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Extracellular Zinc and ATP Restore Chloride Secretion Across Cystic Fibrosis Airway Epithelia By Triggering Calcium Entry

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Summary

Cystic fibrosis (CF) is caused by defective cyclic AMP-dependent cystic fibrosis transmembrane conductance regulator (CFTR) 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 (CaCCs). We hypothesized that stimulation of Ca^{2+} entry from the extracellular space could trigger a sustained Ca^{2+} signal to activate CaCCs. 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 (P2XRs). P2XR-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 re-acquirable upon removal and re-addition of agonists. Our data suggest that activation of P2X calcium entry channels may have profound therapeutic benefit for CF independent of CFTR genotype.

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Introduction

Because morbidity in cystic fibrosis (CF) 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 (ENaC)-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 (CaCCs)(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 ENaCs (2). Many laboratories have shown that stimulation of G protein-coupled P2Y nucleotide receptors (P2YRs) increases $[Ca^{2+}]_i$ derived from intracellular stores and may affect both ion transport mechanisms (2,5). Although P2YRs are currently a target for CF pharmacotherapy (6,7), they trigger transient increases in $[Ca^{2+}]_i$ and desensitize or down-regulate 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

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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 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.

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Material and Methods

Cell Cultures. IB3-1 cells are CF human bronchial epithelial cells carrying two different mutations of the CFTR gene (ΔF508/W1282X)(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 DMEM/Ham's F12 medium supplemented in a manner similar to the media 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 (Fura-2-AM), as previously described in detail (8). At the beginning of each experiment, cells were perfused with Ringer 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 (SPQ) (2 mg/ml) fluorescent dye overnight. At the beginning of

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each experiment, cells were perfused with Ringer 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 baseline fluorescence over 3 minutes. Then, NMDG-containing solution (0 mM NaCl, 3 mM $CaCl_2$ and 0 mM $MgCl_2$) at pH 7.9 was added for 3 minutes, 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 SPQ 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 Ω/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.

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Measurement of Nasal Potential Difference (NPD). A 3-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 solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes and amiloride (50 μM) (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 ± SEM and tested for significance using paired or unpaired Student's t-test, with ANOVA 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.

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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. 1A). 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. 1A) 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. 1B). Titration of external pH in a range of 7.9 and 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. 1C). 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. 1D), 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

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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 similar sustained manner (Fig. 2A) as observed in combination with ATP (compare to Fig. 1A above). The Ca^{2+} plateau was abolished by removal of external Ca^{2+} ($\Delta[Ca^{2+}]_i$ = 11 ± 5 nM, n = 4)(Fig. 2B), reducing external pH to 7.3 ($[Ca^{2+}]_i$ was not significantly different from basal value) or replenishment of 140 mM external Na^+ ($\Delta[Ca^{2+}]_i$ = 10 ± 6 nM, n = 4)(Fig. 2C). 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. 2D). 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 U/ml) and apyrase (1 U/ml), caused a partial but not complete inhibition of zinc-induced Ca^{2+} entry ($\Delta[Ca^{2+}]_i$ = 312 ± 22 nM, n = 10 vs. $\Delta[Ca^{2+}]_{i\ hexok.+\ apyr.}$ = 145 ± 15 nM, n = 4; $p<0.05$). Interestingly, in Ca^{2+}-free medium, zinc-induced increases in $[Ca^{2+}]_i$ were transient ($\Delta[Ca^{2+}]_{i\ peak}$ = 123 ± 16 nM, n = 5)(Fig. 2E), an effect which was completely abolished by thapsigargin pretreatment (no significant changes in $[Ca^{2+}]_i$)(Fig. 2F). These 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.

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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 (ZAC) 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 $\pm$ 23 nM, n = 4)(Fig. 3A). 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 $\pm$ 51 nM, n = 4)(Fig. 3B). Store-operated Ca^{2+} channels (SOCs) 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 SOCs (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 SOC Ca^{2+} entry channels ($[Ca^{2+}]_i$ = 685 $\pm$ 34 nM before and after zinc exposure vs. $[Ca^{2+}]_i$ = 94 $\pm$ 21 nM during zinc exposure, n = 5; $p<0.01$) stimulated by thapsigargin depletion of intracellular endoplasmic reticulum (ER) Ca^{2+} stores (Fig. 3C). 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

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fluorescence until manganese was subsequently added, showing that zinc triggered Mn^{2+} entry and Mn^{2+}-dependent Fura-2 quenching (Fig. 3D). Taken together, these data show that P2XR-independent Ca^{2+} entry mechanisms are likely not involved in zinc-induced Ca^{2+} entry. Our 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 SPQ 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. 4A), 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 similar manner 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

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cell monolayers in Ussing chambers. In the presence of amiloride (20 μM) and a "basolateral towards 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. 5A). 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 re-addition of agonists (Fig. 5B). 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 at al. has recently reported that HCO_3^-/CO_2 removal inhibits Cl^- secretion in Calu-3 monolayers (30). Thus, we hypothesized that, in 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 vs. 16.4 ± 1.2 μA/cm^2, n = 18; $p<0.01$) and sustained currents (76.6 ± 6.8 μA/cm^2, n=5 vs. 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. 5D). 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+}.

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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 1 and Fig. 6A). Addition of $ZnCl_2$ (40 μM) and ATP (100 μM) induced further hyperpolarization that was sustained and indicative of Cl^- secretion (Fig. 6A). This magnitude of hyperpolarization was as large as that elicited by isoproterenol stimulation of CFTR-mediated Cl^- secretion in our hands (data not shown). The hyperpolarization was transient and markedly attenuated by removal of extracellular Ca^{2+} (Table 1). We also tested this protocol in a ΔF508-CFTR homozygous CF mouse (32) and a bitransgenic mouse where the lungs are null for CFTR but intestinal dysfunction was corrected with a fatty acid-binding protein promoter (FABP)-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 1 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 are key issues, especially one that targets an endogenous receptor. Therefore, we tested the duration

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of effect as well as removal and re-administration 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 minutes (Fig. 6D). This long-lasting stimulation was reversible upon removal of agonists (Fig. 6D). In addition, multiple exposures to agonists elicited similar Cl^- secretory responses, suggesting that P2XRs do not desensitize or inactivate (Fig. 6E). 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 1 and Fig., 6D 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 1 and Fig. 6F). Comparison of Fig. 6E with Fig. 6F 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).

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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 (L. Liang and E.M. Schwiebert, unpublished observations). 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 ER 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 baseline $[Ca^{2+}]_i$, suggesting that the overall Ca^{2+} buffering capacity is not affected by zinc. Interestingly, zinc alone stimulated sustained

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cell Ca^{2+} 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 CaCC 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 CaCCs (44) and ciliary beat (45,46). Silberberg and coworkers hypothesized that the latter effects were conferred by a "P2X cilia" (46). Of note, a low Na^+ environment and extracellular ATP potentiated P2XR-modulated ciliary beat in their hands (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 ENaC (2). K^+ efflux would lead to a hyperpolarization of cell membrane potential,

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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, that 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 re-acquirable after re-addition of agonists. These features indicate that P2XRs and CaCCs do not desensitize or inactivate 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, 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

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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 common cold. Oral zinc sulfate is also FDA-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 (ASL) in the large ciliated airways (56) might reduce Na^+ concentration and increase pH of the ASL, 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.

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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 independent of CFTR genotype. Zinc-based therapy for CF, other airway diseases and common cold could also be improved by delivery in an optimized saline vehicle inhaled as a solution.

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Abbreviations

CF, cystic fibrosis; **CFTR**, cystic fibrosis transmembrane conductance regulator; **CaCC**, Ca^{2+}-activated Cl^- channel; **P2XR**, P2X purinergic receptor channel; **ENaC**, epithelial Na^+ channel; **P2YR**, P2Y nucleotide receptor; **$[Ca^{2+}]_i$**, cytosolic calcium concentration; **ZAC**, zinc-activated cation channel; **NMDG**, N-methyl D-glucamine; **SPQ**, 6-methoxy-N-(3-sulfopropyl)quinolinium; **NPD**, nasal potential difference; **SOC**, store-operated Ca^{2+} channel; **FABP**, fatty acid-binding protein promoter; **ECaC**, epithelial Ca^{2+} entry channels; **ASL**, airway surface liquid

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Figure Legends

Figure 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 absence (**black trace**) and presence of 140 mM extacellular Na^+ (**red trace**). Cells were perfused with Na^+-containing Ringer solution (pH_e 7.3). 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 Panel 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 Panels 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 Panels **A-C** was performed 4-6 times with similar results.

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Figure 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 Panel **A-D**, early changes of external ionic composition were performed as described in Fig. 1A. **(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 continuous presence of zinc. **(D)** $ZnCl_2$ was added in increasing concentrations. **(E)** $ZnCl_2$ was added in Ca^{2+}-free medium followed by re-addition of external Ca^{2+}. **(F)** Cells were pretreated with thapsigargin in the absence of external Na^+ and Ca^{2+}, then zinc was added. In the end of the experiment, external calcium was replenished. Each experiment shown in Panels **A-F** was performed 4-6 times with similar results.

Figure 3. P2XR-independent Ca^{2+} entry is not involved in zinc-induced sustained $[Ca^{2+}]_i$ increases.

Original traces showing 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

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changed significantly by $ZnCl_2$ (98 ± 2%). Each experiment shown in Panels **A-D** was performed 4 times with similar results.

Figure 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 ± SEM (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 pH 7.3. Ionic composition of the solutions was changed as described for Panel 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 ± SEM (n = 17 cells in each group).

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Figure 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 absence of HCO_3^-/CO_2 using Calu-3 monolayers. ATP and $ZnCl_2$ were added apically, followed by washout and by re-addition 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 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.

Figure 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 solution (pH 7.3) in 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 approx. 2 min after changing

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solutions. ATP (100 μM) and $ZnCl_2$ (40μM) were added in Na^+-free medium that was pH 7.9. Please note an additional hyperpolarization in the presence of agonists. Typical experiments in a ΔF508 homozygous CF mouse (in **(B)**) and in a bitransgenic CF mouse (in **(C)**) using the same protocol as in Panel 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. Then, ATP and $ZnCl_2$ were added. Please note that the time lag between switching to agonist-containing solution and hyperpolarization is shorter (approx. 1 min) and the amplitude of the response is greater than that achieved in Panel 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 re-addition 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 was perfused with Na^+-free low $[Cl^-]_e$ solution at pH_e 7.9. Note the transient nature of the response.

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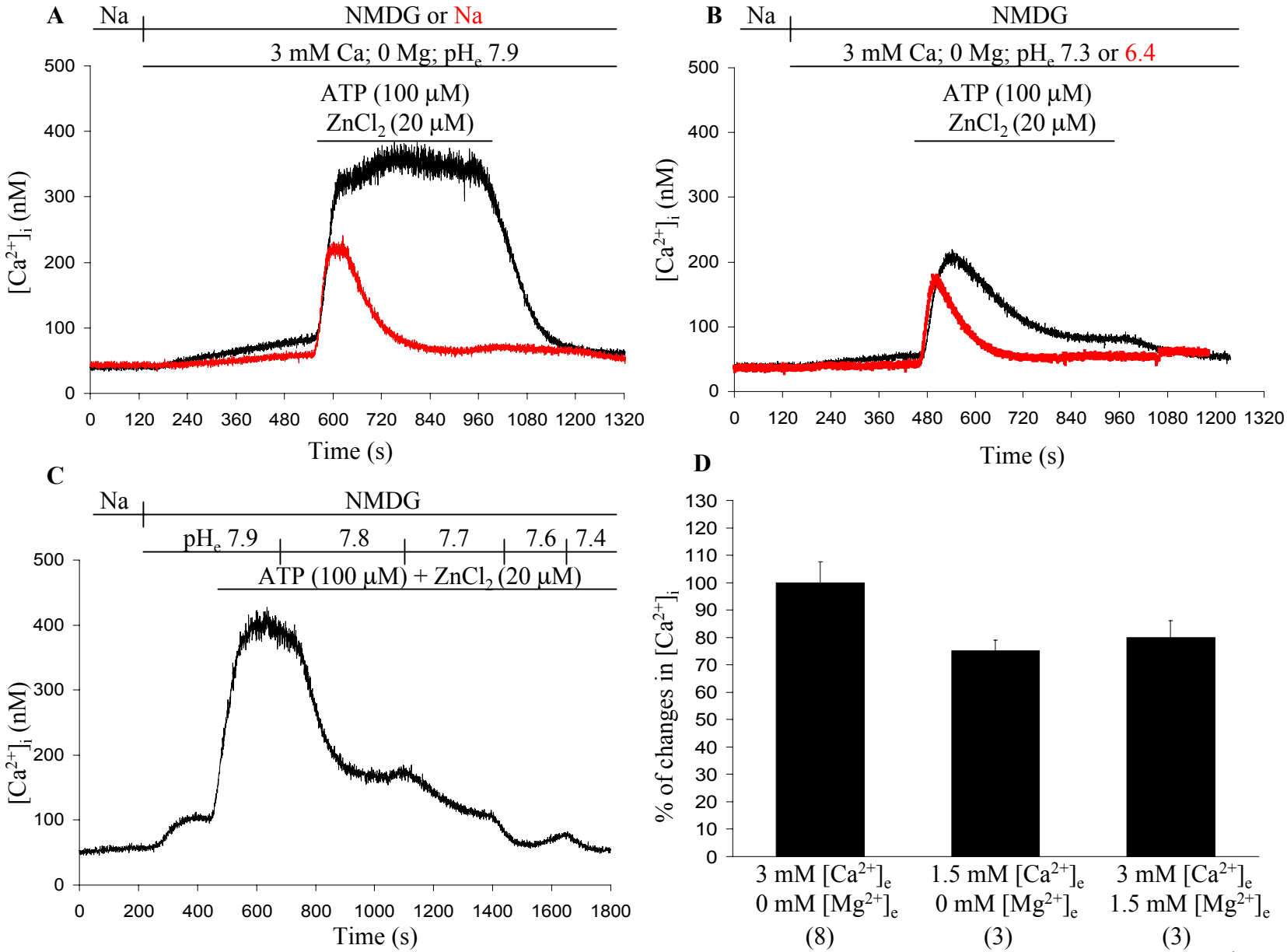
A
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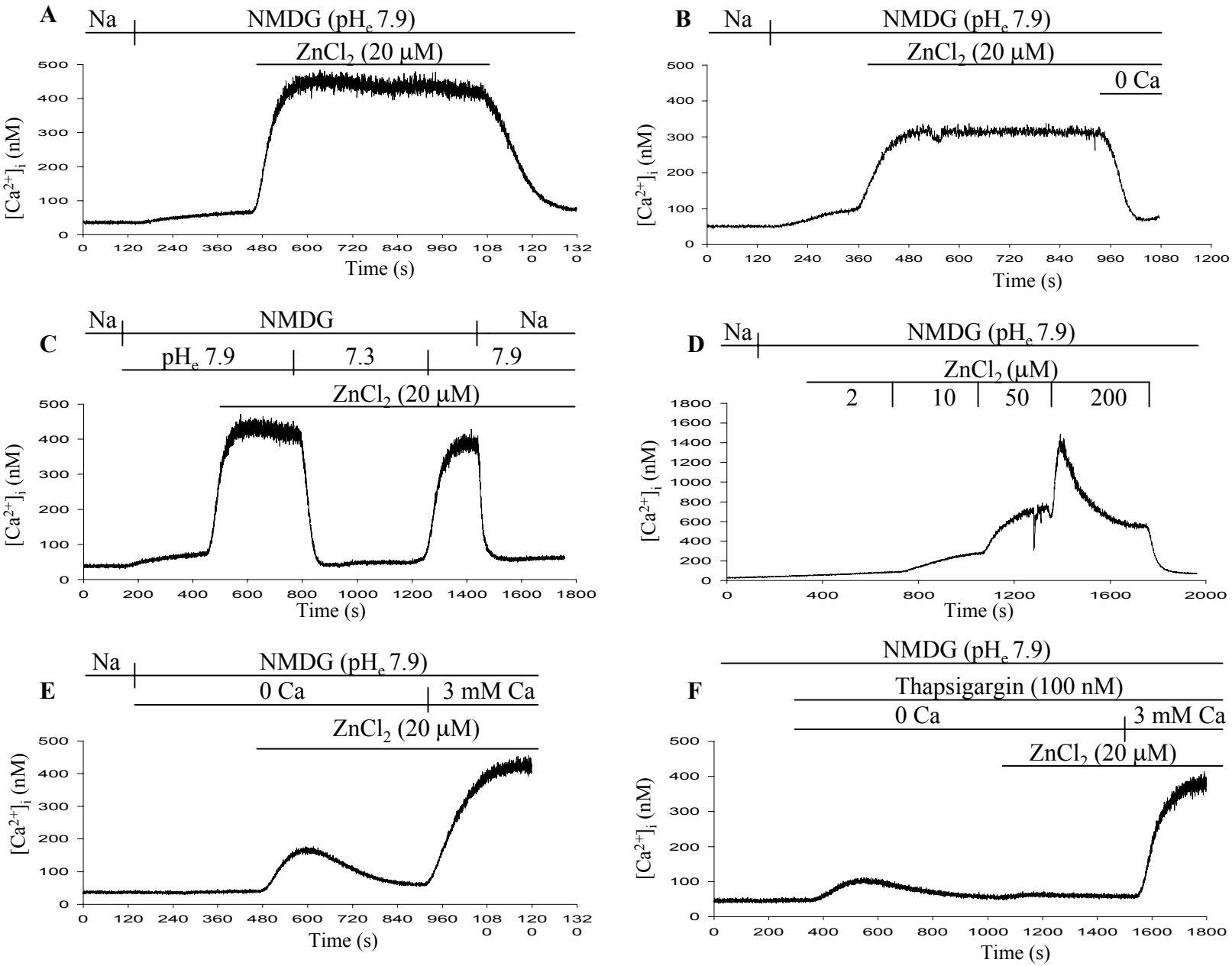
NMDG or Na
3 mM Ca; 0 Mg; pHe 7.9
ATP (100 µM)
ZnCl2 (20 µM)
[Ca2+]i (nM)
Time (s)
B
Na
NMDG
3 mM Ca; 0 Mg; pHe 7.3 or 6.4
ATP (100 µM)
ZnCl2 (20 µM)
[Ca2+]i (nM)
Time (s)
C
Na
NMDG
pHe 7.9
7.8
7.7
7.6
7.4
ATP (100 µM) + ZnCl2 (20 µM)
[Ca2+]i (nM)
Time (s)
D
% of changes in [Ca2+]i
3 mM [Ca2+]e
0 mM [Mg2+]e
(8)
1.5 mM [Ca2+]e
0 mM [Mg2+]e
(3)
3 mM [Ca2+]e
1.5 mM [Mg2+]e
(3)

Fig. 1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NMDG (pHe 7.9)
ZnCl2 (20 μM)
[Ca2+]i (nM)
Time (s)
B
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NMDG (pHe 7.9)
ZnCl2 (20 μM)
0 Ca
[Ca2+]i (nM)
Time (s)
C
Na
NMDG
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pHe 7.9
7.3
7.9
ZnCl2 (20 μM)
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Time (s)
D
Na
NMDG (pHe 7.9)
ZnCl2 (μM)
2
10
50
200
[Ca2+]i (nM)
Time (s)
E
Na
NMDG (pHe 7.9)
0 Ca
3 mM Ca
ZnCl2 (20 μM)
[Ca2+]i (nM)
Time (s)
F
NMDG (pHe 7.9)
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3 mM Ca
ZnCl2 (20 μM)
[Ca2+]i (nM)
Time (s)

Fig. 2

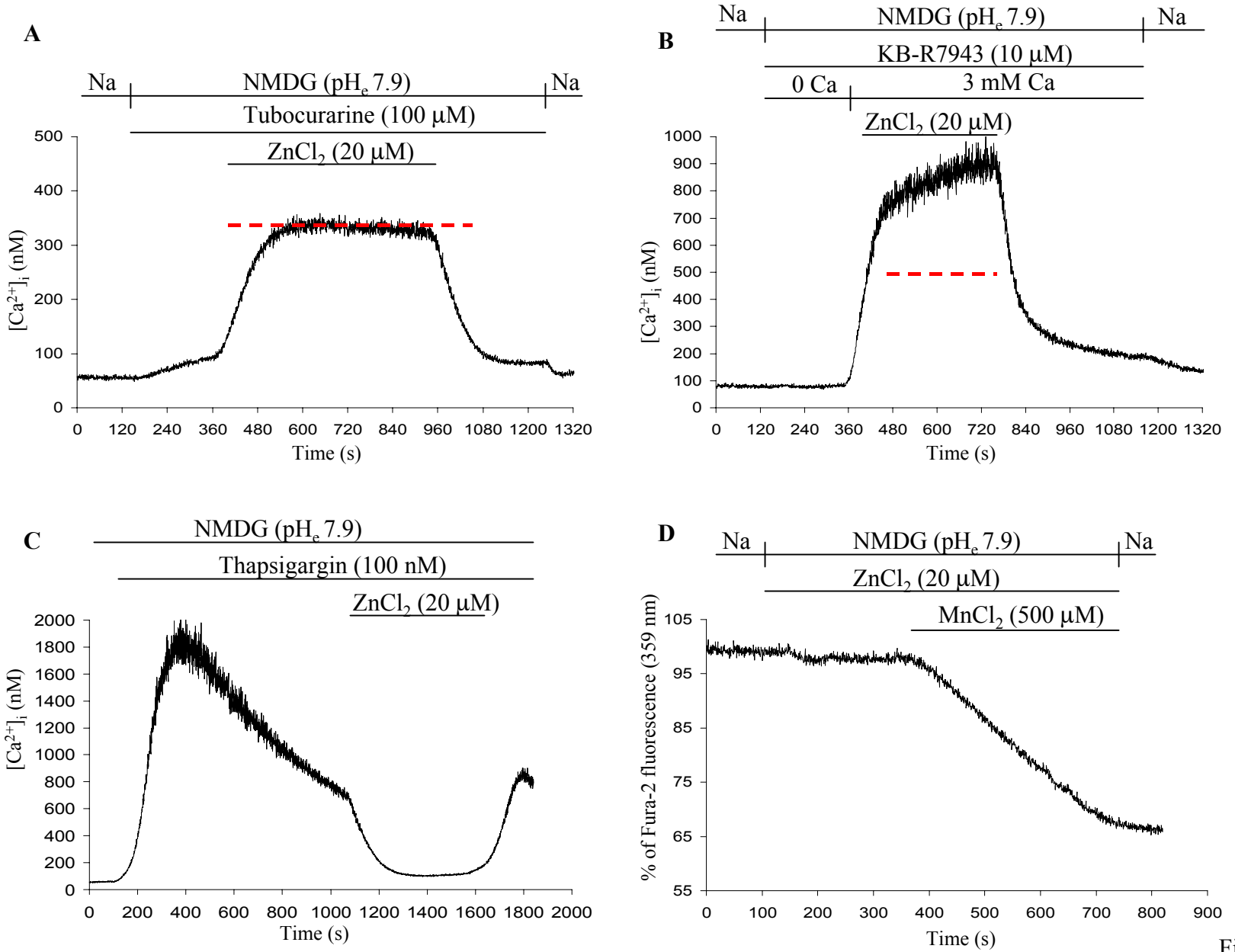
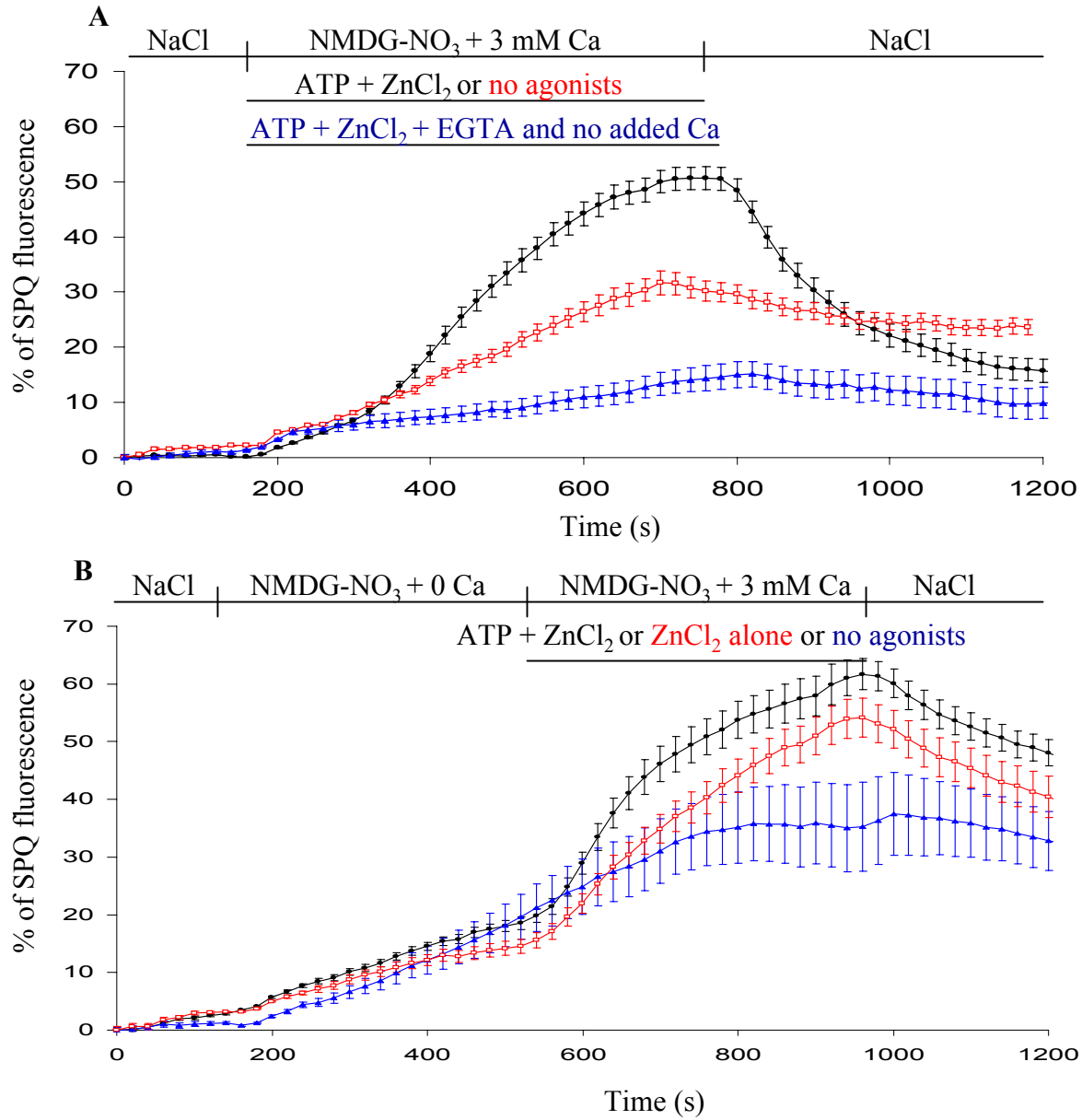

A
Na
NMDG (pHe 7.9)
Na
Tubocurarine (100 μM)
ZnCl2 (20 μM)
[Ca2+]i (nM)
Time (s)
B
Na
NMDG (pHe 7.9)
Na
KB-R7943 (10 μM)
0 Ca
3 mM Ca
ZnCl2 (20 μM)
[Ca2+]i (nM)
Time (s)
C
NMDG (pHe 7.9)
Thapsigargin (100 nM)
ZnCl2 (20 μM)
[Ca2+]i (nM)
Time (s)
D
Na
NMDG (pHe 7.9)
Na
ZnCl2 (20 μM)
MnCl2 (500 μM)
% of Fura-2 fluorescence (359 nm)
Time (s)

Fig. 3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NaCl
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ATP + ZnCl2 + EGTA and no added Ca
% of SPQ fluorescence
Time (s)
B
NaCl
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NMDG-NO3 + 3 mM Ca
NaCl
ATP + ZnCl2 or ZnCl2 alone or no agonists
% of SPQ fluorescence
Time (s)

Fig. 4

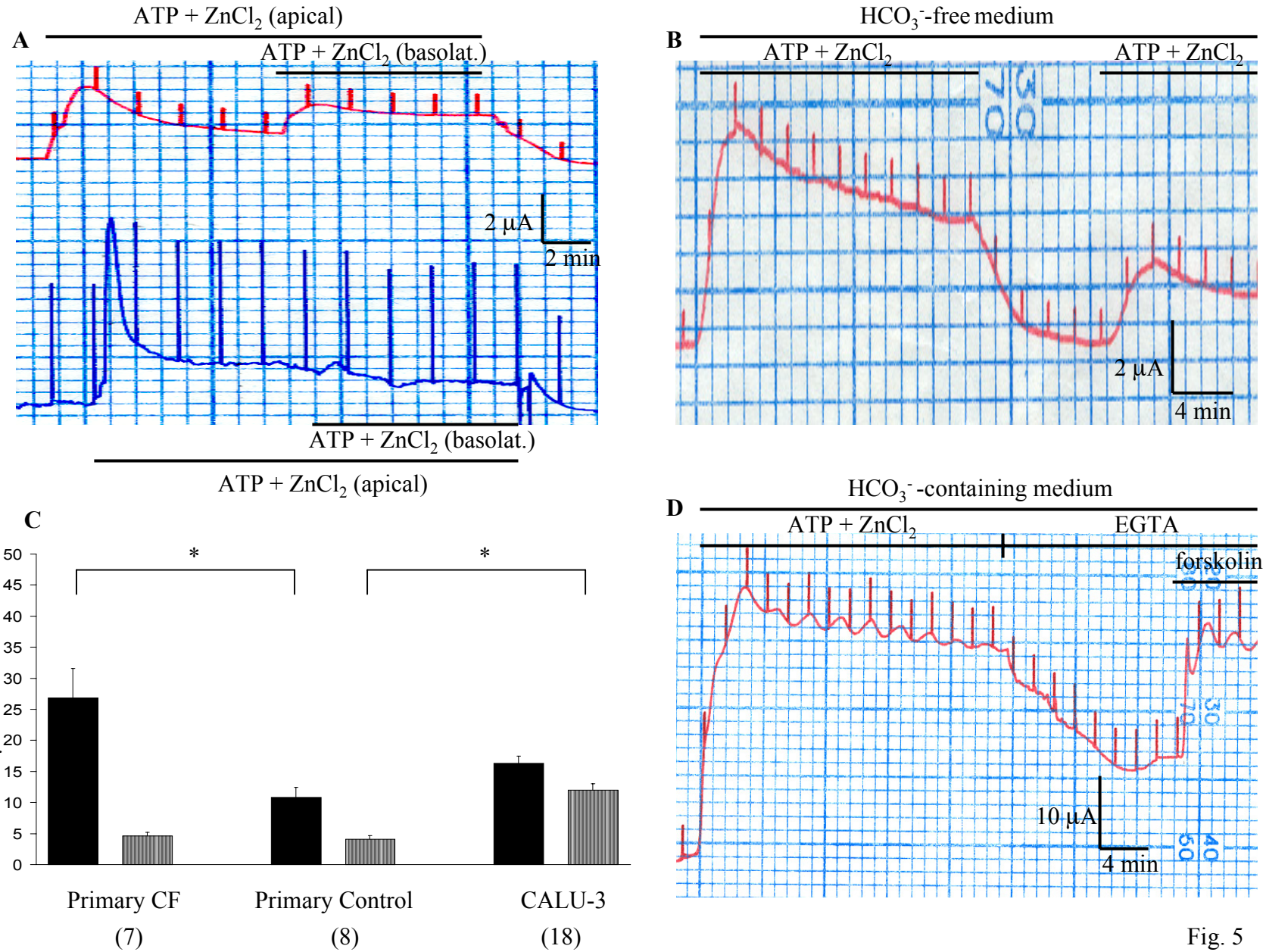
A
ATP + ZnCl2 (apical)
ATP + ZnCl2 (basolat.)
2 µA
2 min
ATP + ZnCl2 (basolat.)
ATP + ZnCl2 (apical)
B
HCO3--free medium
ATP + ZnCl2
ATP + ZnCl2
2 µA
4 min
C
µA/cm2
50
45
40
35
30
25
20
15
10
5
0
*
*
Primary CF
(7)
Primary Control
(8)
CALU-3
(18)
D
HCO3- -containing medium
ATP + ZnCl2
EGTA
forskolin
10 µA
4 min

Fig. 5

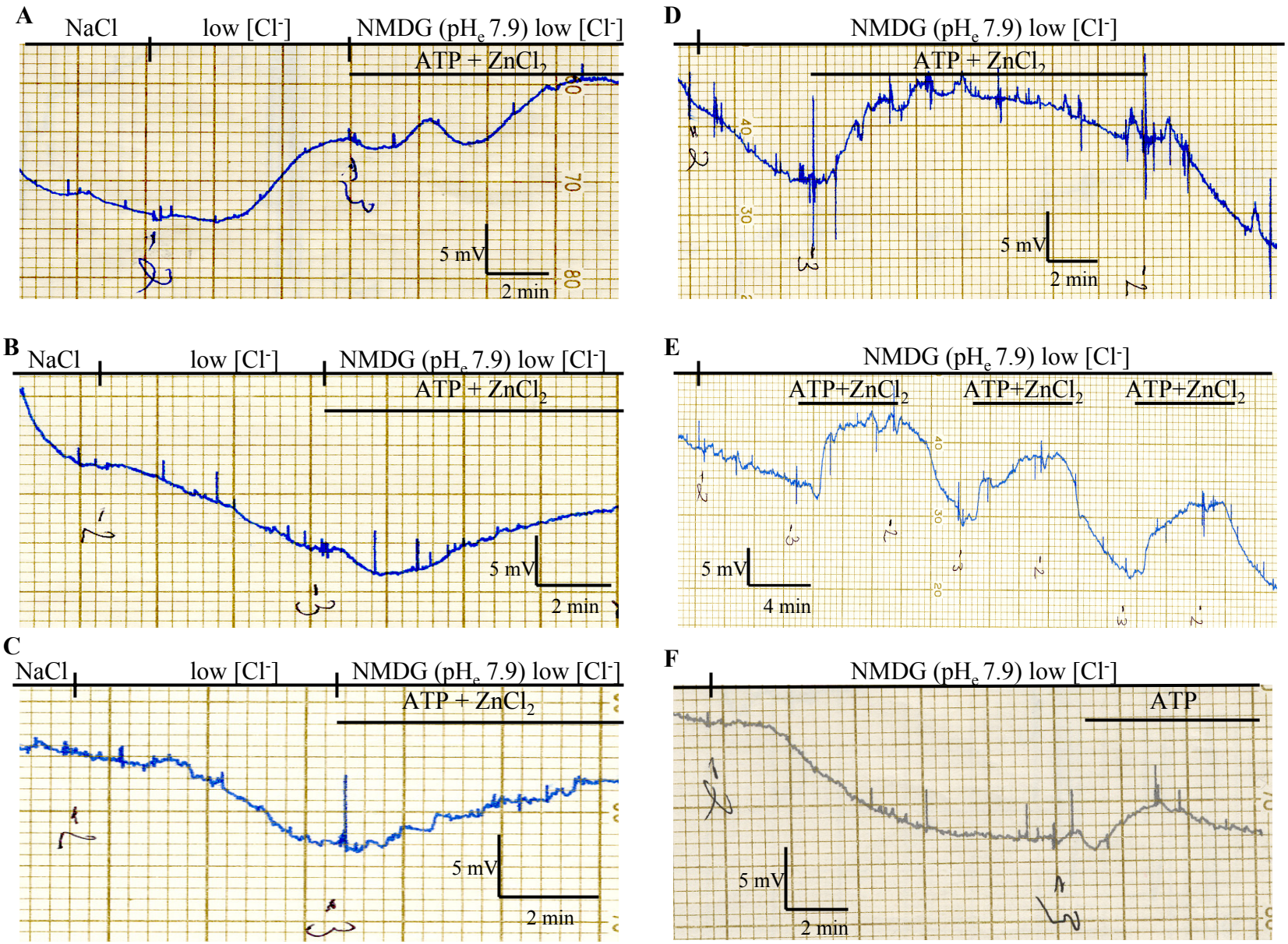
A
NaCl
low [Cl-]
NMDG (pHe 7.9) low [Cl-]
ATP + ZnCl2
5 mV
2 min
B
NaCl
low [Cl-]
NMDG (pHe 7.9) low [Cl-]
ATP + ZnCl2
5 mV
2 min
C
NaCl
low [Cl-]
NMDG (pHe 7.9) low [Cl-]
ATP + ZnCl2
5 mV
2 min
D
NMDG (pHe 7.9) low [Cl-]
ATP + ZnCl2
5 mV
2 min
E
NMDG (pHe 7.9) low [Cl-]
ATP+ZnCl2
ATP+ZnCl2
ATP+ZnCl2
5 mV
4 min
F
NMDG (pHe 7.9) low [Cl-]
ATP
5 mV
2 min

Fig. 6

Table 1. Transepithelial Nasal Potential Difference Values of Control, Δ508 CF and Bitransgenic CF Mice

	Control Cftr(+/-)	n	CF Cftr(ΔF508/ΔF508)	n	Bitransgenic CF Cftr(-/-)	n
Starting point	-18.7 ± 1.5	19	-26.3 ± 2.2*	11	-26.1 ± 1.0*	14
Low $[Cl^-]_e$ (Na; pH:7.3)	-5.5 ± 0.5	8	+3.7 ± 0.9*	3	+4.8 ± 0.9*	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*	7	+6.7 ± 2.3*	3
ATP + $ZnCl_2$ (NMDG; pH:7.9)	-6.0 ± 1.0	2	-9.4 ± 0.6*#	8	-9.7 ± 1.8*&	3
Low $[Cl^-]_e$ (NMDG; pH:7.9)	-4.8 ± 1.5	5			+5.8 ± 1.0*	4
ATP + $ZnCl_2$ (NMDG; pH:7.9)	-5.7 ± 0.7	3			-10.2 ± 0.5*&	6
ATP alone (NMDG; pH:7.9)					-2.3 ± 0.5§	4
Low $[Cl^-]_e$ (NMDG; no added Ca^{2+}; pH:7.9)	-7.3 ± 0.3	3			+6.0 ± 0.4*	4
ATP + $ZnCl_2$ (NMDG; no added Ca^{2+}; pH:7.9)	-1.3 ± 0.3$	3			-2.0 ± 0.6$	4

Starting points represent values (mV) obtained in Ringer 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 towards 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. *p<0.05 vs. control animals, # p<0.05 vs. CF animals ATP + $ZnCl_2$ after low $[Cl^-]$ response with Na^+ (pH:7.3), & p<0.05 vs. Bitransgenic CF animals (generous gift from Dr. Jeffrey A. Whitsett) ATP + $ZnCl_2$ after low $[Cl^-]$ response with Na^+ (pH:7.3), $ p<0.05 vs. ATP + $ZnCl_2$ with NMDG in presence of extracellular Ca^{2+}. § Effect of ATP alone was assessed by the peak of the hyperpolarization response because of the transient nature of the response.

Extracellular zinc and ATP restore chloride secretion across cystic fibrosis airway epithelia by triggering calcium entry

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