Activation of Chloride Secretion in Cystic Fibrosis Cells and Tissues by the Substituted Imidazole SRI 2931[†]

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ABSTRACT: Recent interest in nucleotides and related agents as part of clinical trials in cystic fibrosis (CF) therapy have elicited efforts to identify novel compounds capable of activating transepithelial chloride (Cl^-) transport in CF cells and tissues. From a library of nucleosides, bases, and other substituted heterocycles, 341 compounds were screened for their ability to activate anion transport in CF cells grown on permeable supports. One compound, SRI 2931, was found to confer prolonged and potent activity when administered to the apical surfaces of CF pancreatic epithelial cells, primary CF nasal epithelial cells, non-CF human colonic epithelial cells, and intact tissue taken from mouse models for CF. Concentrations of SRI 2931 (20 μM), which activated Cl^- transport, had minimal effect on cell proliferation. SRI 2931 was not calcium (Ca^{2+}) or cAMP dependent, suggesting important differences from conventional chloride secretagogues. The compound selectively released ATP from the apical, but not basolateral, surfaces of CF cells grown on permeable supports. The magnitude, longevity, and mechanism of action of the response provide a tool for dissecting pathways of epithelial ATP extracellular signaling and Cl^- permeability.

Cystic fibrosis is an autosomal recessive disorder caused by defects in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR),[1] a cAMP-regulated epithelial chloride channel. Diminished CFTR channel activity leads to alterations in the volume and composition of epithelial surface liquid in CF-affected organs including the intestines, pancreas, vas deferens, sweat ducts, and airways (*1*, *2*). The pathological changes in CF that contribute most prominently to morbidity and mortality occur in the airways, which become impacted with viscous mucus. Chronic bacterial infections in CF evoke neutrophil-mediated inflammatory responses and cumulatively damage lung parenchyma. Because defects in Cl^- secretion are believed to contribute to CF pulmonary pathophysiology, there is a substantial need to develop therapies to restore and/or bypass defective CFTR Cl^- channel activity in the disease.

One strategy for development of new CF therapeutics is to identify compounds that correct specific defects in CFTR. For example, the common ΔF508 mutation in CFTR is misfolded and poorly expressed at the apical cell surface. Efforts are underway to increase levels of ΔF508-CFTR in the plasma membrane and/or increase open probability of the mutant ion channel. Ma et al. (*3*) have recently identified a number of novel compounds that act as high-affinity activators of both wild type (wt) and ΔF508-CFTR channels in vitro.

A second strategy regarding development of therapies for CF airway disease is to identify compounds that directly or indirectly increase Cl^- transport through mechanisms that are independent of CFTR. This approach might be applied to a multitude of alternative chloride permeability pathways and includes both channels and regulatory proteins as possible therapeutic targets. CF patients as a group might benefit from therapies that successfully bypass defects in CFTR anion transport, regardless of genotype. Previously, ATP, UTP, adenosine, and related heterocyclic compounds have been shown to elicit transient increases in Cl^- secretion across normal and CF airway epithelia. These compounds utilize a variety of mechanisms, including functions independent of CFTR (*4*−*10*). On the basis of these earlier observations, we screened a synthetic library of nucleosides and related compounds for the ability to activate transepithelial Cl^- transport in cells functionally null for CFTR (i.e., ΔF508 homozygous). Initially, we tested for possible effects using CF cells grown on filters and mounted in Ussing chambers. The advantage of this approach is that filter-grown cells are often more highly differentiated as compared to cells grown on plastic. Once an active compound was identified in the Ussing chambers assay, we further quantified the effect and possible mechanism of action using cells grown on filters or plastic and intact murine tissues. We report the discovery

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[1] Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator.

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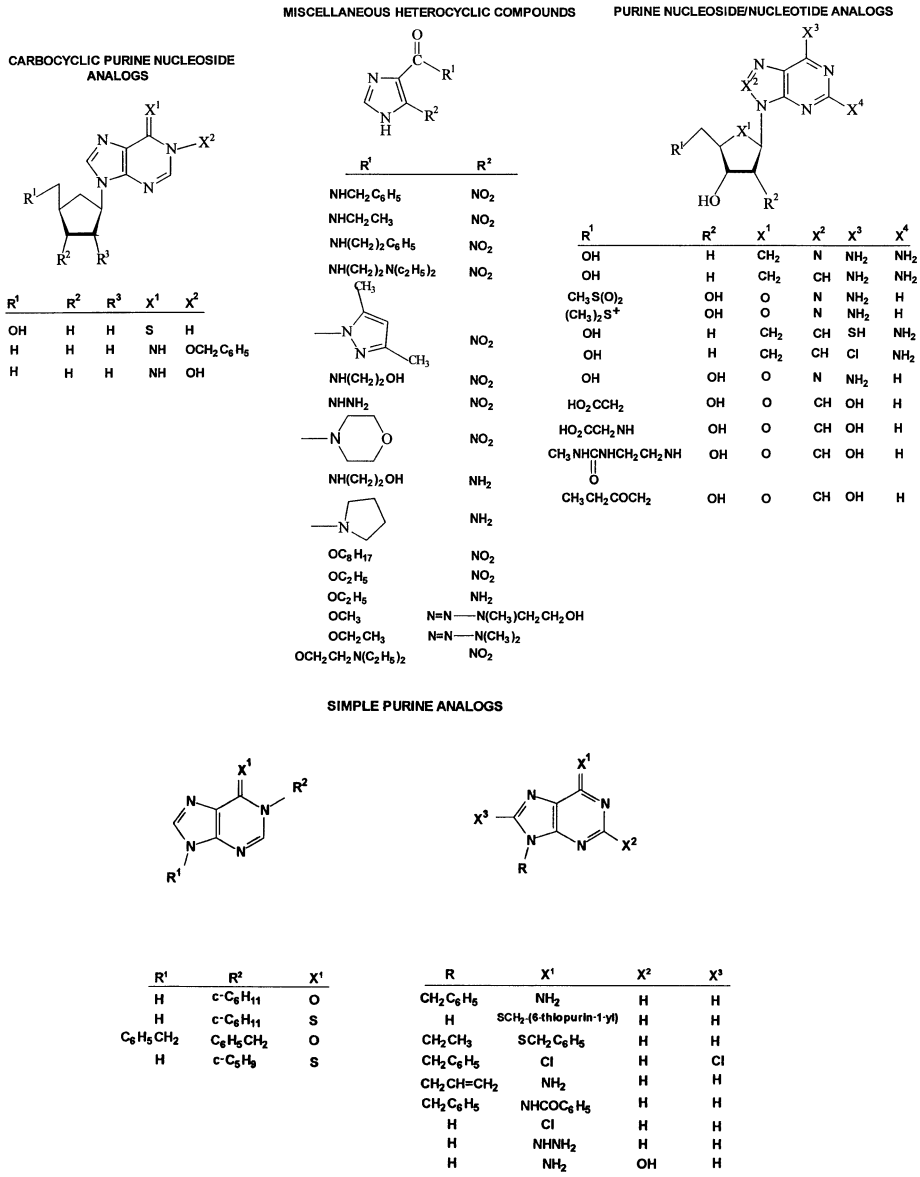

FIGURE 1: Library of compounds. Examples of compound diversity from which 341 drugs were screened for their ability to activate anion transport in CFPAC-1 cells.

of an agent with the unusual property of increasing both ATP and chloride release selectively from apical surfaces of epithelial cells.

EXPERIMENTAL PROCEDURES

Screening of Compounds. The Kettering−Meyer laboratory at Southern Research Institute, Birmingham, AL reviewed their drug repository of unique, synthetic compounds and provided a panel of over 1000 drugs, which included purine and pyrimidine nucleosides, bases, and related molecules. Examples of the types of compounds available in the repository are shown in Figure 1. From this library, 341 drugs representative of small families of compound diversity were screened for their ability to activate a Cl^- dependent current in cystic fibrosis pancreatic adenocarcinoma cells (CFPAC-1) (*11*, *12*). The cells were grown to confluency on permeable Millipore supports (area = 0.6 cm^2) for 10 days. Filters were mounted in an Ussing chamber, and current measurement was carried out as previously described (*13*). Filters were bathed in Ringer's solution (in mM: 145 Na^+, 5 K^+, 124.8 Cl^-, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 25 HCO_3^-, 4.2 PO_4^{2-}, and 10 glucose; pH = 7.4) on the basolateral surface and 6 mM Cl^- Ringer's solution (118.8 mM Cl^- replaced by the impermeant anion gluconate) on the apical surface. Drugs were screened by adding 10 and 100 μM concentrations of each compound to the apical bathing solution. To ensure that cells were capable of ion transport, 100 μM UTP was added to the apical surface at the end of each experiment. Experiments in which no UTP response was observed were disregarded, and the experiment was repeated using new filters. Each compound was tested at least twice. The effect of forskolin (10 μM) was also investigated.

Ussing Chamber Experiments. CFPAC-1 cells and T84 cells were obtained from ATCC and cultured on Millipore millicell HA or Costar transwell filters, respectively. Filters at confluency were mounted in Ussing chambers (Jim's Instrument Mfg Co., Iowa City, IA) and voltage clamped to 0 mV (Warner Instrument Co., Hamden, CT). The Ussing chambers were water-jacketed to maintain the bath temper-

ature at 37 °C, and solutions were stirred via integral gas lifts. Initially, all cells were bathed in symmetric Ringer's solutions as described previously. In certain experiments, the apical bathing solution was replaced with a low (6 mM) Cl^- Ringer's solution to facilitate anion secretion. Other experiments were performed with a reversed Cl^- gradient (i.e., with Ringer's solution on the apical side and 6 mM Cl^- solution on the basolateral side) or with 6 mM Cl^- present in both bath solutions. All Ussing chamber solutions were gassed using a mixture of 95% O_2 and 5% CO_2 to maintain the bath pH at 7.4.

Primary Airway Cell Culture: Nasal polyps from CF and non-CF patients were obtained as remnant polypectomy tissues from The Children's Hospital of Alabama following approval from the UAB Institutional Review Board. Specimens were rinsed in ice cold, sterile phosphate buffered saline (PBS) several times to remove mucus, debris, and blood clots. Tissue was dissociated by Pronase digestion and incubated in 5% CO_2 at 37 °C in SABM media (Clonetics, Walkersville, MD) containing the following: bovine pituitary extract (30 μg/mL), hydrocortisone (0.5 μg/mL), bovine insulin (0.5 μg/mL), epinephrine (0.5 μg/mL), transferrin (10 μg/mL), triiodothyronine (T_3) (6.5 ng/mL), epidermal growth factor (0.5 ng/mL), bovine serum albumin (0.5 mg/mL), retinoic acid (0.1 ng/mL), gentamicin sulfate (50 μg/mL), and amphotericin B (50 ng/mL). Cells were collected by centrifugation (200*g*, 5 min), resuspended in SABM media with supplements as described previously, and cultured on plastic dishes.

Measurement of Iodide Efflux. CFPAC-1 cells or primary CF nasal epithelial cells homozygous for the ΔF508 mutation were grown on 35 mm culture dishes, and effluxes were performed when the cells were 80−100% confluent using a HEPES-phosphate buffered Ringer's solution (HPBS). Cells were loaded with ^{125}I (2−5 μCi/mL) for 30 min and then washed with HPBS to remove extracellular ^{125}I. Efflux was detected by measuring the radioactivity present in bath solutions that were exchanged every 15−30 s. After five to seven time points, test compounds were added to the replacement solutions. Forskolin (10 μM) and ionomycin (3 μM) were used as negative and positive controls, respectively. Excel was used to express the isotope remaining in the cells as a percent of the total at time zero and to calculate the efflux rate for each sample period based on the following equation: $r = \ln(^{125}I_1/^{125}I_2)/(t_1 - t_2)$, which was derived from an exponential decay function (*14*). The counts in the cells at intervals 1 (t_1) and 2 (t_2) are given by $^{125}I_1$ and $^{125}I_2$ respectively.

Studies of CF Mouse Intestinal Ion Transport. CF mice encoding the ΔF508 mutation (*cftr*^{tm1Kth}) were generously provided by Dr. Kirk Thomas's laboratory at the University of Utah. Genotyping of progeny was performed by PCR using the following primers: CF26 (5′-TTCAAGCCCA AGCTTTCGGGAG-3′), CF27 (5′-CTCCCTTCTTCTAGT-CACAACCG-3′), and CF28 (5′-CATCCTGATAGAGCC ACGGTGC-3′). Primers CF26 and CF27 amplify a 300-bp product from the ΔF508 allele, and primers CF27 and CF28 amplify a 430-bp product from the wild type allele.

Small sections (6 × 10 mm) of distal colon were removed from euthanized *cftr*^{tm1Kth} (homozygous normal or homozygous ΔF508) mice. These sections were mounted as flat sheets in modified Ussing chambers, and the current was recorded as described previously. Both mucosal and serosal surfaces were bathed in Ringer's solution (omitting glucose to minimize contribution of sugar dependent ion transport pathways), and tetrodotoxin (TTX) (1.54 nM) was added

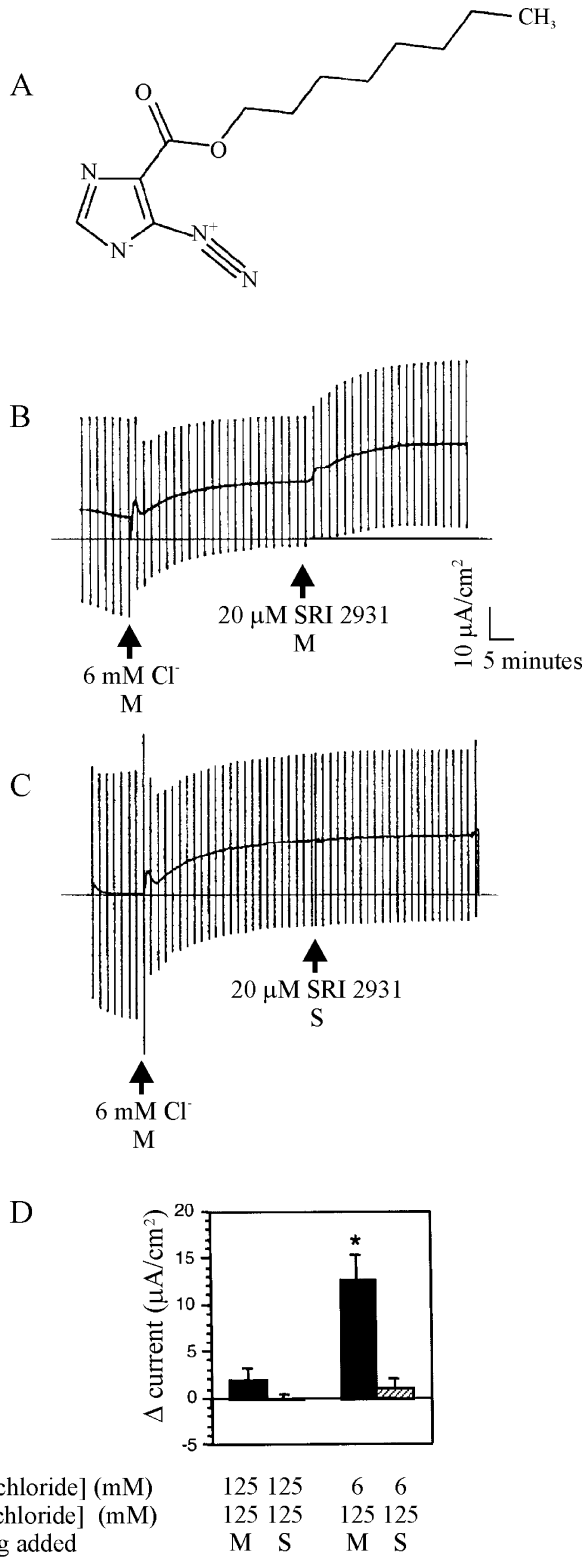

FIGURE 2: Structure of SRI 2931 and current measurements in CFPAC-1 cells. As shown in panel A, SRI 2931 is a substituted imidazole. The tracings (B and C) and summary data (D) describe the effects of SRI 2931 on activation of anion transport after addition to the mucosal (M) vs serosal (S) surface (*$p < 0.05$, as compared with untreated control; $n = 5$ filters per condition). Error bars = standard deviation (SD).

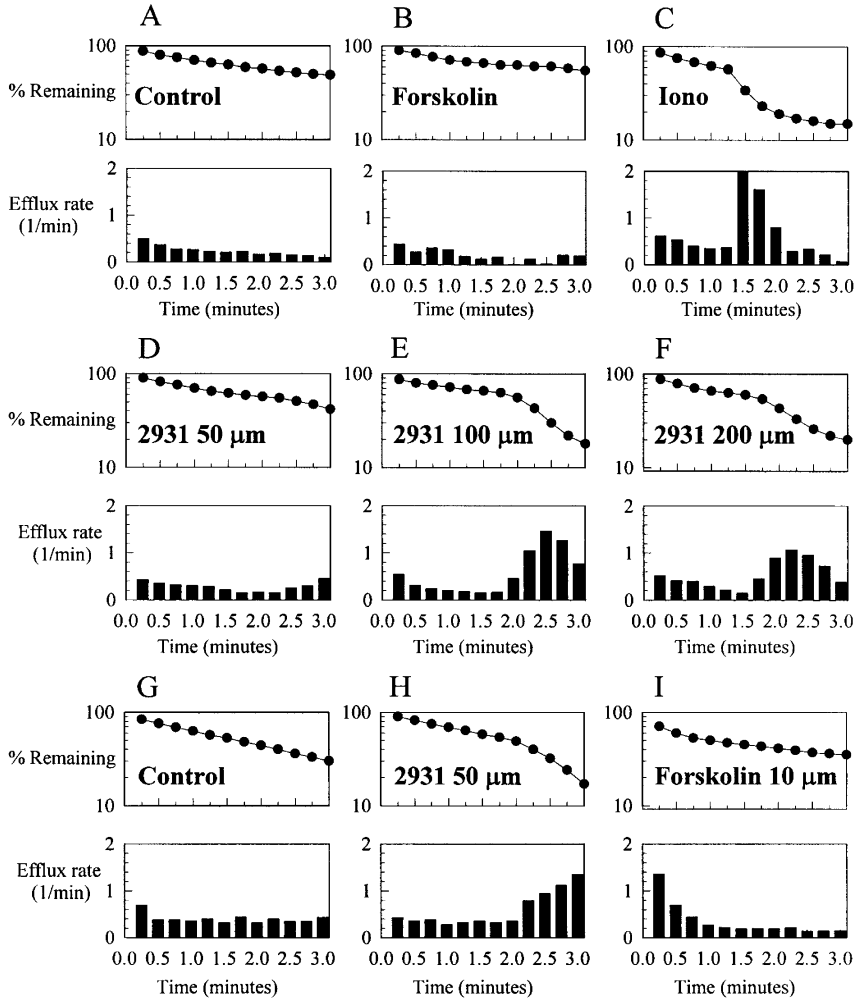

FIGURE 3: Iodide efflux in CF cells. Panels A−F, iodide efflux in CFPAC-1 cells. Panel A, untreated control cells; panel B, cells treated with forskolin (10 μM); panel C, cells treated with ionomycin (3 μM); panels D−F, cells treated with increasing concentrations of SRI 2931. Each panel is representative of three repeat experiments. Panels G−I, iodide efflux in primary CF nasal epithelial cells. Each panel is representative of three experiments. Panel G, untreated control cells; panel H, cells treated with SRI 2931 ($p = 0.01$, as compared with untreated control); and panel I, cells treated with forskolin. Compounds were added to the cells at 1.5 min.

immediately to the serosal side of the tissue to block endogenous (voltage sensitive) sodium channels present in submucosal neurons. After a steady state was achieved, amiloride (100 μM) was added to the mucosal bath to block epithelial Na^+ channels. SRI 2931 (100 μM) was added first to the mucosal and then to the serosal bath solutions. Four wild type and five ΔF508 mice were used in this study.

Signal Transduction and Inhibitor Studies. Pharmacologic inhibitors were tested as probes for the pathway activated by SRI 2931. The assays included both transepithelial current and iodide efflux measurements. Filters were mounted in Ussing chambers and initially equilibrated in Ringer's solution (added to both sides). The apical solution was then exchanged for a low Cl^- buffer (6 mM Cl^-), and transepithelial current measurements were performed as described previously. The compounds were added 10−30 min prior to SRI 2931 and included BAPTA, AM (40 or 100 μM added to both surfaces), acetazolamide (100 μM added to both surfaces), staurosporine (50 or 100 nM added to the apical side), amiloride (10 μM added to the apical side), suramin (100 μM added to both sides), hexokinase (10 units/mL added apically), and ATP at various concentrations. Iodide effluxes were performed in the presence or absence of ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (1 mM added bilaterally).

ATP Release Assay. ATP release assays were performed in CFPAC-1 cells using the protocol developed by Taylor and colleagues (*15*). This assay is based on the ATP catalysis of luciferin to luciferase, which generates a photon of light that can be detected in a luminometer. Cells were seeded on Millipore filters and bathed in media on both sides for 2 days. The media were then removed from the apical surface, and only basolateral surfaces were continuously fed. When no leakage of media from the basolateral to apical surface was observed, the cells were studied for ATP release. SRI 2931 at 10, 20, or 50 μM was added to each surface of the filter. The vehicle alone (1, 2, and 5% DMSO) served as a negative control.

Bioluminescence detection of ATP release was performed as described previously (*15*). Briefly, cells grown on filters were washed gently in OptiMem-I serum-free medium (Gibco-BRL/Invitrogen) devoid of ATP. Luciferase:luciferin (Sigma) was resuspended from a lyophilized reagent into this medium at 1 μg/mL. Cells were then lowered into a Turner TD 20/20 luminometer chamber, and continuous, 15 s collection of photons was performed in real-time.

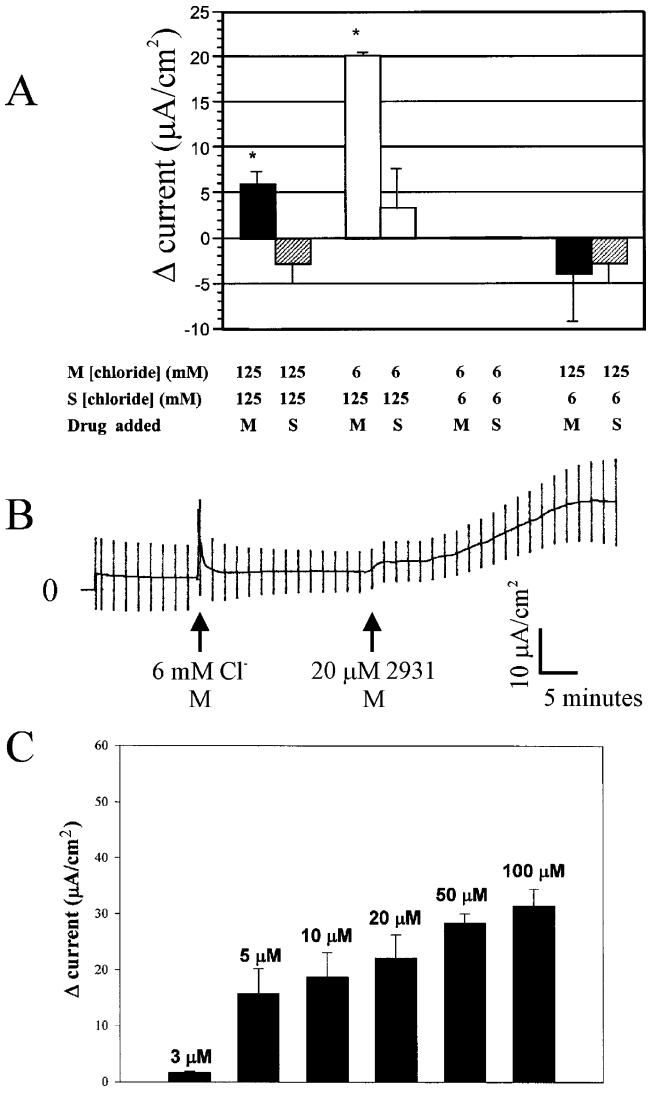

FIGURE 4: Changes in current due to SRI 2931 are dependent on the Cl^- gradient in T84 cells. Ringer's solutions containing either 125 or 6 mM Cl^- were used to bathe cells. SRI 2931 (20 μM) was added to either the mucosal (M) or serosal (S) surface. Panel A, summary data and panel B, representative tracing in the presence of Cl^- gradient. Asterisks represent statistically significant differences from the untreated control condition ($p < 0.05$). Error bars = SD; n = at least 4 filters per condition. The average basal currents prior to drug addition were as follows: 125 mM Cl^- (M and S), 0.52 ± 1.5 μA; 6 mM Cl^- (M), 3.9 ± 2.7 μA; 6 mM Cl^- (M and S), 1.2 ± 1.1 μA; and 6 mM Cl^- (S) = -2.4 ± 2.9 μA. Panel C, dose dependence of apical SRI 2931, performed in an apically directed chloride gradient. Half-maximal activation was approximately 7.7 μM derived from a nonlinear curve fit of the data to the Michaelis−Menten equation (Hill coefficient = 1.3). The observed current with SRI 2931 (20 μM) was 15−20% of that obtained with forskolin (10 μM).

Cell Proliferation and cAMP Assays. CFPAC-1 cells were grown in 96-well trays. Cells were incubated for 2 h or continuously exposed to 20 μM SRI 2931 for up to 6 days. Proliferation was measured for 6 days using a commercially available kit (Promega CellTiter 96) based on the conversion of a tetrazolium salt to blue formazan product detected using a 96-well plate reader at 595 nm. cAMP levels were measured using a Cyclic AMP Enzyme Immunoassay Kit (Cayman Chemical) according to the manufacturer's protocol. Briefly, cells were grown on 35 mm dishes ($\sim 7 \times 10^6$ cells/dish) and were stimulated for 15 min at 37 °C with forskolin (10 μM) or SRI 2931 (100 or 500 μM). Cells were then lysed, and levels of cAMP were detected based on the competition between free cAMP and cAMP tracer for a limited number of cAMP-specific rabbit antiserum binding sites.

Statistical Analyses. The unpaired, two-tailed student's t test was used for all statistical analyses.

RESULTS

Screening of Compounds. Of 341 compounds screened for their ability to activate the current in CFPAC-1 cells, one compound, SRI 2931, led to a strong response indicative of Cl^- secretion. SRI 2931 is a substituted imidazole with a diazo group at the 5′-position, a carboxylic acid at the 4′-position, and an ester linked octyl side chain (Figure 2A). Activation of transepithelial Cl^- secretion by SRI 2931 occurred specifically following addition of the compound to the apical but not basolateral cell surface (Figure 2B−D).

Iodide Efflux in CFPAC-1 and CF Nasal Epithelial Cells. Figure 3 depicts activation of anion transport in CFPAC-1 cells treated with SRI 2931 (50, 100, or 200 μM). Each curve is representative of three experiments. Significant increases in iodide effluxes were observed for SRI 2931 at 100 and 200 μM ($p = 0.03$ and $p < 0.01$, respectively), and the efflux elicited by the two concentrations of SRI 2931 did not differ from each other ($p = 0.55$). Iodide efflux due to SRI 2931 is similar but delayed slightly and smaller in magnitude as compared to ionomycin, a calcium ionophore that increases intracellular calcium and activates chloride transport in CF cells. CFPAC-1 and other CF epithelial cells characteristically fail to secrete anions in response to increased levels of cAMP. The addition of forskolin (10 μM), an activator of adenylate cyclase, served as a negative control. Ionomycin, a positive control, elicited a strong response in CFPAC-1 cells. SRI 2931 (50 μM) also stimulated anion transport in primary airway epithelial cells homozygous for the ΔF508 mutation (Figure 3G−I).

Current Measurements across a Secretory Epithelium. SRI 2931 was next tested in well-characterized Cl^- secretory (T84, human colonic) epithelial cells. Establishment of a Cl^- gradient revealed a dependence of the current on the direction of the driving force for Cl^- transport (Figure 4). Experiments performed in an apically directed Cl^- gradient indicated a strong activation of current when SRI 2931 was added to the luminal surface but not the basolateral bath (Figure 4A). An individual tracing and dose dependence are shown in Figure 4B,C. Glybenclamide (an inhibitor of CFTR and other ion channels) added at 200 μM to both sides of the filter did not block activation by SRI 2931 (data not shown).

Lowering the Cl^- concentration on both surfaces abrogated the effects of SRI 2931, further indicating the Cl^- dependence of the pathway. Similar observations following the omission of Cl^- were noted in CFPAC-1 cells. When an apical to basolateral Cl^- gradient was imposed, the direction of the current was reversed. Prolonged exposure to 20 μM SRI 2931 in these cells led to mild decreases in tissue resistance (approximately 10−20% over a 30 min observation period, possibly due to an increase in paracellular permeability), which may in part explain the reversal of the current in the setting of an apical to basal Cl^- gradient.

Current Measurements in Freshly Excised Tissues from CF Mice. Voltage clamp measurements were also performed

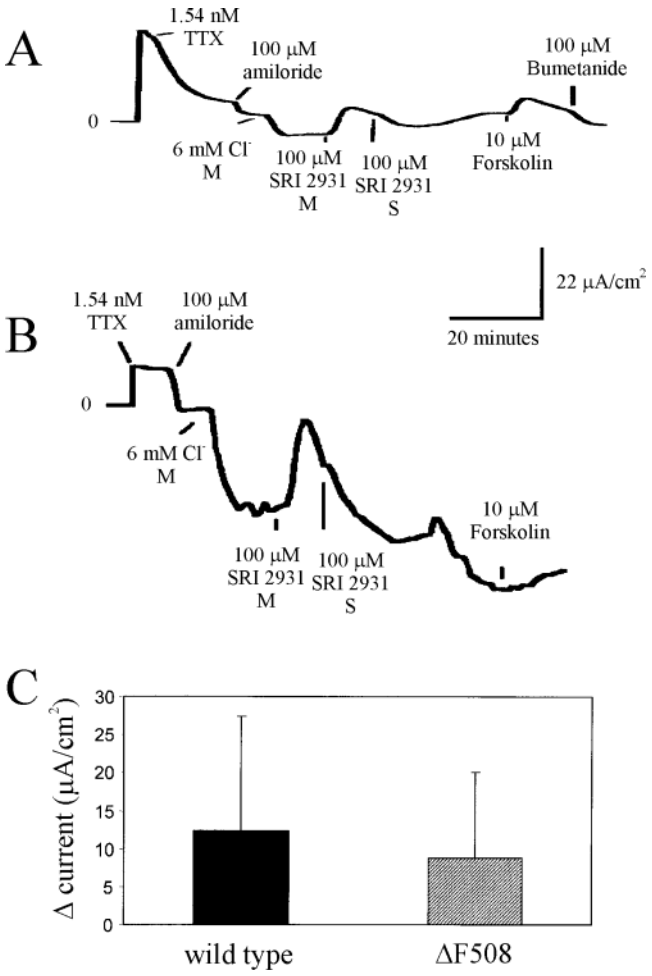

FIGURE 5: Transepithelial current in wild type and ΔF508 murine intestinal epithelia. Distal colon was removed from homozygous normal (A) and homozygous ΔF508 (B) mice and mounted in Ussing chambers. Transepithelial current measurements were recorded with the establishment of a serosal-to-mucosal Cl^- gradient (M, mucosal; S, serosal). Summary data are shown in panel C ($n = 4$ normal and 5 ΔF508 mice, error bars = SD). When means were examined, there were no significant differences in the effects of SRI 2931, TTX, or removal of mucosal chloride between normal and CF mice.

in intestinal tissue taken from normal and CF mice (Figure 5). Again, the activation of the currents was observed only when the compound (100 μM) was added to the apical bathing solution of the tissue.

Signal Transduction and Inhibitor Studies. BAPTA, AM (100 μM), a Ca^{2+} chelator used to examine the dependence of SRI 2931 upon Ca^{2+}-signaling pathways, had no effect on Cl^- transport mediated by SRI 2931 (Figure 6A,B). Further evidence that the effect of SRI 2931 is not dependent on Ca^{2+} was obtained from CFPAC-1 experiments in which cells were pretreated with EGTA (1 mM) and ^{125}I effluxes performed with bath solutions containing EGTA and no added Ca^{2+}. EGTA, which chelates extracellular Ca^{2+}, markedly diminished the ionomycin-induced iodide efflux (causing an 88% reduction, $p < 0.05$; $n = 4$ filters per condition). In contrast, this treatment had relatively little effect on SRI 2931-induced iodide efflux, ($p = 0.18$ as compared to control, $n = 4$) (Figure 6C−F). To test the possible role of protein kinase C, staurosporine (100 nM) was added to the apical bath prior to the addition of 20 μM SRI 2931. This compound had no effect on current activation. Acetazolamide (100 μM), an inhibitor of carbonic anhydrase, also did not block the effects of SRI 2931, suggesting that the current was not due to bicarbonate generation (data not shown).

To determine whether SRI 2931 could be mediating effects through changes in cAMP, intracellular cAMP levels were next measured in CFPAC-1 cells. In contrast to the increased levels of cAMP observed in CFPAC-1 cells treated with forskolin, no rise in intracellular cAMP was detected above the baseline in the presence of SRI 2931 (100 or 500 μM) (Figure 7).

We also tested for possible effects of SRI 2931 on extracellular ATP signaling. Pretreatment with hexokinase (an ATP scavenger) abrogated activity of SRI 2931, suggesting that ATP contributes to the activation of currents by this compound (Figure 8A,B). On the basis of these findings, the addition of SRI 2931 was examined for effects on cellular ATP release. SRI 2931 elicited ATP release from epithelial cells specifically following drug addition (10, 20, or 50 μM) to the apical (Figure 8C) but not basolateral surface (Figure 8D). These data, together with published reports indicating that extracellular nucleotides can activate (non-CFTR) epithelial Cl^- secretion (*4*−*10*), suggested that SRI 2931 might signal through extracellular nucleotides. To explore this further, we tested for effects of ATP directly and found that Cl^- secretion was activated in a dose dependent fashion by the nucleotide (Figure 9A) but that SRI 2931 was not blocked by 100 μM suramin (a purinergic antagonist, Figure 9B). Hexokinase abrogated the effects of SRI 2931 (20 μM) (*$p < 0.0007$, as compared with the SRI 2931 treated control).

Effects of SRI 2931 on Cellular Proliferation. Concentrations of SRI 2931 that activated chloride current had minimal effect on CFPAC-1 proliferation. When cells were incubated with 20 μM SRI 2931 for 2 h, no effect on cell proliferation was observed for the first 3 days, and by day 6, proliferation was increased by 24% as compared to cells grown in media alone. In a more stringent assay, cells were continuously exposed to 20 μM compound for 6 days. This led to an initial 30% decline in cell proliferation that was overcome by day 6 and subsequently returned to a rate similar to cells grown in media without drug.

DISCUSSION

The primary objective of this paper is to report our discovery of a new compound capable of stimulating transepithelial chloride secretion in CF cells and tissues. Identification of chloride secretagogues (including those that act independently of CFTR) represents an important aspect of cystic fibrosis therapeutic development (*3*, *4*, *10*, *17*, *19*, *20*). The compound identified in the present experiments augments anion permeability as judged by efflux in CF respiratory and pancreatic cells (Figures 3 and 6), indicating effects at the plasma membrane (as opposed to junctional complexes) that occur in a CFTR independent fashion. Chloride dependent currents stimulated under conditions of symmetrical bathing solutions (Figure 4) further establish a transcellular (as opposed to paracellular) route of action. The drug activates Cl^- secretion specifically when administered to apical but not basolateral membranes in epithelial cells (Figures 2, 4, and 5), suggesting that a particular apical membrane target (protein or lipid) is necessary for activity.

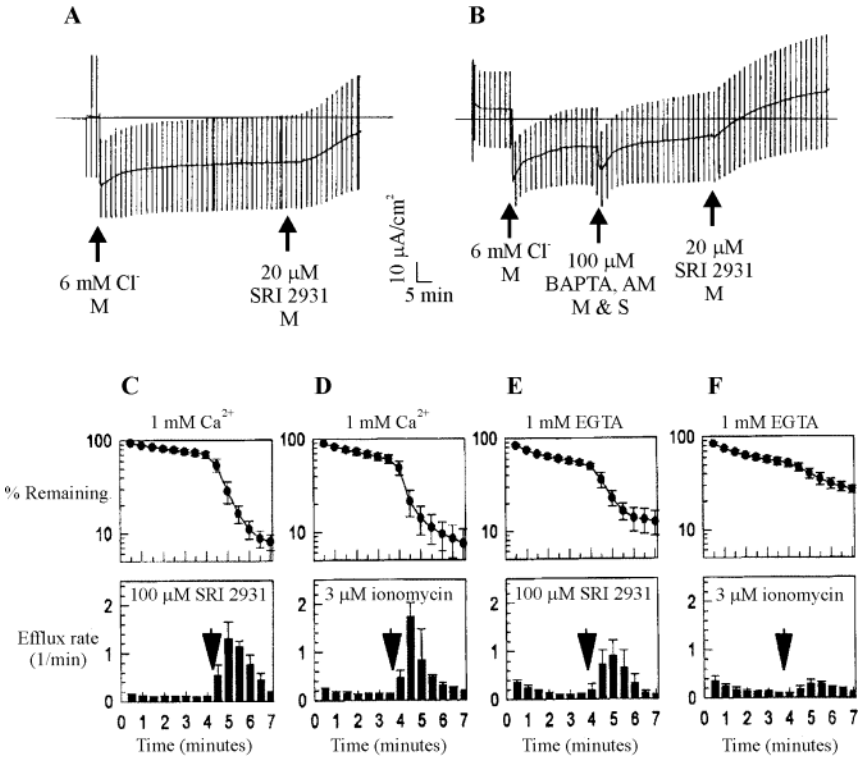

FIGURE 6: Signal transduction studies. Panels A and B, Ussing chamber studies in CFPAC-1 cells in an apically directed Cl^- gradient in the absence (A) or presence (B) of BAPTA, with AM added to both surfaces. The experiment shown was performed three times with similar results. Panels C−F, studies of extracellular Ca^{2+} depletion on iodide efflux due to SRI 2931 in CFPAC-1 cells. Agonists were added at time points indicated by arrows. All experiments were performed four times. Experiments were performed in the presence (E and F) or absence (C and D) of EGTA. In studies using EGTA, calcium was not added to the bathing solutions. The rate of efflux was significantly different in panels E and F ($p < 0.05$) but not in panels C and E ($p = 0.18$). Error bars = SD.

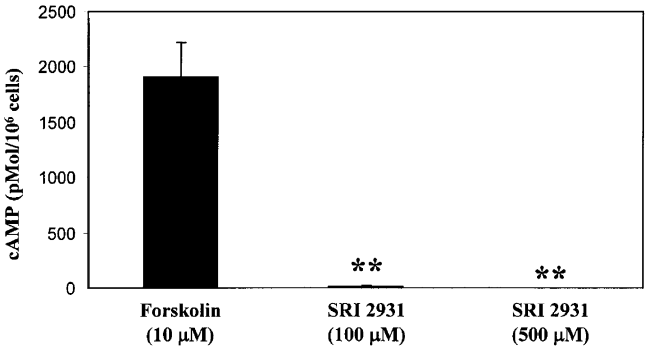

FIGURE 7: SRI 2931 does not increase cellular cyclic AMP. This figure shows the effect of SRI 2931 on intracellular cAMP levels in CFPAC-1 cells. Forskolin, an adenylate cyclase agonist, was used as a positive control. Mean values (±SD) are as follows: forskolin (10 μM) = 1911 ± 301 pmol/10^6 cells; SRI 2931 (100 μM) = 16 ± 7 pmol/10^6 cells; and SRI 2931 (500 μM) = 4 ± 1 pmol/10^6 cells. The levels of cAMP, including forskolin stimulated measurements, were similar to those observed previously in IB3-1 (human airway), Calu-3 (human airway submucosal gland screens), and other cell types (e.g., see refs *16* and *17*). $^{**}p < 0.01$ as compared to forskolin ($n = 4$ experiments per condition).

We considered the possibility that SRI 2931 might increase the permeability of the apical membrane of epithelial cells by acting as a chloride ionophore. The effect of the drug specifically at the apical and not the basolateral surface, taken together with ATP dependence, serves as evidence against a simple membrane insertion type mechanism. The alkyl side chain of SRI 2931 is also too short (eight carbons) (Figure 2A) to span the plasma membrane. Whether SRI 2931 binds a specific apical receptor or directly disrupts the apical membrane so as to elicit ATP release is not known. The functional diazo moiety in SRI 2931 provides a means by which covalent binding of a photoactivated compound could be tested in the future to identify potential receptor-binding molecules.

Both purinergic and adrenergic receptor type pathways have been shown to activate chloride transport in epithelia. Although SRI 2931 leads to the release of ATP, and drug action is blocked by hexokinase, our studies with suramin and other compounds that typically disrupt purinergic signaling (e.g., BAPTA, AM, EGTA, and staurosporine) do not suggest dependence on P2Y receptors. Similarly, adrenergic receptors are not expressed in CFPAC-1 cells, and the addition of conventional adrenergic agonists such as isoproterenol does not elicit chloride secretory responses in these cells. We therefore suggest that SRI 2931 action (and its ATP dependence) occurs through a pathway that differs from most conventional (purinergic or other) modes of Cl^- channel activation and might utilize release of metabolic energy from ATP, a different receptor, or binding of ATP to epithelial ion channels. For example, if SRI 2931 and ATP both bound directly to a channel (so as to allow activation in a P2Y independent fashion), or if the SRI 2931 receptor directly utilizes ATP as a cofactor, such pathways might not be sensitive to the P2Y blockade.

Five chemical analogues closely related to SRI 2931 are shown in Figure 10. Each of the compounds was tested two to three times, and none were found to activate Cl^- secretion in either normal or CF cells. The failure of SRI 4050 and SRI 2627 to activate the transepithelial current indicates the importance of the proper substituent (a carboxylic acid with

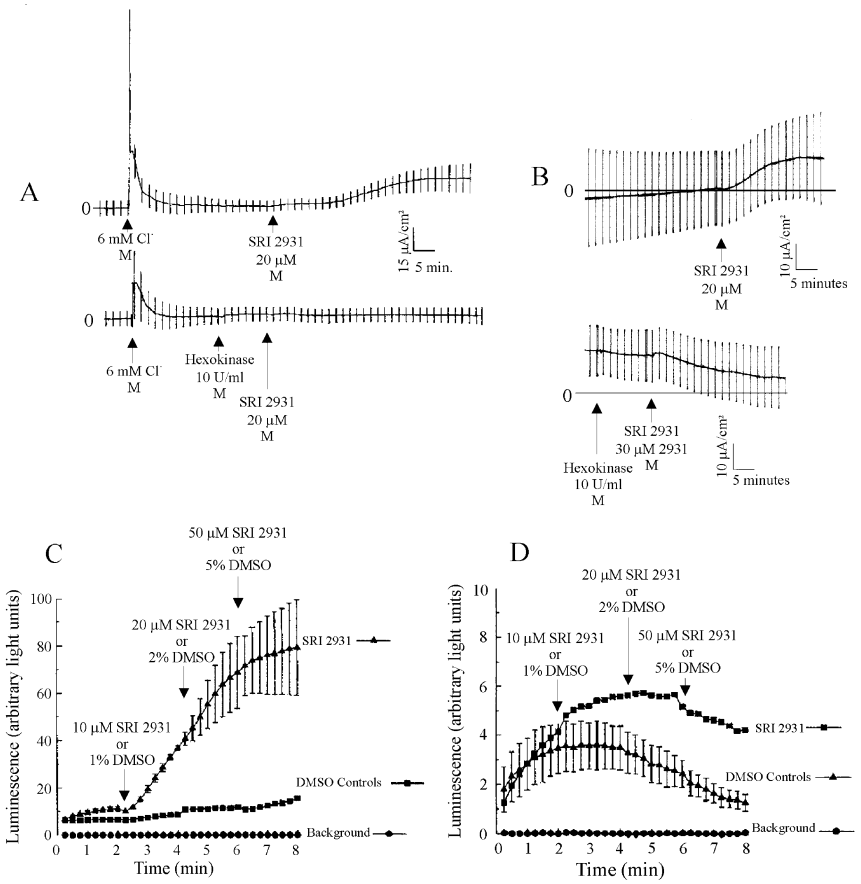

FIGURE 8: SRI 2931 acts through extracellular ATP. Panels A and B, the effect of hexokinase on current activation due to SRI 2931 is shown. These experiments were performed on T84 cells in an apically directed Cl^- gradient (A). Similar results were obtained in CFPAC-1 cells (B). Representative results from five tracings per condition are shown. A luciferase assay was used to measure the release of ATP from CFPAC-1 cells following the addition of SRI 2931 to the apical (C) or basolateral (D) surfaces. (Note different scales in experiments depicted in panels C and D).

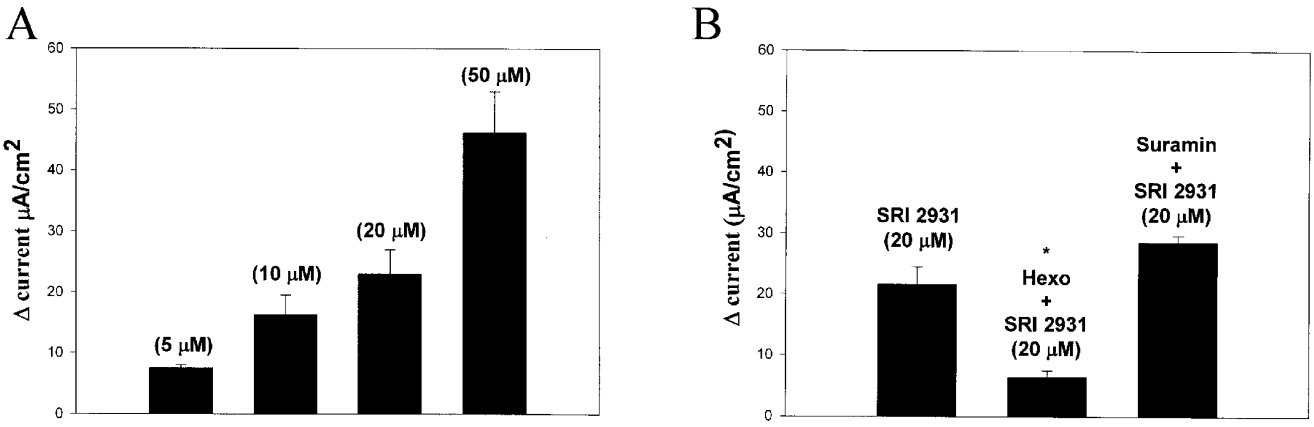

FIGURE 9: Studies of ATP dependence in T84 cell line. Panel A, dose dependence of ATP on Cl^- secretion. Panel B, summary data for the effects of suramin and hexokinase. The change in current observed due to suramin (100 μM) plus SRI 2931 (20 μM) did not differ significantly from SRI 2931 alone (20 μM, $p = 0.115$).

an ester linked octyl side chain or a diazo group) at the 4′- or 5′ position, respectively. The 5′-position substituent seems particularly important since SRI 2926, SRI 2927, and SRI 2928 are identical to SRI 2931 at the 4′-position but showed no activity. The importance of a reactive diazo moiety at the 5′-position suggests a covalent link between SRI 2931 and a specific cellular membrane receptor, and the limited reversibility of drug action (10−20% over a 30 min observation period, unpublished observations) provides support for this view. The additional bulk of the 5′-triazeno substituent in these three analogues may also impose unacceptable steric constraints on interactions with the macromolecular target, preventing drug binding.

In summary, from a library of unique synthetic compounds, a smaller, focused subset of 341 drugs was screened for activation of transepithelial Cl^- secretion using assays of anion transport. One compound, SRI 2931, activated anion secretion in CFPAC-1 cells, T84 cells, primary CF nasal airway cells, and distal colon from normal and CF mice. In all of the tissues and cell types studied, the activity of the

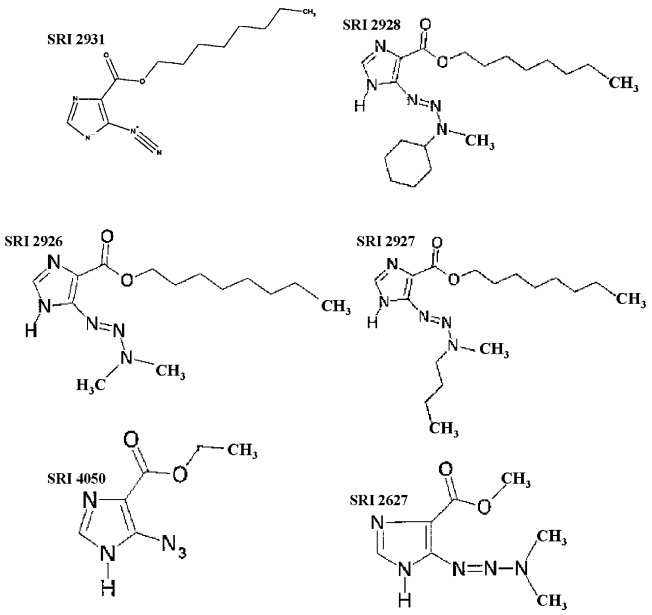

FIGURE 10: Structures of SRI 2931 and five analogues. Five chemical analogues of SRI 2931 were tested for their ability to activate anion transport in CFPAC-1 cells.

compound was observed specifically after administration to the apical, but not basolateral, surface. Because the pathway stimulated by SRI 2931 leads to the release of ATP, an extracellular signaling mechanism dependent upon nucleotides or their metabolic products is suggested. In either case, the compound may be useful for correcting cystic fibrosis defects including Cl^- secretion (*1*, *2*, *18*), ATP release (*19*), nucleotide signaling of other epithelial Cl^- channels (*21*), and fluid secretion. The present results indicate that SRI 2931 is a Cl^- secretagogue well-suited for future studies of epithelial chloride secretion and ATP release.

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