Interruption of *Escherichia coli* Heat-stable Enterotoxin-induced Guanylyl Cyclase Signaling and Associated Chloride Current in Human Intestinal Cells by 2-Chloroadenosine*

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Diarrhea induced by *Escherichia coli* heat-stable enterotoxin (ST*) subtilis* is mediated by a receptor guanylyl cyclase cascade. The present study establishes that an intracellular nucleotide-dependent pathway disrupts toxin-induced cyclic GMP (cGMP) production and the associated chloride (Cl*) flux that underlie intestinal secretion. Incubation of Caco 2 human intestinal epithelial cells with the nucleoside analog 2-chloroadenosine (2ClAdo) resulted in a concentration- and time-dependent inhibition of toxin-induced cGMP production. Inhibition of cGMP production correlated with the metabolic conversion of 2ClAdo to 2-chloroadenosine triphosphate. The effect of 2ClAdo did not reflect activation of adenosine receptors, inhibition of adenosine deaminase, or modification of the binding or distribution of ST* subtilis* receptors. Guanylyl cyclase activity in membranes prepared from 2ClAdo-treated cells was inhibited, in contrast to membranes from cells not exposed to 2ClAdo, demonstrating that inhibition of guanylyl cyclase C (GCC) was mediated by a noncompetitive mechanism. Treatment of Caco 2 cells with 2ClAdo also prevented ST* subtilis*-induced Cl* current. Application of 8-bromo-cGMP, the cell-permeant analog of cGMP, to 2ClAdo-treated cells reconstituted the Cl* current, demonstrating that inhibition of Cl* flux reflected selective disruption of ligand stimulation of GCC rather than the chloride channel itself. Thus, the components required for adenine nucleotide inhibition of GCC signaling are present in intact mammalian cells, establishing the utility of this pathway to elucidate the mechanisms regulating ST-dependent guanylyl cyclase signaling and intestinal fluid homeostasis. In addition, these data suggest that the adenine nucleotide inhibitory pathway may be a novel target to develop antisecretory therapy for enterotoxigenic diarrhea.

Guanylyl cyclase C (GCC), the receptor for *Escherichia coli* heat-stable enterotoxin (ST*) subtilis* expressed in intestinal mucosa cells, is a member of the receptor guanylyl cyclase family that possesses receptor and catalytic domains on a single transmembrane protein (1, 2). Occupancy of ST* subtilis* by the extracellular receptor domain induces catalytic conversion of intracellular GTP to cyclic GMP (cGMP), resulting in sequential alterations in epithelial cell chloride flux, electrolyte and fluid secretion, and diarrhea (3–7). Interventions that specifically interrupt the ST* subtilis*-induced GCC-mediated signal sequence have not been defined. In cell-free systems, GCC is allosterically inhibited by 2-substituted adenine nucleotides (8, 9). Yet, the impermeance of intact cells to phosphorylated nucleotides and the absence of endogenous 2-substituted nucleotides has precluded the disruption of ST* subtilis*-induced signaling in intestinal cells through this inhibitory pathway. However, intestinal cells express transporters, which carry 2-substituted nucleosides into the cytosol, and adenosine kinase, which catalyzes conversion of 2-substituted nucleosides to 2-substituted nucleotides (10). The present studies examine whether that mechanism can be exploited to interrupt transmembrane signaling and alterations in chloride flux induced by ST* subtilis* in intact intestinal epithelial cells.

**EXPERIMENTAL PROCEDURES**

Cyclic GMP Accumulation in Intact Cells—Caco 2 cells, well differentiated human colon carcinoma cells, were seeded in 24-well plates, allowed to reach confluence, and grown for an additional 14–21 days to ensure differentiation of these cells into colonic enterocytes. HEK293 cells, human embryonic kidney cells expressing recombinant GCC, were seeded in 24-well plates, allowed to reach confluence, and used for assays at least 5 days after seeding (1, 11). Cells were incubated in OPTI-MEM serum-free media (Life Technologies, Inc.) (0.5 ml/well) containing indicated concentrations of the test substances for the given period of time. Cells were washed three times with OPTI-MEM, then incubated in OPTI-MEM (0.2 ml/well) containing 0.12 mM isobutylmethylxanthine to inhibit endogenous phosphodiesterases for 10 min. ST* was added to a final concentration of 0.5 μM for 10 min. Trichloroacetic acid (0.2 ml of 1% solution) was added to the wells to lyse the cells and terminate the reaction. Wells were collected and centrifuged 5 min in a microcentrifuge to separate pellet and supernatant (8). The supernatant was collected, the trichloroacetic acid was removed by ether extraction, and the sample was used for cGMP determination by radioimmunoassay (12). Pellets were saved for determination of protein content by the method of Bradford (Bio-Rad).

Guanylyl Cyclase Assay—Cells were treated in OPTI-MEM media containing test substances as described above. Wells were washed three times with a Tris buffer (50 mM, pH 7.5) containing 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (T EBD buffer) (8, 9). Cells were collected in TED and homogenized on ice. Homogenates

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‡ The abbreviations used are: GCC, guanylyl cyclase C; cGMP, cyclic GMP; ST*, E. coli heat-stable enterotoxin; 2ClAdo, 2-chloroadenosine; 2ClATP, 2-chloroadenosine triphosphate.
were centrifuged at 100,000 × g for 60 min at 4 °C. Membranes were resuspended in TED with a final concentration of approximately 1 mg of protein/ml. Membranes were incubated at 37 °C for 5 min in 0.1 ml of a Tris buffer (50 mM, pH 7.5) containing 500 mM isobutylmethylxanthine, 7.5 mM creatine phosphate/20 mM creatine phosphokinase and either 10 mM MgGTP and 1 mM STα or 1 mM MnGTP. Enzyme reaction was terminated by addition of 0.5 ml NaAc (50 mM, pH 4.0) and boiling for 5 min. Cyclic GMP was quantified by radioimmunoassay as described previously (12).

STα Binding Assay—Following membrane preparation as described above, 30 μl of membrane were incubated in 50 mM Tris, pH 7.6, containing 1 mM EDTA, 150 mM KCl, 0.1% bacitracin, and 0.67 mM cystamine (binding buffer). Binding was initiated by the addition of 125I-labeled STα (10−11 to 5 × 10−8 M) (13). Reactions were incubated for 120 min at 37 °C and terminated by filtration on Whatman GF/B glass fiber filters presoaked with 0.3% polyethyleneimine. Filters were washed three times with 5 ml of buffer containing 150 mM NaCl, 20 mM phosphate (pH 7.2), and 1 mM EDTA at 4 °C. Specific binding was determined by subtracting nonspecific binding (1000-fold excess of cold STα) from total binding. Assays were performed in quadruplicate. Analysis of ligand binding was performed using CIGALE, written by M. Bordes (Sophia Antipolis, France; Ref. 13).

Nucleoside Uptake Assays—Cells were incubated with [8-3H]2ClAdo (NECA) and adenosine deaminase inhibitors, deoxycoformycin (DCF) and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), did not mimic the effect of 2ClAdo on STα-induced cGMP accumulation in Caco 2 cells (n = 3; bars, S.E.). In b, treatment of Caco 2 cells with 2ClAdo did not significantly alter STα receptor binding characteristics. A representative experiment demonstrating that Caco 2 cells not treated (−) or treated with 2ClAdo for 20 h (+) exhibited high and low affinity STα binding is shown. c, time course of specific uptake of [8-3H]2ClAdo by Caco 2 cells (n = 3; bars, S.E.).
Treatment of either Caco 2 cells natively expressing GCC or HEK293 cells heterologously expressing recombinant GCC with the nucleoside 2ClAdo, a metabolic precursor of 2ClATP, suppressed STₐ-induced cGMP accumulation (Fig. 1a). The effect of 2ClAdo was concentration (Kᵥ = 101 ± 21 µM; Fig. 1b) and time-dependent (t½ of 10 h; Fig. 1c). The 2ClAdo effect appeared temporally biphasic, because inhibition of STₐ-induced cGMP accumulation was preceded by a transient increase in STₐ-induced cGMP accumulation at early (t ≤ 4 h) timepoints (Fig. 1c). Although the mechanisms underlying this initial transient rise in cGMP remain unclear, 2ClAdo is a potent ligand for adenosine receptors, and activation of other signaling mechanisms through these receptors could activate GCC (18). There was no significant difference in the number of cells or the amount of recovered protein in control or 2ClAdo-treated cells. Removal of 2ClAdo restored STₐ-dependent cGMP accumulation (t½ of 6 h; Fig. 1c, inset), suggesting that inhibition of cGMP synthesis did not reflect cell death.

Adenosine analogs such as 2ClAdo are potent agonists for extracellular purinergic receptors. Furthermore, 2ClAdo is a low potency inhibitor of adenosine deaminase (19), an enzyme that regulates intracellular nucleotide concentrations. However, the effects of 2ClAdo could not be mimicked by N-ethylcarboxamidoadenosine, a purinergic P₁ agonist with similar receptor potencies to 2ClAdo, nor by reversible (erythro-9-(2-hydroxy-3-nonyl)adenine) or irreversible (deoxycoformycin) adenosine deaminase inhibitors (Fig. 2a; Ref. 20). These data suggest that 2ClAdo-dependent inhibition of GCC signaling does not reflect the potency of this nucleoside for purinergic receptors or competitive inhibition of adenosine deaminase. 125I-labeled STₐ bound to membranes prepared from Caco 2 cells incubated in the absence and presence of 2ClAdo in a concentration-dependent and saturable fashion. Scatchard analyses yielded curvilinear isotherms, suggesting the presence of high and low affinity ligand-binding sites in both 2ClAdo-treated and control cells (Fig. 2b). Equilibrium binding parameters derived from Scatchard analyses suggested that 2ClAdo treatment did not significantly alter the number or affinity of ligand receptors (Fig. 2b). Thus, in membranes from control and treated cells, respectively, the numbers of high affinity (Bₚₒᵢₘₓ, 2.1 ± 1.3 pmol/mg of protein) and low affinity (Bₚₒᵢₘₓ, 0.03 ± 0.02 versus 0.08 ± 0.05 pmol/mg of protein) binding sites were closely comparable. Similarly, the affinities of high (Kᵥᵦᵢₜ, 0.9 ± 0.5 versus 6.5 ± 6.1 pm) and low (Kᵥᵦᵢₜ, 1.3 ± 1.1 versus 4.5 ± 2.0 nm) affinity binding sites compared favorably in membranes from control and treated cells, respectively. Equilibrium binding parameters (values ± S.E.) obtained in the present studies compare closely with those reported previously for high and low affinity STₐ binding sites (13, 21–23). Similarly, 2ClAdo neither decreased the number of 125I-labeled STₐ binding sites on the cell surface nor increased the rate of 125I-labeled STₐ internalization in intact cells (data not shown; Ref. 24). Therefore, inhibition of GCC signaling could not be attributed to alterations in distribution, sequestration, or ligand binding characteristics of the receptor.

Caco 2 cells incorporated [8-3H]2ClAdo in a time-dependent fashion (Fig. 2c). Uptake of [8-3H]2ClAdo was not dependent on extracellular Na⁺, suggesting that intracellular accumulation was mediated by an equilibrative nucleoside transport mechanism (10). Iodotubercidin, an adenosine kinase inhibitor, did not alter the initial rate of uptake but prevented further increases in intracellular [8-3H]2ClAdo (data not shown). These data suggest that cellular nucleoside accumulation was dependent on transport coupled to metabolic conversion to a phosphorylated product (10, 20).
Although 2ClAdo inhibited ST$_a$-induced cGMP accumulation in intact cells, this nucleoside did not suppress the activity of GCC in intestinal cell membranes (Fig. 3a). However, membranes prepared from intact cells pretreated with 2ClAdo did exhibit persistent inhibition of GCC (Fig. 3b). These data further suggest that 2ClAdo undergoes intracellular metabolic conversion into the proximal allosteric inhibitor of GCC. High-performance liquid chromatography analysis of 2ClAdo-treated human intestinal cells revealed time-dependent accumulation of 2ClATP (Fig. 3c), which correlated closely with nucleoside inhibition of ST$_a$-induced cGMP accumulation. In contrast to 2ClAdo (Fig. 3a), 2ClATP directly inhibited the activity of GCC in intestinal cell membranes (Fig. 3c, inset). Iodotubercidin, a competitive inhibitor of phosphorylation of 2ClAdo by adenosine kinase, decreased the potency of 2ClAdo to inhibit ST$_a$-induced cGMP production (Fig. 3d; Ref. 20). Thus, 2ClAdo inhibits ST$_a$-induced cGMP accumulation in intact cells following intracellular phosphorylation by adenosine kinase, ultimately to 2ClATP, an effective allosteric inhibitor of GCC (8, 9).

To determine the consequence of disrupting cGMP accumulation with 2ClAdo on ST$_a$-induced postreceptor signals, alterations in chloride current were examined in human intestinal cells. In Caco 2 cells, ST$_a$ induced an outward current (135 ± 33 pA at a membrane potential of +10 mV, n = 4), which was suppressed by removal of extracellular chloride or by the addition of glyburide (Fig. 4a). The selectivity for Cl$^-$ outward rectification reversal potential at −70 mV and pharmacological properties (Fig. 4, a and a$_1$) were all consistent with the presumed role of the cystic fibrosis transmembrane conductance regulator in mediating ST$_a$-induced alterations in chloride conductance in intestinal cells (25–28). However, in Caco 2 cells treated with 2ClAdo, ST$_a$ could no longer induce a chloride current (Fig. 4, b and c). Yet, in the same 2ClAdo-treated cells, 8-bromo cGMP, a membrane-permeant cGMP analog (6), produced an outward current that was abolished by removal of extracellular chloride (Fig. 4b). Thus, 2ClAdo treatment specifically blocked ST$_a$-dependent signaling by inhibiting GCC and accumulation of cGMP, rather than altering the ability of cGMP to generate chloride currents.

The present studies establish an intracellular nucleotide-dependent pathway for inhibition of GCC signaling in intact human intestinal cells (Fig. 5). Uptake and phosphorylation of 2ClAdo results in accumulation of 2ClATP, which inhibits ST$_a$-mediated guanylyl cyclase, accumulation of cGMP, and subsequent chloride fluxes mediating toxin-induced diarrhea. These data demonstrate that the components required for 2-substituted adenine nucleotide inhibition of GCC signaling are present in intact mammalian cells, establishing this path-
way as a tool for elucidating the molecular mechanisms regulating GCC and intestinal fluid homeostasis. In addition, these data suggest that the adenine nucleotide inhibitory pathway may be a novel target for developing antisecretory therapy to treat enterotoxigenic diarrhea.

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