cancer employing dietary  $Ca^{2+}$  supplementation.**

## Introduction

Colorectal cancer is most prevalent in the developed world, representing the second leading cause of cancer-related mortality (1,2). Although the epidemiology of this disease is poorly understood, there is an unexplained inverse relationship between the worldwide incidence of colorectal cancer and enterotoxigenic *Escherichia coli* infections (3,4). Enterotoxigenic *E.coli* produces heat-stable enterotoxins (STs), exogenous ligands for the intestine-specific receptor guanylyl cyclase C (GCC) (5) and a principle cause of secretory diarrhea in endemic populations and animal herds (6–8). Regions of the world with the highest incidence of enterotoxigenic *E.coli*-associated diarrhea exhibit the lowest incidence of colon cancer (4). In that context, activation of GCC inhibits human colon carcinoma cell proliferation (4,9,10) and adenoma formation in mice (3).

Reduced expression of the endogenous paracrine hormones for GCC, guanylin and uroguanylin, represents an early mutational event in colorectal carcinogenesis (11–13). GCC signaling through its second messenger cGMP promotes fluid and electrolyte secretion (5), opposes cell cycle progression and proliferation (4,9) and regulates migration, differentiation and apoptosis along the crypt–villus axis (14,15). Importantly, targeted GCC deletion ( $GCC^{-/-}$ ) in mice

**Abbreviations:** APC, adenomatous polyposis coli;  $Ca^{2+}_o$ , extracellular  $Ca^{2+}$ ; CaR, calcium-sensing receptor; CNG, cyclic nucleotide-gated; GCC, guanylyl cyclase C; ST, heat-stable enterotoxin.

increased intestinal tumorigenesis induced by the carcinogen azoxymethane or adenomatous polyposis coli (APC) mutations by corrupting homeostatic crypt proliferation and genomic integrity (16). These observations suggest a model in which colorectal cancer is a disease of hormone insufficiency where dysregulation of GCC signaling, following loss of guanylin and uroguanylin, promotes tumorigenesis by disrupting mucosal homeostasis (16). However, beyond production of cGMP, molecular mechanisms by which GCC regulates processes underlying carcinogenesis remain undefined.

Like GCC, extracellular  $Ca^{2+}$  ( $Ca^{2+}_o$ ) opposes proliferation and promotes differentiation of intestinal mucosa cells (17–20). Moreover,  $Ca^{2+}$  supplementation abrogates intestinal hyperproliferation and tumor formation induced by a western-style diet in  $APC^{Min/+}$  mice (21). In part, these antitumorigenic effects may reflect GCC-induced activation of cyclic nucleotide-gated (CNG) channels, inducing cytostasis through  $Ca^{2+}_o$  influx (4). Notably,  $Ca^{2+}_o$  in the colonic lumen may increase to  $\geq 20$  mM that activates calcium-sensing receptors (CaRs) (19,22), G protein-coupled receptors (23) expressed in apical membranes of colonocytes (24), an event resulting in reduced proliferation and tumorigenesis through inhibition of  $\beta$ -catenin/Tcf-4 signaling, and increased cell maturation through p21 and p27 activities (25,26). Conversely,  $Ca^{2+}_o$  supports colorectal cancer cell proliferation by capacitative entry through store-operated  $Ca^{2+}$  channels (27,28), which opposes antiproliferative GCC signaling through CNG and  $Ca^{2+}_o$  entry (28). Thus, opposing mechanisms regulating proliferative balance by  $Ca^{2+}_o$  comprise functional units reciprocally orchestrated in colon cancer cells (28).

Here, the functional relationship between GCC and  $Ca^{2+}_o$  was explored in human colon carcinoma cells preconditioned with ST, in the context of a dynamic range of  $Ca^{2+}_o$ , and in  $GCC^{-/-}$  mice. These studies revealed a previously unappreciated role for GCC in regulating CaR signaling, offering a novel paradigm for the prevention and treatment of human colorectal cancer employing hormone replacement therapy with GCC ligands in combination with oral  $Ca^{2+}$  supplementation.

## Materials and methods

### Tumor cell proliferation

Proliferation of cancer cells was quantified in 96 wells per plate by [methyl- $^3H$ ]thymidine (0.2  $\mu$ Ci/well) incorporation into DNA (9). Cells were pulse labeled (3 h) with  $^3H$ -thymidine at the end of 24 h periods of proliferation induced by 10 mM L-glutamine. Following  $^3H$ -thymidine labeling, media was aspirated, cells were incubated for 15 min with ice-cold 10% trichloroacetic acid and rinsed sequentially with 10% trichloroacetic acid and 100% methanol. The acid-insoluble material containing  $^3H$ -labeled DNA was solubilized in 100  $\mu$ l of 0.2 N NaOH, 80  $\mu$ l aliquots were dissolved in 1 ml ScintiVerse and radioactivity quantified in a Packard  $\beta$ -scintillation spectrometer. All experiments were conducted on exponentially growing tumor cells.

### Tumor cell toxicity

Cytotoxicity, including occurrence of apoptosis or necrosis, was assessed by flow cytometry (9). Cancer cells ( $\sim 10^6$  cells per well in six-well plates) were treated (24 h) with the indicated reagents. Then, cells were placed in suspension by trypsinization, pelleted by centrifugation, washed with phosphate-buffered saline and fixed in ice-cold 75% ethanol (30 min). After another wash with phosphate-buffered saline, cells were resuspended in the staining solution (50  $\mu$ g/ml propidium iodide, 100  $\mu$ g/ml RNase A, 1 mM ethylenediaminetetraacetic acid and 0.1% Triton X-100) and analyzed on a Coulter EPICS XL-MCL flow cytometer. Distribution in different phases of the cell cycle was analyzed using WinMDI software (version 2.8) provided by Joseph Trotter, Scripps Research Institute (La Jolla, CA). Twenty thousand cells, cleared from doublets, were analyzed from each sample.

### Cyclic GMP assay

GCC-induced intracellular cGMP accumulation was assessed after treating cancer cells in triplicate with ST (15 min in six well per plate) employing

Eagle's minimal essential medium supplemented with 2 mM L-glutamine. Reactions were terminated by adding ice-cold 100% ethanol, each well was washed twice with ice-cold 100% ethanol and supernates separated from pellets by centrifugation (12 000g, 15 min at 4°C). Supernates containing cGMP were evaporated in a Savant SVC-100H concentrator (Thermo Electron Corporation, Waltham, MA) and reconstituted with 50 mM sodium acetate (pH 4.0), and cGMP was quantified in each sample in triplicate by radioimmunoassay (4).

#### Immunoblot analysis

Proteins from total cell lysates, cytosol or membrane extracts (28) prepared in sodium dodecyl sulfate sample buffer were separated by electrophoresis on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred on nitrocellulose membranes and then probed with rabbit polyclonal antibodies directed against CaR (dilution 1:1000) or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000) or with goat anti-villin antibody [Villin(C-19); 1:1000] in Tris-buffered saline–Tween (5% milk) overnight at 4°C. Then, membranes were probed with horseradish peroxidase-conjugated secondary antibody (dilution 1:5000) for 1 h at room temperature, and specific bands were visualized employing West Pico Chemiluminescent Substrate and subjected to densitometry. Immunoblots, performed under reducing conditions, exhibited only one specific band of ~125 kDa corresponding to the monomer form of CaR.

#### Immunostaining analyses

For immunohistochemistry, tumor cells were washed two times with cold phosphate-buffered saline and immediately fixed (30 min in 4% paraformaldehyde) at room temperature followed by quenching with 3% H<sub>2</sub>O<sub>2</sub>. CaR or CD104 at the cell surface was visualized employing (overnight at 4°C) rabbit anti-CaR (1:100) or mouse monoclonal anti-human CD104 (1:100), respectively, and the Histostain-plus kit. For immunofluorescence, GCC<sup>-/-</sup> mice, generated by neomycin-resistant gene insertion on an I-129 background (29) and backcrossed with a C57BL/6 strain for seven or more generations, were employed. All animals were treated in compliance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and under a protocol approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. GCC<sup>+/+</sup> and GCC<sup>-/-</sup> littermates (males 2 months old; *N*, three per genotype) were killed, the intestine collected, divided into anatomically comparable segments and processed for paraffin-based archiving. Paraffin-embedded tissue sections (5 μm) were rehydrated, heated (two times for 5 min in citrate buffer, pH 6) for antigen retrieval, incubated overnight (4°C) with rabbit anti-CaR (1:100) or goat anti-villin (1:50) followed by incubation for 30 min (room temperature) with Alexa fluor 555 anti-rabbit IgG (for CaR) or Alexa fluor 488 anti-goat IgG (for villin). Digital images were acquired by computers attached to a light microscope or a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany).

#### Cell transductions

A 5' 327 bp CaR fragment containing 8 bp of upstream DNA was cloned into MSCV-puro retroviral vectors in both sense and antisense orientations (30), confirmed by DNA sequencing. Antisense, sense and empty vectors were transfected along with the packaging vector pCL Amphi into HEK 293 cells employing Eugene. Viral supernatants harvested at 48 and 72 h after transfection were used to transduce T84 cells (for 72 h). Then, tumor cells stably transduced with CaR antisense, CaR sense or empty vector were selected employing 5 μg/ml puromycin in Dulbecco's modified Eagle's medium/F12.

#### Statistical analysis

Data are expressed as the mean ± SEM of a representative of at least three experiments performed in triplicate. Data were analyzed employing the unpaired two-tailed Student's *t*-test, and significance was assumed for *P* < 0.05.

## Results

### Colorectal cancer cells are insensitive to Ca<sup>2+</sup><sub>o</sub>-dependent cytostasis

Whereas intestinal epithelial cells along the crypt–villus axis transition from proliferation to differentiation at luminal Ca<sup>2+</sup><sub>o</sub> ~2–3 mM (22), neoplastic transformation progressively desensitizes these cells to the cytostatic effects of Ca<sup>2+</sup><sub>o</sub> (20), with ultimate loss of Ca<sup>2+</sup><sub>o</sub>-induced cell cycle arrest (31). Accordingly, Ca<sup>2+</sup><sub>o</sub> induced cytostasis (Figure 1A), but not cytotoxicity (Figure 1B), in moderately and well-differentiated colon cancer cells. However, Ca<sup>2+</sup><sub>o</sub> failed to induce cell cycle arrest and a substantial proliferating cell population (~20–30%) persisted at 50 mM Ca<sup>2+</sup><sub>o</sub> (Figure 1A), consistent with a phenotype of insensitivity to proliferative restriction by Ca<sup>2+</sup><sub>o</sub> in human colon cancer cells.

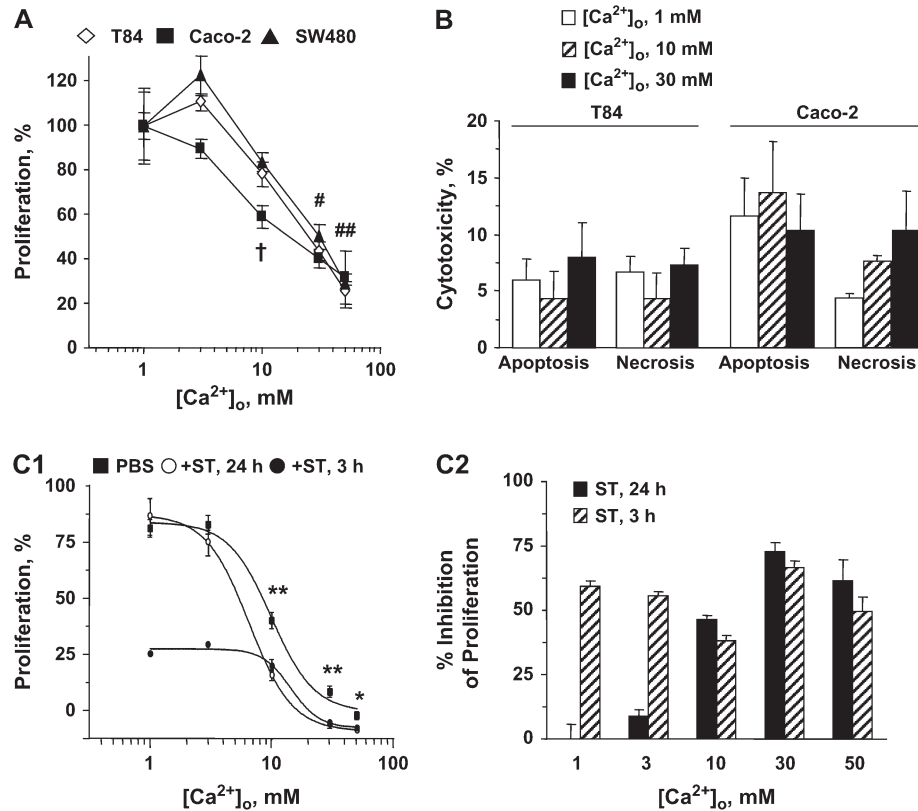
### ST potentiates Ca<sup>2+</sup><sub>o</sub>-dependent cytostasis by inducing GCC signaling through CNG channels

Acute (3 h) ST exposure, which restricts cell cycle progression of colon cancer cells by activating GCC signaling (4,9), induced cytostasis that was additive with Ca<sup>2+</sup><sub>o</sub> (Figure 1C1). Indeed, cytostasis induced by acute ST exposure was not altered by Ca<sup>2+</sup><sub>o</sub> over a range of 1–50 mM (Figure 1C2), suggesting that the antiproliferative effects of ST and Ca<sup>2+</sup><sub>o</sub> are mediated by distinct, non-interfering mechanisms. In contrast, ST preconditioning (24 h), which induces desensitization to GCC-dependent cytostasis in colon cancer cells (10), potentiated the inhibition of proliferation by Ca<sup>2+</sup><sub>o</sub> (Figure 1C1). Thus, ST synergistically increased the potency (IC<sub>50</sub>: Ca<sup>2+</sup><sub>o</sub> alone, 9.91 ± 1.09 mM; ST plus Ca<sup>2+</sup><sub>o</sub>, 6.49 ± 1.13 mM) and efficacy (mean percentage of proliferation: Ca<sup>2+</sup><sub>o</sub> alone, 61.30 ± 1.84; ST plus Ca<sup>2+</sup><sub>o</sub>, 49.47 ± 2.14) of Ca<sup>2+</sup><sub>o</sub> to inhibit colon cancer cell proliferation (*P* < 0.001; Figure 1C1). Importantly, while failing to inhibit cell proliferation, reflecting desensitization to cGMP signaling (10) (see conditions at 1 mM Ca<sup>2+</sup><sub>o</sub> in Figure 1C1), ST preconditioning revealed a novel antiproliferative mechanism that required ≥3 mM Ca<sup>2+</sup><sub>o</sub> (Figure 1C2).

Potential of Ca<sup>2+</sup><sub>o</sub>-induced cytostasis by ST preconditioning was mediated by GCC since ST, but not an inactive analog, inhibited (IC<sub>50</sub>, 62.22 ± 3.21 nM; Figure 2A) proliferation of human colon carcinoma cells expressing GCC, but not of cells lacking GCC (32) (Figure 2B). Similarly, the effects of ST preconditioning were greater in T84 cells, which exhibits the highest GCC expression (32), compared with Caco-2 cells (Figure 2B). Further, two membrane-permeant cGMP analogs mimicked (Figure 2B), whereas an inhibitor of cyclic nucleotide-hydrolyzing phosphodiesterases enhanced (Figure 2C), the effects of ST preconditioning on tumor cell proliferation. Conversely, two inhibitors of CNG channels and an intracellular Ca<sup>2+</sup> chelator, but not inhibitors of cGMP- or cAMP-dependent protein kinase, blocked ST-mediated potentiation of Ca<sup>2+</sup><sub>o</sub>-dependent cytostasis (Figure 2C). Notably, ST did not induce cytotoxicity in colon cancer cells exposed to high Ca<sup>2+</sup><sub>o</sub> (Figure 2D). Rather, ST delayed the progression of these tumor cells through the cell cycle (Figure 2D), consistent with the notion that GCC agonists are cytostatic agents for colon cancer (4,9,10). Together, these observations suggest that in colon cancer cells induction of Ca<sup>2+</sup><sub>o</sub>-dependent cytostasis by ST preconditioning is mediated by cGMP-dependent activation of CNG channels and Ca<sup>2+</sup><sub>o</sub> entry, the same effector mechanism mediating GCC-induced cell cycle delay (4).

### GCC regulates the function of CaRs in normal and malignant colonocytes

Increased antiproliferative effects of Ca<sup>2+</sup><sub>o</sub> following ST preconditioning did not reflect enhanced GCC-dependent cytostasis by Ca<sup>2+</sup><sub>o</sub> because increased Ca<sup>2+</sup><sub>o</sub> did not enhance cytostasis induced by GCC signaling (+ST for 3 h in Figure 1C2) and did not prevent GCC-mediated desensitization in cGMP signaling (10) (Figure 3A). Rather, ST preconditioning enhanced Ca<sup>2+</sup><sub>o</sub>-dependent cytostasis by coupling increased Ca<sup>2+</sup><sub>o</sub> with intracellular signaling mechanisms induced by cGMP signaling (Figure 2A–C). Ca<sup>2+</sup><sub>o</sub> induces cytostasis in colonocytes, in part, by activating CaR signaling (22), a mechanism typically lost during neoplastic transformation (26,33). ST preconditioning (≥3 h) augmented the complement of total cellular (supplementary Figure 1A is available at *Carcinogenesis* Online) and membrane-bound CaRs (Figure 3B) without significantly altering cytosolic CaRs (Figure 3B) or CaR messenger RNA levels (data not shown). Further, examination of specific cell fractions (supplementary Figure 1B is available at *Carcinogenesis* Online) revealed that ST treatment (3 h) induces ~80-fold increase of CaR at tumor cell membranes (supplementary Figure 1C is available at *Carcinogenesis* Online), reflecting translocation of ~60% of the total complement of CaR protein in cancer cells from the cytosol to the membrane compartment (supplementary Figure 1D is available at *Carcinogenesis* Online). Importantly, GCC signal deprivation, either in ligand-free tumor cells (Figure 4, *in vitro* panels on the left showing CaR surface staining



**Fig. 1.** Bacterial enterotoxin ST potentiates  $\text{Ca}^{2+}_o$ -mediated cytotaxis in colorectal cancer cells. (A) Dose responses of cytotaxis by  $\text{Ca}^{2+}_o$  in various human colon carcinoma cells.  $^\dagger P < 0.01$  versus control for Caco-2 cells;  $^\# P < 0.05$  and  $^\#\# P < 0.01$  versus respective controls in each of the three cell lines. (B) Absence of cytotoxicity by  $\text{Ca}^{2+}_o$  in human colon cancer cells. Cytotoxicity was assessed as the proportion of tumor cells identified by flow cytometry in the sub- $G_1$  fractions, which correspond to cells undergoing apoptosis or necrosis, of the cell cycle. Data are mean  $\pm$  SEM from three independent experiments. (C1) Concentration dependence of T84 cell proliferation on  $\text{Ca}^{2+}_o$  in the presence or absence of 1  $\mu\text{M}$  ST. Results in (A) and (C1) are expressed as the percentage of respective control incubations treated with 1 mM  $\text{Ca}^{2+}_o$ .  $^* P < 0.05$  and  $^{**} P < 0.01$ , comparing respective conditions treated with  $\text{Ca}^{2+}_o$  alone and  $\text{Ca}^{2+}_o$  plus ST for 24 h. In (C2), data obtained in (C1) are expressed as  $\{100 - [(\text{condition treated with ST plus } \text{Ca}^{2+}_o)/(\text{respective control condition treated with } \text{Ca}^{2+}_o \text{ alone}) \times 100]\}$ .

of T84 cells) or in enterocytes of  $\text{GCC}^{-/-}$  mice (Figure 4, *in vivo* panels on the right), reduced CaRs in cell membranes. Induction of GCC signaling with bacterial enterotoxin in tumor cells or endogenous paracrine hormonal circuitry in enterocytes of  $\text{GCC}^{+/+}$  mice, in turn, increased the complement of CaRs at the cell surface (Figure 4). Effects of ST preconditioning on CaR staining at the surface of cancer cells were selective (supplementary Figure 2 is available at *Carcinogenesis Online*), independent of elevated  $\text{Ca}^{2+}_o$  but dependent upon cGMP signaling through CNG (Figure 4). Moreover, compared with  $\text{GCC}^{+/+}$  mice, CaR expression in brush-border membranes of  $\text{GCC}^{-/-}$  enterocytes was uniformly attenuated along the entire rostrocaudal axis of the intestine in a GCC-specific fashion since the expression of a marker for absorptive cells, villin, remained unchanged in the same anatomical locations (Figure 4, *in vivo* panels showing representative sections from the jejunum). Thus, GCC signaling physiologically regulates posttranscriptional expression of CaRs in enterocytes, and ST preconditioning induces cell surface translocation of CaR receptors in human colon cancer cells.

*ST preconditioning, with restoration of CaR signaling, is a novel cytotaxic therapeutic strategy in colon cancer cells*

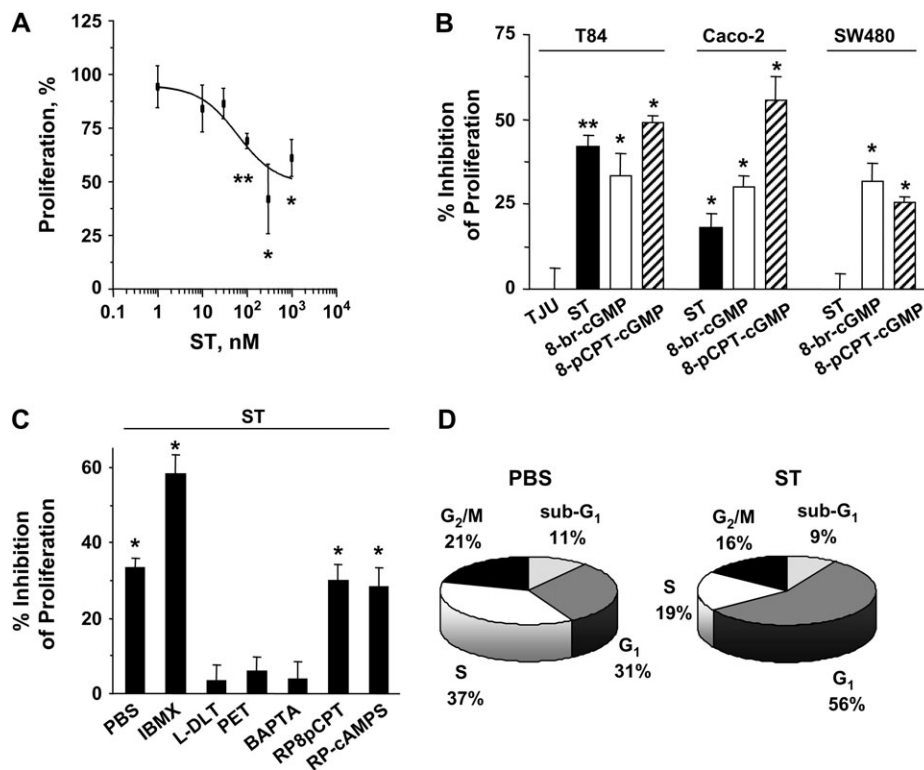
To explore whether posttranscriptional regulation of CaRs mediates  $\text{Ca}^{2+}_o$ -dependent antiproliferation induced by ST preconditioning, CaR expression in colon cancer cells was eliminated employing specific antisense constructs (30) (supplementary Figure 3 is available at *Carcinogenesis Online*;  $\sim 70\%$  inhibition of CaR expression by densitometry, normalized to the respective GAPDH, in CaR antisense-transfected cells compared with CaR sense controls). Whereas the

response of tumor cells to  $\text{Ca}^{2+}_o$  was not altered by CaR antisense (Figure 5A), reflecting lack of effective CaR signaling in colon cancer cells, CaR antisense eliminated the response to  $\text{Ca}^{2+}_o$  of cancer cells preconditioned with ST (Figure 5B). Further, ST preconditioning of tumor cells produced a novel sensitivity of DNA synthesis to acute inhibition by 10 mM, but not 1 mM,  $\text{Ca}^{2+}_o$  (supplementary Figure 4 is available at *Carcinogenesis Online*), consistent with membrane translocation of low-affinity CaRs that typically sense 3–10 mM  $\text{Ca}^{2+}_o$  (34). Moreover, beyond  $\text{Ca}^{2+}_o$ , other CaR agonists, including  $\text{Mg}^{2+}$ ,  $\text{Gd}^{3+}$  and spermine, selectively inhibited proliferation of cancer cells preconditioned by ST (Figure 5C). Thus, ST preconditioning restores the sensitivity of human colon cancer cells to  $\text{Ca}^{2+}_o$ -dependent antiproliferation through CaRs, suggesting GCC-induced CaRs as previously unrecognized therapeutic targets for cytotaxic strategies in patients with colorectal cancer.

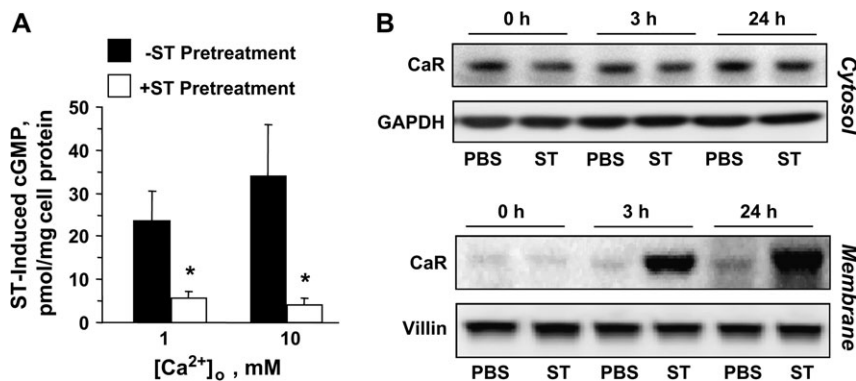
## Discussion

Enterotoxins STs (18 amino acid long) and endogenous paracrine hormones guanylin and uroguanylin (15/16 amino acid long) are structurally homologous peptides (35,36), which specifically bind to and activate GCC at enterocyte brush-border membranes inducing cGMP-dependent protein kinase-dependent ion channel currents (36). In this way, endogenous hormones regulate body fluid homeostasis, whereas STs, principle diarrheagenic agents maximally activating that pathway, permit enterotoxigenic bacteria to propagate in the environment (7,36–38). Of significance,  $\text{GCC}^{-/-}$  mice exhibit increased proliferation, migration and apoptosis and decreased





**Fig. 2.** ST potentiates  $\text{Ca}^{2+}_o$ -mediated cytoostasis by inducing GCC signaling through CNG. (A) Dose response of ST-induced potentiation of antiproliferation by  $\text{Ca}^{2+}_o$  in T84 cells. (B) ST (1  $\mu\text{M}$ ) actions on  $\text{Ca}^{2+}_o$  are mediated by GCC and cGMP in colorectal cancer cells. TJU, 1  $\mu\text{M}$  of the inactive ST analog Thomas Jefferson University 1–103; 8-br-cGMP (5 mM) and 8-pCPT-cGMP (0.5 mM), membrane-permeant cGMP analogs. (C) CNG channel and  $\text{Ca}^{2+}_o$  entry are the proximal molecular effectors of enhanced  $\text{Ca}^{2+}_o$  cytoostasis by ST (1  $\mu\text{M}$ ) in T84 cells. IBMX (50  $\mu\text{M}$ ), the general phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine; L-DLT (*L-cis*-diltiazem, 10  $\mu\text{M}$ ) and PET (8-br-PET-cGMPs, 50  $\mu\text{M}$ ), two inhibitors of CNG channels; BAPTA (2  $\mu\text{M}$ ), the intracellular  $\text{Ca}^{2+}$  chelator BAPTA-AM; RP8pCPT (50  $\mu\text{M}$ ), the cGMP-dependent protein kinase inhibitor Rp-8-pCPT-cGMP; Rp-cAMPS (50  $\mu\text{M}$ ), an inhibitor of cAMP-dependent protein kinase. Inhibitors were employed at concentrations that completely inhibit their target enzymes (4). Results in (A) are expressed as the percentage of respective control incubations treated with 10 mM  $\text{Ca}^{2+}_o$ , whereas in (B) and (C) are expressed as in Figure 1C2. \* $P < 0.05$  and \*\* $P < 0.01$  versus respective parallel control conditions treated with 10 mM  $\text{Ca}^{2+}_o$ . (D) ST (1  $\mu\text{M}$ ) induces cytoostasis, but not cytotoxicity, in synchronized T84 cells exposed to 10 mM  $\text{Ca}^{2+}_o$ . The percentage of tumor cells in each phase of the cell cycle, including the sub-G<sub>1</sub> fraction of apoptotic/necrotic cells, was quantified by flow cytometry. Data are from a representative experiment.

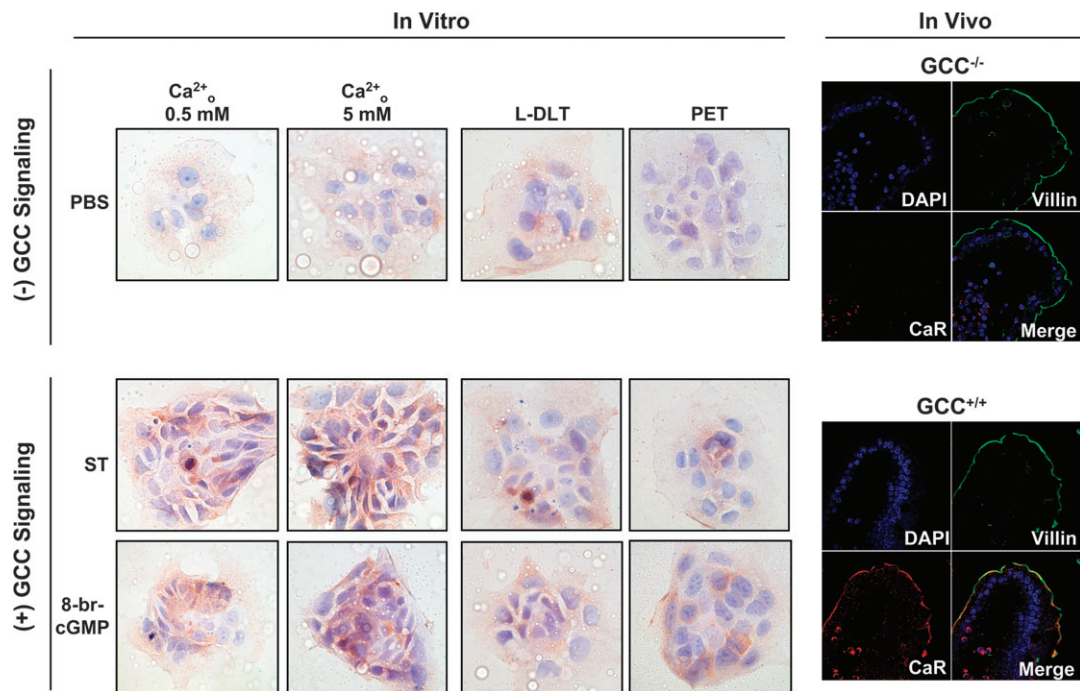


**Fig. 3.** ST induces CaR translocation in colon cancer cells. (A) Intracellular cGMP accumulation induced by ST (1  $\mu\text{M}$  for 15 min) in T84 cells pretreated with ST (1  $\mu\text{M}$  for 24 h) or the vehicle control in the presence of the indicated  $\text{Ca}^{2+}_o$  concentration. \* $P < 0.05$  versus respective control conditions not pretreated with ST. (B) Time course of ST (1  $\mu\text{M}$ ) effects on cytosol (upper panel) or membrane (lower panel) CaR expression in T84 cells. Phosphate-buffered saline (PBS), the vehicle control; GAPDH and villin, the loading controls.

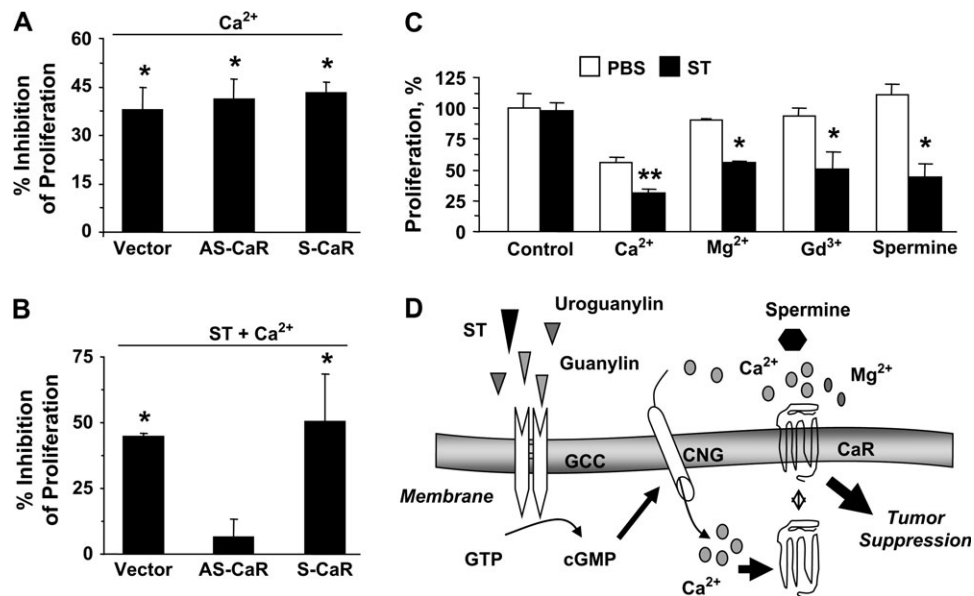
differentiation along the crypt–villus axis (14) and are more susceptible to intestinal tumorigenesis induced by azoxymethane or APC mutations (16). Moreover, expression of guanylin and uroguanylin is uniformly lost early in intestinal carcinogenesis (11–13), loss of guanylin expression resulted in crypt hyperproliferation (15) and administration of GCC ligands inhibits intestinal tumor formation in APC<sup>Min/+</sup> mice (3) and cell cycle progression of colon cancer cells

(9). Thus beyond fluid homeostasis, GCC controls proliferation and tumorigenesis in intestine.

Mechanisms mediating antiproliferation by GCC appear diverse. GCC signaling through unidentified effectors engages colonic cell quality control mechanisms assuring genomic integrity and tumor suppression (16). Also, GCC- and cGMP-dependent  $\text{Ca}^{2+}$  influx through CNGs induces colon cancer cell cytoostasis by reducing



**Fig. 4.** Ligand-dependent GCC signaling upregulates surface CaR receptors in normal and transformed intestinal epithelial cells. Membranes of non-permeabilized T84 cells (*in vitro*) were stained (brown) with specific rabbit polyclonal anti-CaR in the presence of low (0.5 mM; left column) or high (5 mM; right three columns)  $\text{Ca}^{2+}_o$  (magnification  $\times 40$ ). In blue, counterstaining of nuclei with hematoxylin. Tumor cells were treated for 24 h with phosphate-buffered saline (PBS) (vehicle control; upper row), ST (1  $\mu\text{M}$ ; middle row) or 8-br-cGMP (5 mM; lower row) in the absence (left two columns) or presence (right two columns) of the indicated CNG inhibitors (see Figure 1 legend for keys). Also, epithelial cells in villus tips from the jejunum of  $\text{GCC}^{+/+}$  and  $\text{GCC}^{-/-}$  mice were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue, nuclei) and specific antibodies against villin (green, enterocyte brush-border membranes) and CaR (red) and subjected to confocal microscopy (magnification  $\times 63$ ).



**Fig. 5.** CaR induced by ST is a novel therapeutic target in human colon cancer cells. (A and B) The proliferation of T84 cells stably expressing the empty vector (vector, control), the CaR antisense vector (AS-CaR) or the CaR sense vector (S-CaR) was examined. Cells were treated for 24 h with  $\text{Ca}^{2+}_o$  (5 mM) alone (A) or ST (1  $\mu\text{M}$ ) plus  $\text{Ca}^{2+}_o$  (5 mM) (B). Results are expressed as in Figure 1C2; the controls were low (0.5 mM; A) or high (5 mM; B)  $\text{Ca}^{2+}_o$ . \* $P < 0.05$  versus parallel control conditions. (C) T84 cell proliferation exposed to CaR agonists was measured as the percentage of respective control incubations treated with 0.5 mM  $\text{Ca}^{2+}_o$  (first two columns on the left). CaR agonists were added to tumor incubations for 24 h in the presence of the vehicle control (PBS) or ST (1  $\mu\text{M}$ ), and include  $\text{Ca}^{2+}$ , 10 mM;  $\text{Mg}^{2+}$ , 20 mM;  $\text{Gd}^{3+}$ , 100  $\mu\text{M}$  and spermine, 100  $\mu\text{M}$ . CaR agonists are used at concentrations that maximally induce CaR signaling (34,40). \* $P < 0.05$  and \*\* $P < 0.01$  versus respective control [phosphate-buffered saline (PBS)] conditions. (D) Proposed molecular mechanism for restoration of CaR signaling by GCC associated with colorectal cancer suppression (see Discussion for description).

nuclear rates of DNA synthesis (4,9). Here, a previously unappreciated mechanism contributing to tumor suppression by GCC is described (Figure 5D). ST preconditioning induces cGMP-mediated CNG signaling and synthesis and cell surface delivery of CaRs. Increased CaRs at the plasma membrane, in turn, provide new tumor-specific targets for antiproliferative signaling by CaR agonists, including  $\text{Ca}^{2+}_o$ ,  $\text{Mg}^{2+}$ ,  $\text{Gd}^{3+}$  and spermine. Although mediated by the same proximal effector,  $\text{Ca}^{2+}_o$  influx through CNG, inhibition of DNA synthesis (4) and activation of CaR signaling (Figure 5D) represent two distinct, non-overlapping GCC-dependent antiproliferative mechanisms, distinguished by acute (minutes) (9) and chronic ( $\geq 3$  h Figure 3B) kinetics, respectively. They may represent sequential temporal arms of an integrated antiproliferative strategy in which acute cytostasis, silenced by phosphodiesterase-dependent desensitization of cGMP signaling (10), is propagated by CaR-dependent signaling to ensure enduring colon tumor cytostasis imposed by GCC. Interestingly, CaR signaling also promotes phosphodiesterase-dependent hydrolysis of cGMP (39), a mechanism that may contribute to desensitize cGMP-mediated inhibition of DNA synthesis and represents a negative feedback loop for cGMP-induced CaR.

First discovered in parathyroid cells where it senses blood  $\text{Ca}^{2+}$  levels and regulates parathyroid hormone release (40), CaRs are expressed in many cell types, including osteoclasts, neurons and hematopoietic cells (23,41,42). In colonocytes, CaRs sense 1–10 mM  $\text{Ca}^{2+}_o$  in fecal water by interacting with the N-terminal extracellular domain, inducing intracellular signaling through heterotrimeric G proteins (23,43). One emerging function of CaRs in colonocytes is the regulation of proliferation and differentiation and its putative role as a tumor suppressor (22,24,44). Although the CaR gene is not lost or mutated in colon cancer (33), CaR expression is inexplicably reduced during disease progression (25,26,33). The present finding that GCC regulates the posttranscriptional expression of CaRs in normal and malignant intestinal cells offers a mechanistic explanation for that observation since GCC signaling is silenced early during colorectal tumorigenesis following loss of ligand expression (11–13). Accordingly, elimination of GCC signaling in mice abolished the expression of CaRs in enterocytes (Figure 4). Moreover, CaRs are primarily expressed in the apex and central regions of human crypts (26) where expression of endogenous GCC ligands and cGMP levels are greatest (45,46), suggesting that CaR signaling may be conditionally regulated along the crypt–villus axis by GCC- and cGMP-dependent mechanisms. In this model, the common abilities of CaRs and GCC to promote the transition from proliferation to differentiation and inhibit intestinal carcinogenesis may represent convergent, rather than parallel, signaling pathways. Finally, although they remain to be fully characterized, GCC effects on CaRs, including induction of translation, posttranslational processing or trafficking to cell membranes, represent a previously unrecognized mechanism of transcriptionally independent regulation of CaR surface expression and signaling (47).

Beyond CaR,  $\text{Ca}^{2+}_o$  opposes tumorigenesis by forming benign insoluble complexes with toxic ionized fatty acids and bile acids (48) and promoting *cis*-dimerization of E-cadherin molecules underlying cell–cell adhesion (49) and growth suppression (50). This latter mechanism, which occurs at  $\text{Ca}^{2+}_o > 0.5$  mM (49) and induces the function of p27 (50), may explain, in part, the cytostatic effects of high  $\text{Ca}^{2+}_o$  in colon cancer cells not exposed to ST observed here. Indeed, these cells were not affected by CaR antisense delivery (Figure 5A) or application of CaR agonists (Figure 5C), indicating that in the absence of GCC activation CaRs are functionally silent in colon cancer cells. Loss of GCC (11–13) and CaR (25,26,33) signaling, in turn, may underlie the resistant phenotype of colon cancer cells to  $\text{Ca}^{2+}_o$ -induced cytostasis (20,31). These observations are significant since levels of 3–10 mM  $\text{Ca}^{2+}_o$ , required for significant CaR activation (34), are typically achieved in the fecal colonic water as a result of dietary  $\text{Ca}^{2+}$  intake, absorption and secretion (19,22). Moreover, allosteric CaR activators present in the intestinal lumen such as L-amino acids (23) may further increase the CaR sensitivity for  $\text{Ca}^{2+}_o$ . Importantly, CaRs sense other polyvalent cations (i.e.  $\text{Gd}^{3+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ni}^{2+}$  and polylysine), including polyamines (spermine, spermidine and putrescine)

endogenously produced by colonic bacteria (23), suggesting that GCC-induced CaRs may subserve diverse tumor suppressor pathways in intestine.

Strategies for cancer control include chemoprevention and chemotherapy. In contrast to its endogenous ligands, GCC is universally overexpressed in colorectal tumors (51,52), and oral administration of GCC ligands is a novel targeted approach to prevent tumor initiation and arrest disease progression in patients (3,4,9). Limitations to these therapies include adverse effects associated with the use of diarrheagenic bacterial enterotoxins (7,37,38). Conversely, dietary  $\text{Ca}^{2+}$  supplementation has been proposed as a chemoprevention strategy against colon cancer (53). However, caution has been suggested based on the reduced efficacy of  $\text{Ca}^{2+}_o$  to inhibit intestinal cell proliferation following neoplastic transformation probably reflecting reduced CaR expression (33). In that context, dietary  $\text{Ca}^{2+}_o$  could potentially promote growth of  $\text{Ca}^{2+}_o$ -insensitive tumor cells while suppressing proliferation of normal adjacent cells, which retain  $\text{Ca}^{2+}_o$  sensitivity (22). The present observations that GCC preconditioning restores antitumorigenic CaR signaling in human colon carcinoma cells may offer a solution to the limitations of  $\text{Ca}^{2+}_o$  supplementation strategies. Oral  $\text{Ca}^{2+}_o$  therapy, in turn, may prevent adverse effects of GCC-targeted strategies because activation of CaR opposes secretory diarrhea by bacterial enterotoxins (39). Taken together, these data suggest that combinatorial therapies including dietary  $\text{Ca}^{2+}_o$  supplementation and GCC ligand replacement represent a previously unrecognized paradigm for the prevention and treatment of human colorectal cancer.

#### Supplementary material

Supplementary Figures 1–4 can be found at <http://carcin.oxfordjournals.org/>

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