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Lubiprostone Activates CFTR, but not CIC-2, via the Prostaglandin Receptor (EP₄)

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Abstract

The goal of this study was to determine the mechanism of lubiprostone activation of epithelial chloride transport. Lubiprostone is a bicyclic fatty acid approved for the treatment of constipation [1]. There is uncertainty, however, as to how lubiprostone increases epithelial chloride transport. Direct stimulation of CIC-2 and CFTR chloride channels as well as stimulation of these channels via the EP₄ receptor has been described [2; 3; 4; 5]. To better define this mechanism, two-electrode voltage clamp was used to assay *Xenopus* oocytes expressing CIC-2, with or without co-expression of the EP₄ receptor or β adrenergic receptor (β AR), for changes in conductance elicited by lubiprostone. Oocytes co-expressing CFTR and either β AR or the EP₄ receptor were also studied. In oocytes co-expressing CIC-2 and β AR conductance was stimulated by hyperpolarization and acidic pH (pH=6), but there was no response to the β adrenergic agonist, isoproterenol. Oocytes expressing CIC-2 only or co-expressing CIC-2 and EP₄ did not respond to the presence of 0.1, 1, or 10 μ M lubiprostone in the superfusate. Oocytes co-expressing CFTR and β AR did not respond to hyperpolarization, acidic pH, or 1 μ M lubiprostone. However, conductance was elevated by isoproterenol and inhibited by CFTR_{inh}172. Co-expression of CFTR and EP₄ resulted in lubiprostone-stimulated conductance, which was also sensitive to CFTR_{inh}172. The EC₅₀ for lubiprostone mediated CFTR activation was ~ 10 nM. These results demonstrate no direct action of lubiprostone on either CIC-2 or CFTR channels expressed in oocytes. However, the results confirm that CFTR can be activated by lubiprostone via the EP₄ receptor in oocytes.

Keywords

chloride channel; lubiprostone; CFTR; CIC-2; prostaglandin

INTRODUCTION

Apical anion channels are a key element in salt and water secretion by epithelia [6]. CIC-2 is a voltage-gated, pH sensitive epithelial chloride channel implicated in the regulation of cell volume [7; 8]. CIC-2 is of particular interest as a CFTR bypass channel in Cystic Fibrosis because of reports of localization in the apical membrane of respiratory epithelia [9; 10; 11].

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Lubiprostone, a bicyclic fatty acid derived from prostaglandin E1, has been reported to stimulate apical epithelial ion transport via activation of the CIC-2 chloride channel [2; 3], and the compound has been approved by the FDA for the treatment of constipation in humans [1]. However, some studies have reported that lubiprostone effects epithelial ion transport via activation of the CFTR channels rather than CIC-2 [4; 5]. The first report of CIC-2 activation by lubiprostone employed CIC-2 heterologously expressed in HEK293 cells which otherwise exhibit minimal, native chloride conductance. Using whole cell patch clamp techniques, it was found that HEK293 cells expressing CIC-2 exhibited increased current in the presence of lubiprostone compared to control [2]. The activation of CIC-2 was found not to be pKA dependent. No direct or indirect action of lubiprostone on the conductance of HEK293 cells expressing CFTR was detected [2]. In contrast, a study of amphibian A6 renal epithelial cells via cell-attached patch clamp reported that lubiprostone activated two separate distinct conductances, one with properties consistent with CIC-2 and a second consistent with CFTR [3]. These authors reported no increase in cytosolic cyclic AMP in response to lubiprostone. Although both studies reported lubiprostone activation of the CIC-2 channel, lubiprostone's actions on CFTR were less clear. Neither report suggested an increase in cAMP or pKA in response to lubiprostone.

Lubiprostone stimulation gastric muscle contraction suggested the compound may possess additional activity [12]. The observed contraction was inhibited by pre-treatment with an EP prostaglandin receptor antagonist selective for subtype 4 (EP₄). EP₄ is one of four prostaglandin E₂ (PGE₂) receptors and is a G-protein coupled receptor whose activation is associated with extensive second messaging via cAMP/protein kinase A (pKA) dependent pathways and phosphatidylinositol 3-kinase (PI3K) dependent pathways [13].

Later work found lubiprostone very selectively targets the EP₄ prostanoid receptor [4; 14], resulting in increased cellular cAMP and CFTR dependent activity in gut cells and tissue [4]. To date, no studies have investigated possible lubiprostone-mediated CIC-2 conductance when specifically co-expressed with the EP₄ receptor. Here we report the results of experiments designed to assay for possible activation of CIC-2 and CFTR by lubiprostone using an unambiguous model of heterologous channel and receptor expression in the *Xenopus* oocyte. We used two-electrode voltage clamp electrophysiology to determine if lubiprostone acts alone on CIC-2 or CFTR or if co-expression of the EP₄ receptor is required for channel activation.

METHODS

Ethical Approval

Approval for harvesting of *Xenopus Laevis* oocytes was granted by the Animal Care and Use Committee of the Oregon Health and Sciences University.

Preparation and Microinjection of Oocytes

Female *Xenopus Laevis*, were anesthetized by immersion in cold water containing Tricaine, 3mg/ml (Sigma Chemical Co., St. Louis, MO). The oocytes were removed through a small abdominal incision which was then closed by 4.0 nylon suture. Frogs were then recovered in their tanks. The follicular membranes were removed by mechanical agitation (1–2 h) in a Ca²⁺-free solution containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES (pH 7.5), and 0.2 Wünsch units/mL Liberase Blendzyme TM (Roche Molecular Biochemicals, Indianapolis, IN). We selected Stage V and VI defolliculated oocytes which were then washed and incubated at 18° C in a modified Barth's solution (MBSH) containing 88 mM NaCl, 1 mM KCl, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 2.4 mM NaHCO₃, 10 mM HEPES-Hemi-Na, and 250 mg/L Amikacin with 150 mg/L Gentamicin

(pH 7.5) until injection the next day. Oocytes were injected with 0.1–15 ng of cRNA (50nl volume) of human CFTR or rabbit CIC-2 cRNA (gift of N. McCarty) in conjunction with cRNA encoding the human β_2 -adrenergic receptor (β AR) or the human EP₄ (PTGER4) receptor (Missouri University Science and Technology Resource Center, Rolla MO) using a microinjector (Drummond Scientific Co., Broomhall, PA). Injected oocytes were incubated at 18° C in 12-well plates containing MBSH. Injection pipettes were pulled from filamented glass capillary tubes (Sutter Instrument, Novato CA) on a P-97 Flaming – Brown micropipette puller (Sutter Instrument, Novato CA). Typically, CFTR oocytes were used 3 days after injection, while CIC-2 oocytes were used 5 days after injection.

Whole Cell Recordings

Individual oocytes were placed in a 200 μ L RC-1Z recording chamber (Warner Instruments, Hamden CT) and continuously perfused with Frog Ringer's solution (1.5mL/min) via a syringe pump. The Ringer's solution contained 98 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES (hemi-Na) (pH 7.4). For those experiments in which the bath pH was modified, HEPES was replaced with MES (2-(N-morpholino) ethanesulfonic acid) which buffered the pH to 6.0. Oocytes were perfused continuously with the experimental solutions. Oocytes were initially maintained in the experimental chamber under open circuit conditions and experiments began when the transmembrane voltage was between –25mV and –40 mV. The membrane potential was held at –160 mV to +40 mV with steps at 25mV intervals for a period of 9 s. Three replicates of the voltage step protocol were performed for each of the perfusing solutions per oocyte. β AR receptors were activated by 10 μ M isoproterenol (a β -adrenergic agonist) (Sigma Chemical Co., St. Louis, MO) and a phosphodiesterase inhibitor 1 mM 3-isobutyl-1-methylxanthine [IBMX] (Sigma Chemical Co., St. Louis, MO) [referred to as I+I in the figures]. Experiments utilizing EP₄ receptor expression were initially performed with 1 μ M of lubiprostone (a kind gift of Sucampo Pharmaceuticals, Bethesda, MD) with 0.1% DMSO final concentration in the perfusate. A subset of CIC-2 – EP₄ expressing oocytes were subjected to 0.1 μ M and 10 μ M lubiprostone containing perfusing solutions. In later CFTR - EP₄ oocyte experiments, the membrane potential was ramped from –120 to +60 mV over a period of 1.8 s to construct whole-cell I – V plots while increasing concentrations of lubiprostone in the superfusate from 1 pico molar to 1 μ molar to determine dose response with 5 minutes exposure followed by 2 minutes of washout with Ringer's solution. Conductance was then calculated from the slope of the I–V plot at the reversal potential ($V_m = E_{rev}$) using a voltage range from $V_m = E_{rev} = 10$ mV to $V_m = E_{rev} + 10$ mV. The logarithm of the concentration was then plotted against normalized conductance and the EC₅₀ was determined.

Membrane currents were recorded from oocytes with a two-electrode voltage clamp using an amplifier (TEV-200; Dagan, Minneapolis, MN) at a room temperature of ~ 22°C. Current-injecting and potential-measuring electrodes had resistances of ~0.5–2.0 and ~1.0–3.0 M Ω , respectively, when filled with 3 M KCl. The bath solution was connected to the ground via a low-resistance agarose bridge containing 2% agarose in 3 M KCl. A second reference electrode was used to avoid polarization errors. Current measurements were low-pass filtered at 0.5 kHz. Data acquisition and analysis were done on a Pentium-based microcomputer using pCLAMP software and an analog-to-digital converter (Axon Instruments, Foster City, CA). Comparison of mean current at the –110 mV membrane potential was performed by paired t-test.

RESULTS

CIC-2 and CFTR expressed in *Xenopus* oocytes are activated by hyperpolarization and cyclic AMP respectively

Figure 1A illustrates the response of oocytes co-expressing CIC-2 and β AR (n=6) to step changes in membrane potential, revealing voltage- and time-dependence at hyperpolarized potentials characteristic of CIC-2 channels [15; 16]. CIC-2 activation was enhanced by reducing the pH of the perfusate to 6.0 (Figure 1B) (p=0.0006). Exposure of the oocyte to isoproterenol and IBMX (I+I) in order to raise cytosolic cAMP did not alter CIC-2 activation (Figure 1C, p=0.3).

Oocytes expressing CFTR and β AR (n=4) failed to demonstrate significant voltage dependent activation or response to pH 6 perfusate (p=0.32) at negative membrane potentials (Figure 2A). In contrast, exposure of the oocyte to isoproterenol and IBMX (I+I) produced an increase in conductance and previously characterized voltage-dependent inhibition was seen at values of V_m exceeding 100 mV (Figure 2B)[17]. Un-injected oocytes (n=3) did not exhibit significant responses to the voltage step protocol, acidic pH, I +I, or 1 μ M lubiprostone (data not shown).

Lubiprostone activates CFTR in oocytes co-expressing the EP₄ receptor

The conductance of oocytes co-expressing CFTR and β AR was not altered by exposure to 1 μ M lubiprostone (Figure 3A). However, in oocytes co-expressing CFTR and EP₄ receptor, exposure to lubiprostone elicited rapid increases in conductance (n=4) (p=0.03), similar to those evoked by I+I. This current was promptly inhibited by addition of the CFTR-specific inhibitor, CFTR_{inh}-172 (10 μ M, Figure 3B) to the perfusate, consistent with activation of CFTR-mediated chloride channels. The half maximal concentration (EC₅₀) ~ 10 nM for lubiprostone activation of CFTR channels was estimated by plotting the change in normalized conductance (μ S) versus concentration (Figure 3C). CFTR/EP₄ co-expressing oocytes exhibited responses from 10 picomolar to a maximal effect at 1 μ M.

Lubiprostone does not activate conductance in CIC-2 expressing oocytes

There was no change in conductance in CIC-2 only or CIC-2 and EP₄ co-expressing oocytes exposed to 1 μ M lubiprostone (Figures 4A and 4B) when compared to Ringers perfusate at each voltage step. Because a previous report suggested CIC-2 activation at lubiprostone doses lower than that for seen CFTR [3], we also examined current with 0.1 μ M as well as 10 μ M concentrations of lubiprostone, however, no change in conductance was noted. (data not shown).

DISCUSSION

The goal of this study was to determine if a direct action of lubiprostone on CIC-2 and/or CFTR channels could be detected using a relatively simple, heterologous expression system, the *Xenopus* oocyte. A second goal was to use this expression system to test for a role of the EP₄ receptor in previously reported CIC-2 channel activation [2; 3] by lubiprostone. Although our results clearly demonstrate the voltage and pH dependent activation expected for CIC-2 channels expressed in oocytes [7; 16; 18], no action of lubiprostone, either alone or in the context of the co-expressed receptors was detectable. In contrast, oocytes expressing CFTR and co-expressing either the β AR or EP₄ receptor exhibited conductance increases in response to isoproterenol or lubiprostone, respectively, consistent with cAMP-dependent activation [19].

Previous reports suggest modification of CIC-2 channel conductance by pKA phosphorylation [20; 21], however, others report no change in channel activity despite CIC-2 phosphorylation [22]. While we did not measure cAMP or pKA directly, activation of CFTR by both I-I via β AR and lubiprostone via EP₄, suggest a robust elevation of cAMP and increased pKA activity was achieved in oocytes. There was no change in current in CIC-2 – expressing oocytes in response to either β AR or EP₄ receptor stimulation, consistent with the observation that c-AMP-dependent phosphorylation of CIC-2 does not activate the channel.

Lubiprostone application of 0.1 μ M, 1 μ M, or 10 μ M did not activate any CIC-2 current in oocytes expressing the channel. However, CIC-2-expressing oocytes did respond to hyperpolarization and mildly acidic pH, as expected. Reported lubiprostone activation of HEK293 cells expressing CIC-2 was assayed by whole cell patch clamp [2; 3]. While direct comparison is difficult, our model appears to generate greater current at each hyperpolarizing voltage step and is consistent with other published data on CIC-2 in oocytes [16; 23]. Jentsch [24], in a comprehensive review of CIC chloride channels, indicated that the I-V plots published for lubiprostone activation of CIC-2 were not consistent with previously demonstrated channel properties. To our knowledge, no data examining lubiprostone activation of CIC-2 expressed in oocytes has been published. Joo et al [25] describe personal observations of lubiprostone activating CIC-2 expressing oocytes, however, we did not achieve those results.

One report of presumed lubiprostone activation of CIC-2 utilized short circuit current determinations in T84 cells [2] where CIC-2 was reported to be expressed in the apical membrane. However, the membrane localization of CIC-2 in this cell line is highly controversial [4]. Our experiments remove the uncertainty of CIC-2 localization and expression and demonstrate a lack of CIC-2-mediated conductance by lubiprostone in a simplified physiological system.

We did not find evidence for activation of CFTR by lubiprostone alone. This differs from the findings of Bao et al [3] who reported lubiprostone activated CFTR channels in A6 cells absent an increase in cytosolic cAMP. We did detect vigorous activation of CFTR, however, when EP₄ was co-expressed and lubiprostone was applied. This confirms other reports of CFTR activation by lubiprostone via EP₄ [4; 5; 14].

There are several studies suggesting that non-CFTR-mediated anion conductance is activated by lubiprostone. In airway cells, lubiprostone activated currents despite CFTR knockdown by siRNA and the presence of a CFTR specific inhibitor [26]. In excised sheep tracheas, lubiprostone was reported to increase short circuit current, and the current was sensitive to Cd⁺⁺, a non specific CIC-2 channel inhibitor [25]. MacDonald et al demonstrated small increases in transepithelial voltage in the nares of wild type and transgenic CFTR knockout mice in response to large 40 μ M doses of lubiprostone delivered by continuous perfusion [27]. Oral dosing of lubiprostone in CFTR knockout mice ameliorated the intestinal phenotype by increasing intestinal transit time and mucus secretion [28]. A clinical case series and pilot study [29; 30], suggested lubiprostone was effective in treating constipation of Cystic Fibrosis patients. These reports suggest CFTR channel function is not required for the proposed increased epithelial secretion associated with lubiprostone although the molecular identity of the ion transport was not ascertained.

Lubiprostone activates CFTR expressing oocytes when the EP₄ receptor is coexpressed, but in the absence of the receptor lubiprostone was without effect. Lubiprostone caused no change in conductance in CIC-2 - expressing oocytes either alone or when the EP₄ receptor

was co-expressed. Our data suggest CIC-2 may not be the anion conductance activated by lubiprostone when CFTR is absent or inhibited.

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Highlights

- Lubiprostone chloride transport was examined in *Xenopus* oocytes
- We studied ClC-2 and CFTR chloride channels
- We examined ClC-2 co-expressed with prostaglandin receptor sub-type 4
- We demonstrate lack of ClC-2 activation by lubiprostone in oocytes

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Figure 1.

A. Voltage step protocol demonstrating activation of current at each increasingly negative voltage step in a ClC-2 expressing oocyte. B. I – V plot of ClC-2 - βAR expressing oocyte showing enhanced activation with pH 6.0 superfusate. C. I – V plot of ClC-2 and βAR expressing oocyte demonstrating no response to isoproterenol and IBMX.

A

B

Figure 2.

A. I – V plot of CFTR - β AR expressing oocyte demonstrating no activation by membrane hyperpolarization or pH 6.0 perfusate. B. I – V plot of CFTR - β AR expressing oocyte demonstrating activation by isoproterenol and IBMX. Note the inhibition as the transmembrane voltage becomes more negative.

Figure 3.

A. I – V plot of CFTR - β AR expressing oocyte demonstrating no activation by 1 μ M lubiprostone with subsequent activation by isoproterenol and IBMX. B. I – V plot of CFTR - EP₄ expressing oocyte activated by lubiprostone and then inhibited by CFTR_{inh}-172. C. Plot of conductance versus concentration of lubiprostone used to determine EC₅₀ of lubiprostone activation of CFTR – EP₄ oocytes.

A

B

Figure 4.

A. ClC-2 expressing oocyte with typical voltage activation followed by 1 μ M lubiprostone added to the superfusate showing no change. B. ClC-2 – EP₄ oocyte showing voltage activation remains unchanged after 1 μ M lubiprostone added to the superfusate.