Inhibition of enterotoxin-induced porcine colonic secretion by diarylsulfonylureas in vitro

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O’Donnell, Erin K., Roger L. Sedlacek, Ashvani K. Singh, and Bruce D. Schultz. Inhibition of enterotoxin-induced porcine colonic secretion by diarylsulfonylureas in vitro. Am J Physiol Gastrointest Liver Physiol 279: G1104–G1112, 2000.—Muscle-striped piglet colon was used to evaluate changes in short-circuit current (I sc) as an indicator of anion secretion. Mucosal exposure to Escherichia coli heat-stable (STa) or heat-labile enterotoxins (LT) stimulated I sc by 32 ± 5 and 42 ± 7 μA/cm2, respectively. Enterotoxin-stimulated I sc was not significantly affected by either 4,4’-diaminostilbene-2,2’-disulfonic acid or CdCl2, inhibitors of Ca2+-activated Cl− channels and ClC-2 channels, respectively. Alternatively, N’(4-methylphenylsulfonyl)-N’-(4-trifluoromethylphenyl)urea (DASU-02), a compound that inhibits cystic fibrosis transmembrane conductance regulator (CFTR)-mediated Cl− secretion, reduced I sc by 29 ± 7 and 34 ± 11 μA/cm2, respectively. Two additional diarylsulfonylurea (DASU)-based compounds were evaluated for their effects on enterotoxin-stimulated secretion. The rank order of potency for inhibition by these three closely related DASU structures was identical to that observed for human CFTR. The degree of inhibition by each of these compounds was similar for both STa and LT. The structure- and concentration-dependent inhibition shown indicates that CFTR mediates both STa- and LT-stimulated colonic secretion. Similar structure-dependent inhibitory effects were observed in forskolin-stimulated rat colonic epithelium. Thus DASUs compose a family of inhibitors that may be of therapeutic value for the symptomatic treatment of diarrhea resulting from a broad spectrum of causative agents across species.

cystic fibrosis transmembrane conductance regulator; diarrhea; pharmacology; LY-295501

CONSIDERABLE SOCIOLOGICAL and economic cost is associated with intestinal hypersecretion. Secretory diarrhea ranks as one of the world’s top infectious killers, affecting mostly children in third world countries (1) and causing an estimated 5 million human deaths annually (33). In the United States, children under the age of 5 years experience 20–35 million bouts of diarrhea every year, resulting in 2–3.7 million doctor visits, >200,000 hospitalizations, and 500 deaths (12, 20). Although significant focus might be placed on neonatal and pediatric diarrhea, >50% of diarrhea-related deaths in the United States occur in persons over the age of 74, a growing portion of the population (19). Thus significant incentive is present to develop safe, effective, and broad-spectrum treatments for diarrhea.

Vibrio cholerae and Escherichia coli are among the major agents responsible for infectious diarrhea in both humans and animals (14). Enterotoxins of E. coli are classified as either heat stable (STa and STb) or heat labile (LT) (13). Cholera toxin (CT) is virtually identical to LT in both structure and function (29, 34). These toxins are not cytotoxic, but they interact directly with intestinal epithelial cells to stimulate profound fluid and electrolyte secretion into the lumen of the gut. Severe dehydration compromises multiple physiological systems in affected individuals and can ultimately lead to death or can set the individual up for opportunistic infection by other organisms.

The biochemistry of enterotoxins and the cellular pathways that contribute to enterotoxin stimulation of electrolyte transport are relatively well characterized (see Refs. 5, 13, 15, 29, and 34 for review). A key player in the response is thought to be the cystic fibrosis transmembrane conductance regulator (CFTR), an apical membrane anion channel. Treatments for diarrhea aimed at blocking this ion channel have been theoretically proposed (7). Indeed, attempts have been made to reduce toxin-stimulated secretion by blocking anion channels (10), although the compounds available proved not to be effective. The widespread and successful use of oral rehydration therapy in humans indicates that simply circumventing or preventing dehydration will greatly reduce morbidity and mortality associated with enterotoxigenic infections. Thus an intervention that acutely reduces enterotoxin-stimulated intestinal fluid loss will be of great value in both human and veterinary medicine.

Results from the present study document that LT and STa functionally share a common anion conductive component in porcine colon. Pharmacological evidence indicates that the channel most likely responsible for enterotoxin-induced Cl− secretion in neonatal porcine

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colon is the CFTR Cl\(^{-}\) channel, which is blocked by diarylsulfonylureas (DASUs). Comparative results are presented for the inhibition of rat colonic secretion. Thus pharmacological intervention to selectively inhibit CFTR is a means by which a broad spectrum of secretory diarrheas might be managed across species.

**MATERIALS AND METHODS**

**Tissue acquisition.** Twenty-seven mixed-breed suckling 7- to 11-day-old pigs were purchased from reputable local sources for use as tissue donors for in vitro studies. Pigs were euthanized by an overdose of pentobarbital sodium in accordance with protocols approved by the Kansas State University Institutional Animal Care and Use Committee. Female Sprague-Dawley rats were anesthetized and then euthanized by cervical dislocation. The spiral colon was removed from each pig, linearized, and flushed with ice-cold Ringer’s solution of the following composition (in mM): 120 NaCl, 25 NaHCO\(_3\), 1.2 MgCl\(_2\), 1.2 CaCl\(_2\), 3.3 KH\(_2\)PO\(_4\), and 0.8 K\(_2\)HPO\(_4\). Indomethacin (50 mM; Sigma Chemical, St. Louis, MO) was included in all solutions to preclude prostaglandin synthesis. Distal rat colon was prepared by the same method. In all cases, the colon was split along the mesenteric margin and the muscularis was carefully separated from the epithelial mucosa.

**Apparatus.** Mucosal epithelium was mounted in modified Ussing chambers (model DCV9, Navecyle, San Diego, CA) with 0.64 cm\(^2\) of exposed surface area. Mucosal and serosal compartments contained 5 ml of Ringer solution with 10 mM mannitol and glucose, respectively. Chambers were maintained at 39°C and continually mixed with a bubble lift system (95% O\(_2\)-5% CO\(_2\)) that maintained pH at 7.4. Tissues were clamped to zero transepithelial voltage (model 558C, Department of Bioengineering, University of Iowa, Iowa City, IA), and the short-circuit current (I\(_{sc}\)), which represents the algebraic sum of active ion-transport processes of the tissues, was recorded. Electrical conductance was determined using Ohm’s law by exposing the tissues to a 1-mV bipolar pulse (5-s duration) at 100-s intervals and recording the current deflections. I\(_{sc}\) was digitally acquired using an MP100A-CE interface and AcqKnowledge software (version 3.2.6; BIOPAC Systems, Santa Barbara, CA) on a Macintosh computer (Apple Computer, Cupertino, CA).

**General protocol.** After mounting, tissues were allowed to acclimate for 5–10 min before the transepithelial voltage was clamped. Once a stable baseline was observed, TTX (1 \(\mu\)M; Sigma Chemical) was added to the mucosal chambers to eliminate the residual activity of any nerves remaining associated with the mucosa. Amiloride (10 \(\mu\)M; Sigma Chemical) was then added to the apical compartment to reduce variation between tissues caused by electrogenic Na\(^{+}\) absorption. Three anion transport inhibitors were used throughout the study [CdCl\(_2\), DNDS, and N\(-(4-methylphenyl)sulfonyl)-N\'-4(trifluoromethyl)phenylurea (DASU-02)].

**RESULTS**

In nonstimulating (i.e., basal) conditions, porcine spiral colon epithelium exhibited an I\(_{sc}\) of 48.8 \(\pm\) 6.2 \(\mu\)A/cm\(^2\) (mean \(\pm\) SE; 27 pigs, 110 tissues), which is consistent with either net anion secretion or cation absorption. Basal resistance in these tissues was 117 \(\pm\) 8 \(\Omega\)-cm\(^2\). Treatment of the tissues with TTX to inhibit the activity of any adherent enteric nerves resulted in a modest but significant reduction in I\(_{sc}\) (\(-3.6 \pm 0.9 \mu\)A/cm\(^2\); 24 pigs, 98 tissues). The average change in I\(_{sc}\) caused by the addition of amiloride was \(-16.8 \pm 5.1 \mu\)A/cm\(^2\) (\(n = 24\) pigs, 98 tissues), indicating that a portion of the basal current could be attributed to electrogenic Na\(^{+}\) absorption. Three anion transport inhibitors were used throughout the study [CdCl\(_2\), DNDS, and N\-(4-methylphenyl)sulfonyl)-N\'-4(trifluoromethyl)phenylurea (DASU-02)]. CdCl\(_2\) and DNDS were consistently without effect on basal I\(_{sc}\). DASU-02 was sometimes associated with a reduction in basal I\(_{sc}\) (see, e.g., Fig. 4), although statistical significance was not achieved (\(P > 0.06\)) with observations on four tissues.

**LT-induced secretion is selectively inhibited by DASUs.** Depicted in Fig. 1 are results from experiments demonstrating that LT stimulates porcine colonic I\(_{sc}\) in a manner consistent with anion secretion and that this secretion is selectively sensitive to anion conductance blockers. As expected, TTX and amiloride reduced I\(_{sc}\). LT was added to the apical compartment and, after a delay of 1 h, caused a slowly mounting increase in I\(_{sc}\). An increase in current to 95 \(\mu\)A/cm\(^2\) was observed after an additional 80 min. Anion channel blockers were then evaluated for their effects on LT-stimulated I\(_{sc}\). CdCl\(_2\), an inhibitor of CIC-2 channels, and DNDS, an inhibitor of outwardly rectifying and Ca\(^{2+}\)-activated Cl\(^{-}\) channels, were virtually without effect. Alternatively, DASU-02, a DASU that was previously reported...
(26, 27) to inhibit CFTR Cl⁻ channels, caused an immediate and significant reduction in $I_{sc}$. It should be noted that the order of addition had no impact on the outcomes; CdCl₂ and DNDS were without effect and DASU-02 caused profound inhibition. Bumetanide, a loop diuretic that inhibits intestinal Cl⁻ secretion by blocking the loading step at the basolateral membrane, was added to determine the magnitude of anion secretion remaining. A modest, although statistically significant, inhibition was observed. Data from a total of 12 similarly treated tissues are summarized in Fig. 1B. The data show that, compared with the previous conditions, amiloride ($P \leq 0.02$), LT ($P \leq 0.001$), DASU-02 ($P \leq 0.005$), and bumetanide ($P \leq 0.003$) significantly altered $I_{sc}$, whereas TTX, CdCl₂, and DNDS were without effect.

Anion conductance modulators had similar effects on forskolin-stimulated $I_{sc}$. Shown in Fig. 2A are results recorded in the presence of TTX and amiloride. In this tissue, forskolin caused a 118 µA/cm² increase in $I_{sc}$ that then modestly decreased over time. Neither the addition of CdCl₂ nor DNDS caused any inflection in the $I_{sc}$ recording. Alternatively, DASU-02 caused an immediate and marked decrease in $I_{sc}$ after which bumetanide was associated with a modest increment of inhibition. The results presented in Fig. 2A are representative of results from 11 tissues (5 pigs), which are summarized in Fig. 2B. Because the forskolin-induced increase in $I_{sc}$ tended to decline slowly, there was a modest but statistically insignificant reduction in $I_{sc}$ that occurred while CdCl₂ and DNDS were being evaluated. In every case, however, dramatic inhibition of $I_{sc}$ accompanied exposure to DASU-02. In two additional tissues, substantial inhibition of forskolin-stimulated $I_{sc}$ was observed with a second recognized inhibitor of CFTR, diphenylamine-2-carboxylic acid (1 mM; data not shown).

A third agonist that putatively acts by stimulation of adenyl cyclase, VIP, was also used as a stimulant of $I_{sc}$. The effects of anion channel blockers on VIP-stimulated $I_{sc}$ were virtually identical to those observed in the presence of LT or forskolin. It is obvious from inspection that neither DNDS nor CdCl₂ was associated with any change in $I_{sc}$. However, because the VIP-induced increase in $I_{sc}$ tended to decline slowly, there was a statistically significant reduction in $I_{sc}$ that occurred while CdCl₂ and DNDS were being evaluated. More importantly, DASU-02 caused rapid and nearly complete inhibition of VIP-stimulated $I_{sc}$ (Fig. 2).

STa-induced secretion is selectively inhibited by DASUs. Depicted in Fig. 3 are results from experiments demonstrating that, like LT, STa stimulates colonic $I_{sc}$ in a manner consistent with anion secretion and that this secretion is selectively sensitive to anion conductance blockers. Again, TTX and amiloride reduced basal $I_{sc}$, whereas TTX, CdCl₂, and DNDS were without effect.

Anion channel blockers were then evaluated for their effects on STa-stimulated $I_{sc}$. DNDS and CdCl₂ were without effect. Alternatively, DASU-02 caused an immediate and significant reduction in $I_{sc}$. It should again be noted that the order of addition had no impact on the outcomes; CdCl₂ and DNDS were without effect and DASU-02 caused profound inhibition. Bumetanide again had only a modest effect on $I_{sc}$ after inhibition by DASU-02, indicating that little Cl⁻ secretion remained. Data from a total of 14 tissues (7 pigs) are summarized in Fig. 3B. The data show that, compared with the previous conditions, TTX ($P \leq 0.003$), amiloride ($P \leq 0.001$), STa ($P \leq 0.001$), and DASU-02 ($P \leq 0.001$), significantly altered $I_{sc}$, whereas CdCl₂ and DNDS were without effect.

DASU-02 precludes enterotoxin-induced anion secretion. Experiments were conducted to test the hypothesis that DASU-02 could be effective as a prophylactic treatment for enterotoxin-induced secretion. Data presented in Fig. 4 show that DASU-02, but not DNDS and CdCl₂, can reduce basal anion secretion. It should be noted, however, that such a reduction in $I_{sc}$ was not consistently observed. Subsequent exposure to STa re-
sulted in an increase in $I_{\text{sc}}$, but the maximal change in $I_{\text{sc}}$ in the presence of DASU-02 was <40% of that observed in control tissues ($n = 2$ pairs of tissues from 2 pigs). There was no difference in response to STa between control tissues and tissues pretreated with DNDS and CdCl$_2$ (not shown). Virtually identical results were obtained when VIP was used as the stimulant; maximal response in the presence of DASU-02 was <20% of that observed in control tissues or tissues pretreated with DNDS and CdCl$_2$ ($n = 2$ pairs of tissues from 2 pigs).

The results presented in Fig. 4 also demonstrate that inhibition of intestinal ion transport by DASU-02 is not caused by a nonselective, toxic effect of the compound. In the presence of DASU-02, colonic epithelial cells retain their ability respond to receptor-mediated stimuli from both the apical (STa) and basolateral (VIP) membranes, albeit at a much reduced magnitude. Further evidence of this conclusion is provided by the observation that the mucosa displays a prototypical response to carbamylcholine that is characterized by a transient increase in $I_{\text{sc}}$.

Structure dependence of DASU inhibition. Data presented in Fig. 5 demonstrate that inhibition of enterotoxin-stimulated ion transport by DASUs is structure dependent. All tissues were pretreated with TTX and amiloride before stimulation with either LT (Fig. 5, A, C, E, and G) or STa (Fig. 5, B, D, F, and H). Data from three to five similar experiments for each set of conditions are summarized for LT- and STa-stimulated tissues in Fig. 5, I and J, respectively. Concentration dependence of $I_{\text{sc}}$ inhibition for the benchmark compound DASU-02 is presented in Fig. 5, A and B. It should be noted that the secretory effects of both LT and STa are significantly inhibited by 30 µM DASU-02, the lowest concentration tested ($P \leq 0.053$ for LT; $P \leq 0.048$ for STa; $n = 5$ each); the concentration of 300 µM is maximally effective, as evidenced by the fact that increasing the concentration from 300 to 600 µM had virtually no effect. These data demonstrate a similar concentration dependence for in-
Inhibition of both LT- and STa-stimulated secretion, further documenting that a common target is being affected in tissues stimulated by the two enterotoxins. Typical control data are presented in Fig. 5, A and B. The solvent vehicle DMSO had no effect on enterotoxin-stimulated I_{sc}, although nearly complete inhibition of enterotoxin-stimulated secretion was achieved in these tissues by the addition of 300 μM DASU-02. LY-295501 is a structurally related compound that is reported to be oncolytic and is in Phase II clinical trials. Like DASU-02, LY-295501 reduced I_{sc} in enterotoxin-stimulated tissues, although statistically significant inhibition (P < 0.05) was observed only at the highest concentration used (Fig. 5, C and D). The maximal magnitude of inhibition was similar to that observed with DASU-02, and little inhibition was observed when tissues were exposed to DASU-02 in the presence of LY-295501. In contrast, exposure to CH3-DASU-H, which differs from DASU-02 only by the para-substituent of the urea phenyl (H vs. CF3), resulted in virtually no inhibition of enterotoxin-stimulated I_{sc}, even at the highest concentration tested (300 μM; Fig. 5, E and F). Subsequent exposure to DASU-02 resulted in profound and immediate reversal of the enterotoxin-stimulated increase in I_{sc}, ruling out the possibility that the tissues had become unresponsive to DASUs. The rank order of potency (DASU-02. LY-295501. CH3-DASU-H) and the relative proportion of inhibition were identical, regardless of whether the stimulant was LT or STa. DASU-02 was shown to be the most potent inhibitor of LT- and STa-stimulated colonic secretion, with effects consistently observed at the lowest concentration tested.

Structure-dependent inhibition of rat colonic secretion. Results from experiments using rat colonic epithelium are presented in Fig. 6. It should be noted that these results closely parallel the observations presented in Fig. 5. A total of 10 closely related DASU-based structures were evaluated, and results from the three most instructive compounds are presented here. In the presence of indomethacin, TTX, and amiloride, basal I_{sc} was 50 ± 7 μA/cm² (n = 32 tissues). Forskolin caused an increase in
Isc of 192 ± 10 μA/cm² that was subsequently reversed by selected DASUs. Once again, DASU-02 was the most potent of the compounds tested, with inhibitory effects observed at the lowest concentration tested, 10 μM. More than 80% of the forskolin-stimulated increment in Isc was inhibited by 100 μM DASU-02. Inhibitory effects were likewise observed when LY-295501 was used as the antagonist. Modest inhibition was observed in the presence of 30 μM, whereas exposure to 100 μM yielded significant reduction in Isc (P < 0.001). Neither CH₃-DASU-H nor the carrier vehicle (DMSO) had any observable effect on forskolin-stimulated ion secretion in rat colon. Additional experiments indicated that LY-295501 had no effect on basal Isc but reduced the subsequent effects of VIP, serotonin, and forskolin (data not shown). These results further document that DASUs are effective inhibitors of intestinal secretion across a broad spectrum of stimulants and across species.

**DISCUSSION**

The results of this study have two major implications. First, a lead compound for the symptomatic treatment of secretory diarrhea is identified. Second, the results confirm previous indications that STa and LT affect a common final pathway of electrolyte secretion, CFTR.
Enterotoxigenic diarrhea depends on the activity of endogenous host mechanisms, which culminates in activation of an apical anion conductance. Thus interruption of the host response would limit fluid loss resultant from colonization and toxin production. Any required epithelial component would be a logical target for intervention. Zhang et al. (38) reported that inhibition of the guanylyl cyclase cascade precluded the effect of STa on intestinal epithelial cells. Such an approach provides proof of the concept, but specificity might be an issue in a clinical setting; either multiple cascades must be blocked or a common element in all cascades must be identified. Alternatively, it has been suggested (7, 21) that blockage of apical anion channels would be a logical therapeutic target. In support of this proposal are numerous studies (6, 11, 30) that showed that CFTR −/− mice were insensitive to enterotoxins, indicating that a CFTR channel blocker would render the gut resistant to enterotoxin stimulation. Such an approach was previously taken by Forsyth and Gabriel (10), although the compounds available at the time of their studies did not prove to be effective when used in vivo.

Results presented in this study suggest that a newly identified family of compounds, DASUs, could prove to be effective in the treatment of enterotoxigenic diarrhea. The results demonstrate a similar pharmacological profile of ion-transport inhibition for LT, CT, forskolin, VIP, and STa. The possibility that receptor antagonism, G protein inhibition, or inhibition of adenylyl cyclase could account for the inhibition is ruled out by the spectrum of stimulants used. It remains possible that DASUs could mediate their effects by modulation of endogenous kinases or phosphatases. However, it was previously reported (25, 28) that DASUs reversibly block CFTR Cl− channels in excised membrane patches. A reversible effect in an excised membrane patch strongly suggests a direct interaction with the ion channel rather than a regulatory protein. Thus the results presented indicate that each of these agonists stimulates a cascade that culminates in the activation of CFTR.

The possibility of other anion conductances contributing to the enterotoxin-induced secretion was evaluated with the experimental paradigms used here. ClC Cl− channels are reported to be present in a variety of epithelial cells including those of the gastrointestinal tract (17). At least some members of this gene family of Cl− channels are blocked by Cd2+ in the micromolar range (3, 9, 23). Additionally, ClC-2g channels expressed in Xenopus oocytes are reversibly inhibited by 300 μM Cd2+ (B. D. Schultz, unpublished observations). Ca2+-activated Cl− channels (CaCC) and outwardly rectifying Cl− channels (ORCC) are likewise reported to be present in a variety of epithelia, including those of the gastrointestinal tract. Although these channels are not yet fully described at the molecular level, both classes of Cl− channels are widely reported to be inhibited by disulfonic stilbenes (2, 4, 18, 22, 32, 36, 37). Thus the complete lack of inhibition by both Cd2+ and DNDS indicates that ClC, CaCC, and ORCC...
channels do not participate in enterotoxin-stimulated anion secretion in the colon, thus eliminating them as possible targets for therapeutic intervention.

DASUs show promise in the symptomatic treatment of enterotoxigenic diarrhea across species. Because oral rehydration therapy is effective in the treatment of enterotoxigenic diarrhea, it is obvious that antibiotic intervention is not required to treat the disease. Rather, if adequate hydration can be maintained, normal physiological processes can bring about a complete cure. We (25) previously found that DASUs inhibited ion transport in epithelial cells of human colonic origin (T84 cells) and here report their effectiveness in pig and rat colon. Thus DASUs hold therapeutic promise for the treatment of secretory diarrhea across a variety of species.

Sheppard and Welsh (31) first reported that sulfonylureas could inhibit CFTR-mediated anion transport. The compounds that they (31) identified, glibenclamide and tolbutamide, were subsequently shown (24, 35) to directly modulate CFTR channel activity in excised membrane patches. However, these compounds are not viable candidates for the therapeutic treatment of diarrhea because they are widely used as antidiabetic agents and are known to induce hypoglycemia. Alternatively, DASUs do not universally cause hypoglycemia (16) and were thus investigated for effects on CFTR. Preliminary evidence has shown that this family of compounds exhibit structure-dependent inhibition of CFTR channel gating (25). The structure and concentration dependence of CFTR inhibition in excised membrane patches was similar to the inhibition of intestinal secretion demonstrated in the present study. Furthermore, the compounds that were most effective in reducing enterotoxin-stimulated anion secretion are known to have modest (DASU-02) or no (LY-295501) effect on insulin secretion or blood glucose levels (16, 25). More importantly, LY-295501 is currently in Phase II clinical trials as an oncolytic, which indicates that it can be safely administered to humans. This compound can be delivered orally and is expected to exhibit a relatively long half-time of elimination based on observations in other species (8). Because CFTR exhibits little variation in structure across species, it is reasonable to predict that similar therapeutic effects would be observed in all species. Certainly, additional studies will be required to determine the in vivo efficacy. At the very least, a lead structure has been identified that is not antimicrobial and appears to be therapeutic for treatment of diarrhea in a variety of species.

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