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Extracellular Zinc and ATP Restore Chloride Secretion across Cystic Fibrosis Airway Epithelia by Triggering Calcium Entry*

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Cystic fibrosis (CF) is caused by defective cyclic AMP-dependent cystic fibrosis transmembrane conductance regulator Cl^- channels. Thus, CF epithelia fail to transport Cl^- and water. A postulated therapeutic avenue in CF is activation of alternative Ca^{2+}-dependent Cl^- channels. We hypothesized that stimulation of Ca^{2+} entry from the extracellular space could trigger a sustained Ca^{2+} signal to activate Ca^{2+}-dependent Cl^- channels. Cytosolic $[Ca^{2+}]_i$ was measured in non-polarized human CF (IB3-1) and non-CF (16HBE14o$^-$) airway epithelial cells. Primary human CF and non-CF airway epithelial monolayers as well as Calu-3 monolayers were used to assess anion secretion. *In vivo* nasal potential difference measurements were performed in non-CF and two different CF mouse (ΔF508 homozygous and bitransgenic gut-corrected but lung-null) models. Zinc and ATP induced a sustained, reversible, and reproducible increase in cytosolic Ca^{2+} in CF and non-CF cells with chemistry and pharmacology most consistent with activation of P2X purinergic receptor channels. P2X purinergic receptor channel-mediated Ca^{2+} entry stimulated sustained Cl^- and HCO_3^- secretion in CF and non-CF epithelial monolayers. In non-CF mice, zinc and ATP induced a significant Cl^- secretory response similar to the effects of agonists that increase intracellular cAMP levels. More importantly, in both CF mouse models, Cl^- permeability of nasal epithelia was restored in a sustained manner by zinc and ATP. These effects were reversible and reacquirable upon removal and readdition of agonists. Our data suggest that activation of P2X calcium entry channels may have profound therapeutic benefit for CF that is independent of cystic fibrosis transmembrane conductance regulator genotype.

Because morbidity in cystic fibrosis (CF)[1] often results from lung disease (1), several approaches have been contemplated to control and cure CF in the lung and airways. They include introduction of the wild-type cystic fibrosis transmembrane conductance regulator (CFTR) gene, repair of mutated CFTR proteins, attenuation of airway inflammation, stimulation of Cl^- channels alternative to CFTR, and/or inhibition of epithelial sodium channel-mediated Na^+ hyperabsorption (2). While methods of gene and protein therapy are defined, pharmacological intervention remains feasible in CF for dysregulated NaCl transport and airway inflammation (3). Ca^{2+}-activated Cl^- channels (4) have been proposed to substitute for cyclic AMP-dependent CFTR Cl^- channels, offering a target for CF pharmacotherapy to rescue anion transport.

An ideal therapeutic compound would ameliorate multiple defects in ion transport as well as quell airway inflammation. Increases in cytosolic calcium concentration ($[Ca^{2+}]_i$) activate epithelial Cl^- channels (2, 5) but inhibit epithelial Na^+ channels (2). Many laboratories have shown that stimulation of G protein-coupled P2Y nucleotide receptors increases $[Ca^{2+}]_i$ derived from intracellular stores and may affect both ion transport mechanisms (2, 5). Although P2Y nucleotide receptors are currently a target for CF pharmacotherapy (6, 7), they trigger transient increases in $[Ca^{2+}]_i$ and are desensitized or down-regulated via multiple mechanisms (8–10).

Our laboratory has shown that airway epithelia express another subclass of nucleotide receptors, the P2X receptor channels (P2XRs) (8, 11). P2XRs function as extracellular ATP-gated, Ca^{2+}-permeable, non-selective cation channels (8, 12). Recently we have reported that stimulation of airway epithelial P2XRs leads to sustained Ca^{2+} entry from extracellular stores (8). The magnitude and sustained nature of the $[Ca^{2+}]_i$ increase was dependent on extracellular pH and the presence or

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[1] The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; P2XR, P2X purinergic receptor channel; NMDG, *N*-methyl-D-glucamine; NPD, nasal potential difference.

absence of extracellular calcium, sodium, and zinc. Moreover the degree of alkaline pH potentiation of zinc and ATP stimulation and the lack of desensitization or inactivation of the receptor or channel properties was novel. In this study, we hypothesized that a combination of zinc and ATP could induce a prolonged Ca^{2+} signal that may rescue impaired Cl^- secretion in CF airway epithelium in a sustained and reversible manner.

MATERIALS AND METHODS

Cell Cultures—IB3-1 cells[2] are CF human bronchial epithelial cells carrying two different mutations of the CFTR gene (ΔF508/W1282*X*) (13). 16HBE14o$^-$ cells are non-CF or normal bronchial epithelial cells expressing wild-type CFTR (14). Culture of these two cell lines has been described previously (15). Human airway epithelial cell monolayers (primary CF isolated from patients homozygous for ΔF508 mutations, primary non-CF, and immortalized Calu-3 cells) were grown in air/fluid interface culture on Costar 6.5-mm-diameter permeable filter supports in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented in a manner similar to the medium used for IB3-1 cells.

Fura-2 Imaging and Quenching—Cytosolic Ca^{2+} concentration was measured with dual excitation wavelength fluorescence microscopy after cells were loaded with the Fura-2-acetoxymethyl ester as described previously in detail (8). At the beginning of each experiment, cells were perfused with Ringer's solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Hepes at pH 7.3 adjusted with NaOH. The effects of hexokinase and apyrase solutions were tested in solutions containing 5 mM glucose. In all Na^+-substituted solutions, *N*-methyl-D-glucamine (NMDG) was used as a replacement cation. In NMDG-containing solutions, $CaCl_2$ was raised to 3 mM, while $MgCl_2$ was reduced to 0 mM, and external pH was adjusted with HCl. Fura-2 quenching experiments with $MnCl_2$ were performed as described previously (8).

Measurement of Cl^- Permeability—Cells were loaded with 6-methoxy-*N*-(3-sulfopropyl)quinolinium (2 mg/ml) fluorescent dye overnight. At the beginning of each experiment, cells were perfused with Ringer's solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Hepes at pH 7.3 adjusted with NaOH to establish a base-line fluorescence over 3 min. Then NMDG-containing solution (0 mM NaCl, 3 mM $CaCl_2$, and 0 mM $MgCl_2$) at pH 7.9 was added for 3 min followed by addition of zinc alone or with ATP. Effects of the agonists were tested in both Ca^{2+}-free and Na^+-containing medium. Specifics concerning the 6-methoxy-*N*-(3-sulfopropyl)quinolinium assay and system have been published previously (16).

Measurement of Transepithelial Anion Current—Primary cultures of human CF and non-CF as well as immortalized Calu-3 cells grown as monolayers on 6.5-mm collagen-coated permeable supports were studied as described previously (11). Monolayers had electrical resistance at least 1,000 ohms/cm^2. When HCO_3^-/CO_2-free solutions were used, the apical side of the monolayers were bathed in a 140 mM NMDG- and 20 μM amiloride-containing solution with 3 mM $CaCl_2$ and 0 mM $MgCl_2$ at pH 7.9 (adjusted with gluconic acid). Basolaterally we added a 140 mM NaCl-containing solution with 1.5 mM $CaCl_2$ and 1 mM $MgCl_2$ at pH 7.3 (adjusted with NaOH). Both solutions contained 10 mM Hepes. In HCO_3^-/CO_2-containing solutions, apical and basolateral solutions contained 125 mM NMDG, 25 mM choline-Cl^- and 100 mM NaCl, 50 mM $NaHCO_3$, respectively. Both solutions were gassed with 5% CO_2. The pH of the apical solution was adjusted to 7.9 with gluconic acid. Concentrations of $CaCl_2$ and $MgCl_2$ were the same as described for HCO_3^-/CO_2-free experiments.

Measurement of Nasal Potential Difference (NPD)—A three-step protocol was used as described previously (11); however, the solutions were modified as below. First, the nasal cavity of anesthetized mice was perfused with Ringer's solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, and 50 μM amiloride (pH 7.3 adjusted with NaOH). Second, we switched to low Cl^--containing solution (6 mM) either in Na^+- or NMDG-containing solution (pH was adjusted to 7.3 with NaOH and to 7.9 with gluconic acid, respectively). Third, zinc (40 μM) and ATP (100 μM) were added in Na^+-free, NMDG-containing solution at pH 7.9. Because of the continuous presence of amiloride (50 μM) and the complete replacement of Na^+ with a membrane-impermeant cation, NMDG (140 mM), in the perfusion solution, hyperpolarization reflects only Cl^- secretion rather than cation absorption.

Data Analysis—Data are expressed as mean ± S.E. and tested for significance using paired or unpaired Student's *t* test with analysis of variance as appropriate. Results with $p < 0.05$ were considered significant. Values given in the text that refer to $\Delta[Ca^{2+}]_i$ or absolute $[Ca^{2+}]_i$ refer to the sustained plateau of cytosolic Ca^{2+} concentration measured 5 min after peak stimulation except where noted.

RESULTS

Extracellular Zinc and ATP Trigger a Sustained Increase in $[Ca^{2+}]_i$ in CF and Non-CF Airway Epithelial Cells—Based on our previous observations (8, 11), we hypothesized that combined stimulation of P2XRs by ATP and zinc in sodium-free medium at pH 7.9 could induce a more robust and sustained increase in cytosolic calcium than achieved previously (8). In IB3-1 cells, administration of ATP (100 μM) and $ZnCl_2$ (20 μM) stimulated a rapid increase in $[Ca^{2+}]_i$ followed by a sustained plateau (Fig. 1*A*). The plateau was markedly higher than basal $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$ = 317 ± 23 nM, n = 8). Similar results were also obtained in 16HBE14o$^-$ non-CF cells ($\Delta[Ca^{2+}]_i$ = 444 ± 28 nM, n = 6). The sustained Ca^{2+} plateau was abolished in the presence of 140 mM extracellular sodium ($\Delta[Ca^{2+}]_i$ = 32 ± 9 nM, n = 4) (Fig. 1*A*) or by reducing external pH to 7.3 ($\Delta[Ca^{2+}]_i$ = 45 ± 7 nM, n = 4) or 6.4 ($\Delta[Ca^{2+}]_i$ = 12 ± 5 nM, n = 4) (Fig. 1*B*). Titration of external pH in a range of 7.9 to 7.4 revealed a gradual decrease in Ca^{2+} plateau levels, exhibiting the largest decline between pH 7.9 ($[Ca^{2+}]_i$ = 383 ± 11 nM, n = 4) and pH 7.7 ($[Ca^{2+}]_i$ = 135 ± 8 nM, n = 4) (Fig. 1*C*). Because external Mg^{2+} inhibits $P2X_4$Rs (17), we hypothesized that removal of Mg^{2+} from the superfusion medium might further support Ca^{2+} entry mechanisms. In addition, we predicted that increasing external Ca^{2+} from 1.5 mM to 3 mM would enhance Ca^{2+} entry. Because our data show that ATP- and zinc-induced Ca^{2+} entry was potentiated in Mg^{2+}-free and Ca^{2+}-enriched medium (Fig. 1*D*), we studied Ca^{2+} entry under these ionic conditions (see below).

Zinc Alone Is an Agonist for Ca^{2+} Entry in IB3-1 Cells—Zinc has been reported to trigger an increase in $[Ca^{2+}]_i$ in many cell models (18–20). Thus, we tested the effects of zinc alone on cytosolic $[Ca^{2+}]$. Addition of $ZnCl_2$ (20 μM) to Na^+-free medium that was pH 7.9 increased $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$ = 312 ± 22 nM, n = 10) in a sustained manner (Fig. 2*A*) similar to that observed in combination with ATP (compare with Fig. 1*A* above). The Ca^{2+} plateau was abolished by removal of external Ca^{2+} ($\Delta[Ca^{2+}]_i$ = 11 ± 5 nM, n = 4) (Fig. 2*B*), reducing external pH to 7.3 ($[Ca^{2+}]_i$ was not significantly different from the basal value), or replenishment of 140 mM external Na^+ ($\Delta[Ca^{2+}]_i$ = 10 ± 6 nM, n = 4) (Fig. 2*C*). To define the concentration range in which zinc stimulates sustained Ca^{2+} entry, we exposed IB3-1 cells to $ZnCl_2$ in increasing concentrations from 2 to 200 μM. Significant stimulation was achieved at 10 μM ($\Delta[Ca^{2+}]_i$ = 187 ± 12 nM, n = 4) with maximal effects at 50 μM ($\Delta[Ca^{2+}]_i$ = 551 ± 42 nM, n = 4) (Fig. 2*D*). Although zinc alone caused robust Ca^{2+} entry from extracellular solution, we postulated that superfusion of cells might cause mechanically induced release of endogenous ATP that could act synergistically with exogenous zinc. Indeed elimination of ATP from the superfusion medium by ATP scavengers hexokinase (5 units/ml) and apyrase (1 unit/ml) caused a partial but not complete inhibition of zinc-induced Ca^{2+} entry ($\Delta[Ca^{2+}]_i$ = 312 ± 22 nM, n = 10 *versus* $\Delta[Ca^{2+}]_{i\ \text{hexok. + apyr.}}$ = 145 ± 15 nM, n = 4; $p < 0.05$). Interestingly, in Ca^{2+}-free medium, zinc-induced increases in $[Ca^{2+}]_i$ were transient ($\Delta[Ca^{2+}]_{i\ \text{peak}}$ = 123 ± 16 nM, n = 5) (Fig. 2*E*), an effect that was completely abolished by thapsigargin pretreatment (no significant changes in $[Ca^{2+}]_i$) (Fig. 2*F*). These

[2] Because all human cells and mice were handled by the University of Alabama at Birmingham Cystic Fibrosis Center, University of Alabama at Birmingham Cystic Fibrosis Center human subjects (assurance of compliance number M1149) and vertebrate animals (animal welfare assurance number A3255-01) protocols were followed.

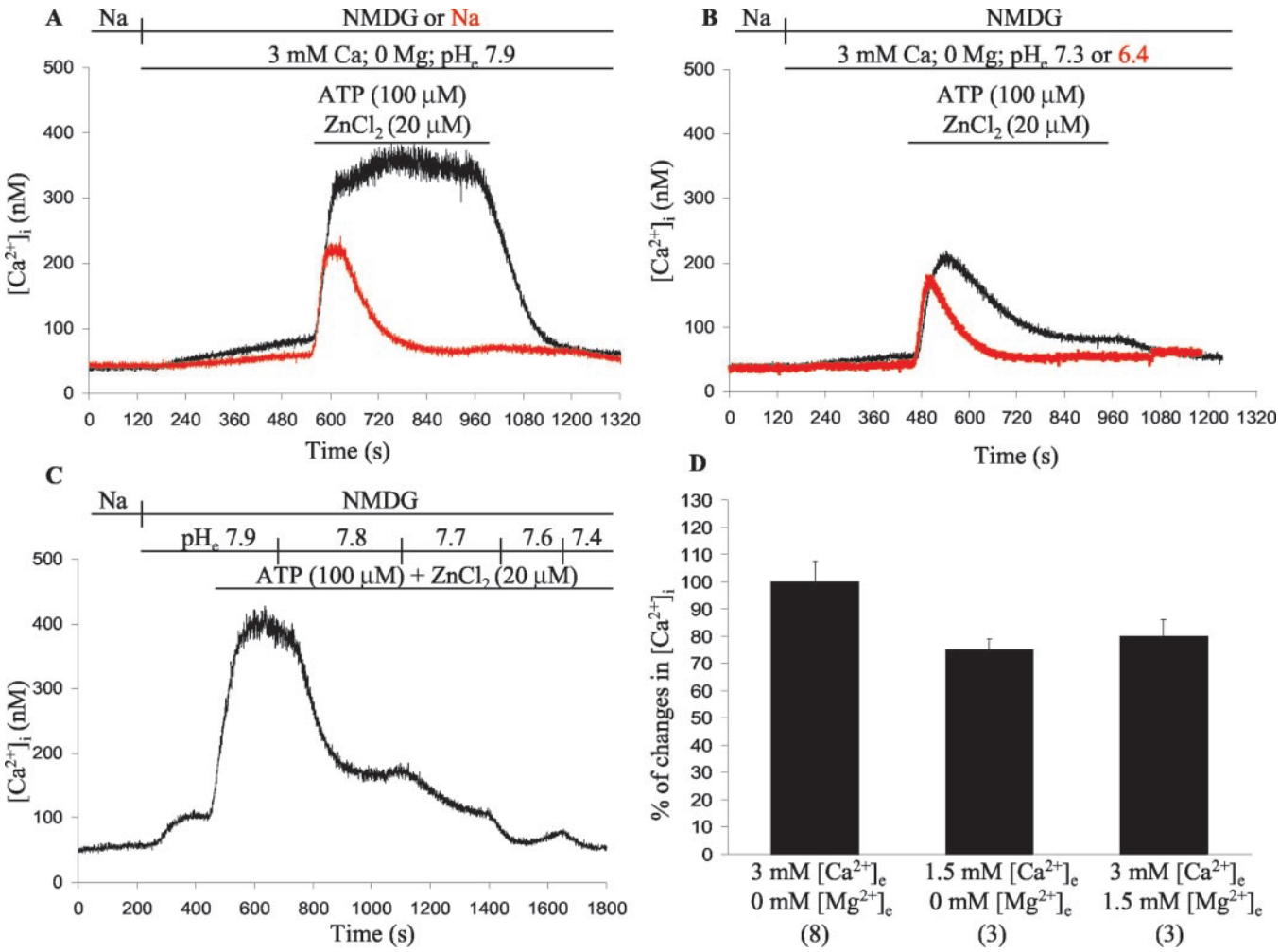

FIG. 1. **Effects of ATP and zinc on $[Ca^{2+}]_i$ in IB3-1 cells.** *A*, original traces showing the effect of combined administration of ATP (100 μM) and $ZnCl_2$ (20 μM) in the absence (*black trace*) and presence of 140 mM extracellular Na^+ (*red trace*). Cells were perfused with Na^+-containing Ringer's solution (pH_e 7.3), and then extracellular pH was raised to 7.9, and extracellular $[Ca^{2+}]$ was increased from 1.5 to 3 mM in Mg^{2+}-free medium. At the same time, extracellular Na^+ was substituted by NMDG in Na^+-free experiments. *B*, combined administration of ATP and $ZnCl_2$ in the absence of external Na^+ at pH_e 7.3 (*black trace*) and 6.4 (*red trace*). Cells were perfused with the same medium as in *A*. Extracellular $[Ca^{2+}]$ was increased to 3 mM in a Na^+- and Mg^{2+}-free medium. At the same time, pH_e was dropped to 6.4 (*red*). *C*, effects of ATP and $ZnCl_2$ on the sustained Ca^{2+} plateau in a pH_e range between 7.9 and 7.4. External pH was raised to 7.9, and Na^+ was removed as indicated. Changes in $[Ca^{2+}]_e$ and $[Mg^{2+}]_e$ were similar to those indicated for *A* and *B*. After addition of agonists, pH_e was decreased in a stepwise manner. *D*, ATP- and $ZnCl_2$-induced sustained increase in $[Ca^{2+}]_i$ relative to basal $[Ca^{2+}]_i$. In optimized solution (3 mM Ca^{2+} and 0 Mg^{2+}), a sustained increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$ = 317 ± 23 nM) was considered 100%. A reduction in $[Ca^{2+}]_e$ or an increase in $[Mg^{2+}]_e$ attenuated the Ca^{2+} plateau. *Numbers* of experiments are indicated in *parentheses*. Each experiment shown in *A–C* was performed four to six times with similar results.

data show that zinc increases $[Ca^{2+}]_i$ from cytosolic calcium stores and extracellular solution. However, a sustained Ca^{2+} plateau was only observed when external calcium was present, suggesting that activation of Ca^{2+} entry mechanisms plays a key role in this prolonged Ca^{2+} signal.

P2XR-independent Ca^{2+} Entry Pathways Are Not Involved in Zinc-induced Ca^{2+} Entry—Because Ca^{2+} entry was stimulated by zinc even in the absence of ATP, we were required to investigate whether P2XR-independent mechanisms were involved in this process. First, we assessed the effects of the zinc-activated cation channel inhibitor tubocurarine (21) on zinc-induced Ca^{2+} entry. Tubocurarine (100 μM) had no effect on Ca^{2+} entry in IB3-1 cells ($\Delta[Ca^{2+}]_i$ = 282 ± 23 nM, n = 4) (Fig. 3*A*). We have shown previously that activation of the reverse operation mode of Na^+/Ca^{2+} exchange did not contribute to ATP-induced Ca^{2+} entry in Na^+-free solution (8). Zinc also inhibits Na^+/Ca^{2+} exchange in rat brain (22). However, we could not exclude the possibility that zinc might influence activity of the Na^+/Ca^{2+} exchanger under Na^+-free experimental conditions. Thus, we tested the effects of KB-R7943, a selective inhibitor of the reverse operation mode of this exchanger. Surprisingly, instead of inhibition of zinc-induced Ca^{2+} entry, KB-R7943 (10 μM) potentiated zinc stimulation ($\Delta[Ca^{2+}]_i$ = 727 ± 51 nM, n = 4) (Fig. 3*B*). Store-operated Ca^{2+} channels play an important role in Ca^{2+} entry in non-excitable cells (23, 24) and in epithelial cells (25–27). Although zinc has been described as an inhibitor of store-operated Ca^{2+} channels (23–27), we tested its effects on thapsigargin-induced Ca^{2+} entry. At concentrations shown to activate sustained Ca^{2+} entry in human airway epithelial cells, zinc inhibited store-operated Ca^{2+} entry channels ($[Ca^{2+}]_i$ = 685 ± 34 nM before and after zinc exposure *versus* $[Ca^{2+}]_i$ = 94 ± 21 nM during zinc exposure, n = 5; $p < 0.01$) stimulated by thapsigargin depletion of intracellular endoplasmic reticulum Ca^{2+} stores (Fig. 3*C*). Because zinc has been reported to modify the properties of Fura-2 (28), we tested whether zinc enters the cells and affects Fura-2 when added at the concentration that induces sustained Ca^{2+} entry. Zinc did not change Fura-2 fluorescence until manganese was subsequently added, showing that zinc triggered Mn^{2+} entry and Mn^{2+}-dependent Fura-2 quenching (Fig. 3*D*). Taken together, these data show that P2XR-independent Ca^{2+} entry mechanisms are likely not involved in zinc-induced Ca^{2+} entry. Our

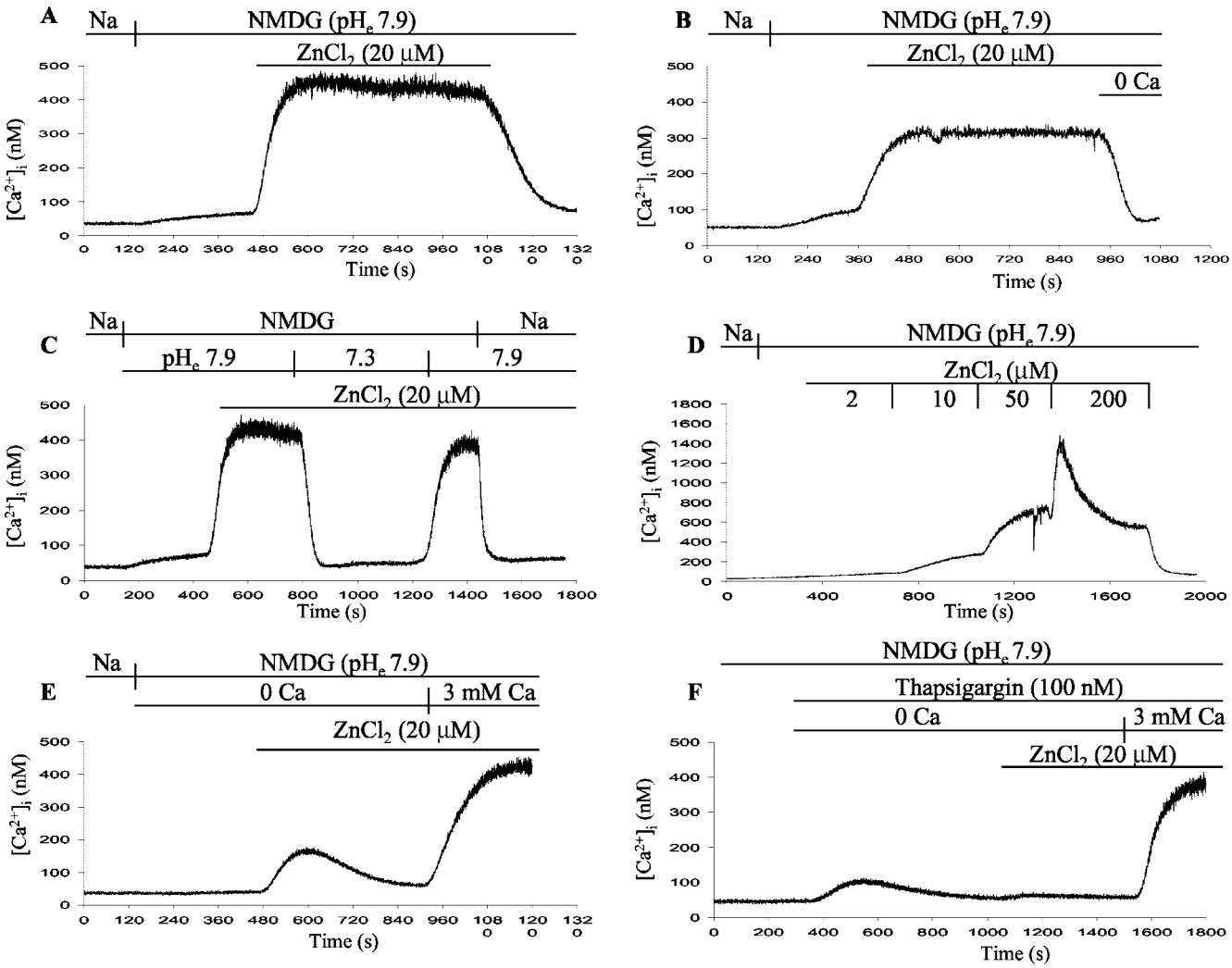

FIG. 2. **Effects of zinc alone on $[Ca^{2+}]_i$ in IB3-1 cells.** Original traces show the effects of zinc alone. In experiments shown in *A–D*, early changes of external ionic composition were performed as described in Fig. 1*A*. *A*, addition and removal of zinc as indicated. *B*, external Ca^{2+} was removed in the continuous presence of zinc. *C*, external pH was dropped from 7.9 to 7.3 and restored again to 7.9 followed by replenishment of sodium in the continuous presence of zinc. *D*, $ZnCl_2$ was added in increasing concentrations. *E*, $ZnCl_2$ was added in Ca^{2+}-free medium followed by readdition of external Ca^{2+}. *F*, cells were pretreated with thapsigargin in the absence of external Na^+ and Ca^{2+}, and then zinc was added. At the end of the experiment, external calcium was replenished. Each experiment shown in *A–F* was performed four to six times with similar results.

previous results (8) and the properties of zinc- or zinc and ATP-induced Ca^{2+} entry argue for a prominent role for P2X receptor Ca^{2+} entry channels.

Zinc Alone or in Combination with ATP Restores Cl^- Transport in IB3-1 Cells—To test the hypothesis whether a sustained increase in cytosolic Ca^{2+} of greater than 300 nM could restore Cl^- transport in IB3-1 CF cells grown on collagen-coated glass coverslips, we assessed Cl^- efflux using 6-methoxy-*N*-(3-sulfopropyl)quinolinium halide fluorescence assay. $ZnCl_2$ (20 μM) alone or in combination with ATP (100 μM) stimulated Cl^- efflux when administered in Na^+-free alkaline (pH 7.9) medium (Fig. 4, *A* and *B*). These effects were dependent upon the presence of extracellular Ca^{2+} (Fig. 4*A*), suggesting a key role of Ca^{2+} entry mechanisms in stimulating Cl^- transport. It is important to note that stimulation of Cl^- efflux was achieved under the same conditions that resulted in a prolonged cytosolic Ca^{2+} increase. It is also probable that flow-induced release of endogenous ATP contributed to zinc-induced rescue of Cl^- efflux in a manner similar to the Fura-2 imaging assays (see above).

Zinc and ATP Stimulate Cl^- Secretion in Polarized CF and Non-CF Human Airway Epithelial Cell Monolayers—We next tested the efficacy of zinc and ATP in rescuing transepithelial Cl^- transport in primary human CF and non-CF airway epithelial cell monolayers as well as in Calu-3 immortalized human non-CF submucosal gland serous cell monolayers in Ussing chambers. In the presence of amiloride (20 μM) and a "basolateral toward apical" Cl^- gradient, apical ATP (100 μM) and $ZnCl_2$ (40 μM) in Na^+-free solution (pH 7.9) stimulated transepithelial chloride current in both CF and non-CF airway epithelial cells (Fig. 5*A*). This Cl^- current was biphasic, showing transient and sustained components (Fig. 5, *A–D*). Removal of the agonists abolished the sustained Cl^- current, which was stimulated again upon readdition of agonists (Fig. 5*B*). Calu-3 cell monolayers are a preferred respiratory cell model system to study anion and water transport. It has been shown that forskolin-stimulated anion current is carried mainly by bicarbonate in these monolayers (29). Furthermore Cuthbert *et al.* (30) have recently reported that HCO_3^-/CO_2 removal inhibits Cl^- secretion in Calu-3 monolayers. Thus, we hypothesized that, in the presence of HCO_3^-/CO_2, ATP and zinc could stimulate a more robust and sustained anion secretion than we observed in Hepes-buffered solution. Indeed, under these conditions, ATP (100 μM) and $ZnCl_2$ (40 μM) elicited significantly higher peak (100.4 ± 10.0 μA/cm^2, $n = 5$ *versus* 16.4 ± 1.2 μA/cm^2, $n = 18$; $p < 0.01$) and sustained currents (76.6 ± 6.8 μA/cm^2, $n = 5$

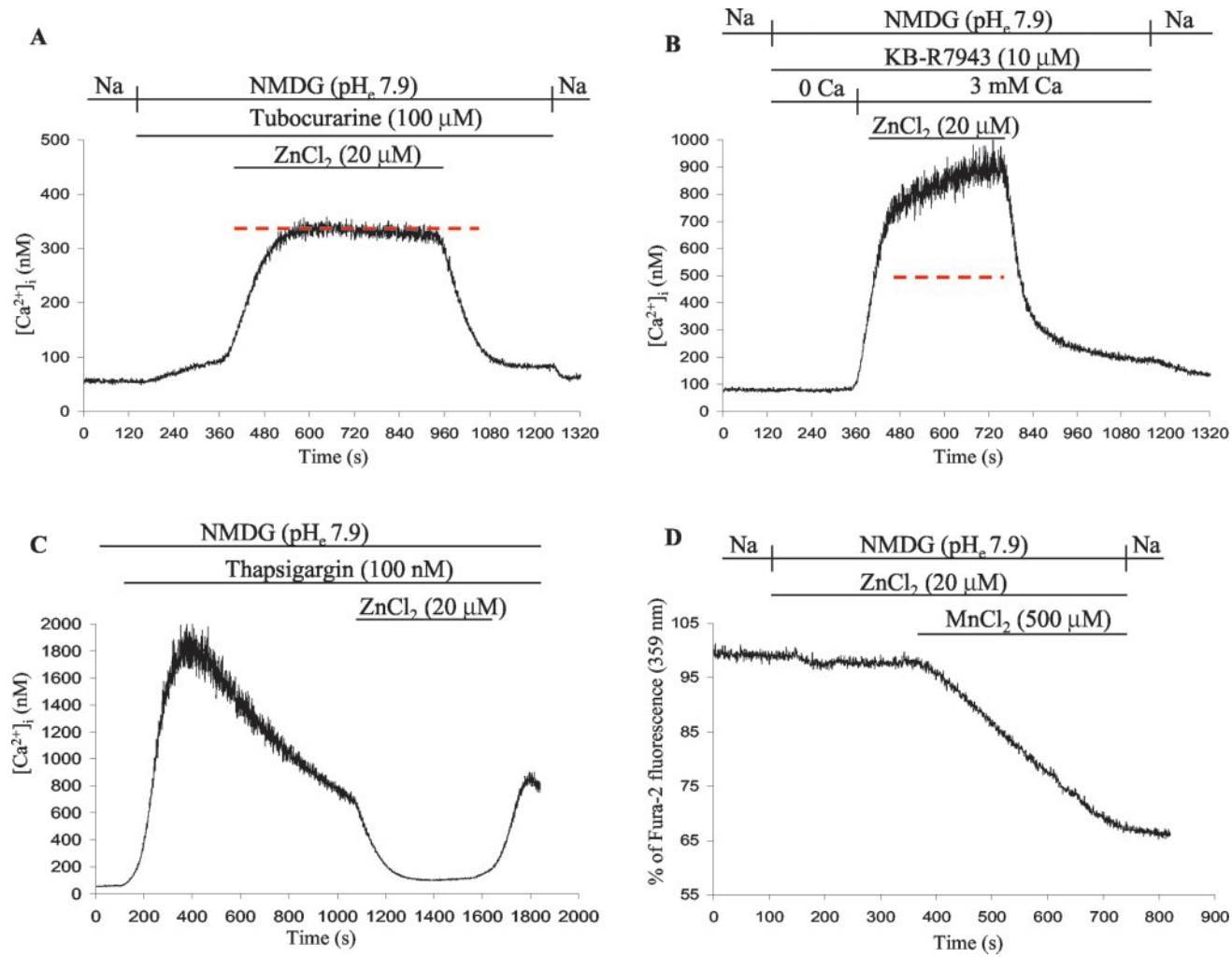

FIG. 3. **P2XR-independent Ca^{2+} entry is not involved in zinc-induced sustained $[Ca^{2+}]_i$ increases.** Original traces show the effects of tubocurarine (*A*) and KB-R7943 (*B*) on zinc-induced $[Ca^{2+}]_i$ increases. The *dashed red lines* indicate the levels of the zinc-induced sustained Ca^{2+} plateau in parallel experiments performed on the same day in the absence of tubocurarine and KB-R7943, respectively. *C*, thapsigargin-induced increase in $[Ca^{2+}]_i$ in Na^+-free, Ca^{2+}-containing (3 mM) medium that was pH 7.9. Addition of $ZnCl_2$ inhibited Ca^{2+} influx as indicated, while withdrawal of zinc revealed residual store-operated Ca^{2+} entry channel activity. *D*, quenching of Fura-2 was assessed in the presence of $ZnCl_2$ (20 μM) followed by addition of $MnCl_2$. At the beginning of each experiment Fura-2 fluorescence was considered 100%, which was not changed significantly by $ZnCl_2$ (98 ± 2%). Each experiment shown in *A–D* was performed four times with similar results.

versus 12.0 ± 1.0 μA/cm^2, $n = 18$; $p < 0.01$) (Fig. 5, *B* and *D*). The sustained current was inhibited by chelation of extracellular Ca^{2+}, a maneuver that did not prevent the forskolin-stimulated anion secretion (Fig. 5*D*). These data show that zinc and ATP under optimal Ca^{2+} entry conditions stimulated sustained Cl^- and/or HCO_3^- secretion in polarized CF and non-CF human airway and submucosal gland serous cell epithelia by a mechanism that, at least in part, requires extracellular Ca^{2+}.

Zinc and ATP Correct Defective Cl^- Transport in NPD Assays of Mice—A critical test for these agonists and vehicle was the NPD assay in anesthetized mice (31). We applied zinc and ATP onto the nasal mucosa of different strains of control and CF mice in the identical saline vehicle optimized for marked Ca^{2+} entry. In mice with at least one wild-type CFTR allele, the NPD depolarized with gradual decay in the presence of amiloride (50 μM). Under these conditions, reduction of mucosal $[Cl^-]$ caused significant hyperpolarization, indicating Cl^- secretion by nasal epithelial cells (Table I and Fig. 6*A*). Addition of $ZnCl_2$ (40 μM) and ATP (100 μM) induced further hyperpolarization that was sustained and indicative of Cl^- secretion (Fig. 6*A*). This magnitude of hyperpolarization was as large as that elicited by isoproterenol stimulation of CFTR-mediated Cl^- secretion in our studies (data not shown). The hyperpolarization was transient and markedly attenuated by removal of extracellular Ca^{2+} (Table I). We also tested this protocol in a ΔF508-CFTR homozygous CF mouse (32) and a bitransgenic mouse in which the lungs are null for CFTR but intestinal dysfunction was corrected with a fatty acid-binding protein promoter-driven CFTR construct. In the presence of amiloride (50 μM), reduction of mucosal Cl^- was without effect (Fig. 6, *B–F*), illustrating the loss of Cl^- permeability in CF. However, administration of $ZnCl_2$ and ATP caused marked and sustained hyperpolarization in both CF models (Table I and Fig. 6, *B–E*). This degree of rescue of Cl^- permeability and the sustained nature of this rescue are novel to the CF NPD field.

The duration, reversibility, and reproducibility of a potential therapeutic compound are key issues, especially one that targets an endogenous receptor. Therefore, we tested the duration of effect as well as removal and readministration of agonists. To our knowledge, these are the first CF NPD assays in which such protocols have been performed. In CF mice, administration of ATP and zinc hyperpolarized the NPD in a sustained manner for 15 min (Fig. 6*D*). This long lasting stimulation was reversible upon removal of agonists (Fig. 6*D*). In addition, multiple exposures to agonists elicited similar Cl^- secretory responses, suggesting that P2XRs do not become desensitized

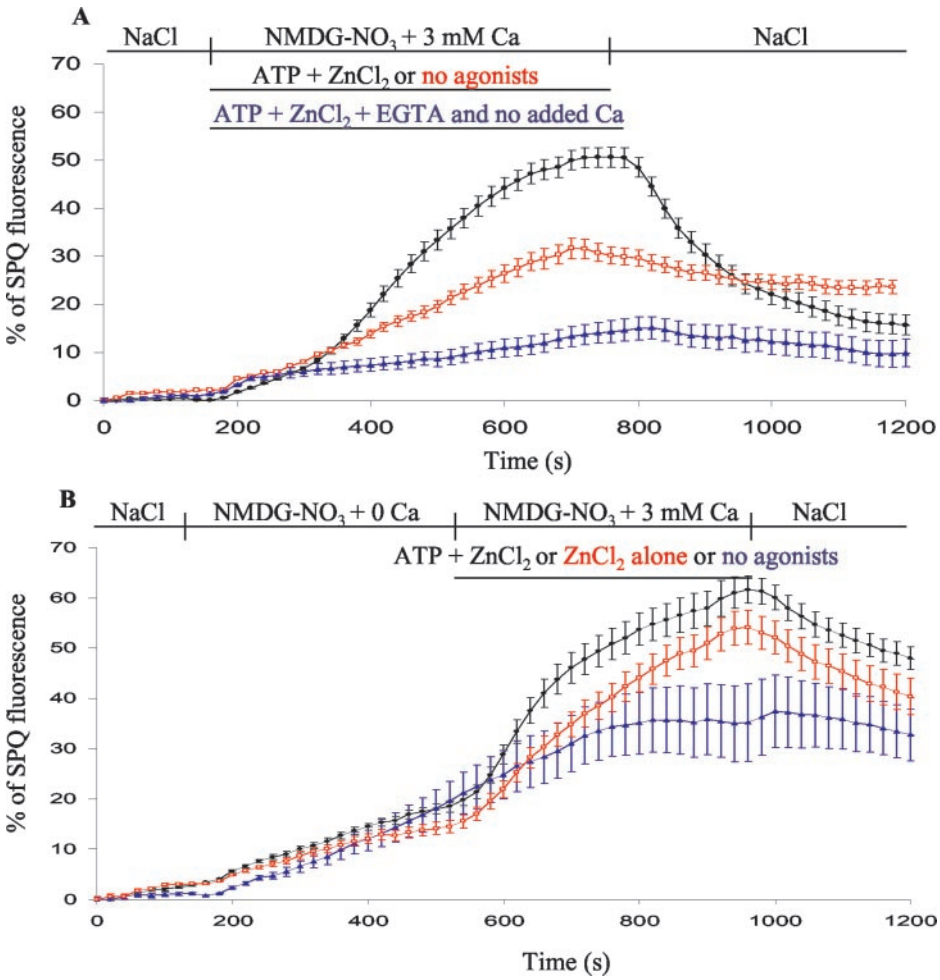

FIG. 4. **Effects of zinc and ATP on Cl^- efflux in IB3-1 cells.** *A*, effects of combined administration of ATP and $ZnCl_2$ on Cl^- efflux. Extracellular Na^+ and Cl^- were replaced by NMDG and NO_3^-, respectively. At the same time, the concentration of $CaCl_2$ was increased to 3 mM, and pH_e was elevated to 7.9 in the presence of ATP and $ZnCl_2$ (*black trace*) or in the absence of the agonists (*red trace*). In the *blue trace*, Na^+, Cl^-, and pH_e were changed as described above, but $CaCl_2$ was removed, and EGTA was added in the presence of ATP and $ZnCl_2$. Na^+-containing solution without agonists was given back as indicated, although the point at which it was given in the three separate traces was slightly different (the deflections in the traces show when agonist-free solutions affected the cells in the different experiments). Values are means ± S.E. (n = 17 cells in each group). *B*, effects of combined administration of the agonists *versus* $ZnCl_2$ alone on Cl^- efflux. Cells were superfused with Ca^{2+}-free, Na^+-containing solution that had a pH of 7.3. Ionic composition of the solutions was changed as described for *A* with the exception that no added $CaCl_2$ was present in NMDG-containing solutions. ATP and $ZnCl_2$ (*black trace*), $ZnCl_2$ alone (*red trace*), or no agonists (*blue trace*) were added with $CaCl_2$. Values are means ± S.E. (n = 17 cells in each group). *SPQ*, 6-methoxy-*N*-(3-sulfopropyl)quinolinium.

or inactivated (Fig. 6*E*). Notably Cl^- secretion induced by ATP and zinc was more rapid when the low Cl^- solution added prior to the agonist-containing solution was also pH 7.9, independent of the absence or presence of extracellular Na^+ (Table I and Fig. 6, *D* and *E*). Interestingly ATP, when administered alone in Na^+-free, alkaline solution, caused significantly smaller and transient hyperpolarization responses in bitransgenic CF mouse NPD (Table I and Fig. 6*F*). Comparison of Fig. 6*E* with Fig. 6*F* illustrates the pivotal role for zinc in triggering sustained Cl^- secretion *in vivo*. Finally, as in transepithelial anion current recordings, administration of $ZnCl_2$ alone was not sufficient to produce significant Cl^- secretion in either CF mouse model (data not shown).

DISCUSSION

Having ruled out P2XR-independent Ca^{2+} entry mechanisms (22–27) and zinc-activated channels (21), P2XRs are the most likely candidates to conduct Ca^{2+} into airway epithelial cells when stimulated with zinc and ATP. Because of alkaline pH potentiation of ATP and zinc-induced Ca^{2+} entry, we speculate that the $P2X_4$ subtype is involved in this process. However, it is possible that $P2X_5$ and/or $P2X_6$ may also contribute to Ca^{2+} entry because they co-assemble with $P2X_4$ (12, 33) and are also expressed in human airway epithelial cells.[3] Zinc is an antagonist for $P2X_1$ and $P2X_7$, while $P2X_2$ receptors are stimulated by acidic pH (12). Furthermore $P2X_1$ and $P2X_3$ can also be excluded because of their rapid inactivation (12). Thus, functional, biochemical, and immunohistochemical definition of the relative roles of $P2X_4$, $P2X_5$, and/or $P2X_6$ in alkaline pH-dependent, zinc-induced Ca^{2+} entry is in progress.

The sustained nature of the Ca^{2+} signal induced by zinc and/or ATP was surprising and intriguing. In addition to the fact that Ca^{2+} entry was essential, two additional factors may explain this phenotype. First, both zinc and ATP also cause endoplasmic reticulum Ca^{2+} release. Second, since zinc inhibits the human erythrocyte plasma membrane Ca^{2+} ATPase pump (34), it is conceivable that submicromolar concentrations of zinc could accumulate in the cells that might inhibit Ca^{2+} extrusion without altering Fura-2 properties. Nonetheless we emphasize that removal of zinc or of extracellular Ca^{2+} quickly lowered and reversed the signal back to base-line $[Ca^{2+}]_i$, suggesting that the overall Ca^{2+} buffering capacity is not affected by zinc. Interestingly zinc alone stimulated sustained cell Ca^{2+}

[3] L. Liang and E. M. Schwiebert, unpublished observations.

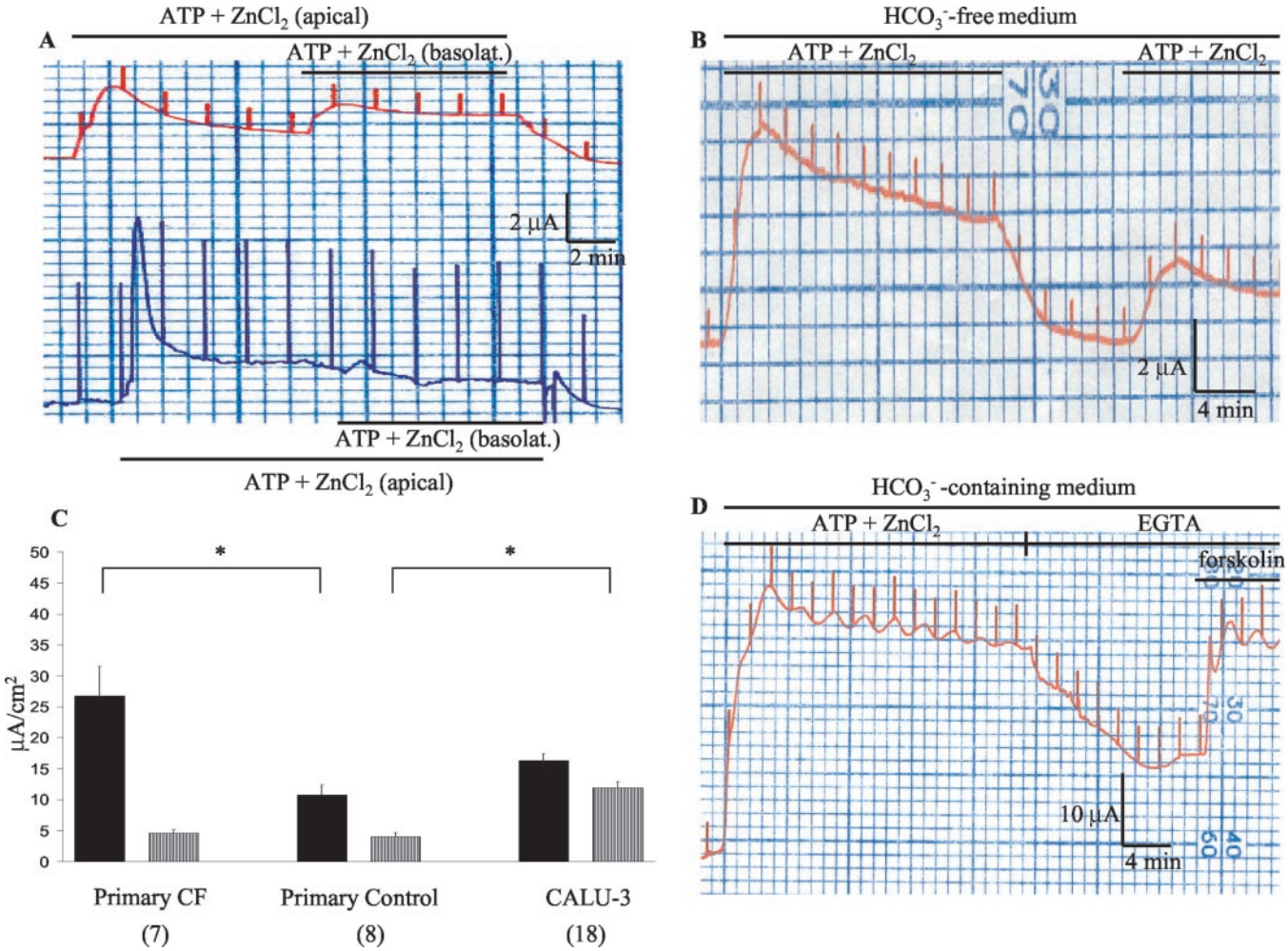

FIG. 5. **Effects of zinc and ATP on secretory Cl^- and HCO_3^- currents in polarized airway epithelia.** *A*, representative tracings of transepithelial chloride current measurement are shown using non-CF (*red trace*) and CF (*blue trace*) human primary airway epithelial cell monolayers. ATP and $ZnCl_2$ were added apically and basolaterally as indicated. *B*, representative tracing of transepithelial chloride current in the absence of HCO_3^-/CO_2 using Calu-3 monolayers. ATP and $ZnCl_2$ were added apically followed by washout and by readdition of the agonists. *C*, summarized data for transepithelial chloride current experiments. *Black columns* represent the peak stimulation by ATP and $ZnCl_2$, while *gray columns* represent currents measured 5 min after the peak. Please note that primary CF cells exhibited the highest peak current component, while Calu-3 cells had the highest sustained current component. *Numbers* of experiments are shown in *parentheses* (*, $p < 0.05$). *D*, representative tracing of transepithelial anion current in the presence of HCO_3^-/CO_2 using Calu-3 monolayers. ATP and $ZnCl_2$ were added apically followed by addition of EGTA (2 mM). Forskolin (5 μM) was given to the apical side of the monolayers as indicated. *basolat.*, basolateral.

TABLE I

Transepithelial nasal potential difference values of control, Δ508 CF, and bitransgenic CF mice

Starting points represent values (mV) obtained in Ringer's solution containing amiloride immediately after the beginning of experiments. Low $[Cl^-]_e$ responses represent the changes in values (mV) reducing $[Cl^-]_e$ to 6 mM in Na^+- or NMDG-containing medium at different pH_e. Negative and positive values reflect changes toward hyperpolarization and depolarization, respectively. Effects of ATP and $ZnCl_2$ were tested in NMDG-containing medium at pH 7.9 following reduction of $[Cl^-]_e$. *n* = number of experiments.

	Control		CF		Bitransgenic CF	
	Cftr (+/−)	*n*	Cftr(ΔF508/ΔF508)	*n*	Cftr(−/−)	*n*
Starting point	−18.7 ± 1.5	19	−26.3 ± 2.2[a]	11	−26.1 ± 1.0[a]	14
Low $[Cl^-]_e$ (Na^+ pH 7.3)	−5.5 ± 0.5	8	+3.7 ± 0.9[a]	3	+4.8 ± 0.9[a]	7
ATP + $ZnCl_2$ (NMDG, pH 7.9)	−4.7 ± 0.7	6	−4.0 ± 1.2	3	−3.8 ± 0.6	12
Low $[Cl^-]_e$ (Na^+ pH 7.9)	−4.8 ± 0.8	6	+5.4 ± 1.1[a]	7	+6.7 ± 2.3[a]	3
ATP + $ZnCl_2$ (NMDG, pH 7.9)	−6.0 ± 1.0	2	−9.4 ± 0.6[a,b]	8	−9.7 ± 1.8[a,c]	3
Low $[Cl^-]_e$ (NMDG, pH 7.9)	−4.8 ± 1.5	5			+5.8 ± 1.0[a]	4
ATP + $ZnCl_2$ (NMDG, pH 7.9)	−5.7 ± 0.7	3			−10.2 ± 0.5[a,c]	6
ATP alone (NMDG, pH 7.9)					−2.3 ± 0.5[d]	4
Low $[Cl^-]_e$ (NMDG, no added Ca^{2+}, pH 7.9)	−7.3 ± 0.3	3			+6.0 ± 0.4[a]	4
ATP + $ZnCl_2$ (NMDG, no added Ca^{2+}, pH 7.9)	−1.3 ± 0.3[e]	3			−2.0 ± 0.6[e]	4

[a] $p < 0.05$ *vs.* control animals.
[b] $p < 0.05$ *vs.* CF animals; ATP + $ZnCl_2$ after low $[Cl^-]$ response with Na^+ (pH 7.3).
[c] $p < 0.05$ *vs.* bitransgenic CF animals (a generous gift from Dr. Jeffrey A. Whitsett); ATP + $ZnCl_2$ after low $[Cl^-]$ response with Na^+ (pH 7.3).
[d] The effect of ATP alone was assessed by the peak of the hyperpolarization response because of the transient nature of the response.
[e] $p < 0.05$ *vs.* ATP + $ZnCl_2$ with NMDG in the presence of extracellular Ca^{2+}.

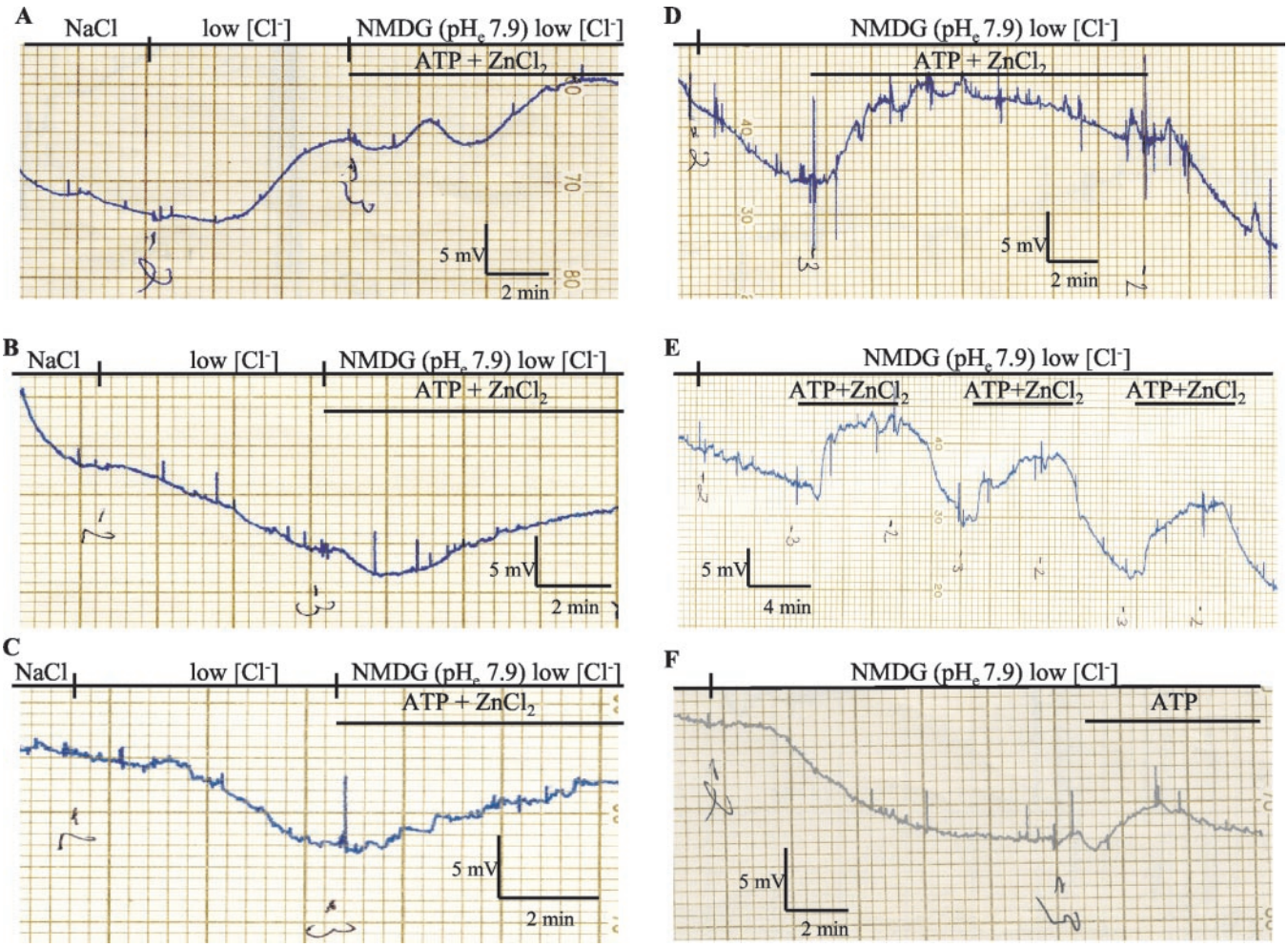

FIG. 6. **Effects of zinc and ATP on Cl^- secretion in mouse NPD measurements.** *A*, typical experiment in control animals. The nasal cavity of the mouse was perfused with Na^+-containing Ringer's solution (pH 7.3) in the presence of amiloride (50 μM) showing a gradual decay in NPD (depolarization). Then we switched to a low Cl^--containing (6 mM) solution. Please note the hyperpolarization upon lowering external $[Cl^-]$. Due to a delay in the perfusion system, hyperpolarization occurred approximately 2 min after changing solutions. ATP (100 μM) and $ZnCl_2$ (40 μM) were added in Na^+-free medium that was pH 7.9. Please note an additional hyperpolarization in the presence of agonists. Shown are typical experiments in a ΔF508 homozygous CF mouse (in *B*) and in a bitransgenic CF mouse (in *C*) using the same protocol as in *A*. Please note that in both CF mouse models hyperpolarization occurred only upon addition of agonists. *D*, long exposure to agonists in a ΔF508 homozygous mouse. Extracellular Na^+ was substituted by NMDG, and pH was raised to 7.9 in low Cl^--containing medium before addition of agonists, and then ATP and $ZnCl_2$ were added. Please note that the time lag between switching to agonist-containing solution and hyperpolarization is shorter (approximately 1 min), and the amplitude of the response is greater than that achieved in *B*. Also note that washout of agonists reversed the response completely. *E*, multiple exposures to ATP and $ZnCl_2$ in a ΔF508 homozygous mouse. Please note that the amplitude of the responses did not decline with the time even upon removal and readdition of agonists two additional times. *F*, a bitransgenic CF mouse was exposed to ATP alone in Na^+-free low $[Cl^-]_e$ solution at pH_e 7.9. Before adding the agonists, nasal epithelia were perfused with Na^+-free low $[Cl^-]_e$ solution at pH_e 7.9. Note the transient nature of the response.

increases and Cl^- efflux in non-polarized cells. In polarized monolayers, however, both zinc and ATP were required to stimulate sustained Cl^- secretion. It is probable that Ca^{2+}-activated Cl^- channel expression is regulated by epithelial polarity (35), which might underlie the different Cl^- secretory responses in polarized and non-polarized airway epithelial cells. Furthermore administration of ATP scavengers inhibited partially the zinc-induced sustained Ca^{2+} plateau, suggesting that rapid perfusion triggers endogenous ATP release in fluorescence-based assays (36, 37) and that addition of both co-agonists provides full stimulation of epithelial P2XRs.

Modifications of the saline vehicle appear essential to activate P2XR Ca^{2+} entry channels. These include a low extracellular Na^+ concentration (which benefits all other Ca^{2+} entry channels (38–43)) and an alkaline extracellular pH (which potentiates $P2X_4$ (8, 12)). Removal of extracellular Mg^{2+} and increased external Ca^{2+} concentrations also potentiated the effects of zinc and ATP. Applying these modifications, P2X agonists induced a sustained increase in $[Ca^{2+}]_i$ of 300–450 nM above basal levels in CF and non-CF airway epithelial cells. This signal would be sufficient for marked stimulation of Ca^{2+}-activated Cl^- channels (44) and ciliary beat (45, 46). Silberberg and coworkers (46) hypothesized that the latter effects were conferred by "P2X cilia." Of note, a low Na^+ environment and extracellular ATP potentiated P2XR-modulated ciliary beat in their studies (46). Together our studies argue for possible improvement in CF mucociliary clearance.

A sustained Ca^{2+} signal also stimulates Ca^{2+}-dependent K^+ channels and may inhibit epithelial Na^+ channels (2). K^+ efflux would lead to a hyperpolarization of cell membrane potential, establishing a favorable electrical gradient for Cl^- secretion. Reduction of Na^+ hyperabsorption would promote rehydration of airway surfaces. Furthermore zinc inhibition of a recently described proton conductance could also alkalinize the airway surface, which may be acidic during airway inflammation (47). Thus, inclusion of zinc might correct multiple airway epithelial ion transport dysfunctions. Interestingly, in Calu-3 submucosal gland serous cells, ATP and zinc

stimulated marked anion secretion (especially in HCO_3^-/CO_2-containing medium), suggesting that P2XRs may also be useful for more general rescue of anion secretion in submucosal glands.

Stimulation of sustained Ca^{2+} entry in CF therapy must also occur in a controlled manner because of possible induction of apoptosis (48). Cytosolic Ca^{2+} imaging, Ussing chamber, and NPD experiments show that zinc- and ATP-stimulated Ca^{2+} entry and Cl^- secretion are reversible upon removal of agonists and reacquirable after readdition of agonists. These features indicate that P2XRs and Ca^{2+}-activated Cl^- channels are not desensitized or inactivated under these experimental conditions. However, it is noteworthy that administration of ATP alone caused only transient Cl^- secretion in CF mouse nasal epithelia, underscoring the importance of zinc co-application along with ATP.

The most novel and compelling aspect of this proposed P2XR-targeted CF therapy is the inclusion of zinc. Zinc is a trace element and transition metal. It is derived from human diets and is required for healthy function of the body, and no chronic disorders are known to be associated with its accumulation (49, 50). Zinc oxide creams alleviate dermatitis, including acrodermatitis enteropathica in at least 30% of CF patients caused by zinc malabsorption and deficiency (51). Defective activity of a zinc transporter, hZip4, in the intestinal mucosa is also linked to this form of dermatitis (52). Of note, homeopathic remedies such as Zicam™ and ColdEeze™, based on zincum gluconicum (53, 54), are available for treatment of the common cold. Oral zinc sulfate is also Food and Drug Administration-approved in milligram quantities as an adjunct therapy for Wilson's disease (55). Despite current therapeutic use, the anti-inflammatory mechanisms of and receptors for zinc are poorly defined. It is possible that luminal epithelial P2XRs function, at least in part, as zinc-sensing receptors and participate in these mechanisms.

One could argue that zinc and a nucleotide would require a low sodium, alkaline environment to activate P2XRs, conditions that would make its application difficult in therapeutic trials. Nevertheless efficacy was achieved in the mouse nasal cavity. We speculate that inhalation of Na^+-free alkaline solution of a volume markedly greater than the estimated volume of the airway surface liquid in the large ciliated airways (56) might reduce Na^+ concentration and increase pH of the airway surface liquid, allowing zinc to exert its beneficial effects. CF aerosol administration of drugs, such as tobramycin, is often administered in markedly diluted saline (75% diluted saline in water in the case of Tobi™; information is provided in *Physician's Desk Reference*). There is also precedence for an inhaled isotonic alkaline solution (pH 8.0–9.0) containing bicarbonate that improved radioaerosol clearance significantly in patients with chronic cough (57). Importantly, in contrast to acidic aerosols, alkaline aerosols do not trigger bronchoconstriction (58). These previous observations suggest that properly compiled aerosols may have significant influence on the efficacy of zinc in future human studies. Thus, we propose that zinc and ATP (or an equivalent nucleotide) added to the nasal passages and airways may be of significant benefit to CF pharmacotherapy that is independent of CFTR genotype. Zinc-based therapy for CF, other airway diseases, and the common cold could also be improved by delivery in an optimized saline vehicle inhaled as a solution.

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