

# **Lubiprostone Activates non-CFTR dependent Respiratory Epithelial Chloride Secretion in Cystic Fibrosis Mice**

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Running Head: Activation of nasal epithelial ClC2

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**ABSTRACT**

Periciliary fluid balance is maintained by the coordination of sodium and chloride channels in the apical membranes of the airways. In the absence of the cystic fibrosis transmembrane regulator (CFTR), chloride secretion is diminished and sodium reabsorption exaggerated. CIC-2, a pH and voltage-dependent chloride channel is present on the apical membranes of airway epithelial cells. We hypothesized that CIC-2 agonists would provide a parallel pathway for chloride secretion. Using nasal potential difference (NPD) measurements, we quantified lubiprostone-mediated Cl<sup>-</sup> transport in sedated Cystic Fibrosis null (gut-corrected), C57Bl/6, and A/J mice during nasal perfusion of lubiprostone (a putative CIC-2 agonist). Baseline, amiloride-inhibited, chloride-free gluconate-substituted Ringer's with amiloride, and low chloride Ringer's plus lubiprostone (at increasing concentrations of lubiprostone) were perfused and the NPD was continuously recorded. A clear dose-response relationship was detected in all murine strains. The magnitude of the NPD response to 20 μM lubiprostone was  $-5.8 \pm 2.1$  mV (CF, n=12),  $-8.1 \pm 2.6$  mV (C57Bl6 wildtype, n=12), and  $-5.3 \pm 1.2$  mV (AJ wildtype, n=8). A cohort of CIC-2 knockout mice did not respond to 20 μM lubiprostone (n=6, p=0.27). In C57Bl/6 mice, inhibition of CFTR with topical application of CFTR inhibitor-172, did not abolish the lubiprostone response, thus confirming the response seen is independent of CFTR regulation. RT-PCR confirmed expression of CIC-2 mRNA in murine lung homogenate. The direct application of lubiprostone in the CF murine nasal airway restores nearly normal levels of chloride secretion in nasal epithelia.

Keywords: nasal potential difference, cystic fibrosis, CIC-2, lubiprostone, murine

**INTRODUCTION**

Cystic fibrosis (CF) is one of the most common autosomal recessive life-span shortening diseases that affect almost 30,000 individuals in the US. CF results from mutations in the gene that encodes the membrane glycoprotein CFTR (cystic fibrosis transmembrane regulator). In CF, mutant CFTR impairs epithelial apical chloride secretion and fails to inhibit ENaC-mediated sodium reabsorption(15), leading to dehydrated viscous mucus that obstructs respiratory, gastrointestinal, and reproductive tracts(18). Nasal and bronchial airway epithelial cells also co-express other apical chloride channels (2) which may be therapeutic targets in CF. CIC-2, a member of the pH and voltage-activated chloride channel family, is highly expressed in fetal airways and reduced at birth(20, 21) similarly to CFTR(6, 28). Lipecka et al (17) demonstrated significant expression of CIC-2 at the apex of ciliated cells in both rat and human airways. We previously (25) demonstrated that overexpression of CIC-2 corrected the chloride transport defect in immortalized CF airways cells. No human lung disease has yet been attributed to mutations in CIC-2, however, we previously demonstrated functional response to acidic pH in adult human nasal epithelia *in vivo* by nasal potential difference (NPD) consistent with CIC-2 (29). Therefore, we propose that pharmaceutical activation of CIC-2-mediated chloride transport may compensate for the chloride transport defect in CF. Several novel prostones or PGE series-derived molecules developed by Sucampo Pharmaceuticals Inc. (Bethesda, MD) including SPI-0211 (lubiprostone) target CIC-2 channels (4, 7). Lubiprostone was recently approved by the FDA for the treatment of idiopathic constipation (16). The proposed mechanism of action is stimulation of intestinal chloride channels and resultant fluid secretion into the gut.

In this paper, we hypothesized that activation of CIC-2 with topical lubiprostone in CF murine nasal airways would activate sufficient chloride secretion to restore near normal levels of epithelial chloride transport. CFTR<sup>tm1Unc</sup>-Tg(FABPCFTR)1Jaw/J; gut corrected bi-transgenic, here after known as CFKO, wild type C57Bl/6 and, A/J mice underwent a modified Nasal

Potential Difference (NPD) protocol designed to quantify the contribution of lubiprostone mediated activation of chloride transport in murine airway epithelia. We also employed a transgenic CIC-2 knock-out mouse as an important negative control. We demonstrate that chloride transport can be restored in CF mice with topical application of a putative CIC-2 agonist. Activation of CIC-2 persists in wildtype mice in the presence of CFTR inhibitor. Finally, using RT-PCR techniques, we examined whole lung homogenates of CFKO, C57BL/6, A/J and CIC-2 KO mice strains. We confirmed that CIC-2 mRNA is expressed in lungs from the three mouse models that responded to lubiprostone, but not in the CIC-2 knock out.

## **METHODS**

### **Animals**

All studies were approved by the Johns Hopkins University Animal Care and Use Committee. The double transgenic CFKO mice (31) were obtained from the Pediatric Animal Core at Case Western Reserve University (JAX #002364). The Fatty Acid Binding Protein (FABP) promoter drives human CFTR expression in the mouse gastrointestinal tract which improves survival. C57Bl/6 (JAX #000664) and A/J (JAX #000646) strains of female mice were obtained from JAX Mice (Bar Harbor, MA). The CIC-2 null mice (22) were generated on site from breeding pairs generously provided by Dr. James Melvin (U. of Rochester, Rochester NY). Because we have previously demonstrated CIC-2 expression is down regulated after birth(21), only mature female mice, aged between 16 and 28 weeks at the time of study were employed. The weights of each cohort appear in the data tables. All mice were maintained in the JHU mouse core facility until study in the laboratory. Mice that later underwent tissue harvest were euthanized by anesthetic overdose and cervical dislocation in accordance with university guidelines.

### **Genotyping**

CIC-2 null genotype was determined from murine tail DNA. Primers were forward 5'-ATGTATGGCCGGTACACTCAGGAACTC-3', reverse 5'-

ACACCCAGGTCCCTGCCCAATCTGG-3' and reverse 5'-CCTGGAAGGTGCCACTCCCCTGTCC-3' to amplify a 380 bp region. A 25 µl reaction mixture containing diluted genomic DNA, 10 µM dNPT (dATP, dGTP, dCTP and dTTP), primers, MgCl, Taq DNA polymerase, and 10 X buffer from Qiagen PCR kit was assembled. The DNA was initially denatured at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 68°C for 15 sec and extension at 72°C for 30 sec. In the last cycle, extension at 72°C was performed for 7 min. Positive, negative, and heterozygous controls were included with each run. Amplified DNA underwent electrophoresis on a 1.5% ethidium bromide-stained agarose gel along with DNA markers.

CFKO PCR was performed on mouse tail DNA using primers forward 5'-GAGAACTGGAAGCTTCAGAGG-3', reverse 5'-TCCATGTAGTGGTGTGAACG-3', and Neo 5'-TCCATCTTGTTCAATGGCC-3' to amplify a 357 bp region. Amplification of the gene was done in a 20 µl reaction mixture containing diluted genomic DNA, 10 µl REExtract-N-Amp Readymix (Sigma, St. Louis, MO), 4 µl sterile H<sub>2</sub>O, and 1 µl each forward, neo, and reverse primers. DNA was initially denatured at 94°C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 58 °C for 45 s and extension at 72 °C for 45 s. In the last cycle, extension at 72 °C was allowed for 2 min. Positive, negative, and heterozygous controls were run with each experiment. Amplified DNA underwent electrophoresis on a 1.5 % ethidium bromide-stained agarose gel along with DNA molecular mass markers and photographed under UV transillumination.

### **Nasal Potential Difference (NPD)**

All mice were anesthetized with an IP injection of a ketamine and xylazine mixture (100 µg/10 µg per gram of body weight). After reaching a steady plane of anesthesia (absent toe pinch), oral intubation was performed beginning with direct visualization of the vocal folds with an otoscope with 2 mm speculum (model #20200, Welch Allyn, Skaneateles Falls, NY). A flexible

guide wire was advanced through the vocal folds, and a 20 GA IV catheter was passed over the wire (BD Medical, Franklin NJ). Spontaneous ventilation via a breathing circuit containing free flow oxygen at 0.25 mL/min occurred throughout the procedure. Mice were placed head down on a 15 degree incline. Body temperature was monitored rectally (TH-5, Physiotemp Inc, Clifton, NJ) and maintained with a phase change heating pad (Braintree Scientific, Braintree, MA) and heat lamp as needed. NPD measurements were undertaken using a modification of methods originally described by Grubb et al (10). A high impedance voltmeter (World Precision Instruments, Sarasota, FL) was connected by silver-chloride pellet electrodes to an exploring nasal bridge and a reference subcutaneous bridge. The nasal bridge, a single polyethylene tube (PE10, 0.28 mm ID, Clay-Adams, BD, Sparks, MD) was pulled to approximately one-half its original diameter and cut at an acute angle to maximize surface area. The resulting orifice is ~ 0.7 mm in diameter. The tubing was marked at 3 and 5 mm from the tip. The tubing was inserted into the nare to 3mm, and after steady state, was advanced to the point of maximum voltage, but never beyond 5 mm. The subcutaneous bridge was a 25 GA Butterfly® (Abbott, Chicago, IL) needle containing Ringers solution inserted subcutaneously in the right abdominal wall. Each solution was warmed to 37°C and perfused to the nare for at least 3 minutes at 8 µl/min using a perfusion pump. Following the procedure, the oral cavity was gently suctioned of perfusate and the mice were recovered in their cages.

**Reagents**

Baseline Ringer's (solution 1) was composed of 135 mM NaCl, 2.25 mM CaCl<sub>2</sub> x 2H<sub>2</sub>O, 1.2 mM MgCl<sub>2</sub> x 6H<sub>2</sub>O, 2.4 mM K<sub>2</sub>HPO<sub>4</sub>, and 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4. The 100 µM amiloride (solution 2) was generated by adding amiloride directly to solution 1. The amiloride containing zero chloride (solution 3) solution was a gluconate substituted balanced Ringer's produced by sequential substitution with 135 mM Na Gluconate, 1.2 mM MgSO<sub>4</sub> x 7H<sub>2</sub>O, 2.2 mM Ca Gluconate, 2.4 mM K<sub>2</sub>HPO<sub>4</sub>, and 0.4 mM K<sub>2</sub>H<sub>2</sub>PO<sub>4</sub> adjusted to pH 7.4. The lubiprostone-

containing solutions (solutions 4-6) utilized stock drug supplied as aliquots of 2 mM lubiprostone in DMSO (Sigma, Japan) as well as a stock bottle of same DMSO (provided by MTA with Sucampo Pharmaceuticals Inc. (Bethesda, MD)). Lubiprostone was added to a stock of solution 3 and underwent serial dilution to final 1, 10, and 20  $\mu\text{M}$  concentrations. The resulting DMSO concentration was less than 1% by volume. The pH remained  $> 7.1$  following addition of lubiprostone to stock solutions. Isoproterenol (solution 7) containing solution was prepared fresh with isoproterenol hydrochloride (1:5000) added to a stock of solution 3, to a final concentration of 100  $\mu\text{M}$  and protected from light. The 100  $\mu\text{M}$  ATP (solution 8) was diluted in solution 7 just prior to use. The CFTR channel inhibitor 172 (24) (CalBioChem, San Diego, CA) was added to a stock of solution 3 to reach 50  $\mu\text{M}$  concentration with final DMSO concentration  $< 0.1\%$ . Control solutions were prepared with 1% DMSO (Sigma, Japan). Solutions were sterilized through a 0.22  $\mu\text{m}$  filter (Millipore, Bedford, MA).

### **Data Collection and Analysis**

Voltmeter data (mV) were recorded by a PowerLab A/D converter connected to a PC running Chart software at 20 samples per sec (AD Instruments, Colorado Springs, CO). A minimum of 400 data points ( $\sim 20$  sec) were used to calculate the mean voltage values for Ringers,  $\Delta \text{Cl}^-$  (the voltage difference between peak low  $\text{Cl}^-$  mV and peak amiloride mV) and  $\Delta \text{LUBI}$  (the voltage difference between peak lubiprostone mV and peak Zero  $\text{Cl}^-$  mV). Statistical analysis was performed with SPSS software for Windows (SPSS, Chicago, IL) and included paired t-test, one-way ANOVA, and Tukey post-hoc multiple comparisons. Significance was set at  $p < 0.05$  for all tests. Voltage tracings were generated by exporting raw data from Chart software at a reduction ratio of 1:25 and imported to Microsoft Excel.

### **Total RNA isolation and RT-PCR**

Lungs were harvested by a ventral chest incision. The trachea was identified and then using careful cranial to caudal dissection, the trachea, heart and lung were removed as one unit. Each lung was then divided at the main stem bronchus and immediately placed in RNAlater (Ambion, Austin, TX) and stored at -80 °C for later homogenization. Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Following isolation, the RNA was purified twice using RNeasy spin columns with an on-column DNase I treatment to remove genomic DNA (Qiagen, Valencia, CA). A one-step RT-PCR reaction to detect CIC-2 mRNA was performed using the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen). Pre-validated Quantitect primers were used for the specific amplification of murine CIC-2 (Qiagen, qt00141876, product size: 90 bp) and beta-actin control primers were (+) 5'-TGGACTTCGAGCAAGAGATG -3' and (-) 5'GAAGGAAGGCTGGAAGAGTG-3' (product size: 137 bp). A touch down PCR approach was used for the CIC-2 amplification and included a five minute incubation at 50° C (cDNA synthesis) followed by 10 cycles with annealing temperatures of 65-60° C (decreasing .5° per cycle) and 30 cycles at 59° C. The one-step amplification of actin included a 5 minute incubation at 50° C followed by 35 cycles at 60° C. A No-RT control was performed by replacing Superscript III with 5 U Taq Polymerase (Invitrogen). 20 µl of each PCR reaction was separated on a 2% agarose gel and products were examined under UV fluorescence.

## **RESULTS**

### **Baseline electrical properties of murine nasal epithelium resemble human nasal potential difference responses.**

The baseline potential difference (PD) during perfusion with solution 1 (Ringers) is primarily a measure of the sodium potential difference across the nasal epithelium. The PDs of both non-CF strains, C57Bl/6 ( $n=12$ ) is  $-5.2 \pm 4.2$  mV and A/J ( $n=8$ )  $-4.74 \pm 1.8$  mV, are significantly less

polarized than the CFKO PD ( $-23.6 \pm 3.6$  mV),  $p < 0.05$  (Table 1). Although we observed slightly lower baseline voltages than previously reported in these strains of mice(5, 10), the expected depolarization with amiloride blockade and subsequent hyperpolarization with zero chloride and isoproterenol solutions, faithfully replicated both the human NPD waveform and previous reports in the two wildtype strains of mice. Depolarization induced with solution 2 (amiloride) was significantly greater in the CF mice than either wild type strain,  $p < 0.05$  (Table 1). Replacement of chloride with gluconate in the continued presence of amiloride (solution 3) increases the chemical gradient for chloride exit and the PD will hyperpolarize if chloride channels are open. Multiple chloride channel species may participate including the calcium-activated chloride channel CaCC, the outwardly rectifying chloride channel ORCC, CIC species, and CFTR, if present. Wild type C57Bl/6 mice ( $n=12$ ) repolarized to a mean of  $-13.5 (\pm 2.3)$  mV, whereas CFKO mice ( $n=12$ ) continued to depolarize to a mean of  $-10.6 (\pm 3.1)$  mV, which is consistent with other reports. The A/J mouse strain has been reported to demonstrate lower polarization as compared with C57Bl/6 in response to Zero Cl<sup>-</sup> solution, yet share a nearly identical forskolin response (5). We also observed a lower mean Zero Cl<sup>-</sup> response in the A/J mice of  $-9.2 (\pm 1.8)$  mV (vs.  $-13.5$  mV), suggesting a possible reduction in the population of one or more chloride channel species in A/J mice.

### **Lubiprostone activates chloride transport in a dose-dependent relationship in wild type and CF mice**

There has been controversy regarding the subcellular localization of CIC-2 protein, particularly in GI epithelia(23). We sought to confirm an apical location in respiratory epithelia by measuring activation of CIC-2 through a non-absorbable putative CIC-2 agonist, lubiprostone. Nanomolar concentrations of lubiprostone in solution 4 which had shown activity *in vitro*(7), did not lead to detectable hyperpolarization in murine NPD (data not shown). However, 20  $\mu$ M concentrations of lubiprostone led to consistently significant polarization of the NPD (Table 1 and Figure 1) in

all mouse strains. Separate observations in two CFKO mice showed that following topical lubiprostone, reperfusion with non lubiprostone containing solution 3 resulted in a slow depolarization suggesting that the agent must remain in contact with the channel to activate chloride conductance (Figure 2). The vehicle control for lubiprostone (1% DMSO in solution 3) did not affect the potential difference ( $n=3$ ) in C57Bl/6 mice tested (Figure 3). Perfusion of isoproterenol (solution 7) after the lubiprostone escalation in C57Bl/6 mice resulted in further repolarization Table 1, which was significant ( $p = 0.01$ ) when compared to the voltage generated by 20  $\mu\text{M}$  lubiprostone.

Mean lubiprostone responses in C57Bl/6 mice ( $n=12$ ) were;  $-3.4 (\pm 2.5)$  mV at 1  $\mu\text{M}$ ,  $-6.2 (\pm 2.1)$  mV for 10  $\mu\text{M}$  and  $-8.1 (\pm 2.6)$  mV for 20  $\mu\text{M}$ . Analysis by one-way ANOVA was significant at  $p=0.0001$ , however, post hoc Tukey analysis demonstrated significance only for the 10  $\mu\text{M}$  and 20  $\mu\text{M}$  lubiprostone concentrations when compared to Zero  $\text{Cl}^-$ . In CFKO mice ( $n=12$ ), the mean lubiprostone responses were;  $1.2 (\pm 1.1)$  mV for 1  $\mu\text{M}$ ,  $-3.7 (\pm 1.5)$  mV for 10  $\mu\text{M}$  and  $-5.8 (\pm 2.1)$  mV for 20  $\mu\text{M}$ . Analysis by one-way ANOVA was significant at  $p=0.0001$ , however, post hoc Tukey analysis demonstrated significance only for the 10  $\mu\text{M}$  and 20  $\mu\text{M}$  lubiprostone concentrations when compared to Zero  $\text{Cl}^-$ . Mean lubiprostone responses in A/J mice ( $n=8$ ) are included in Table 1. Analysis by one-way ANOVA was significant at  $p=0.0001$ , and post hoc Tukey analysis demonstrated significance for all 3 lubiprostone concentrations when compared to Zero  $\text{Cl}^-$  ( $p=0.035$ ,  $p=0.0001$ ,  $p=0.0001$ ). In data not shown CFKO mice generated increased, response at higher lubiprostone concentration (100  $\mu\text{M}$ ). Separate observations in two CFKO mice showed that removal of lubiprostone led to rundown of chloride transport over 10 minutes, again suggesting that the compound must remain in contact with the epithelium to continue the response (Fig. 4).

**Topical lubiprostone has no effect on NPD in CIC-2 null mice**

If the majority of the chloride transport response elicited by topical lubiprostone is through CIC-2, then mice lacking CIC-2 should not have a significant response to lubiprostone. We challenged six CIC-2 ( $n=6$ ) null mice by NPD with 20  $\mu\text{M}$  concentrations of lubiprostone and observed no significant additional chloride transport following the low chloride (solution 3) (Table 2 and Figure 5). These mice exhibited a significant response to isoproterenol (solution 7) (Table 2) suggesting CFTR was further activated following lubiprostone perfusion. One-way ANOVA was significant at  $p=0.01$ , Tukey post hoc analysis was significant when comparing 20  $\mu\text{M}$  lubiprostone and isoproterenol ( $p=0.013$ ).

**CFTR is not required for lubiprostone activated chloride transport**

Lubiprostone stimulates chloride transport in CFTR null mice, however the magnitude of the response is less than in wildtype C57Bl/6. C57Bl/6 mice respond to both lubiprostone and isoproterenol suggesting that CFTR can respond to lubiprostone at high dose, (4) or increase the response of other Cl<sup>-</sup> channels to the compound. To confirm that functional CFTR is not an absolute requirement for lubiprostone activity in the C57Bl/6 strain, CFTR inhibitor-172 was included in solution 3 in a subset of experiments to inhibit CFTR prior to application of lubiprostone. In C57Bl/6 mice ( $n=7$ ), topical inhibition of CFTR did not block subsequent lubiprostone activity (Table 3). We observed a lack of polarization of the PD with the addition of CFTR-172inh to the Zero Cl<sup>-</sup> (solution3) perfusate (Table 3). In earlier reports of murine NPDs, CFTR 172 inhibitor was applied after hyperpolarization was induced with chloride free ringers and resulted in only minor depolarization(24). This demonstrates *in vivo* a requirement for CFTR Cl<sup>-</sup>-activation potential being required for apical Cl<sup>-</sup> transport in response to a Cl<sup>-</sup> ion gradient even if CFTR is not the principal anion channel in mouse NPDs as the authors suggest . Further addition of 100  $\mu\text{M}$  ATP to the final perfusate resulted in continued polarization (Table 3), suggesting further activation of calcium activated channels.

**CIC-2 mRNA is expressed in murine whole lung homogenates**

Figure 6 demonstrates the expression of CIC-2 mRNA (157 bp) by RT-PCR in mature mice of all 3 genotypes that had a positive response to lubiprostone. The CIC-2 knock-out mouse lacked detectable CIC-2 mRNA expression.

**DISCUSSION****Physiologically active concentrations of lubiprostone are in the  $\mu\text{M}$  range when applied to the murine nasal luminal surface**

Previous studies of lubiprostone in cultured cell systems suggested that chloride secretion could be induced with nM concentrations in the cell medium(7, 19). We anticipated a similar dose-response profile in the murine nose; however, in our study of murine nasal airways *in vivo*,  $\mu\text{M}$  concentrations were required. Cuppoletti et al (7) employed T-84 colon carcinoma cells which are known to have significantly increased expression of CIC-2 mRNA compared to the immortalized human bronchial epithelial cell line IB3-1 (25). T84 monolayers were treated with 1-ethyl-2-benzimidazolinone (1-EBIO) followed by permeabilization of the basal lateral membrane with nystatin, eliminating basolateral potassium exchange transport as a limit to apical chloride secretion. In intact airway epithelial Calu-3 monolayers, lubiprostone stimulated short circuit current at roughly twice the maximum concentration of the T84 experiments (19). However, the location and amount of expression of CIC-2 in Calu-3 is unclear(8). The T84 and Calu-3 cell lines employed by the previous authors are both primary secretory epithelia, without detectable levels of sodium absorption via ENaC. Mouse respiratory epithelium has active ENaC channels and chloride secretion detection by NPD requires the presence of amiloride. Amiloride reduces the influx of sodium so that a repolarization due to chloride exit is detectable as a change in NPD in the negative direction, and is not necessary during measurements of

whole cell currents *in vitro*. However, as we have shown experimentally, the nasal potential difference in CFKO following amiloride is not zero and remains significantly polarized compared to non-CF mice, suggesting a higher gradient and more drug needed to make effective measurements. We do not expect amiloride will be required therapeutically and only use it here to make the electrical measurements. In fact, the opposite therapeutic maneuver, aerosolization of hypertonic sodium chloride to the lumen has been shown to improve lung function in CF patients. In that study, amiloride added no further benefit (9). Furthermore, not all tissues affected in CF depend on epithelial sodium reabsorption, making lubiprostone potentially useful in CF gut related distal intestinal obstruction syndrome (DIOS). To date lubiprostone has only been critically evaluated in adult functional constipation(12).

The nasal potential difference measurement is a dynamic process requiring constant perfusion of drug to maintain electrical contact with the epithelium. We demonstrate that when lubiprostone perfusion is removed from the NPD circuit (Figure 4), the effect runs down. Additionally, time of drug exposure was severely limited in our model due constraints of multiple doses and limited duration of animal anesthesia.

Although the precise mechanism of action of lubiprostone is unclear, MacVinish et al noted that ion channel properties when studied *in vitro*, often differ when assayed *in vivo* (19). Variability is introduced when the drug must be solubilized at the final concentrations prior to placement in the syringe and tubing. It is possible that solubilization, even with DMSO, is not identical in perfusion solution as cell culture medium to the process used *in vitro*. A reduction in potency is also expected when moving from a single cell layer *in vitro* to a living host *in vivo* with intact airway defenses. An editorial by J.G. Widdicombe (30) reviews the pitfalls of airway drug delivery including penetration and diffusion of the airway surface layer as well as drug-mucin interactions, supporting the observations that higher concentrations are required for the mouse nasal airway. Our data clearly demonstrate a dose related response to topical lubiprostone in 3 distinct mouse strains, suggesting it may be useful as an airway hydration agent.

**CFTR is not required for CIC-2 mediated chloride secretion**

Lubiprostone had no significant effect on chloride secretion in CIC-2 null mice, and inhibition of CFTR in C57Bl/6 mice with CFTRinh-172 did not prevent lubiprostone-mediated repolarization of the NPD. However, the magnitude of the chloride secretory change in NPD in CFKO mice was lower than in C57BL/6 mice, but similar to the A/J strain. This suggests a strain or genetic variation in Zero Cl<sup>-</sup> PD (5) and subsequent strain related lubiprostone responses. The CFKO double transgenic CF mouse(31) used here, is a robust animal, however, it is in a mixed strain background (mostly C57BL/6 and SVJ129 ) making differences due to strain difficult to quantify. Other explanations for these observed voltage differences might include; 1) lubiprostone induced chloride secretion may lead to increased cellular cAMP and indirect stimulation of CFTR in wild type mice. However, in our data, when CFTR was inhibited prior to 20  $\mu$ M lubiprostone, the response was similar: - 7.4 mV versus - 8.1 mV without CFTR inhibition in C57BL/6 mice. Also, Bao et al(4) have demonstrated that lubiprostone does not lead to increased cAMP. Lubiprostone might induce calcium entry into the cell, activating the CaCC pathway; however, to our knowledge this has not been shown. We are actively examining CIC-2 specific antagonists (27) to examine lubiprostone channel selectivity. 2) Our lung homogenate data suggest reduced expression of CIC-2 mRNA in CFKO and A/J mice by PCR as compared to the C57BL/6 mice, however, quantitative studies would be required to verify this hypothesis. 3) Lubiprostone induced chloride secretion might further down-regulate ENaC activity in the CFKO mouse, thus blunting the negative change. This last explanation would be desirable in CF but would require additional experiments to confirm the effect on ENaC. König et al (13) demonstrate inhibition of ENaC in oocytes by co-expression of CIC-0, as well as high extracellular Na<sup>+</sup> and Cl<sup>-</sup> in partially permeabilized oocytes. They conclude that inhibition of ENaC is not specific to CFTR and seems to be mediated by intracellular Cl<sup>-</sup> levels. Bachhuber et al showed that activation of CIC-0 in oocytes increased intracellular Cl<sup>-</sup> concentration leading

to ENaC inhibition (3). Kunzelmann had similar findings (14). Mo and Wills concluded co-expression of CIC-5 and ENaC in oocytes resulted in decreased trafficking activity of ENaC.

Continuous exposure to lubiprostone appears to be desirable since either removal of lubiprostone from the nasal perfusion or washout led to rundown in the chloride secretory PD (Figures 2 and 4). Lubiprostone is not absorbed from the GI tract when given for idiopathic constipation and therefore is not likely absorbed through the airways either. Airway delivery of a prostone for CF might benefit from a slow release formulation or dry powder aerosol to sustain the effect in the airway lumen.

### **CIC-2 is expressed in tissues affected by CF**

Given the co-expression of CFTR and CIC-2 in airways and gastrointestinal epithelia including the biliary tract(17, 26), specific CIC-2 agonists that are under development for liver and biliary tract disease (1), might also have some utility in CF. The NPD is widely accepted as a surrogate marker of tracheal and bronchial ion transport. However measurement of ion transport in other affected tissues is much more difficult, and with the exception of the sweat chloride test, there are no surrogate measures for CFTR function in other locations in the body. CFTR is normally expressed in the sweat gland secretory coil and duct, the sinuses, airway submucosal glands, liver, reproductive tract, and small intestines. Aerosol delivery would only assist the lung epithelium and possibly penetrate to the submucosal glands, but would not treat sweat glands, the sinuses or gastrointestinal tracts. Topical delivery of a CIC-2 agonist would be inaccessible in tissues such as the pancreas, liver, where CIC-2 is expressed in the biliary epithelium, and the small intestines (26). Realistically, the pancreas and male reproductive tract are likely to be resistant to therapies that increase chloride secretion because the damage from dysfunctional CFTR begins early *in utero* and results in irreversible structural changes prior to birth. Fortunately, systemic delivery of a prostone is feasible. One example, SPI-8811, is a novel prostone developed by Sucampo Pharmaceuticals for treatment of biliary cirrhosis via

activation of ClC-2 (1). This investigational agent has oral bioavailability and has been studied in a Phase I trial in CF.

**ClC-2 function is not required for normal lung development or airways defense.**

ClC-2 mutations have been identified in people suffering from idiopathic generalized seizures (11). These persons do not have a history of lung disease. In our experience, the ClC-2 KO mouse does not develop these seizures or spontaneous lung disease, however, the male mice are sterile. We have previously shown in the rat that ClC-2 is abundant in fetal lung airways and that in the perinatal period, there is a transition from predominantly chloride secretion to sodium absorption across airway epithelial cells (21). ClC-2 immunoreactivity is strongly positive in the fetal lung, and is markedly down regulated at birth. The fetal lung liquid is acidic, and acidic pH on the luminal surface of fetal airway cells activates chloride secretion. Thus, it is likely that ClC-2 contributes to the driving force for lung liquid generation in utero. Surprisingly, the ClC-2 KO mouse undergoes normal lung development, probably due to the presence of multiple alternative chloride channel conductance pathways including the CFTR, the ORCC, and the CaCC, at a minimum.

In summary, our data demonstrate lubiprostone mediated chloride channel activation in murine nasal epithelium that is independent of the function of CFTR. This work shows *in vivo* the potential of prostones as a new therapeutic class for cystic fibrosis.

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## Figure Legends

Figure 1: Effect of transgenic mouse strain on the NPD response to lubiprostone. The standard NPD protocol was modified to replace the isoproterenol perfusion with a dose escalation of lubiprostone. Data are expressed as mean mV PD (Y axis) in each perfusate (X axis). Error bars mark the SD. Pink (CFKO, N= 12), Blue (C57Bl/6, N =12), Black (A/J, N = 8). The A/J and CFKO strains have nearly identical lubiprostone responses.

Figure 2: Lubiprostone mediated chloride transport is sustained after 4 minute perfusion (X axis units in hr:min elapsed), but runs down when perfusion of the compound is halted. The NPD in a CFKO mouse (one of two experiments) is shown to illustrate that perfusion with lubiprostone sustained chloride secretion (~ 10 minutes) before diminishing.

Figure 3: Effect of 1% DMSO vehicle control on an NPD in C57Bl/6 mouse. DMSO was added to the Zero Cl<sup>-</sup> (solution 3) and run for at least 3 minutes. DMSO free solution 3 was then applied for 3 minutes and then 1% DMSO in solution 3 was reapplied. No significant voltage difference was seen in 3 mice tested. (X axis units in hr:min elapsed),

Figure 4: Effect of “wash out” of lubiprostone. (X axis units in hr:min elapsed), 10  $\mu$ M lubiprostone (solution 5) supplied to a CFKO mouse produces the characteristic hyperpolarization (N=2). However, substitution with Zero Cl<sup>-</sup> (solution 3) containing 1% DMSO (vehicle control) results in a loss of effect or “wash out”. This can be repeated several times in the same mouse.

Figure 5: Lubiprostone challenge in CIC-2 knockout mouse. (X axis units in hr:min elapsed), After baseline NPD was recorded, amiloride superfusion led to a modest depolarization consistent with the presence of wildtype CFTR. Shortly after switching to solution 3, chloride transport was induced, consistent with the presence of functional CFTR. Addition of 20  $\mu$ M lubiprostone did not activate further chloride transport, consistent with the absence of CIC-2. Confirmation of CFTR function occurred with the subsequent exposure to 100  $\mu$ M isoproterenol in perfusate 7.

Figure 6: (top panel) Photograph of RT PCR gel examining CIC-2 mRNA (90 bp). Left to right C57Bl/6, CFKO, A/J, CIC-2 KO, C57Bl/6 no RT control, no template control. (bottom panel)  $\beta$ -actin control( 130 bp). Left to right C57Bl/6, CFKO, A/J, CIC-2 KO, C57Bl/6 no RT control, no template control.

Table 1: Effect of topical lubiprostone on NPD in three mouse strains\*

Genotype (n)	Weight (g)	Baseline (mV)	Amiloride (mV)	Zero Cl <sup>**</sup> (mV)	Δ lubi 1μM# (mV)	Δ lubi 10 μM (mV)	Δ lubi 20 μM (mV)	Δ iso## (mV)
CFKO (12)	24.0 ±3.1	-23.6 ±3.6	-14.2 ±3.0	-10.6 ±3.4	-1.2 ±1.1	-3.7 ±1.5	-5.8 ±2.1	-
<i>significance</i>					<i>p=0.01</i>	<i>p=0.0001</i>	<i>p=0.0001</i>	
C57Bl/66 (12)	23.6 ±2.9	-5.2 ±4.0	-2.3 ±2.0	-13.5 ±3.3	-3.4 ±2.5	-6.2 ±2.1	-8.1 ±2.6	-9.7 ±3.9
<i>significance</i>					<i>p=0.001</i>	<i>p=0.0001</i>	<i>p=0.0001</i>	<i>p=0.001</i> <sup>†</sup>
A/J (8)	21.2 ±2.3	-4.7 ±1.8	-3.3 ±2.0	-9.2 ±1.8	-2.2 ±1.6	-4.1 ±1.2	-5.3 ±1.2	-
<i>significance</i>					<i>p=0.005</i>	<i>p=0.0001</i>	<i>p=0.0001</i>	
Genotype (n)	Weight (g)	Baseline (mV)	Amiloride (mV)	Zero Cl (mV)	1% DMSO (mV)	Zero Cl (mV)	1% DMSO (mV)	-
C57Bl/6 (3)	22.8 ±1.9	-3.8 ±0.5	-0.8 ±0.6	-12.3 ±1.8	-12.2 ±2.0	-12.2 ±1.8	-12.1 ±1.9	

\*Data are mean ± SD.

\*\*Δ Zero Cl<sup>-</sup> is the difference between the maximal change in lubiprostone (lubi) and the stable amiloride inhibited voltage.

#Δ lubi 1, 10, or 20 μM is the difference between the maximal change in lubi and the stable Zero Cl<sup>-</sup> voltage.

## Δ iso is the difference between the maximal change in iso and the stable Zero Cl<sup>-</sup> PD.

<sup>†</sup>Paired t-test for lubi 20 μM and iso was significant at *p*= 0.001.

The DMSO vehicle control data set is at the bottom of the table.

Table 2: Absence of chloride response to 20µm lubiprostone in CIC-2 KO mice.\*

Genotype (n)	Weight (g)	Baseline (mV)	Amiloride (mV)	Zero Cl (mV)	lubi 20µM (mV)	isoproterenol 100 µM (mV)	Δ lubi 20 µM (mV)	Δ iso (mV)
CIC-2 KO (6)	25.4 ±2.1	-3.2 ±1.0	-0.9 ±0.7	-11.0 ±2.5	-10.3 ±2.8	-15.9 ±3.4	0.7 ±2.0	-4.6 ±1.6
							$p=0.413^{**}$	$p=0.001^{**}$

\*Data are mean ± S.D.

\*\*Paired t-test was not significant between zero Cl- PD and lubiprostone ( $p=0.413$ ), however, it was significant between Zero Cl- PD and iso PD ( $p=0.001$ ). *Data are consistent with the absence of CIC-2 and the presence of CFTR.*

Table 3: Inhibition of CFTR does not abolish the chloride transport activated by lubiprostone or ATP.

Genotype (n)	Weight (g)	Baseline (mV)	Amiloride (mV)	Zero Cl <sup>-</sup> (mV) + 50μM CFTRinh 172**	Δ lubi 20 μM (mV)#	Δ 100μM ATP (mV)##
C57Blk6 (7)	24.7 ±0.5	-4.0 ±0.71	-1.6 ±0.51	-0.7 ±0.84	-6.5 ±1.8	-10.0 ±3.7
					<i>p=0.0001</i>	<i>p=0.003</i> <i>p=0.007†</i>

C57Bl/6 mice (N=7) underwent NPD testing with Zero Cl<sup>-</sup> solution containing CFTR inh 172 prior to lubiprostone stimulation.

\*\*Application of CFTR inhibitor before the Zero Cl<sup>-</sup> response resulted in near isoelectric PD.

#The value Δ lubi 20 μM reflects the difference of the maximal change induced by lubiprostone and the stable Zero Cl<sup>-</sup> value.

##The Δ 100μM ATP value is the difference of maximal ATP response and the stable Zero Cl<sup>-</sup> value, which represents the calcium activated chloride conductance. Significance was tested by Paired t-test for each response compared with Zero Cl<sup>-</sup> containing CFTRinh 172

† is the Paired t-test result for lubiprostone and ATP.

Figure 1

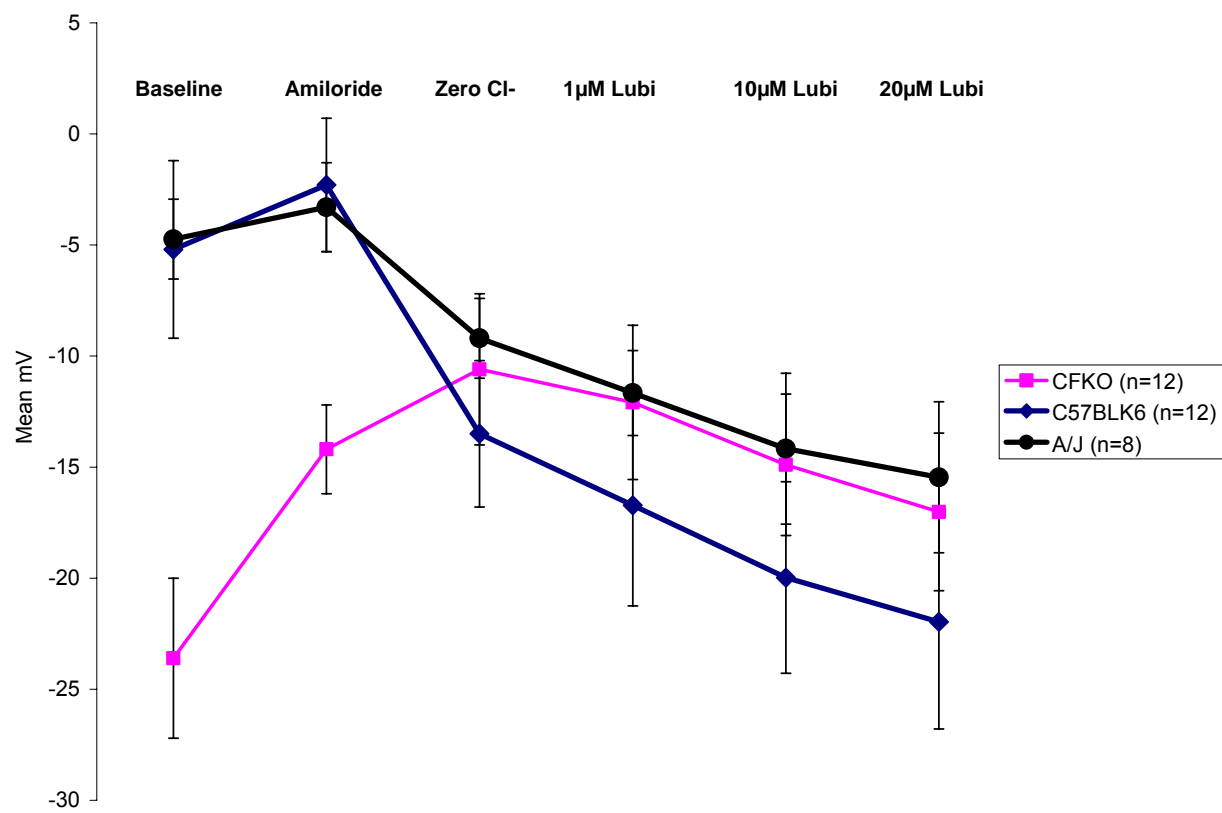


Figure 2

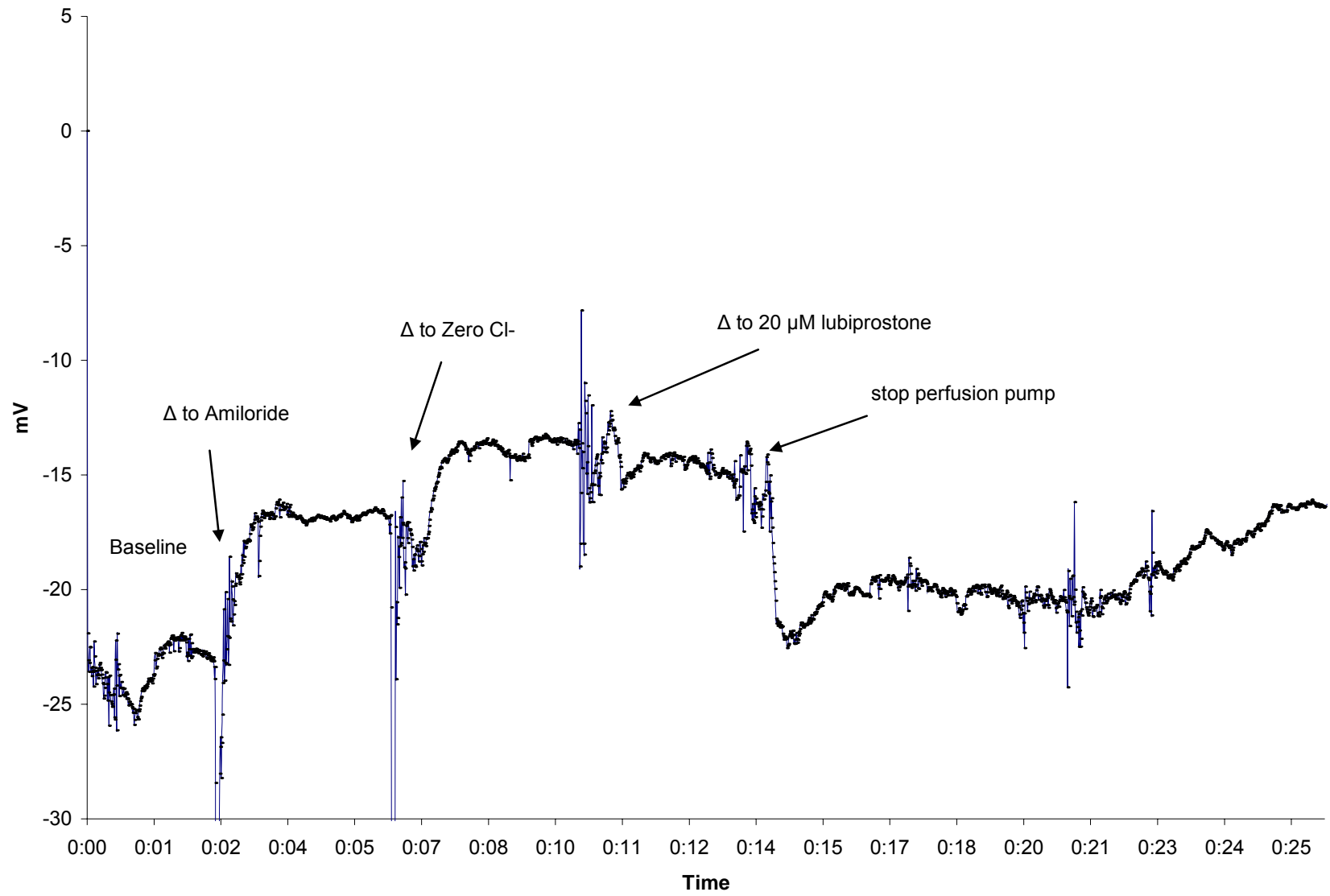


Figure 3

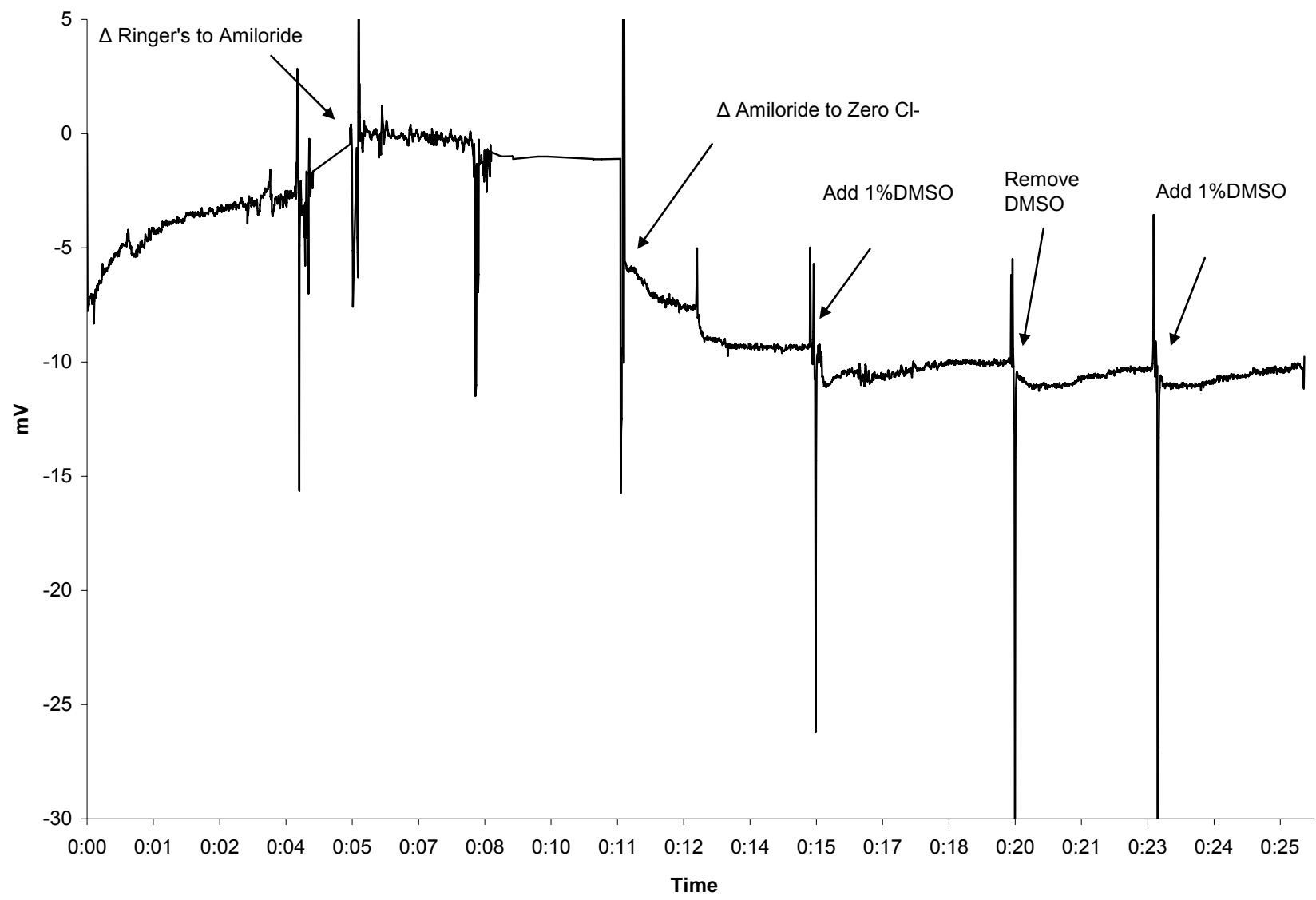


Figure 4

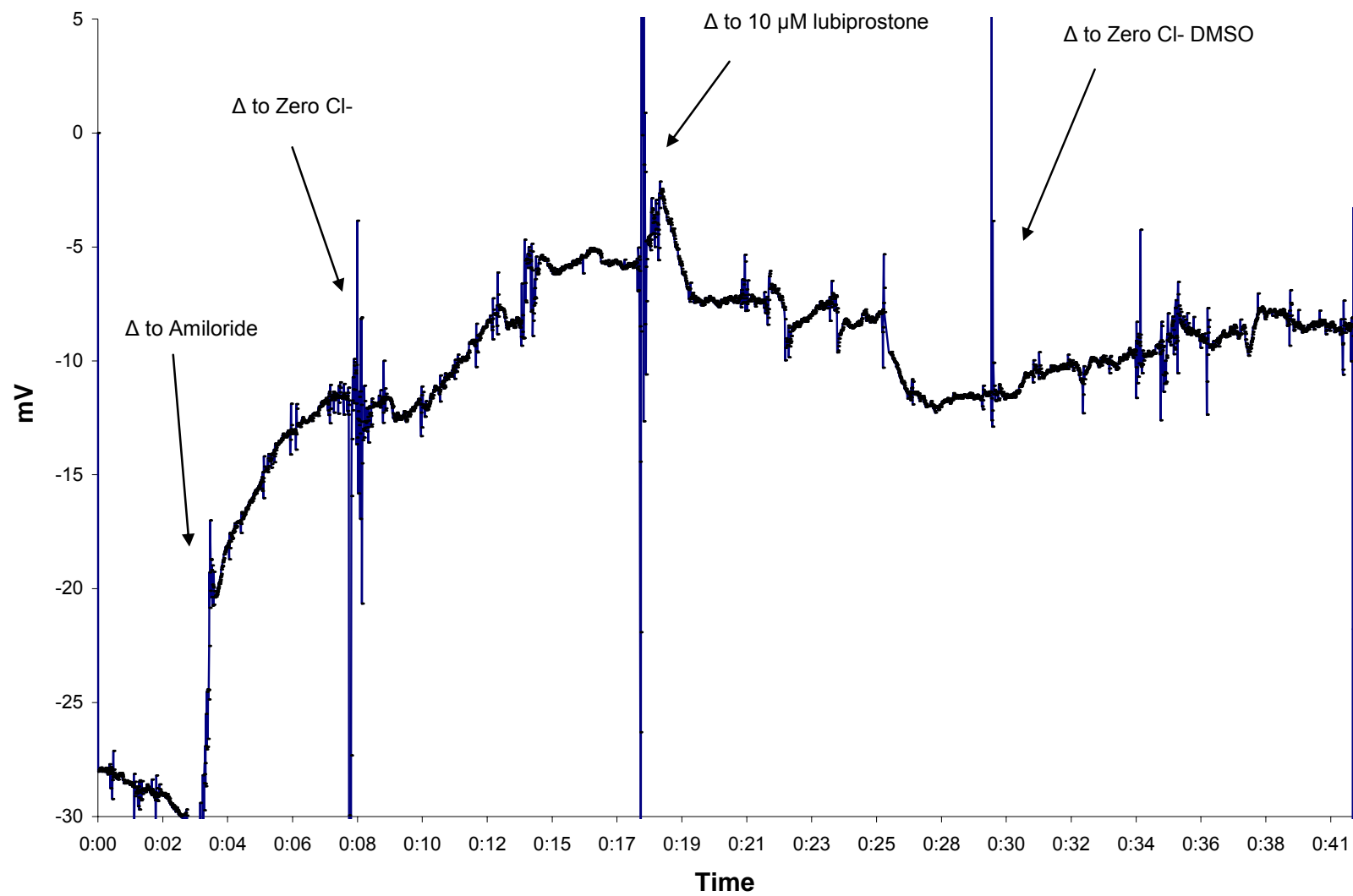


Figure 5

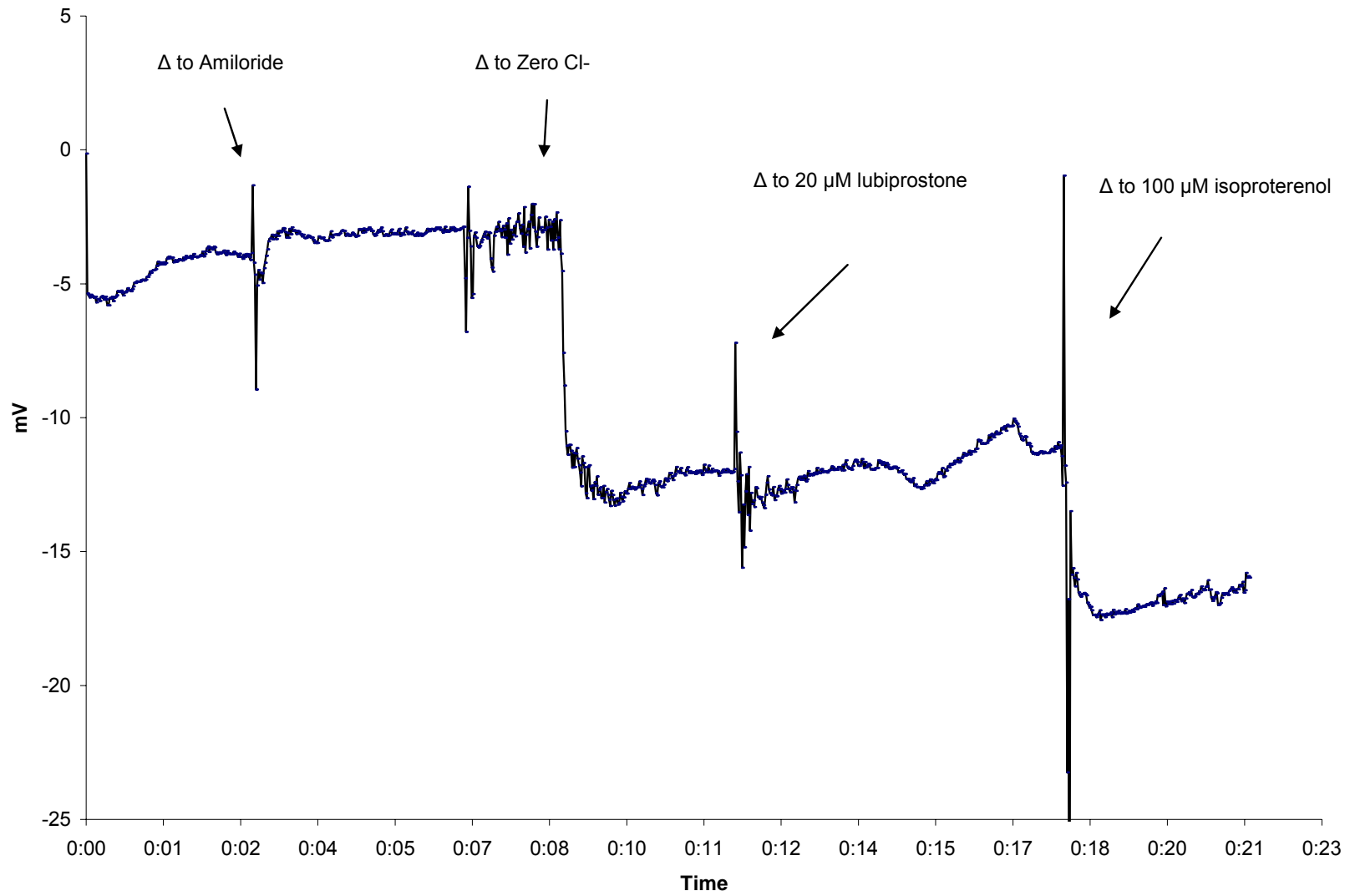


Figure 6

