



A novel plant-derived inhibitor of cAMP-mediated fluid and chloride secretion

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Gabriel, S. E., S. E. Davenport, R. J. Steagall, V. Vimal, T. Carlson, and E. J. Rozhon. A novel plant-derived inhibitor of cAMP-mediated fluid and chloride secretion. *Am. J. Physiol.* 276 (*Gastrointest. Liver Physiol.* 39): G58–G63, 1999.—We have identified an agent (SP-303) that shows efficacy against in vivo cholera toxin-induced fluid secretion and in vitro cAMP-mediated Cl⁻ secretion. Administration of cholera toxin to adult mice results in an increase in fluid accumulation (FA) in the small intestine (FA ratio = 0.63 vs. 1.86 in control vs. cholera toxin-treated animals, respectively). This elevation in FA induced by cholera toxin was significantly reduced (FA ratio = 0.70) in animals treated with a 100 mg/kg dose of SP-303 at the same time as the cholera treatment. Moreover, when SP-303 was administered 3 h after cholera toxin, a dose-dependent inhibition of FA levels was observed with a half-maximal inhibitory dose of 10 mg/kg. In Ussing chamber studies of Caco-2 or T84 monolayer preparations, SP-303 had a significant effect on both basal current and forskolin-stimulated Cl⁻ current. SP-303 also induced an increase in resistance that paralleled the observed decrease in current. These data suggest that SP-303 has an inhibitory effect on cAMP-mediated Cl⁻ and fluid secretion. Thus SP-303 may prove to be a useful broad-spectrum antidiarrheal agent.

cystic fibrosis transmembrane conductance regulator; secretory diarrhea; chloride channel

SECRETORY DIARRHEA REMAINS one of the major causes of infant morbidity and mortality around the world, accounting for over a billion episodes per year in children under 5 years of age (13). In comparison to other mechanisms of diarrheal disease, secretory diarrheas caused by bacterial enterotoxins [e.g., *Vibrio cholerae* toxin and *Escherichia coli*, heat stable (ST), heat labile (LTa), and LTb toxins] are not associated with severe intestinal histopathology. Rather, secretory diarrheas appear to function by stimulating transepithelial Cl⁻ secretion, thereby increasing the osmotic impetus for fluid secretion (4). Cholera toxin (CT) is likely the most recognizable enterotoxin that causes secretory diarrhea. After colonization of the small intestine by *Vibrio cholerae*, binding of the cholera enterotoxin to the intestinal enterocyte leads to ADP-ribosylation of the α -subunit of a stimulatory G protein, G_s α (7, 11). This covalent modification activates adenylate cyclase for the lifetime of the enterocyte during its migration up

the villus axis. As a consequence of this irreversible elevation of cAMP, an apical membrane Cl⁻ channel is activated that results in a voluminous Cl⁻ and fluid secretion that can be fatal if untreated (4, 7).

Cystic fibrosis (CF) is a disease characterized by defective cAMP-regulated Cl⁻ secretion of the apical membrane of epithelial cells (12, 14). Cloning of the CF gene (10, 16) and expression studies of the protein product, the cystic fibrosis transmembrane conductance regulator (CFTR), reveal that it is a cAMP-regulatable Cl⁻ channel (1, 2, 18). CFTR is widely expressed in many epithelial tissues and is prevalent in the mammalian small intestinal epithelium in both crypt and villus cells (20). Gene-targeting approaches have successfully been used to create several CF mouse models (reviewed in Ref. 9), all of which show that a primary pathophysiology occurs in the gastrointestinal tract. This pathophysiology can be explained by the finding that CFTR is the predominant mechanism for apical Cl⁻ secretion in the murine gastrointestinal tract (5). Thus a functional absence of CFTR in the CFTR(-/-) mouse results in a severely dehydrated intestinal epithelium that is the catalyst for constrictions, obstructions, and ultimately death (17).

SP-303 is derived from the latex of the plant *Croton lechleri* and comprises a mixture of naturally occurring proanthocyanidin oligomers with an average molecular mass of 2,200 Da (21). This naturally occurring latex has been used by the indigenous people of South America to treat various kinds of watery diarrheas, including diarrhea caused by cholera. In this study, we examine the ability of SP-303 to inhibit CT-induced fluid secretion in the murine intestine. We also test whether this compound is capable of inhibiting Cl⁻ secretion across a human intestinal epithelial cell monolayer.

MATERIALS AND METHODS

Fluid accumulation. Fluid accumulation (FA) was measured using the sealed adult mouse model for secretory diarrhea as described previously (8, 15). Briefly, 8- to 10-wk-old C57Bl/6 mice of either sex with body masses that ranged from 12.1 to 36.0 g were used. Initially, mice were given single doses of SP-303 dissolved in 7% wt/vol NaHCO₃ delivered by an orogastric feeding tube, followed immediately by 10 μ g CT (this is referred to as simultaneous addition). Subsequent experiments involved administration of 10 μ g CT at *time 0* followed by administration of varying doses of SP-303 at 3 h (one set of experiments, involving SP-303 prepared as an enteric-coated bead). Under either of the experimental conditions, mice were killed 6 h after the administration of CT. The entire small intestine, from the pylorus to the cecum, was

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isolated with care to avoid tissue rupture and loss of fluid. The attached mesentery and connective tissues were removed, and the mass of the tissue and enclosed fluid was determined. The tissue was then opened longitudinally, the fluid was removed, and the tissue was patted dry. The mass of the dry tissue was then reweighed. FA was measured as a ratio of the mass of accumulated fluid in the small intestine and cecum vs. the mass of the small intestine minus the mass of the fluid. Both the CT dose administered and the time of incubation were selected to ensure that a maximal amount of fluid would be secreted into the intestinal lumen. Data were analyzed to determine whether SP-303 administration resulted in a statistically significant reduction in CT-induced FA ratios compared with CT-treated animals given only NaHCO_3 as a control solution. Baseline (e.g., nonsecretory) animals were administered a bolus of 7% NaHCO_3 solution in the absence of CT, and FA values were measured 6 h later.

Cell culture. Human colonic epithelial cell lines, either Caco-2 or T84, were grown in either DMEM (Caco-2) or DMEM-Ham's F-12 (T84) supplemented with 8 mM L-glutamine, 100 U/ml penicillin G, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10–15% FCS, in an atmosphere of 95% air and 5% CO_2 . Cell monolayers were plated at high density (2×10^4 cells per mm^2) onto tissue culture inserts (collagen matrix supports with a 4.5-mm plating diameter) as described previously (6). For all experiments, cells were evaluated for confluence by daily monitoring of transepithelial resistance (R_t) and potential difference (PD). Only monolayers generating at least a 0.5 mV PD and a 150 $\Omega \cdot \text{cm}^2$ resistance were used for Ussing chamber studies.

Ussing chamber studies. Electrical measurements, PD, and short-circuit current (I_{sc}) were made on cultured epithelial cell monolayers mounted in Ussing chambers (4). Unless otherwise stated, the bathing medium used for all studies was a standard Krebs-Ringer bicarbonate solution (KRB) on the basolateral side and a modified KRB, high- K^+ and low- Cl^- solution (gluconate was substituted for Cl^-) on the apical side (6). In some experiments, an apical Na^+ -free solution (*N*-methyl-D-glucamine was substituted for Na^+) was used to eliminate any contribution of absorptive Na^+ channels. In these experiments, ion solutions were balanced with the exception of Na^+ (140 mM Na^+ in the basolateral solution and 0 mM Na^+ in the apical solution). All bathing solutions were bubbled with 95% O_2 -5% CO_2 and maintained at 37°C. PD was clamped to zero, and the I_{sc} was continuously displayed on a pen recorder. R_t was calculated from the magnitude of the current deflections in response to a voltage pulse imposed on short-circuited cell sheets every 60 s with a duration of 0.5 s. Equivalently, electrometer output was digitized online, and I_{sc} , R_t , and calculated transepithelial potential were displayed on a video monitor and stored on a computer hard drive. Drugs were added from concentrated stock solutions to either apical and/or basolateral bathing solutions. All compounds were added cumulatively, i.e., drugs were not removed before addition of subsequent doses or compounds. Data are expressed as means \pm SE for *n* (no. of experiments).

cAMP measurements. T84 cell monolayers were plated and cultured on tissue culture inserts as described for Ussing chamber studies. Cells were bathed in KRB apical and basolateral solutions, and CT or SP-303 was administered from concentrated solutions to the apical bath. T84 cells were administered a single dose of 10 μg CT at *time 0* followed by 300 μM SP-303 3 h later. Bathing solutions were removed, and 6% ice-cold TCA was added to the cells at *hour 6* to stop the reaction. Precipitated protein and supernatant were collected and centrifuged (1,000 *g* for 5 min). Ether extracts of

the supernatants were reconstituted and assayed for cAMP content by an acetylated RIA kit (Biomedical Technologies, Stoughton, MA). All samples were measured in duplicate and titrated to lie within the linear range of the standard curve. Student's *t*-test was used to determine statistically different means.

Materials. All biochemicals used were obtained from commercial sources and were of tissue culture grade or better. SP-303 was prepared as described previously and verified to be at least 95% pure by HPLC (21).

RESULTS

We have previously shown that CT-induced fluid and Cl^- secretion is directly proportional to the expression level of the CFTR Cl^- channel (8). In this study, we investigate the effects of SP-303 on CT-induced FA in the small intestine of mice expressing normal levels of CFTR. Control mice treated with only a bolus of 7% NaHCO_3 solution in the absence of either CT or SP-303 show an FA ratio of 0.63 ± 0.07 (Fig. 1, dashed line in *A* and *C*). CT (10 μg)-treated animals show an FA level (1.86 ± 0.2) that is nearly three times greater than control values. Simultaneous administration of SP-303 with CT results in an inhibition of FA in a concentration-dependent manner (Fig. 1*A*). Gavage administration of 100 mg/kg SP-303, simultaneously with CT treatment, restores FA to near normal levels (0.70 ± 0.19). To exclude the possibility that SP-303 may be either interacting with CT or preventing binding of CT to the ganglioside receptor, we studied SP-303-mediated inhibition of FA in mice prestimulated by CT. Mice that received a dose of SP-303 3 h after exposure to CT showed a significant decrease in FA ratios (Fig. 1*B*). The dose-response curve showed inhibition with an apparent half-maximal inhibitory concentration (IC_{50}) of ~ 10 mg/kg. The highest SP-303 doses administered brought about a nearly complete inhibition of CT-induced FA levels [i.e., 50 mg/kg SP-303-treated mice had FA values that were not significantly different from untreated control FA values (0.78 ± 0.08 vs. 0.63 ± 0.07)]. In an attempt to maximize efficient delivery of SP-303 to the small intestinal lumen and in anticipation of future clinical trials, SP-303 was encapsulated in an alkali-soluble, enteric-coated bead and FA ratios were measured (Fig. 1*C*). Administration of SP-303 encapsulated in this formulation was likewise capable of significantly inhibiting CT-induced FA (0.75 ± 0.10 vs. 1.51 ± 0.08 for CT and 100 mg/kg SP-303 vs. treatment with CT alone, respectively).

Secretory diarrhea as induced by CT is the result of irreversible activation of adenylate cyclase, leading to elevation of cAMP and finally stimulation of Cl^- secretion. Thus we sought to determine if the observed SP-303 inhibition of FA was due to a metabolic inhibition of cellular cAMP levels. We measured the effect of SP-303 on cellular cAMP levels in confluent T84 cell monolayers. Administration of CT (10 $\mu\text{g}/\text{ml}$) resulted in a large increase in cAMP levels sampled 6 h after treatment ($1,031.4 \pm 64$ vs. 4.97 ± 0.3 pmol/ml for CT vs. control, respectively; *n* = 6 for each condition). Addition of 300 μM SP-303 neither significantly reduced the CT-elevated cAMP levels ($1,031.4 \pm 64$ vs.

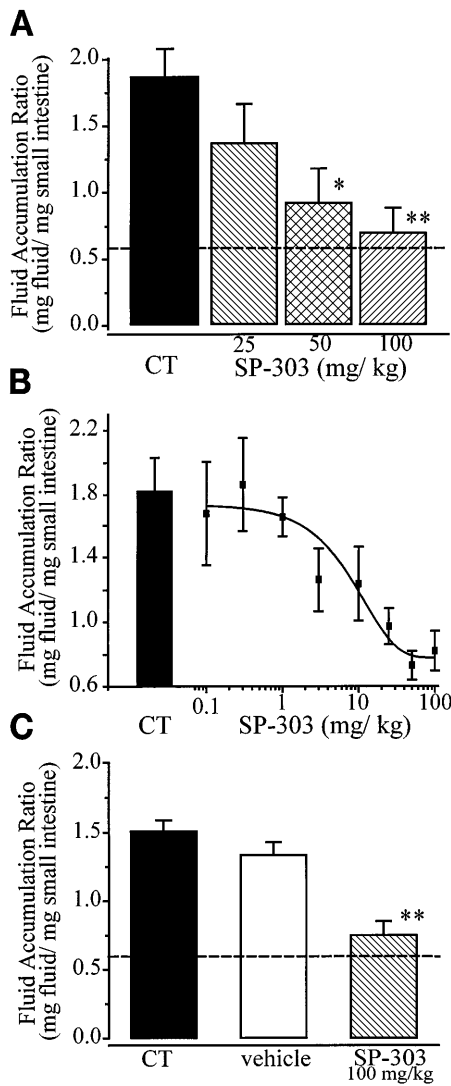


Fig. 1. Effect of SP-303 on cholera toxin (CT)-induced fluid accumulation (FA) ratios in mice. All mice were treated with 10 μ g CT per os, and FA ratios were measured 6 h after CT administration. **A:** CT ($n = 20$) and SP-303 ($n =$ at least 6 mice for each dose studied) were both administered at *time 0* and FA was measured 6 h later. **B:** CT was administered at *time 0* followed 3 h later by either vehicle alone (solid bar; $n = 16$) or varying doses of SP-303 (■; $n =$ at least 8 mice for each dose studied). **C:** CT was administered as in **B** (solid bar; $n = 13$), 3 h after CT, and either enteric-coated beads containing SP-303 were delivered (hatched bar; $n = 18$) or vehicle-coated beads (no SP-303) were delivered (open bar; $n = 20$). Dashed line in **A** and **C** represents the FA ratio in control mice not treated with either CT or SP-303 (FA = 0.63 ± 0.07 ; $n = 20$). All values are represented as means \pm SE. Asterisks indicate statistically significant difference from mice receiving CT alone (* $P < 0.05$; ** $P < 0.001$) as determined by a single-factor ANOVA (**A**) or Student's *t*-test (**C**).

972.5 ± 77 pmol/ml for CT vs. CT and SP-303, respectively; $P = 0.57$) nor reduced the non-CT-treated basal cAMP levels (4.97 ± 0.3 vs. 5.51 ± 0.3 pmol/ml for control vs. control and SP-303, respectively; $P = 0.24$).

The lack of an effect of SP-303 on cAMP levels suggests that the compound is acting at a site distal to elevation of cAMP (i.e., the CFTR Cl⁻ channel itself or a regulator of the channel). We therefore investigated the effects of SP-303 on the basal I_{sc} of Caco-2 epithelial cell

monolayers as a measure of Cl⁻ secretion (Fig. 2). Sequential addition of SP-303 to the solution bathing the apical membrane inhibited the basal I_{sc} by nearly 50%. Moreover, subsequent addition of forskolin (an activator of adenylate cyclase) resulted in a relatively small Cl⁻ secretory response (Fig. 2A). Forskolin-stimulated currents had a mean of 16.9 ± 2.5 μ A/cm² ($n = 8$) after exposure to 300 μ M SP-303, whereas forskolin-stimulated currents in monolayers not exposed to SP-303 showed a mean of 38.3 ± 4.3 μ A/cm² ($n = 16$). Inhibition of basal I_{sc} was seen at all doses of SP-303, with maximal effects observed following administration of 300 μ M SP-303 (Fig. 2B). Importantly, increasing doses of SP-303 resulted in an increase in monolayer resistance that correlated with the decrease in I_{sc} (300 μ M SP-303 showed an increase in resistance of $39 \pm 7\%$ of basal values, $n = 9$).

To further define the effect of SP-303 on cAMP-mediated Cl⁻ secretion, we examined the ability of SP-303 to inhibit the maximum I_{sc} stimulated by forskolin in Caco-2 cells (Fig. 3). Cl⁻ secretion maximally stimulated by the addition of forskolin was inhibited in a stepwise manner by sequential additions of SP-303 to the apical solution (Fig. 3A). The forskolin-

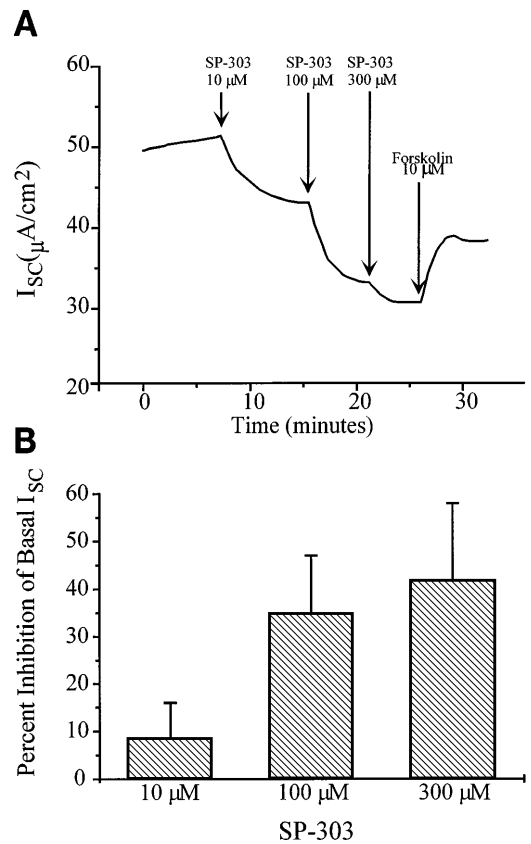


Fig. 2. Effect of SP-303 on basal short-circuit current (I_{sc}) measured across Caco-2 epithelial monolayers. **A:** characteristic inhibition of basal I_{sc} by apical addition of SP-303. Sequential addition of increasing doses of SP-303 results in a decrease in the basal I_{sc} (~49%), and subsequent addition of forskolin shows a significantly blunted response (~55%; $n = 8$). **B:** mean percent inhibition of the basal I_{sc} by increasing doses of SP-303. Values represent means \pm SE for 9 individual measurements.

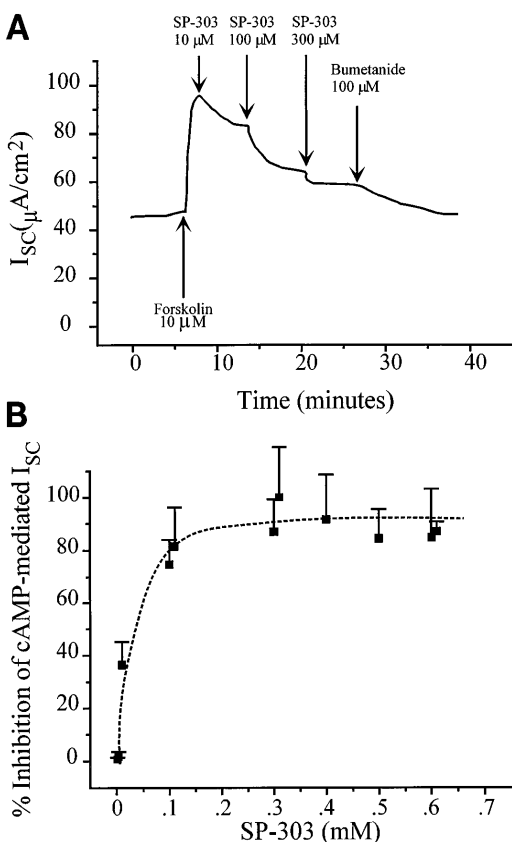


Fig. 3. Effect of SP-303 on I_{sc} of Caco-2 epithelial monolayers after stimulation by forskolin. *A*: characteristic I_{sc} recording showing stimulation of current by 10 μM forskolin and subsequent inhibition by repeated administrations of SP-303. Basolateral addition of 100 μM bumetanide, an inhibitor of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter, inhibits the residual current to prestimulated levels. *B*: dose-dependent inhibition of I_{sc} by SP-303 after stimulation by forskolin. Values represent means \pm SE of at least 6 individual epithelial monolayers treated with each SP-303 dosage and shown as a percent of the forskolin-stimulated I_{sc} . Maximal inhibition of the I_{sc} was achieved by addition of 300 μM SP-303. SP-303 also increased transepithelial resistance by $\sim 40\%$, consistent with an inhibition of Cl^- secretion.

stimulated current, if untreated, was previously shown (6) to be relatively stable for up to 45 min after stimulation. Maximal inhibition of the forskolin-stimulated I_{sc} was achieved by the addition of 300 μM SP-303 (Fig. 3*B*). The IC_{50} for SP-303 was ~ 50 μM . Bumetanide, an inhibitor of the basolateral $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter responsible for Cl^- entry, decreased the residual I_{sc} to prestimulated levels.

To determine that the effect of SP-303 was not restricted to a single intestinal epithelial cell line, we also studied inhibition of Cl^- secretion in another colonic cell line, T84. The T84 cell line is known to express CFTR at relatively high levels, and, when studied as a polarized monolayer in Ussing chambers, cAMP causes a significant increase in I_{sc} that is primarily due to Cl^- secretion (19). Moreover, by bathing the apical surface of the T84 monolayers in a Na^+ -free solution, we more effectively translate I_{sc} into a direct measurement of apical Cl^- secretion. Under these conditions, addition of 500 μM SP-303 to the apical

solution inhibited nearly all the I_{sc} stimulated by forskolin (mean inhibition of 88.7%; $n = 8$) (Fig. 4).

SP-303 did not have any effect on basal or forskolin-stimulated I_{sc} measured in Caco-2 monolayers exposed to bilateral Cl^- -free solutions (data not shown), although under these conditions basal currents were decreased before SP-303 addition. Basolateral addition of SP-303 to T84 monolayers was used as an assessment of nonspecific (metabolic) inhibitory effects of SP-303. T84 monolayers were bathed in bilateral KRB and exposed to 50 μM SP-303 in the basolateral bath followed by apical addition of forskolin (5 μM). Basolateral addition of SP-303 neither had an effect on basal current (28.4 ± 2.4 vs. 27.4 ± 1.1 $\mu\text{A}/\text{cm}^2$ before and after exposure to SP-303, respectively; $n = 3$) nor did it significantly blunt the subsequent increase mediated by forskolin (ΔI_{sc} of 103.7 ± 11.4 vs. 119 ± 13.8 $\mu\text{A}/\text{cm}^2$ with or without pretreatment by SP-303, respectively; $n = 3$). A further addition of 50 μM SP-303 to the basolateral bath (total concentration = 100 μM) did not have an effect on the forskolin-stimulated current. We were not able to reverse the inhibitory effect of apical SP-303 on forskolin-stimulated current in T84 monolayers. After stimulation of I_{sc} by forskolin, addition of 100 μM SP-303 to the apical solution resulted in the expected inhibition of current as previously reported in Fig. 3*A*. The apical bath solution was then replaced with identical Ringer solution devoid of SP-303. This maneuver did not restore I_{sc} levels (68.2 ± 7.8 vs. 67.8 ± 10.1 $\mu\text{A}/\text{cm}^2$, I_{sc} measured after inhibition by SP-303 and after bath replacement of SP-303, respectively; $n = 8$).

DISCUSSION

We have described effects of SP-303 on CT-induced fluid secretion in an *in vivo* mouse model and also on cAMP-mediated Cl^- secretion measured across epithelial cell monolayers. The animal studies demonstrate a potent, nearly complete inhibition of fluid secretion when SP-303 is applied either early (simultaneous to toxin delivery) or late (3 h after CT delivery, when

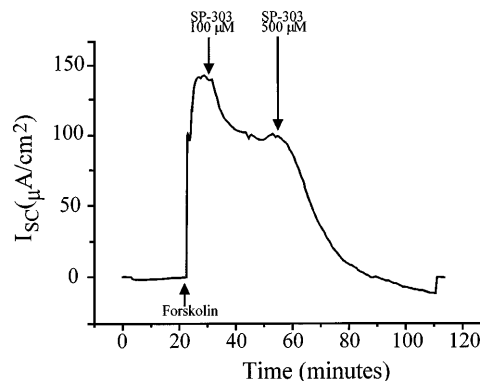


Fig. 4. Effect of SP-303 on Cl^- current in T84 epithelial monolayers. A typical I_{sc} recording of T84 epithelial cells and the effect of SP-303 in an apical Na^+ -free solution. Forskolin (10 μM) stimulation of I_{sc} (Cl^- secretion) is nearly completely inhibited by SP-303. Maximal inhibition ($88 \pm 22\%$) was achieved by apical addition of 500 μM SP-303 ($n = 8$).

cAMP levels are maximal and Cl^- secretion is strongly stimulated; Ref. 8). Interestingly, SP-303 was effective regardless of when it was delivered, suggesting that this agent may have utility as either a postsymptomatic antidiarrheal or as a prophylactic. It remains to be seen if SP-303 truly can function as a prophylactic agent and also how long before the introduction of CT is SP-303 capable of maintaining a protective effect.

Because SP-303 was shown to be efficacious 3 h after administration of CT, it follows reasonably that SP-303 does not interfere with CT binding, internalization, or cAMP elevation. We did, however, verify that SP-303 had no effect on either basal or elevated cAMP levels measured in T84 cells and thereby focused on a more direct effect on Cl^- secretion. Furthermore, preliminary studies in humans have not been able to detect SP-303 in plasma following oral administration, suggesting little or no systemic absorption (SHAMAN Pharmaceuticals, unpublished observations). Consistent with this hypothesis of little or no transcellular flux of SP-303 is the finding that administration of SP-303 to the basolateral solution had no significant effect on either basal or forskolin-stimulated currents. Importantly, we did not observe an effect of SP-303 on other non-CFTR transport processes (e.g., bumetanide-sensitive $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport or carbachol-mediated K^+ secretion; unpublished observations). These data suggest that administration of SP-303 by gavage is likely to have its effects at the apical membrane.

We have observed that the magnitude of Cl^- secretion mediated by forskolin is at least partially dependent on the magnitude of the basal current. Because CFTR is the primary mechanism for Cl^- secretion in both Caco-2 and T84 cells, it follows reasonably that channels that are already open and maximally active in the basal condition are not likely to be further activated by forskolin. Thus it is not surprising that we have observed effects of SP-303 on both basal and forskolin-stimulated Cl^- currents.

These data suggest that SP-303 may be acting at the site of Cl^- secretion, perhaps by directly inhibiting CFTR or by altering regulation of this channel. Interestingly, the active compound contains several exposed hydroxyl and carboxylic acid residues that might be able to penetrate the mouth of the Cl^- channel and occlude the pore and would thereby effectively inhibit various secretory diarrheas. It is still possible, however, that SP-303 may be exerting a general metabolic effect that may deplete cytosolic ATP levels and prevent normal gating of CFTR or other channels. Direct effects of SP-303 on the CFTR Cl^- channel will require more mechanistic studies, involving patch-clamp protocols to definitively determine if there is a direct effect.

Although these data suggest an effect on cAMP-mediated Cl^- conductance, it is not yet clear if this is a specific effect. Previous studies have demonstrated that CFTR is the predominant Cl^- channel in the apical membrane of the murine small intestine (5), but, in other epithelial tissues, SP-303 may interact with other Cl^- channels, i.e., volume, voltage, or intracellular

Ca^{2+} -activated Cl^- channels. If SP-303 does show high specificity to CFTR, by either inhibition and/or binding, this compound will be of great utility as a tool in understanding CFTR structure-function relationships, aside from its use as an antidiarrheal drug.

Although cholera is the classic example of a secretory diarrhea, other bacterial toxins such as *E. coli* ST and LT are also capable of causing fluid secretion. As shown previously, agents such as *E. coli* STa, regardless of initial second messenger activation, ultimately result in elevation of cAMP and stimulation of CFTR (3). Therefore, a specific inhibitor acting directly at CFTR could prove to be very useful as it would inhibit the final step associated with most if not all known bacterial agents causing secretory diarrhea.

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