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(54) Title: METHODS FOR USING LUBIPROSTONE TO ABSORB FLUID FROM THE SUBRETINAL SPACE

(57) Abstract: Particular aspects of the invention provide methods for decreasing the amount of fluid present in the subretinal space of the eye by administering a prostone to the basolateral side of the retinal pigment epithelium. Adverse ocular conditions associated with the accumulation of fluid in the subretinal space (like age-related macular degeneration, chronic macular edema, diabetic retinopathy, retinal detachment, glaucoma, uveitis) can be treated by administering an amount of a prostone (preferably lubiprostone, unoprostone, cobiprostone) to the basolateral side of the retinal pigment epithelium effective to remove excess fluid from the subretinal space.



METHODS FOR USING LUBIPROSTONE TO ABSORB FLUID
FROM THE SUBRETINAL SPACE

TECHNICAL FIELD

[0001] The present description relates to methods of removing fluid from the subretinal space of the eye that comprise administering lubiprostone to the basolateral side of the retinal pigment epithelium. Particular aspects of the description relate to using such methods to treat adverse ocular conditions associated with the accumulation of fluid in the subretinal space.

BACKGROUND

[0002] The retinal pigment epithelium (RPE) is a highly pigmented, terminally differentiated monolayer of cells at the back of the eye that forms the outer blood brain barrier between the neural retina and the blood vessels of the choroid. The RPE performs numerous processes that are critical for the maintenance of photoreceptor health and function. To facilitate efficient exchange of materials between the RPE and photoreceptors, the RPE apical membrane is adorned with numerous microvilli that sheathe the photoreceptor outer segments and enhance the effective surface area. The RPE is separated from the photoreceptors by a small ($\approx 10 \mu\text{L}$) fluid-filled subretinal space (SRS) that serves as a conduit for nutrient and metabolic-waste exchange between the two cell types.

[0003] The role of RPE in mediating nutrient transport is particularly important because photoreceptors are highly metabolically active cells that rely exclusively on the choroid for its glucose supply. Photoreceptors are among the most metabolically active cells in the human body - large amounts of ATP are needed to meet the high energy demands of visual phototransduction. Much of the energy consumed by photoreceptors is spent driving the Na/K ATPase and to keep cGMP-gated Na^+ -channels in the open configuration, which are essential components for visual phototransduction. In addition, energy is devoted to replace aged and UV-damaged photoreceptor outer segments that are constantly shed and phagocytosed by the RPE. Photoreceptors generate significant amounts of ATP via glycolysis, by which at least 80% of all glucose consumed by photoreceptors is converted into lactic acid that is subsequently released into the SRS. This translates to the generation of approximately four molecules of lactic acid (from glycolysis) for every three molecules of CO_2 (from oxidative respiration) produced, all of which are released into the SRS. Since human photoreceptor produces CO_2 at a rate of 0.29 to 0.54 mmole.hr⁻¹/ retina, lactic acid is released into the SRS at a rate of 0.39 to 0.72 mmole.hr⁻¹/ retina. This causes with the high lactate concentration (4 - 13 mM) in the SRS compared to that in blood ($\approx 1 \text{ mM}$). Such a high rate of acid release into an extremely small space necessitates an

efficient lactic acid removal system, without which the resultant SRS acidosis could potentially cause irreversible photoreceptor damage and visual impairment. Since the entry of lactic acid into RPE cells causes intracellular acidification that can disrupt RPE function, pH regulatory mechanisms at the apical membrane are activated to help buffer intracellular pH.

[0004] The RPE transports lactate by H⁺-coupled monocarboxylate transporters at the apical (MCT1) and basolateral membranes (MCT3 & MCT4). These facilitate transepithelial lactate transport from the SRS into the choroidal blood supply.. Lactate-transport via MCT1 can be acid-base coupled with pH-regulatory mechanisms to help buffer incoming lactic acid-load, both to maintain pH-homeostasis and to facilitate lactate transport. Nonetheless, the synergistic interaction between CAs and NBCs, called the “bicarbonate transport metabolon, has been utilized to propose a model for acid-base coupled HCO₃⁻-transport as a driving force for transepithelial fluid transport in the human RPE. Na/H exchanger (NHE) and Na-linked HCO₃⁻-transporters, NBC1 and NBC3, are localized at the apical membrane, which places them in close proximity with MCT1, thus enabling efficient H⁺ buffering of entering lactic acid.

[0005] The RPE actively transport ions and fluid from the SRS to the choroid at a rate of $\approx 11 \mu\text{L}/\text{cm}^2/\text{hr}$ in humans; *in vivo*. At this rate, the RPE replaces the entire volume of the SRS once every ten minutes, which helps maintain volume homeostasis of the SRS and promotes retinal adhesion. Transepithelial Cl⁻-transport is the major driving force for fluid transport. Chloride enters the apical membrane of RPE via the Na/K/2Cl co-transporter (NKCC1) and exits at the basolateral membrane via Ca²⁺-activated Cl-channel and cystic fibrosis transmembrane conductance regulator (CFTR).

[0006] In addition to Ca²⁺-activated Cl-channel and CFTR, there is evidence for expression of ClC-2 chloride channel in RPE (Edwards et al., 2010, Invest Ophthal & Vis Sci 51:3264-72; Wills et al., Invest Ophthal Vis Sci 41: 4247-55, 2000). The physiological function of ClC-2 in the RPE is poorly understood. Heterozygous mice expressing an early stop mutant ClC-2 present with a reduced ERG light peak (Edwards et al., Invest Ophthal Vis Sci 51: 3264-72, 2010), a light-induced response originating from a depolarization of the RPE basolateral membrane (Linsenmeier et al., J Physiol 331:653-73, 1982). These observations point to localization of ClC-2 at the RPE basolateral membrane together with other known Cl-channels in the RPE and open the possibility that ClC-2 may play a role in transepithelial fluid transport. Results in mice show that the knocking out ClC-2 expression significantly decreased RPE resting transepithelial potential (TEP), suggesting a defect in active transepithelial ion-transport (Bosl et al., EMBO J 20:1289-99, 2001), consistent with its activity in other epithelia such as the airway (Blaisdell et al., Am J Physiol Lung Cell Mol Physiol 278:L1248-55, 2000), intestine (Gyomai et al.,

et al., Am J Physiol Cell Physiol 279:C1787-94, 2000), and colon epithelium (Catalan et al., Gastroenterol 126: 1104-14, 2004).

[0007] A variety of diseases are directly linked to edema, disregulation of fluid in the eye, including age-related macular degeneration, chronic macular edema, diabetic retinopathy, retinal detachment, glaucoma, or uveitis, peripheral vitreoretinopathy, inherited retinal degeneration such as retinitis pigmentosa, retinal detachment or injury, and retinopathies, whether inherited, induced by surgery, trauma, a toxic compound or agent, or photically. Glaucoma is not a uniform disease but rather is a heterogeneous group of disorders that share a distinct type of optic nerve damage that leads to loss of visual function. The disease is manifest as a progressive optic neuropathy that, if left untreated, leads to blindness. It is estimated that as many as three million Americans have glaucoma and, of these, as many as 120,000 are blind as a result. Furthermore, it is the number one cause of blindness in African-Americans. Its most prevalent form, primary open-angle glaucoma, can be insidious. This form usually begins in midlife and progresses slowly but relentlessly. If detected early, disease progression can frequently be arrested or slowed with medical and surgical treatment.

[0008] There remains an unmet need to develop drugs for treating edema associated with diseases of the eye by targeting a key site of regulation of fluid transport, and to evaluate whether CIC-2 is such a key site.

SUMMARY

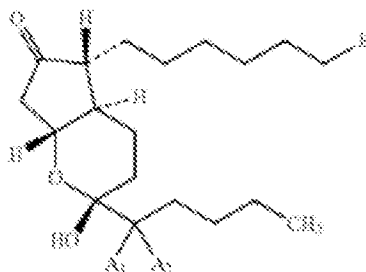
[0009] The accumulation of subretinal fluid occurs in connection with numerous adverse ocular conditions. Applicants have discovered that lubiprostone activates a key enzyme for transporting fluid from the neuroretinal side of the RPE to the choroidal side of the RPE. This leads to absorption of excess fluid from the SRS. Particular aspects of the description thus relate to methods for decreasing the amount of fluid present in the SRS of a patient with ocular edema that comprise administering an amount of lubiprostone to the eye of the patient effective to decrease the amount of fluid present in the subretinal space of the patient.

[0010] Further aspects of the description are directed to methods for treating decreases in visual acuity that are associated with diseases or disorders that cause the accumulation of fluid in the subretinal space. Such methods comprise administering an amount of lubiprostone to the eyes of patients effective to decrease the amount of fluid present in the subretinal space of the patients.

[0011] One aspect of the description is a method for treating a decrease in visual acuity associated with a disease or disorder that causes the accumulation of fluid in the subretinal space

of a patient, comprising administering an amount of a prostone to the eye of the patient effective to decrease the amount of fluid present in the subretinal space of the patient. The disease or disorder may be age-related macular degeneration, chronic macular edema, diabetic retinopathy, retinal detachment, or uveitis. The retinal detachment may be a result of retinal injury or surgery. The disease or disorder may be peripheral vitreoretinopathy, glaucoma, or may be caused by exposure to a toxic compound or a drug.

[0012] In one embodiment of the method, the prostone may consist of a molecule selected from compounds of the structure



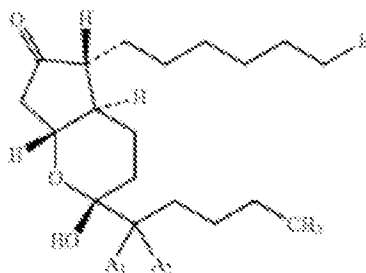
wherein A_1 and A_2 are the same or different halogen atoms and B is $-\text{COOH}$. The prostone preferentially consists of a molecule selected from the group consisting of 11-deoxy-13,14-dihydro-16,16-difluoro-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 isopropyl ester, 2-decarboxy-2-(2-carboxyethyl)-11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 isopropyl ester, 2-decarboxy-2-(2-carboxyethyl)-11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-methyl-PGE1 isopropyl ester, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-methyl-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-ethyl-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 methyl ester, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-ethyl-PGE1 isopropyl ester, and 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGF1 α isopropyl ester. In another embodiment of the method, the prostone may be co-administered with IFN γ . The prostone more preferentially is lubiprostone, unoprostone, or cobiprostone.

[0013] In another embodiment of the method, the prostone may be administered to the basolateral side of the retinal pigment epithelium, preferentially by administration to the anterior surface of the eye. Administration may be by subtenon injection.

[0014] Another aspect of the description is a method for treating age-related macular degeneration, chronic macular edema, diabetic retinopathy, retinal detachment, glaucoma, or uveitis comprising decreasing the amount of fluid present in the subretinal space of a patient suffering from such a disorder by administering an amount of the prostone to the eye of the

patient effective to decrease the amount of fluid present in the subretinal space of the patient. The retinal detachment is a result of retinal injury or surgery, or the patient may suffer from peripheral vitreoretinopathy. The prostone may be administered to the basolateral side of the retinal pigment epithelium, preferentially when administered to the basolateral side of the retinal pigment epithelium by administration to the anterior surface of the eye or by subtenon injection.

[0015] Another aspect of the description is a method for decreasing the amount of fluid present in the subretinal space of a patient, comprising administering an amount of a prostone to the eye of the patient effective to decrease the amount of fluid present in the subretinal space of the patient. The prostone may be administered to the basolateral side of the retinal pigment epithelium preferentially by administration to the anterior surface of the eye, or by subtenon injection. The method may be directed to a patient who suffers from age-related macular degeneration, chronic macular edema, diabetic retinopathy, retinal detachment, or uveitis. The retinal detachment may be a result of retinal injury or surgery. The patient may suffer from peripheral vitreoretinopathy, or from glaucoma. The prostone consists of a molecule selected from compounds of the structure



wherein A₁ and A₂ are the same or different halogen atoms and B is -COOH. The prostone preferentially consists of a molecule selected from the group consisting of 11-deoxy-13,14-dihydro-16,16-difluoro-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 isopropyl ester, 2-decarboxy-2-(2-carboxyethyl)-11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 isopropyl ester, 2-decarboxy-2-(2-carboxyethyl)-11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-methyl-PGE1 isopropyl ester, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-methyl-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-ethyl-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 methyl ester, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-ethyl-PGE1 isopropyl ester, and 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGF1 α isopropyl ester. The prostone more preferentially is lubiprostone, unoprostone, or cobiprostone. The prostone may be

administered to the basolateral side of the retinal pigment epithelium. The prostone may be co-administered with IFN γ .

[0016] Another aspect of the description is a method for treating a decrease in visual acuity associated with a disease or disorder that causes the accumulation of fluid in the subretinal space of a patient, comprising administering an amount of a CLCN2 activator to the eye of the patient effective to decrease the amount of fluid present in the subretinal space of the patient.

[0017] Another aspect of the description is a method for treating age-related macular degeneration, chronic macular edema, diabetic retinopathy, retinal detachment, glaucoma, or uveitis comprising decreasing the amount of fluid present in the subretinal space of a patient suffering from such a disorder by administering an amount of a CLCN2 activator to the eye of the patient effective to decrease the amount of fluid present in the subretinal space of the patient.

[0018] Another aspect of the description is a method for decreasing the amount of fluid present in the subretinal space of a patient, comprising administering an amount of a CLCN2 activator to the eye of the patient effective to decrease the amount of fluid present in the subretinal space of the patient.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] **Fig. 1** shows apical lactate activated NBC1, NBC3, and NHE at the apical membrane. 20 mM lactate was added to the apical bath to obtain initial control response (pH_i, TEP, and R_T). The lactate-induced responses were first obtained in the presence of 10 μ M EZA in the apical bath, then in the presence of both EZA and amiloride in the apical bath. After washout with control Ringer, 20 mM lactate was added to obtain the final control response. Solid bars above the graphs represent solution changes from control Ringer. The lactate-induced pH_i response is separated into two successive phases: a fast acidification phase (R1-black bar) followed by a slow alkalization phase (R2-white bar). The time bar at the bottom-right corner applies to pH_i, TEP, and R_T measurements. In each experiment, pH_i, TEP, and R_T were recorded simultaneously..

[0020] **Fig. 2** shows apical lactate-induced pH_i and TEP changes in the presence vs. absence of CO₂/HCO₃. 20 mM lactate was added to the apical bath (2 min), and the resultant changes in pH_i, TEP, and R_T were recorded. Next, CO₂/HCO₃-rich Ringer was replaced with CO₂/HCO₃-free Ringer and the lactate-induced responses were obtained in the absence of CO₂/HCO₃. Solid bars above the graphs represent solution changes from control Ringer as described in Fig. 1 legend.

[0021] Fig. 3 shows apical lactate activated ClC-2 at the basolateral membrane. 20 mM lactate was added to the apical bath to obtain initial control responses (V_A , V_B , $\Delta R_A/R_B$, TEP, and R_T). The lactate-induced responses were then obtained in the presence of 200 μM Zn^{2+} in the basal bath. After washout with control Ringer, 20 mM lactate was added to obtain the final control response. The apical lactate induced V_A and V_B response is separated into two successive phases: an initial V_A and V_B depolarization (P1) followed by V_A and V_B hyperpolarization (P2). Solid bars above the graphs represent solution changes from control Ringer as described in Fig. 1, legend.

[0022] Fig. 4 shows apical lactate activates apical membrane K-channel. In $\text{CO}_2/\text{HCO}_3^-$ -free condition, 20 mM lactate was added to the apical bath to obtain changes in V_A , V_B , $\Delta R_A/R_B$, TEP, and R_T . The lactate-induced responses were then obtained in the presence of (Fig. 4A) Ba^{2+} (2 mM) in the apical bath or (Fig. 4B) both Ba^{2+} (2 mM; apical & basal baths) & 200 μM Zn^{2+} (basal bath). After washout with control Ringer, 20 mM lactate was added to obtain the final control response. Solid bars above the graphs represent solution changes from control Ringer as described in Fig. 1 legend.

[0023] Fig. 5 shows ClC-2 was activated by extracellular acidification. Low pH_i Ringer (pH 6.8) were perfused to both apical and basal baths (2 min) to obtain control pH_i , TEP, and R_T responses. The lactate-induced responses were then obtained in the presence of 200 μM Zn^{2+} in the basal bath. After washout with control Ringer, low pH_i Ringer was added to obtain the final control responses. Solid bars above the graphs represent solution changes from control Ringer as described in Fig. 1 legend.

[0024] Fig. 6 shows lubiprostone activates ClC-2 at the basolateral membrane. In $\text{CO}_2/\text{HCO}_3^-$ -rich condition, lubiprostone (10 μM) was added to the apical or basal bath to obtain changes in V_A , V_B , $\Delta R_A/R_B$, TEP, and R_T (Fig. 6A). Lubiprostone was added to the apical bath and changes in V_A , V_B , $\Delta R_A/R_B$, TEP, and R_T was obtained in the presence vs. absence of Zn^{2+} (200 μM) in the basal bath (Fig. 6B). Solid bars above the graphs represent solution changes from control Ringer as described in Fig. 1 legend.

[0025] Fig. 7 shows lubiprostone is a highly reversible ClC-2 activator and did not activate CFTR channels in hfRPE. (Fig. 7A) In the presence of $\text{CO}_2/\text{HCO}_3^-$, hfRPE cells were subjected to two consecutive short-term exposures to lubiprostone (10 μM) in the apical bath and the changes in V_A , V_B , $\Delta R_A/R_B$, TEP, and R_T were obtained. (Fig. 7B) Lubiprostone was added to the apical bath and changes in V_A , V_B , $\Delta R_A/R_B$, TEP, and R_T were obtained in the presence vs. absence of CFTR-inh172 (10 μM) in the basal bath. Solid bars above the graphs represent solution changes from control Ringer as described in Fig. 1 legend.

[0026] **Fig. 8** shows apical lactate activates NBC1 in the presence of CO_2/HCO_3 . In CO_2/HCO_3 -rich condition, 20 mM lactate was added to the apical bath to obtain changes in V_A , V_B , $\Delta R_A/R_B$, TEP, and R_T . Solid bars above the graphs represent solution changes from control Ringer as described in Fig. 1 legend.

[0027] **Fig. 9** shows addition of lubiprostone to the basal side of RPE can significantly elevate fluid transport in vitro. The lower panel shows the concomitant electrical measurements (transepithelial potential/TEP in mV and total tissue resistance (R_T) in $\Omega\cdot\text{cm}^2$).

[0028] **Fig. 10** shows that the Ringer-to-Ringer control does not change in fluid transport. Addition of 5 μM methadone to the basal bath decreased J_V by approximately 5 $\mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$. The lower panel shows that methadone decreased TEP.

[0029] **Fig. 11** shows the changes in TEP and R_T produced by the addition of 1 μM lubiprostone to the apical bath in bicarbonate vs. bicarbonate-free (HEPES) Ringers. In HCO_3 , the addition of lubiprostone to the apical bath increased the TEP (1mV) and decreased R_T by 25 $\Omega\cdot\text{cm}^2$ (left-hand panel).

[0030] **Fig. 12** shows that 0.5 μM lubiprostone, added to the apical bath, produced a smaller but reproducible increase in TEP which was partially blocked by the prior addition of Zn^{+2} to the basal bath.

[0031] **Fig. 13** shows the results of qRT-PCR performed to compare *CLCN2* mRNA expression in fully differentiated hRPE cells at 11 days after infection with lentivirus containing *CLCN2* shRNA vs. non-target (control) shRNA (MOI = 4). Sample # 904, 905 and 907 represent individual lentiviral clones that contain siRNA sequence against different region of mRNA for *CLCN2*.

[0032] **Fig. 14** shows that the results of electrical recordings performed on Days 9-11 after transfection with *CLCN2* shRNA (#905) or Non-target control to evaluate lactate-or lubiprostone-induced electrical responses. Continuous traces represent transepithelial potential (TEP), and open squares represent total tissue resistance (R_t). CFTR-inhibitor 172 was added to the basal bath to eliminate any potential effect of CFTR Cl^- channels on the electrical responses. Fig. 14A, Non-target control. Fig. 14B, *CLCN2* shRNA.

[0033] **Fig. 15** shows the results of summary data from seven sets of experiments such as in Fig. 14B.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0034] Retinal pigment epithelial (RPE) cells form a monolayer that regulates the transport of fluids, ions, and metabolites between the sensory retina and the vascular choroidal system. The RPE constitutes the outer part of the blood-retinal barrier, and impairment of this barrier causes subretinal fluid accumulation, which is associated with numerous adverse ocular conditions,.

[0035] Applicants have discovered that administration of lubiprostone to the basolateral side of the RPE unidirectionally increases fluid transport across the RPE, resulting in the absorption of fluid from the SRS.

[0036] Applicants have discovered that the lubiprostone-stimulated increase in fluid transport across is mediated by CIC-2 channels, and is also associated with RPE ionic conductance.

[0037] Applicants have demonstrated that lubiprostone significantly increases fluid absorption (J_V) across the RPE, whether added acutely or chronically, and the effect occurs if lubiprostone is added to the basal bath or to both the apical and basal baths, but not if lubiprostone is added only to the apical bath.

[0038] Applicants have discovered that lactate-induced acidification activated pH-buffering mechanisms at the RPE apical membrane (NBC1, NBC3, and NHE) by a lactate-activated CIC-2 at the RPE basolateral membrane.

[0039] These discoveries point to the application of lubiprostone for treating diseases associated with retinal edema, for example, retinal detachment, chronic macular edema, age-related macular degeneration, diabetic retinopathy, peripheral vitreoretinopathy, glaucoma, and uveitis.

[0040] Described herein are the mechanisms involved in lactate transport and its link to fluid transport through the chloride channel protein 2 (CIC-2).

[0041] Lubiprostone can be administered to the basolateral side of the RPE by application to the anterior surface of the eye for the treatment of adverse ocular conditions in which subretinal fluid accumulation occurs.

[0042] As used herein, the phrase “decrease in visual acuity” refers to any diminishing or lessening of the acuteness or clearness of vision, and can refer to any measurable diminishing or lessening in the acuteness or clearness of form vision, which is dependent on the sharpness of the retinal focus within the eye and the sensitivity of the interpretative faculty of the brain.

[0043] As used herein, the phrase “accumulation of fluid in the subretinal space” refers to an increase in the amount of fluid present in the space that separates the retinal pigment

epithelium (RPE) from the outer segments of the photoreceptors beyond the amount of fluid normally present in that space in healthy eyes. The phrase “decrease the amount of fluid present in the subretinal space,” and all variations thereof, refers to any lessening or diminishing of the amount of fluid present in the SRS, which separates the RPE from the outer segments of the photoreceptors.

[0044] As used herein, the phrase “basolateral side of the retinal pigment epithelium” refers to the side of the retinal pigment epithelium that is adjacent to, borders, or faces, the choroid.

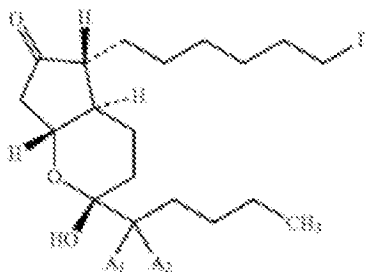
[0045] As used herein, the phrase “anterior surface of the eye” refers to portion of the cornea that comprises the exterior, exposed part of the eye.

[0046] As used herein, the phrase “subretinal injection” refers to the introduction by any means of a substance into the subretinal space.

[0047] As used herein, the phrase “subtenon injection” refers to the introduction by any means of a substance into the area below the Tenon's capsule and above the sclera of the eye at a point posterior to a limbus of the eye.

[0048] As used herein, prostones are a class of compounds which derive from functional 15 keto fatty acids natural to the human body as metabolic products of 15-PGDH.

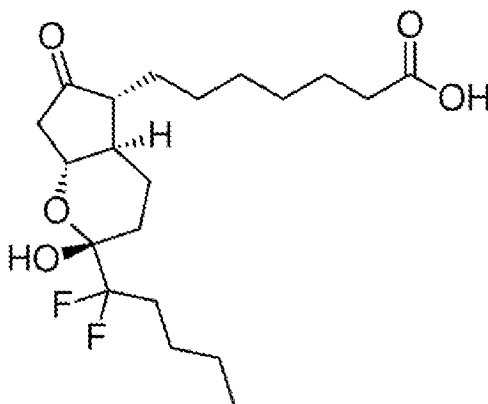
[0049] Prostones include compounds of the structure



where A1 and A2 are the same or different halogen atoms and B is –COOH, e.g., 11-deoxy-13,14-dihydro-16,16-difluoro-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 isopropyl ester, 2-decarboxy-2-(2-carboxyethyl)-11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 isopropyl ester, 2-decarboxy-2-(2-carboxyethyl)-11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-methyl-PGE1 isopropyl ester, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-methyl-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-ethyl-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 methyl ester, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-ethyl-PGE1 isopropyl ester, and 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGF1 α isopropyl ester.

This class of compounds is described in US patent numbers 8,337,891, 8,304,562, 8,236,969, 8,202,909, 8,143,316, 8,114,911, 8,114,890, 8,097,653, 8,097,649, 8,088,934, 8,071,613, 8,026,393, 7,985,770, 7,868,045, 7,812,182, 7,795,312, 7,732,487, 7,459,583, 7,417,067, 7,396,946, 7,253,295, 7,129,272, 7,074,827, 7,064,148, 7,063,857, 7,033,604, 6,982,283, 6,956,056, 6,872,383, 6,864,232, 6,852,687, 6,610,732, 6,596,765, 6,583,174, 6,469,062, 6,458,836, 6,420,422, 6,414,021, 6,414,016, and US patent application publication nos. 20130005995, 20120277299, 20120270945, 20120259008, 20120237598, 20120225938, 20120095090, 20120088824, 20120022152, 20110300211, 20110244037, 20110244036, 20110065784, 20110064748, 20110054016, 20110034424, 20100305203, 20100298424, 20100274032, 20100267832, 20100204491, 20100204489, 20100204332, 20100087540, 20090209643, 20090030072, 20090022787, 20090012165, 20080255227, 20080221050, 20080207759, 20080119462, 20080070979, 20070276006, 20070203228, 20060281818, 20060247317, 20060229346, 20060205725, 20060194880, 20060122411, 20060063830, 20060034892, 20050261375, 20050255500, 20050239813, 20050222265, 20050222195, 20050070468, and 20040235885, hereby incorporated by reference.

[0050] The class of molecules also includes products either being sold or in clinical trials, including lubiprostone, unoprostone, and cobiprostone. Lubiprostone has the following structure

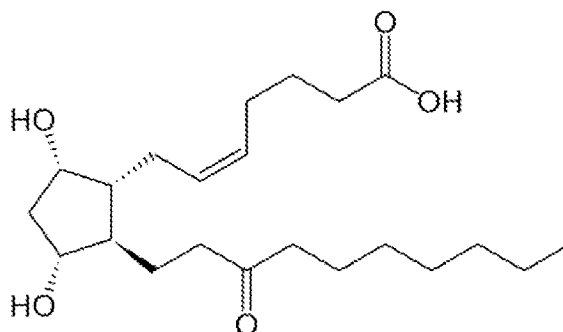


7-[(1R,3R,6R,7R)-3-(1,1-difluoropentyl)-3-hydroxy-8-oxo-2-oxabicyclo[4.3.0]non-7-yl]heptanoic acid.

[0051] Lubiprostone is a bicyclic prostaglandin E1 derivative. Lubiprostone (AMITIZA®, Sucampo Pharmaceuticals, Inc., Bethesda MD; and Takeda Pharmaceuticals America, Lincolnshire, IL). Lubiprostone is FDA-approved for chronic constipation. Lubiprostone works by stimulating CIC-2 chloride channels to drive transepithelial fluid transport across the intestinal epithelia (Lacy et al. Expert Opin Pharmacother 10: 143-52, 2009;

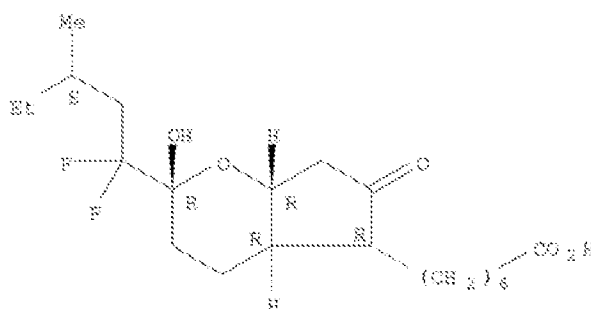
Tuteja et al., Expert Rev Gastroenterol Hepatol 2:727-33, 2008). In addition, lubiprostone induces fluid-secretion in airway epithelium and is currently being developed as a treatment for cystic fibrosis (Joo et al., Am J Physiol Lung Cell Mol Physiol 296:L811-24, 2009; MacDonald et al., Am J Physiol Lung Cell Mol Physiol 295:L933-40, 2008; MacVinish et al. Br J Pharmacol 150:1055-65, 2007; O'Brien et al., Ann Pharmacother 44:577-81, 2010).

[0052] Unoprostone has the following structure



(Z)-7-[(1R,2R,3R,5S)-3,5-dihydroxy-2-(3-oxodecyl)cyclopentyl]hept-5-enoic acid (Rescula[®]).

[0053] Cobiprostone has the following structure



[0054] CLC-2 chloride channel (also known as CLCN2) refers to a protein that is encoded by the *CLCN2* gene. This gene encodes a voltage-gated chloride channel, which is a transmembrane protein that maintains chloride ion homeostasis in various cells. Four transcript variants encoding different CLC-2 isoforms have been found for this gene.

[0055] Certain aspects of the description relate to methods for treating decreases in visual acuity, particularly decreases in visual acuity associated with diseases and disorders that cause the accumulation of fluid in the subretinal space, that involve administering an amount of lubiprostone to the eye of a patient effective to decrease the amount of fluid present in the subretinal space of the patient. Such methods can be used, for example, to treat age-related

macular degeneration, chronic macular edema, diabetic retinopathy, glaucoma, peripheral vitreoretinopathy, uveitis, or retinal detachment caused by, for example, retinal injury or surgery. In this regard, further aspects of the description relate to methods for treating age-related macular degeneration, chronic macular edema, diabetic retinopathy, peripheral vitreoretinopathy, retinal detachment caused by, for example, retinal injury or surgery, glaucoma, or uveitis that comprise decreasing the amount of fluid present in the subretinal space of a patient. In such methods an amount of lubiprostone is administered to the eye of the patient effective to decrease the amount of fluid present in the subretinal space of the patient.

[0056] A wide variety of diseases of the eye may be readily treated or prevented according to particular embodiments of the description, including for example, glaucoma, macular degeneration, diabetic retinopathies, uveitis, inherited retinal degeneration such as retinitis pigmentosa, retinal detachment or injury, and retinopathies, whether inherited, induced by surgery, trauma, a toxic compound or agent, or photically.

[0057] Within other aspects of the description, lubiprostone is administered to a patient's eye to treat or prevent glaucoma. Briefly, glaucoma is not a uniform disease but rather is a heterogeneous group of disorders that share a distinct type of optic nerve damage that leads to loss of visual function. The disease is manifest as a progressive optic neuropathy that, if left untreated, leads to blindness. It is estimated that as many as three million Americans have glaucoma and, of these, as many as 120,000 are blind as a result. Furthermore, it is the number one cause of blindness in African-Americans. Its most prevalent form, primary open-angle glaucoma, can be insidious. This form usually begins in midlife and progresses slowly but relentlessly. If detected early, disease progression can frequently be arrested or slowed with medical and surgical treatment.

[0058] Within yet other embodiments of the description, lubiprostone can be administered to a patient's eye to treat or prevent injuries to the retina, including retinal detachment, photic retinopathies, surgery-induced retinopathies, toxic retinopathies, retinopathies due to trauma or penetrating lesions of the eye.

[0059] The present description also provides methods of treating, preventing, or inhibiting neovascular disease of the eye, comprising the step of administering lubiprostone to a patient's eye. Representative examples of neovascular diseases include diabetic retinopathy, AMD (wet form), and retinopathy of prematurity. Briefly, choroidal neovascularization is a hallmark of exudative or wet Age-related Macular Degeneration (AMD), the leading cause of blindness in the elderly population. Retinal neovascularization occurs in diseases such as diabetic

retinopathy and retinopathy of prematurity (ROP), the most common cause of blindness in the young.

[0060] Further aspects of the description relate to methods for decreasing the amount of fluid present in the subretinal space of a patient. Such methods can be used, for example, to treat patients suffering from diseases and disorders associated with the accumulation of fluid in the subretinal space. Accordingly, in certain aspects of such methods, the patient suffers from age-related macular degeneration, chronic macular edema, diabetic retinopathy, glaucoma, uveitis, peripheral vitreoretinopathy, or retinal detachment caused by, for example, retinal injury or surgery.

[0061] In preferred aspects, such methods involve administering an amount of lubiprostone to the eye of the patient effective to decrease the amount of fluid present in the subretinal space of the patient.

[0062] In preferred embodiments of the methods of the description, lubiprostone is administered to the basolateral side of the retinal pigment epithelium. The lubiprostone can be administered to the basolateral side of the retinal pigment epithelium by administration to the anterior surface of the eye. Alternatively, the lubiprostone can be administered to the basolateral side of the retinal pigment epithelium by subtenon injection or by subretinal injection. In preferred embodiments of the description, lubiprostone is administered to the anterior surface of the eye.

[0063] The present description also provides a composition comprising lubiprostone in a pharmaceutically acceptable carrier, in the form of an aqueous solution, a gel, or a gel-like formulation. The pharmaceutically acceptable carrier is a physiologically compatible vehicle, which may include, for example, one or more water soluble polyethers such as polyethylene glycol, polyvinyls such as polyvinyl alcohol and povidone, cellulose derivatives such as methylcellulose and hydroxypropyl methylcellulose, petroleum derivatives such as mineral oil and white petrolatum, animal fats such as lanolin, polymers of acrylic acid such as carboxypolyethylene gel, vegetable fats such as peanut oil, polysaccharides such as dextrans, glycosaminoglycans such as sodium hyaluronate or hyaluronic acid, salts such as sodium chloride and potassium chloride, lanolin, or glycine. In preferred embodiments of the description, the carrier is a saline solution or is a CELLUVISC[®] solution.

[0064] When the composition is in the form of an aqueous solution, it may comprise physiologically safe excipients formulated to an osmolarity between 250-350 mOsm and pH 5-9; preferably 280-300 mOsm and pH 7.0 -7.6. When the pharmaceutical formulation is in the form

of a gel or gel-like formulation, it is preferably a hyaluronic acid or hyaluronic acid-containing formulation approved for intraocular use.

[0065] The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, and triethanolamine oleate. The composition optionally comprises an intraocular irrigation solution approved for surgical use.

[0066] The concentration of lubiprostone in the compositions can vary from less than about 0.01% to more than about 1 %, and will be determined primarily based upon fluid volumes, viscosities, etc., in accordance with the particular mode of administration used. The concentrations for the lubiprostone preferably ranged from 500 nanomolar (nM) to 10 micromolar (mM). When added in drops onto the anterior surface of the eye, useful concentrations are higher by a factor of 10 since the tear ducts take up so much fluid with each blink. The amount actually delivered around the side of the eye (sub-tenon) to the back of the eye is much less.

[0067] The compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration.

[0068] The compositions can be formulated as polymer matrices, hydrogel matrices, polymer implants, or encapsulated formulations to allow slow or sustained release of the compositions. A particularly preferred formulation is a suspension or solution of the delivery system in a topical ocular formulation, such as eye drops.

[0069] Compositions containing lubiprostone can be administered to the eyes of a patient using any suitable means, but are preferably applied to the anterior surface of the eye using methods familiar to those skilled in the art. For example, in certain embodiments of the description, the compositions are applied to the eye via liposomes. Further, in other embodiments the compositions are infused into the tear film via a pump-catheter system. Another embodiment of the present description relates to the compositions contained within a continuous or selective-release device, for example, membranes such as, but not limited to, those employed in the Ocusert™ System (Alza Corp., Palo Alto, Calif.). As an additional embodiment, the compositions are contained within, carried by, or attached to, contact lenses that are placed on the eye. Another embodiment of the present description involves the compositions contained

within a swab or sponge that is applied to the ocular surface. Further embodiments of the present description involve the compositions contained within a liquid spray that is applied to the ocular surface. Still further embodiments of the present description involve injection of the compositions directly into the lachrymal tissues or onto the eye surface. In particularly preferred embodiments of the description, the compositions are applied to the surface of the eye using conventional eye droppers.

[0070] In some embodiments of the description, the compositions of the description are administered directly into the eye, such as to the subretinal space. In certain of such embodiments, the compositions are administered by subretinal injection using means familiar to those skilled in the art. In other embodiments, the compositions are administered by subtenon injection, as described, for example, in U.S. patent number 6,413,245, incorporated herein by reference in its entirety.

[0071] The compositions of the present description can be administered in a single dose or in multiple doses. For example, the compositions can be administered at the time of eye surgery. Alternatively, the compositions of the present description can be administered over a course of treatment ranging from weeks to years. In certain embodiments of the description, sustained release formulations such as implants are administered for the long-term treatment of diseases and disorders amenable to such modes of administration. In exemplary sustained release formulations, lubiprostone is delivered over a period of 24 to 72 hours. In preferred embodiments of the description, a single dose of the compositions is administered. In alternative embodiments, multiple doses of the compositions are administered, for example, every 12, 24, 36, or 48 hours.

[0072] Within further embodiments of the description, lubiprostone is administered to a patient in combination with other active agents, methods, or therapeutic regimens, including for example, photodynamic therapy (e.g., for wet AMD), laser photocoagulation (e.g., for diabetic retinopathy and wet AMD), and intraocular pressure reducing drugs (e.g., for glaucoma).

EXAMPLES

Example 1: *hfRPE culture model*

[0073] Cultured hfRPE cells were grown on transwell filters using a known method (Maminishkis et al., Invest Ophthal Vis Sci 47: 3612-24, 2006). Briefly, hfRPE cells were trypsinized from a T25 flask (P0) and seeded onto 12-well transwells at $\approx 1.25 \times 10^5$ cells/well. P1 hfRPE cells were cultured for three to four weeks to reach maturity (transepithelial resistance $> 500 \Omega \cdot \text{cm}^2$) prior to experimentation.

Example 2: *Intracellular pH and Ca^{2+} measurements*

[0074] Fluorescence imaging was used to monitor intracellular pH, calcium, and volume as previously described in US 2011/0305668, hereby incorporated by reference in its entirety. Briefly, hfRPE monolayers on transwell filters were incubated (at room temperature and 5% CO_2) in control Ringer solution containing BCECF-AM (8 μM ; 30 minutes) or Fura-2-AM (8 μM ; 1 hour) (Invitrogen, Carlsbad, CA). Following dye-incubation, hfRPE sample was incubated for another 30 minutes in control Ringer before mounting onto a modified Üssing chamber placed on the stage of a Zeiss inverted microscope. For pH_i -imaging, a 20X plan-neofluar objective was used. For Ca^{2+} -imaging (fura-2), a 40X plan-neofluar water-immersion objective was used. Excitation photons (440/480 nm-for BCECF; 350/380 nm for Fura-2) were generated by a Xenon light source and selected with a monochromator, Polychrome IV (Photonics, CA). Emission fluorescence signals were captured with a photomultiplier tube (Thorn, EMI). pH_i -calibration method was previously described (Adijanto et al. 2009). The intrinsic buffering capacity (μ_i ; mM/pH units) of hfRPE was used for calculating proton-flux, $\beta_i = -93.4 \text{pH}_i^3 + 2150.4 \text{pH}_i^2 + 16483.6 \text{pH}_i + 42065.6$ for $\text{pH}_i < 7.35$, and $\beta_i = 9.06$ for $7.35 \leq \text{pH}_i \leq 7.7$. In CO_2/HCO_3 -rich Ringer, the total buffering capacity (μ ; mM/pH units) is $\beta_{\text{total}} = \beta_i + \beta_{\text{HCO}_3} = \beta_i + 2.3[\text{HCO}_3]_i$ where $[\text{HCO}_3]_i$ was estimated from the Henderson-Hasselbalch equation with the assumption that intracellular CO_2 level is 5%. For experiments performed in the absence of CO_2/HCO_3 (HEPES-buffered Ringer), $\beta_{\text{total}} = \beta_i$. H^+ -flux was determined by multiplying the initial rate of pH_i -change (dpH_i/dt) by μ_{total} . Fura-2 signals was calibrated by first perfusing Ca^{2+} -free Ringer solution (containing 2 mM EGTA; apical & basal baths) before adding ionomycin (10 μM ; both baths). When equilibrium was reached, control Ringer solution (1.8 mM Ca^{2+}) containing ionomycin (10 μM) was added to both baths. After $[\text{Ca}^{2+}]_i$ reaches saturation, Ca^{2+} -free Ringer containing 5 mM MnCl_2 was added to quench Fura-2 fluorescence to reveal autofluorescence signals, which were subtracted from the data to obtain the “true” Fura-2 fluorescence signals for analysis.

Example 3: Intracellular microelectrode recording

[0075] Transepithelial potential (TEP) was measured with a pair of calomel electrodes in series with Ringer solution agar (4 %wt/v) bridges placed in the apical and basal baths of the Üssing chamber. The electrophysiology of the RPE has been described in US 2011/0305668. Transepithelial tissue resistance (R_T) was determined by passing a 2 μ A current across the hfRPE monolayer with Ag/AgCl electrodes. Microelectrodes were made from borosilicate glass tubes (0.5 mm I.D. and 1 mm O.D.; with filament) using P-97 Brown-Flaming micropipette puller (Sutter Instrument Co., Novato, CA) and back-filled with 150 mM KCl. The intracellular microelectrode is referenced to the basal bath to measure basolateral membrane potential, V_B . V_A was calculated by using the equation: $V_A = V_B - \text{TEP}$. Microelectrodes with resistances of 120 - 160 M Ω were used. All hfRPE preparations have $R_T \geq 500 \Omega \cdot \text{cm}^2$.

Example 4: Western blot

[0076] For analysis of MCT protein expression, hfRPE monolayers on transwells were lysed as previously described (Philp et al. 2003.). Briefly, 15 μ g total protein lysates were loaded onto a NuPAGE® 4-12% Tris-Acetate Gel (Invitrogen) for electrophoresis. Proteins were subsequently transferred onto PVDF membranes using XCell II™ Blot Module (Invitrogen). Nonspecific binding sites were blocked with TBS (+ 0.1% Tween20) containing 5% w/v powdered milk. Rabbit anti-human MCT1, MCT3, and MCT4 antibodies were generated as previously described (Philp NJ et al., Invest Ophthal Vis Sci 44: 1305-11, 2003B).

Example 5: Immunofluorescence imaging

[0077] Confluent monolayers of pigmented hfRPE on transwells were fixed with 4% paraformaldehyde in PBS for five minutes at room temperature followed by 20 minutes at 4°C. Samples were permeabilized for 10 min with 0.3% TritonX-100, and blocked with PBS + 0.1% Tween20 (PBST) containing BSA (5% w/v). Samples were incubated overnight with antibodies against MCT1, MCT3, and MCT4. Confocal images were taken with Zeiss LSM 510 confocal microscope at 40x (Plan-Neofluar 40x/1.3 Oil DIC) and 0.5 μ m Z-stack intervals.

Example 6: Ringer solutions and chemicals

[0078] CO_2/HCO_3 buffered Ringer used for dye-incubation (pH 7.4 with 5% CO_2) contains (in mM): 133.7 Na^+ , 116.1 Cl^- , 26.2 HCO_3^- , 5 K^+ , 0.5 Mg^{2+} , 1.8 Ca^{2+} , 2 Taurine, 5 Glucose. The CO_2/HCO_3 -free Ringer has the same ionic composition as CO_2/HCO_3 Ringer except: (1) all HCO_3 was replaced with gluconate; (2) 7 mM HEPES acid was added to the CO_2/HCO_3 -free Ringer and titrated with NMDG to reach pH 7.4 (at 37 °C). Cl-free Ringer was prepared by replacing all Cl with gluconate (138.7 mM), Cl-free Ringer solution was reformulated to contain equimolar concentration of CaSO_4 instead of CaCl_2 and mercury sulfate

electrodes (sat. K₂SO₄; Koslow, NJ) were used instead of calomel electrodes. To account for Ca²⁺ and Mg²⁺ chelation by the high concentration of gluconate in the solution, Ca²⁺ and Mg²⁺ concentrations were raised to 5.5 mM and 0.8 mM, respectively. All Ringer solutions also contain 0.5 mM probenecid to reduce the rate of dye-leakage. Sucrose was used to normalize the osmolarity of all Ringer solutions to 305 ± 5 mOsm. DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid) and ionomycin were purchased from Calbiochem (CA, La Jolla). pCMBS (p-chloromercuribenzenesulfonic acid) was purchased from Toronto Research Chemicals (Ontario, Canada). Lubiprostone (AMITIZA ®) was a generous gift from Sucampo Pharmaceuticals, Inc. All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Example 7: Lactate transport

[0079] In RPE, lactate is transported across its apical and basolateral membranes via H⁺-coupled monocarboxylate transporters (MCTs). Current measurements in hRPE cells confirmed that lactate is transported across these membranes, in which MCT1 was polarized to the apical membrane, and that MCT3 and MCT4 were localized at the basolateral membrane. Lactate transport was induced by increasing lactate level in the apical bath (from 0 to 20 mM) and recorded changes in intracellular pH (pH_i), transepithelial potential (TEP), and total tissue resistance (R_T). (See Fig. 1.) The lactate-induced acidification (referred to as the R1 phase), which reflected MCT1 activity, was followed by a progressive alkalinization (R2 phase), which was a collective activity of pH-buffering mechanisms. The rates of acidification or alkalinization (ΔpH_i/Δt) were normalized to the total buffering capacity to obtain “H⁺-fluxes”, which took into account variations in buffering capacity at different baseline pH_i. Using this analysis, the results showed lactate-induced intracellular acidification was inhibited by niflumic acid and blocked by pCMBS (p-chloromercuribenzenesulfonic acid), known inhibitors of MCT1 (Halestrap et al., Biochem J 343: 281-99, 1999). These results are consistent with studies in bovine and human RPE in which the apical lactate-induced intracellular acidification was blocked by other MCT1 inhibitors such as α-CHC (alpha-cyano-4-hydroxycinnamate) and pCMPS (p-chloromercuriphenylsulfonic acid) (Kenyon et al. 1994; la Cour et al. 1994).

Example 8: Lactate activates pH regulatory mechanisms at the apical membrane

[0080] Several Na-linked pH regulatory mechanisms are known to be present at the apical membrane, including Na/H exchanger (NHE) and Na/HCO₃ co-transporters (NBC1 and NBC3). Since NHE, NBC1, and NBC3 are all Na-linked transporters, the alkalinization in R2 reflected the activities of these transporters by removing all Na from both apical and basal baths, which eliminated the R2 alkalinization. NHE activity was blocked using amiloride (1 mM), which significantly decreased R2 alkalinization. The results are shown in Table 1.

Table 1: *Apical lactate-induced pH_i, TEP, and R_T responses (CO₂/HCO₃ rich)*

inhibitor/ condition	Apical lactate-induced pH _i , TEP, and R _T responses ¹						n
		Control	+ Inhibitor/ condition	Recovery	p ²	%Δ ³	
Amiloride (1 mM; Ap)	R1	-13.60 ± 4.81	-14.22 ± 5.17	-10.42 ± 2.91	NS	5	5
	R2	0.77 ± 0.13	0.38 ± 0.22	0.70 ± 0.23	S	-51	
	ΔTEP	0.93 ± 0.15	0.47 ± 0.20	0.87 ± 0.23	S	-49	
	ΔR_T	38 ± 14	40 ± 15	34 ± 19	NS	5	
Na-free (Ap & Ba)	R1	-15.05 ± 3.09	-12.80 ± 6.30	-11.98 ± 5.71	NS	-15	5
	R2	0.91 ± 0.37	-0.13 ± 0.26	0.62 ± 0.42	S	-114	
	ΔTEP	1.08 ± 0.22	0.07 ± 0.24	1.09 ± 0.26	S	-94	
	ΔR_T	37 ± 9	10 ± 11	29 ± 19	S	-73	
DIDS (0.5 mM; Ap)	R1	-10.83 ± 2.55	-9.45 ± 2.73	-8.43 ± 2.90	NS	-13	10
	R2	0.60 ± 0.35	0.77 ± 0.42	0.45 ± 0.36	NS	28	
	ΔTEP	0.82 ± 0.44	0.36 ± 0.33	0.39 ± 0.15	S	-56	
	ΔR_T	26 ± 14	43 ± 14	18 ± 8	S	65	
EZA (10 μM; Ap)	R1	-15.21 ± 4.16	-17.09 ± 4.83	N/A	NS	12	15
	R2	1.12 ± 0.38	0.75 ± 0.33	N/A	S	-33	
	ΔTEP	1.22 ± 0.44	2.59 ± 0.84	N/A	S	112	
	ΔR_T	33 ± 16	58 ± 17	N/A	S	76	
EZA (10 μM; Ap) + Amiloride (1 mM; Ap)	R1	-12.88 ± 1.21	-11.03 ± 3.42	-9.20 ± 2.05	NS	-14	4
	R2	1.03 ± 0.35	0.08 ± 0.31	0.85 ± 0.23	S	-92	
	ΔTEP	1.60 ± 0.55	2.27 ± 0.78	2.14 ± 0.70	S	42	
	ΔR_T	30 ± 19	35 ± 17	26 ± 13	NS	17	
CO₂/HCO₃-free (Ap & Ba)	R1	-8.56 ± 1.06	-17.45 ± 2.48	-8.33 ± 1.23	S	104	4
	R2	0.73 ± 0.31	0.62 ± 0.29	0.74 ± 0.31	NS	-15	
	ΔTEP	0.68 ± 0.19	1.79 ± 0.31	0.72 ± 0.09	S	163	
	ΔR_T	26 ± 9	53 ± 13	22 ± 6	S	104	

¹ R1 and R2 (H⁺-flux) have units of mM·min⁻¹. TEP and R_T responses have units of mV and Ω·cm² respectively. Values are reported as mean ± SD.

² p-values refer to the t-test for statistical significance between the apical lactate-induced control response and the same response in the presence of inhibitor/condition. "S" indicates statistical significance (p < 0.05) and "NS" indicates that the change is not statistically significant (p > 0.05).

³ Δ% refers to the percent change of the lactate-induced responses in the presence of the inhibitor/condition compared to control.

[0081] The results showed that R2 was not completely eliminated, which is evidence that NBC1 and NBC3 are also involved in pH buffering. NBC1 is an electrogenic transporter (1Na:2HCO₃) and is sensitive to DIDS, whereas NBC3 is electroneutral (1:1) and insensitive to DIDS. Therefore DIDS was used as a tool to evaluate the contribution of NBC1 to the pH-buffering in R2. DIDS surprisingly had no effect on R2 alkalization (Table 1), suggesting that NBC1 contributed little to intracellular pH buffering. In this experiment however, we found that lactate also increased TEP (0.8 ± 0.4 mV) and this response was significantly decreased by

apical DIDS (Table 1). The orientation of this TEP response was consistent with apical membrane hyperpolarization due to NBC1 activity (HCO_3^- transport into the cell). These results show that lactate likely activated NBC1 and that the lack of effect of apical DIDS on R2 alkalization may be a result of compensatory action by NHE or NBC3, or both.

[0082] HCO_3^- -transporters such as NBC1 and NBC3 rely on carbonic anhydrases (CAs), which catalyzes the conversion of CO_2 to HCO_3^- (and vice versa), to achieve optimal HCO_3^- transport. NBC1 and NBC3 mediated HCO_3^- -transport in hRPE were previously found to be significantly inhibited by a CA inhibitor, dorzolamide. The current results showed that dorzolamide (250 μM) or ethoxzolamide (EZA; 10 μM) significantly increased lactate-induced acidification (in R1 phase) compared to control (Fig. 1A). Further, blocking carbonic anhydrase activity decreased intracellular buffering capacity by $\approx 40\%$ (54), the calculated H^+ -flux of R1 in the presence of EZA was not statistically different from control. EZA decreased the H^+ -flux of R2 alkalization by 33%. Since EZA alone did not completely block R2 alkalization, addition of both amiloride and EZA to simultaneously block NHE, NBC1, and NBC3 resulted in elimination of R2 (Fig. 1A), which showed NHE was involved. Further, in the absence of $\text{CO}_2/\text{HCO}_3^-$ (no NBC activity), addition of amiloride (1 mM) alone was sufficient to eliminate R2 (Fig. 1B). Collectively, the results showed that lactate transport via MCT1 recruits Na-linked pH regulatory mechanisms at the RPE apical membrane (NHE, NBC1, and NBC3) to help buffer intracellular pH.

Example 9: Lactate activates *ClC-2* at the basolateral membrane

[0083] Lactate increased TEP, and this electrical response was directly linked to the activation of NBC1 (Fig. 1A). Since NBC1 can be inhibited by carbonic anhydrase inhibitors (Adjianto et al. 2009.), addition of EZA should decrease the lactate-induced TEP response. Surprisingly, the lactate-induced TEP response to the contrary increased by more than two-fold in the presence of EZA (Fig. 1A; Table 1). When all HCO_3^- -transport mechanisms were blocked by removing all $\text{CO}_2/\text{HCO}_3^-$ from the Ringer solution (HEPES buffered; pH 7.4), the lactate-induced TEP response was almost three-fold larger in $\text{CO}_2/\text{HCO}_3^-$ -free vs. $\text{CO}_2/\text{HCO}_3^-$ -rich Ringer (Fig. 2; Table 1). This results showed that NBC1 could not have contributed to the TEP response in conditions where $\text{CO}_2/\text{HCO}_3^-$ -buffering was compromised. To determine what caused this TEP response and the localization of the originating mechanism (apical or basolateral), intracellular microelectrodes were used to monitor changes in apical and basolateral membrane voltages (V_A and V_B). The results are shown in Table 3.

Table 3: Apical lactate-induced V_A , V_B , TEP, and R_T responses (CO_2/HCO_3 free)

inhibitor/ condition	Apical lactate-induced V_A , V_B , TEP, R_T , & R_A/R_B responses ¹						
			Control	+ Inhibitor/ condition	p ²	% Δ ₃	n
Zinc (200 μM; Ba)	P1	ΔV_A	4.87 ± 1.40	3.90 ± 0.62	NS	-20	5
		ΔV_B	5.54 ± 1.54	4.11 ± 0.77	S	-26	
	P2	ΔV_A	-3.13 ± 1.27	-0.92 ± 0.55	S	-71	
		ΔV_B	-2.89 ± 1.34	-0.79 ± 0.50	S	-73	
		ΔTEP	0.94 ± 0.30	0.30 ± 0.18	S	-68	
		ΔR_T	74 ± 28	80 ± 30	NS	8	
		$\Delta R_A/R_B$	0.06 ± 0.03	0.02 ± 0.02	S	-67	
Barium (2 mM; Ap)	P1	ΔV_A	4.81 ± 0.97	5.87 ± 1.04	S	22	6
		ΔV_B	5.74 ± 1.20	6.12 ± 1.07	NS	7	
	P2	ΔV_A	-3.08 ± 1.31	-1.68 ± 0.87	S	-45	
		ΔV_B	-3.09 ± 1.24	-1.46 ± 0.74	S	-53	
		ΔTEP	0.98 ± 0.44	0.48 ± 0.09	S	-51	
		ΔR_T	60 ± 30	67 ± 31	NS	12	
		$\Delta R_A/R_B$	0.09 ± 0.05	-0.10 ± 0.06	S ⁴	-211	
Barium (2 mM; Ap & Ba) + Zinc (200 μM; Ba)	P1	ΔV_A	4.76 ± 0.63	3.41 ± 0.46	S	-28	5
		ΔV_B	5.63 ± 0.70	3.52 ± 0.55	S	-37	
	P2	ΔV_A	-2.95 ± 1.42	-0.81 ± 0.63	S	-73	
		ΔV_B	-3.00 ± 1.42	-0.92 ± 0.70	S	-69	
		ΔTEP	1.01 ± 0.34	0.10 ± 0.06	S	-90	
		ΔR_T	49 ± 19	68 ± 18	S	39	
		$\Delta R_A/R_B$	0.09 ± 0.02	-0.02 ± 0.05	S ⁵	-122	

¹ P1 & P2 indicates separate phases in the lactate induced changes in V_A , V_B , and TEP responses, which are presented in mV. R_T response has units of $\Omega \cdot \text{cm}^2$. All values are reported as mean $\pm$ SD.

² p-values refer to the t-test for statistical significance between the apical lactate-induced response in control condition and the same response in the presence of inhibitor/condition. "S" indicates statistical significance ($p < 0.05$), "NS" indicates statistical insignificance ($p > 0.05$).

³ $\Delta\%$ refers to the percent change of the lactate-induced responses in the presence of the inhibitor/condition compared to control.

⁴ Apical lactate had no effect on R_A/R_B in two of the six tissues tested, statistical analysis was performed with data from the remaining four tissues.

⁵ Apical lactate had no effect on R_A/R_B in one of the five tissues tested, statistical analysis was performed with data from the remaining four tissues.

[0084] The lactate-induced changes in membrane potentials can be separated into two distinct consecutive phases (P1 and P2). P1 phase is represented by a rapid V_A and V_B depolarization with a larger change in V_B (ΔV_B) relative to ΔV_A , thus indicating that the depolarization originated from the basolateral membrane and that ΔV_A is a result of electrical shunting. P2 phase begins at the onset of V_A and V_B hyperpolarization with $\Delta V_A > \Delta V_B$, indicating that the hyperpolarization originated from the apical membrane, the mechanism of

which will be presented in the following sections. Since the increase in TEP during P1 phase originated from V_B depolarization, lactate may have activated a Cl-channel at the basolateral membrane.

[0085] Ca^{2+} -activated Cl-channels and CFTR are known to be located at the basolateral membrane (Hughes et al., 1998, in *The retinal pigment epithelium*, ed. Marmor et al. NY: Oxford U Press, p. 103-134). Lactate may stimulate Cl-efflux via one or more of these Cl-channels. Consistent with this notion, removal of all chloride ions from the Ringer solution (HEPES-buffered and CO_2/HCO_3 -free) from both apical and basal baths significantly attenuated the lactate-induced TEP response (Table 4).

Table 4: *Apical lactate-induced pH_i , TEP, and R_T responses (HEPES buffered - CO_2/HCO_3 free)*

inhibitor/ condition	Apical lactate-induced pH_i , TEP, and R_T responses ¹						n
		Control	+ Inhibitor/ condition	Recovery	p^2	% Δ^3	
pCMBS (0.5 mM; Ap)	R1	-8.19 ± 0.94	-1.03 ± 0.09	-1.51 ± 0.20	S	-87	4
	R2	1.09 ± 0.06	0.13 ± 0.08	-0.06 ± 0.08	S	-88	
	ΔTEP	1.99 ± 0.31	0.04 ± 0.08	0.24 ± 0.29	S	-98	
	ΔR_T	27 ± 13	0	0	S	-100	
Niflumic acid (100 μ M; Ap)	R1	-5.93 ± 1.29	-3.22 ± 0.79	-6.24 ± 0.89	S	-46	7
	R2	0.78 ± 0.25	0.49 ± 0.23	0.77 ± 0.26	S	-37	
	ΔTEP	1.58 ± 0.73	1.08 ± 0.52	1.47 ± 0.79	S	-32	
	ΔR_T	48 ± 12	38 ± 13	36 ± 11	NS	-21	
Cl-free (Ap & Ba)	R1	-6.04 ± 1.31	-5.34 ± 0.67	-6.17 ± 0.98	NS	-12	5
	R2	0.63 ± 0.34	0.59 ± 0.09	0.61 ± 0.04	NS	-6	
	ΔTEP	1.72 ± 0.73	0.61 ± 0.36	1.47 ± 1.24	S	-65	
	ΔR_T	38 ± 19	90 ± 48	32 ± 12	S	137	
DIDS (0.5 mM; Ba)	R1	-5.02 ± 1.81	-6.12 ± 1.90	-7.42 ± 3.16	NS	22	5
	R2	0.74 ± 0.10	0.87 ± 0.24	1.00 ± 0.44	NS	18	
	ΔTEP	1.28 ± 0.73	0.46 ± 0.32	0.38 ± 0.39	S	-64	
	ΔR_T	40 ± 8	35 ± 7	26 ± 5	NS	-13	
BAPTA-AM (20 μ M; Ap)	R1	N/A	N/A	N/A	-	-	5
	R2	N/A	N/A	N/A	-	-	
	ΔTEP	1.65 ± 0.50	1.56 ± 0.32	1.43 ± 0.51	NS	-5	
	ΔR_T	70 ± 17	72 ± 19	79 ± 13	NS	3	
CFTR-inh172 (10 μ M; Ba)	R1	-6.98 ± 1.41	-6.78 ± 1.59	-8.33 ± 3.13	NS	-3	3
	R2	0.95 ± 0.14	0.95 ± 0.22	1.10 ± 0.34	NS	0	
	ΔTEP	2.10 ± 0.78	2.06 ± 0.67	2.10 ± 0.65	NS	-2	
	ΔR_T	54 ± 20	53 ± 17	53 ± 12	NS	-2	
Forskolin (40 μ M; Ba)	R1	-7.18 ± 0.69	-7.98 ± 1.37	-8.26 ± 1.49	NS	11	3
	R2	0.28 ± 0.06	0.27 ± 0.14	0.42 ± 0.25	NS	-4	
	ΔTEP	1.16 ± 0.27	1.20 ± 0.31	1.02 ± 0.35	NS	3	
	ΔR_T	64 ± 36	58 ± 38	66 ± 39	NS	-9	

¹ R1 and R2 (H^+ -flux) have units of $mM \cdot min^{-1}$. TEP and R_T responses have units of mV and $\Omega \cdot cm^2$ respectively. Values are reported as mean $\pm$ SD.

² p-values refer to the t-test for statistical significance between the apical lactate-induced control response and the same response in the presence of inhibitor/condition. "S" indicates statistical significance ($p < 0.05$) and "NS" indicates that the change is not statistically significant ($p > 0.05$).

³ $\Delta\%$ refers to the percent change of the lactate-induced responses in the presence of the inhibitor/condition compared to control.

[0086] The TEP response was also inhibited by basal DIDS (Table 2), a known blocker of Ca^{2+} -activated Cl-channels. However, chelating intracellular $[Ca^{2+}]$ using BAPTA-AM did not affect the TEP response, suggesting that the Cl-conductance was not Ca^{2+} dependent. To evaluate the role of CFTR, the effects of a specific CFTR inhibitor (CFTR-inh172; 10 μM) or a CFTR activator (forskolin; 40 μM) were tested. The results showed that neither had any effect on the TEP response (Table 4). These results provided the surprising conclusion that neither CFTR nor Ca^{2+} -activated Cl-channels were responsible for the lactate-induced TEP response.

[0087] ClC-2 expression and activity in RPE are known (Hartzell et al., J Gen Physiol 549:453-69, 2003; Weng et al., Am J Physiol Cell Physiol 283:C839-49, 2002). To test whether lactate activates ClC-2, a ClC-2 inhibitor, zinc (Zn^{2+} ; 200 μM) was added to either the basal or apical side of the RPE cells. Surprisingly, it was found that zinc significantly inhibited the lactate-induced TEP response (CO_2/HCO_3 -free Ringer; Fig. 3) when added to the basolateral membrane, but had no effect when added to the apical bath. This result pointed directly to ClC-2 as the mediator of the lactate-induced TEP response.

[0088] In addition to the TEP response, microelectrode recordings showed that basal Zn^{2+} also significantly inhibited the V_B depolarization in P1 phase, consistent with lactate-induced activation of ClC-2 activity at the basolateral membrane. The P1 phase was followed by the P2 phase, during which V_A and V_B gradually hyperpolarize as TEP continue to increase, consistent with K-efflux at the apical membrane. Kir7.1 K-channels are highly expressed at the RPE apical membrane and are responsible for maintaining the apical membrane resting potential (Hughes et al., Am J Physiol Cell Physiol 294:C423-31, 2008; Shimura et al., J Physiol 531:329-46, 2001). To test whether lactate-induced activation of apical membrane K-channels contributed to the observed V_A depolarization in P2 phase, apical membrane K-conductance was blocked with barium (Ba^{2+} ; 2 mM), which significantly decreased lactate-induced TEP response and the V_A hyperpolarization in P2 (Fig. 4A; Table 3). Furthermore, addition of both Ba^{2+} and Zn^{2+} completely blocked the lactate-induced TEP response and the associated changes in V_A and V_B (Fig. 4B; Table 3). These results showed that lactate activates apical membrane K-channels, likely as a secondary effect by the V_A depolarization during P1 phase.

[0089] CIC-2 is activated by acidic pH (Jordt et al., EMBO J 16:1582-92, 1997). The effect decreasing the pH of the Ringer solution (from 7.4 to 6.8) in both apical and basal baths was tested. The results showed that the pH shift induced similar responses to that caused by apical lactate. Consistent with activation of CIC-2, the switch from control Ringer (pH 7.4) to low pH (6.8) Ringer induced an increase in TEP (by 0.83 ± 0.45 mV) that was completely blocked by basal Zn^{2+} (Fig. 5).

[0090] The effect of a specific CIC-2 activator, lubiprostone (10 μM), on hfrPE was tested. As shown in Fig. 6A and Table 5, adding lubiprostone to either the apical or basal bath increased TEP and depolarized V_B .

Table 5: Apical lubiprostone-induced V_A , V_B , TEP, and R_T responses

inhibitor/ condition	Lubiprostone-induced V_A , V_B , TEP, R_T , & R_A/R_B responses ¹					
		Control	+ inhibitor/ condition	p^2	$\% \Delta^3$	n
Zinc (200 μM; Ba)	ΔV_A	5.10 ± 0.36	1.23 ± 1.10	S	-76	3
	ΔV_B	5.63 ± 0.35	1.40 ± 1.22	S	-75	
	ΔTEP	0.72 ± 0.26	0.17 ± 0.21	S	-77	
	ΔR_T	69 ± 31	10 ± 17	S	-85	
	$\Delta R_A/R_B$	0.27 ± 0.13	0.02 ± 0.03	S	-94	
CFTR- inh172 (10 μM; Ba)	ΔV_A	5.77 ± 2.40	5.83 ± 1.77	NS	-1	3
	ΔV_B	6.07 ± 2.32	6.27 ± 1.86	NS	-3	
	ΔTEP	0.51 ± 0.33	0.507 ± 0.43	NS	1	
	ΔR_T	53 ± 15	50 ± 20	NS	5	
	$\Delta R_A/R_B$	0.30 ± 0.17	0.29 ± 0.18	NS	6	

¹ V_A , V_B , and TEP responses are presented in mV. R_T response has units of $\Omega \cdot \text{cm}^2$. All values are reported as mean $\pm$ SD.

² p-values refer to the t-test for statistical significance between the apical lactate-induced control response and the same response in the presence of inhibitor/condition. "S" indicates statistical significance ($p < 0.05$) and "NS" indicates that the change is not statistically significant ($p > 0.05$).

³ $\Delta\%$ refers to the percent change of the lactate-induced responses in the presence of the inhibitor/condition compared to control.

[0091] These surprising and unpredictable results show that lubiprostone induced activation of CIC-2 at the basolateral membrane. These changes in TEP and V_B were highly reversible (Fig. 7A; Table 4) and they were more robust and larger when lubiprostone was added to the apical membrane compared to the basolateral membrane, indicating that lubiprostone is highly membrane permeable and has better access to CIC-2 when added to the apical bath. The results likely reflected the significantly higher surface area of the apical membrane and the diffusion barrier imposed by the porous transwell filter attached to the hfrPE basal surface.

[0092] To confirm that these responses were specific to ClC-2, Zn^{2+} was added to the basal bath and showed that it completely blocked lubiprostone-induced TEP response and V_B depolarization (Fig. 6B; Table 4). Although earlier studies showed that lubiprostone can activate CFTR at higher concentrations, the results showed that the lubiprostone-induced responses were unaffected by CFTR-inh172 (10 μ M) (Fig. 7B; Table 4). This shows that lubiprostone did not activate CFTR in hfRPE. Collectively, the results show that ClC-2 is expressed at the RPE basolateral membrane, that ClC-2 can be activated by lactate, and that lubiprostone activates ClC-2 as well.

[0093] In addition to pH buffering mechanisms, the results above show that lactate activated ClC-2 chloride channels at the RPE basolateral membrane. This effect is limited to a condition when CO_2/HCO_3 buffering was compromised, either by removal of CO_2/HCO_3 from both apical and basal baths or by addition of carbonic anhydrase inhibitors (dorzolamide or ethoxzolamide). This effect is shown by comparing lactate-induced changes in V_A , V_B , and TEP in the presence and the absence of CO_2/HCO_3 . In the presence of CO_2/HCO_3 , lactate hyperpolarized V_A and increased TEP by activation of NBC1 at the apical membrane (Fig. 8). In the absence of CO_2/HCO_3 (HEPES-buffered), lactate depolarized V_B and increased TEP by activation of ClC-2 at the basolateral membrane (Fig. 2). Lactate-induced activation of ClC-2 occurs only when CO_2/HCO_3 buffering in the cell is compromised. In the absence of CO_2/HCO_3 , decreasing extracellular pH (from 7.4 to 6.8) activated ClC-2 and increased TEP. This response can be blocked by zinc (basal bath). In CO_2/HCO_3 -rich condition, decreasing extracellular pH by increasing CO_2 levels (from 5% to 13%) at either the apical or basal baths had little or no effect on TEP. These results show that pH buffering by CO_2/HCO_3 attenuated lactate-induced acidification and prevented ClC-2 activation, therefore compromising CO_2/HCO_3 buffering allows for lactate-mediated activation of ClC-2. Recombinant ClC-2 expressed in oocytes showed that ClC-2 is activated by acidic pH (Zifarelli et al., *Rev Physiol Biochem Pharmacol* 158:23-76, 2007).

[0094] The results above show the effects of activation of ClC-2 using a specific activator, lubiprostone. The results of the measurements show that activating ClC-2 using lubiprostone induced V_B depolarization and increased TEP, effects that were completely blocked by zinc in the basal bath. Collectively, these results show that lubiprostone is highly membrane permeable and its effects were specific to ClC-2 at the RPE basolateral membrane. The results herein show a novel means for regulating ClC-2 in the RPE. The high specificity of lubiprostone makes it a good candidate for its development as a safe treatment for retinal edema.

Example 10

[0095] Addition of lubiprostone to the basal side of RPE significantly elevates fluid transport in vitro (Fig. 9). This increase can be blocked by addition of methadone, a specific CIC-2 channel blocker. The lubiprostone-induced increase was $10 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$. Persistent responses were obtained at concentrations as low as $1 \mu\text{l}$ lubiprostone. If this increase in fluid transport occurred across the back of a human eye with a retina detached over most of the posterior pole (8 cm^2) it would remove approximately 2 ml of fluid per day -- a clinically significant rate of fluid removal.

[0096] Fig. 9, lower panel, shows the concomitant electrical measurements (transepithelial potential/TEP in mV and total tissue resistance (R_T) in $\Omega\cdot\text{cm}^2$). This experiment shows that lubiprostone added from either the apical or basolateral side of the epithelium increased TEP. This increase is consistent with a lubiprostone-induced increase in basolateral membrane CIC-2 conductance and subsequent membrane depolarization. For technical reasons, the R_T responses are unreliable as evidenced by the apparent changes in R_T in the control to control transition (initial response - left hand side). Methadone (right-hand side), a CIC-2 channel inhibitor caused a TEP decrease consistent with reports for its effect on CIC-2 channel blockade (Cuppolletto et al., Cell Biochem Biophys, 2012).

[0097] The data summarized in Fig. 10 shows that the Ringer-to-Ringer control showed no change in fluid transport. Addition of $5 \mu\text{M}$ methadone to the basal bath decreased JV by approximately $5 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$, consistent with the observation in other systems that methadone is CIC-2 channel blocker and CIC-2 is constitutively active in human RPE. This effect is apparently not reversible. The lower panel shows that methadone decreased TEP, consistent with the closure of anion channels on the basolateral membrane (presumably CIC-2).

[0098] Electrophysiological responses from these tissues are shown in Figs. 11 and 12. Fig. 11 shows the changes in TEP and RT produced by the addition of $1 \mu\text{M}$ lubiprostone to the apical bath in bicarbonate vs. bicarbonate-free (HEPES) Ringers. In HCO_3 , the addition of lubiprostone to the apical bath increased the TEP (1 mV) and decreased RT by $25 \Omega\cdot\text{cm}^2$ (left-hand panel). These changes are consistent with the activation of basolateral CIC-2 channels. In HEPES buffer (pH, 6.8) lubiprostone produced larger (approximately a factor of 2) electrical responses in TEP, consistent with the contribution of extracellular acidification to CIC-2 activation. The data summarized in Fig. 12 (left-hand panel) shows that $0.5 \mu\text{M}$ lubiprostone, added to the apical bath, produced a smaller but reproducible increase in TEP which was partially blocked by the prior addition of Zn^{+2} to the basal bath, consistent with linear,

concentration-dependent lubiprostone-induced changes in TEP. The subsequent responses shown in the right-hand panel show that Zn^{+2} , also a CIC-2 inhibitor, but less specific than methadone, itself significantly decreased TEP ($\approx 3\text{mV}$) and increased RT ($\approx 70\ \Omega\cdot\text{cm}^2$), consistent with partial blockade of the CIC-2 channel. Similar but slightly smaller results were obtained at $0.2\ \mu\text{M}$ but no TEP/RT responses were obtained at $0.1\ \mu\text{M}$ lubiprostone.

Example 11

[0099] A rodent model of retinal reattachment as previously described in US 2011/0305668 can be used to determine if CIC-2 activators can rapidly (within hours) reduce significant amounts of abnormal accumulation of fluid in the SRS. The compounds are either injected into the subtenon or added topically to the anterior surface of the cornea. Both of these delivery routes have been previously employed in clinical trials based on similar pre-clinical animal experiments (Maminishkis et al., IOVS, 2002; Li et al., Am J. Physiology (cell), 2009).

[0100] Physiologically effective concentrations of lubiprostone and similar compounds are determined along with specific blockers and working concentrations. Fluid transport, microelectrode, and fluorescence imaging rigs determine the specificity and localization of CIC-2 activation by measuring JV, TEP, R_T , intracellular Ca^{+2} and pH changes, membrane potential, and resistance.

[0101] The retinal re-attachment model will be used to determine lubiprostone and similar compound efficacies. These experiments will be done by delivering lubiprostone topically (see Li et al., 2009) or by intratenon injections (Maminishkis et al., 2002), both common clinical delivery routes.

[0102] These experiments will also require the measurement of OCT to quantify the rate of fluid removal over time. We will also need to monitor visual function before and after the addition of drug using electrophysiological responses generated following the absorption of light quanta in the retinal photoreceptors and the downstream retinal signaling. These measurements are made using the so-called electroretinogram or ERG. In separate recording sessions, the light-induced electrical responses that specifically occur in the retinal pigment epithelium are recorded. These responses are part of the electrooculogram or DC-ERG.

Example 12: *IFN γ Increases Fluid Transport Across the RPE*

[0103] Fluid transport assays were performed as described above to examine whether IFN γ induced changes in fluid transport across hRPE monolayers. IFN γ did increase fluid transport: IFN γ ($5\ \text{ng/ml}$ in the basal bath) increased J_V by $\sim 8.6\ \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$, reflecting an

increase in steady-state fluid absorption from the retinal to the choroidal side of the tissue. The mean J_V increased from 12.9 ± 1.6 to $20.5 \pm 3.1 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ (mean $\pm$ s.e.m., $P < 0.01$).

[0104] In contrast, addition of IFN γ to the apical bath had no significant effect on J_V . A solution containing anti-IFN γ R1 blocking antibody (2 $\mu\text{g}/\text{ml}$) was used to perfuse an intact monolayer of hFRPE for 30 minutes prior to the addition of IFN γ (10 ng/ml) to the basal bath. In the presence of blocking antibody, no significant J_V changes were observed. JAK inhibitor I (5 μM) was added about 0.5-1 hours prior to the addition of IFN γ . JAK inhibitor significantly blocked the IFN γ increase in J_V across hFRPE.

[0105] Fluid transport in hFRPE was also measured following chronic exposure to IFN γ . Pairs of hFRPE inserts with matching R_T levels were incubated with IFN γ (5 ng/ml) in both the apical and basal baths, or were left in control media, for 24 hours. Fluid transport and electrical parameters were then measured in each pair. The J_V in the control monolayers was $7.6 \pm 1.5 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ and IFN γ treatment increased J_V to $15.2 \pm 2.0 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ (mean $\pm$ SEM, $P < 0.01$). There was a significant IFN γ -induced decrease in R_T from 592 ± 80 to $356 \pm 139 \Omega\cdot\text{cm}^2$ ($n = 5$; $p < 0.05$); at $t = 0$, the pairs had no significant difference in R_T (205 ± 17 compared with $210 \pm 20 \Omega\cdot\text{cm}^2$, $n = 5$, $P = 0.86$).

[0106] Basal bath addition of a specific CFTR inhibitor (CFTR_{inh}-172; 5 μM) decreased the IFN γ -induced J_V increase by $\approx 9.8 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$. In 11 experiments, this inhibitor reversibly decreased mean J_V from 16.6 ± 1.2 to $6.8 \pm 1.0 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ ($P < 0.001$). A similar inhibitory effect of CFTR_{inh}-172 was also observed in RPE cells incubated with IFN γ , in both the apical and basal media, for 24 hours. After addition of CFTR_{inh}-172 (5 μM), J_V decreased from $15.1 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ to $4.8 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$.

[0107] The baseline of J_V ($12.5 \pm 3.1 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$) was also significantly inhibited by basal bath addition of CFTR_{inh}-172 ($3.2 \pm 1.0 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$). DIDS (500 μM) had no effect on spontaneous steady-state J_V ($n = 2$), and the addition of DIDS following CFTR_{inh}-172 inhibition of IFN γ -induced J_V produced no further inhibition of J_V . Furthermore, the IFN γ -induced J_V increase was not affected by the addition of basal DIDS, but the subsequent addition of CFTR_{inh}-172 decreased J_V by $12.6 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$. These results show that the basolateral membrane DIDS sensitive mechanism does not affect IFN γ -induced changes in J_V .

[0108] Whether the IFN γ -induced changes in J_V required *de novo* protein synthesis was tested using cyclohexamide (CHX), a protein synthesis inhibitor. A confluent hFRPE monolayer was perfused with cyclohexamide (62 μM) in both the apical and basal baths for 30 minutes

before the addition of IFN γ (5 ng/ml). CHX *per se* produced no change in J_V , and had no effect on the IFN γ -induced increase in J_V . Also, CHX did not affect the ability of CFTR_{inh}-172 to block the IFN γ -induced J_V increase.

[0109] Since CHX blocked IRF-1 synthesis after 4 hours of incubation, the effects of chronic exposure to CHX on the IFN γ -induced J_V increase were examined. After 4 hours of pre-treatment with CHX, there was no IFN γ -induced J_V increase or further effect of the CFTR inhibitor on J_V . This result was also observed in two additional experiments (data not shown). Next, paired RPE tissues were treated with IFN γ (5 ng/ml; apical/basal) or IFN γ (5 ng/ml, apical/basal) and CHX (62 μ M; apical/basal) for 24 hours. Compared to control, cyclohexamide treatment decreased J_V from 15.9 to 8.8 μ l·cm⁻²·hr⁻¹ across RPE. A similar result was obtained in another experiment which decreased J_V from 14.9 to 9.8 μ l·cm⁻²·hr⁻¹.

[0110] These effects of CHX show that the IFN γ -induced J_V increase has two components, one that involves protein synthesis and one that does not. The results above demonstrate that the latter response is acute and not blocked by CHX. Thus, it is most likely mediated by downstream signals from JAK/STAT or p38 MAPK, or other second messengers that directly activate CFTR. On the other hand, the results above with chronic treatment with CHX indicate that the IFN γ -induced increase in J_V is driven, at least in part, by activation of nuclear transcription factors and protein synthesis.

[0111] These results show that IFN γ -increases fluid transport via a different pathway than lubiprostone. Accordingly, it is likely that a combination treatment that includes both drugs will have an additive effect over the result of treating with either drug alone.

Example 13: *shRNA blocks the effect of lubiprostone*

To further verify that lubiprostone activates the ClC-2 chloride channels, human fetal RPE (hfRPE) cells were transfected with lentivirus carrying shRNA against *CLCN2* or non-target shRNA control. qRT-PCR was used to determine which lentiviral clone produced the desired knockdown effect in *CLCN2* mRNA expression.

Five different lentiviral clones, each expressing shRNA against *CLCN2* mRNA at a distinct coding region, were transfected to mature cultured hfRPE cells (MOI=4). The same amount of Non-target shRNA lentiviral particles were transfected as controls. Cells were collected at Day 11 post-transfection and analyzed by qRT-PCR for mRNA expression. Data were normalized against GAPDH mRNA levels and all experiments were performed in triplicates. The results (Fig. 13) shows shRNA specific to *CLCN2* knocks down expression of the *CLCN2* mRNA. RPE cells transfected with *CLCN2* shRNA had 70-90% lower *CLCN2*

expression as compared to non-target controls at 11 days post-transfection. Clones #905 and #907 in particular, produced the most efficient knockdown by up to 85% and 90% respectively.

The decrease in mRNA expression is also reflected at the protein level, as shown in western blot analysis. CIC2 protein expression in hRPE cells at Day 7 and 11 post-transfection were compared. The results showed that by 11 days after transfection, CICN2 protein expression is substantially knocked down in CICN2 shRNA transfected cells.

Lactate and lubiprostone-induced electrical responses in *CLCN2*-knocked down were compared to control hRPE cells. Electrical recordings were performed on Days 9-11 after transfection with CIC2 shRNA (Fig. 14B) or Non-target control (Fig. 14A) to evaluate lactate-or lubiprostone-induced electrical responses. It was shown that both lactate and lubiprostone-induced TEP responses were significantly reduced in CLCN2 knockdown RPE cells as compared to the non-target controls (1.2 ± 0.2 mV vs. 0.66 ± 0.08 mV, $p < 0.05$ for lactate and 1.33 ± 0.18 mV vs. 0.51 ± 0.08 mV, $p < 0.01$ for lubiprostone, $n=7$). CFTR-inhibitor 172 was added to the basal bath to eliminate any potential effect of CFTR Cl⁻ channels on the electrical responses. Fig. 15 summarizes and averages results from seven experiments.

These results provide strong functional evidence that lubiprostone activates basolateral membrane CIC2 channels in human RPE.

The present results show that CLCN2 activators can be used to activate a special class of Cl channels, independent of Ca or cAMP, to increase fluid absorption across human RPE. This class of activators may be used therapeutically to abrogate the effects of retinal edema, for example caused by diabetic retinopathy, uveitis, or retinal degenerative diseases. These activators can also help mitigate adverse events caused by other therapeutics (MEKi) that lead to the pathological accumulation of retinal fluid. In addition, CIC2 mutations that affect channel activity are possible causes of human disease, such as epilepsy

[0112] Although, lubiprostone is used as an agent of choice to describe various exemplary embodiments of the present description, one or more of other prostones alone or in combination can be used to achieve one or more of the benefits of the description and as such they are within the scope of the description.

[0113] The entire disclosure of each patent, patent application, and publication cited or described in this document is hereby incorporated herein by reference.

[0114] The various features and processes described above may be used independently of one another, or may be combined in various ways. All possible combinations and subcombinations are intended to fall within the scope of this disclosure. In addition, certain method or process blocks may be omitted in some implementations. The methods and processes

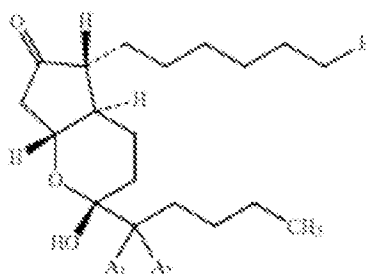
described herein are also not limited to any particular sequence, and the blocks or states relating thereto can be performed in other sequences that are appropriate. For example, described blocks or states may be performed in an order other than that specifically disclosed, or multiple blocks or states may be combined in a single block or state. The example blocks or states may be performed in serial, in parallel, or in some other manner. Blocks or states may be added to or removed from the disclosed example embodiments. The example systems and components described herein may be configured differently than described. For example, elements may be added to, removed from, or rearranged compared to the disclosed example embodiments.

[0115] Conditional language used herein, such as, among others, “can,” “could,” “might,” “may,” “e.g.,” and the like, unless specifically stated otherwise, or otherwise understood within the context as used, is generally intended to convey that certain embodiments include, while other embodiments do not include, certain features, elements, and/or steps. Thus, such conditional language is not generally intended to imply that features, elements and/or steps are in any way required for one or more embodiments or that one or more embodiments necessarily include logic for deciding, with or without author input or prompting, whether these features, elements and/or steps are included or are to be performed in any particular embodiment. The terms “comprising,” “including,” “having,” and the like are synonymous and are used inclusively, in an open-ended fashion, and do not exclude additional elements, features, acts, operations, and so forth. Also, the term “or” is used in its inclusive sense (and not in its exclusive sense) so that when used, for example, to connect a list of elements, the term “or” means one, some, or all of the elements in the list.

[0116] While certain example embodiments have been described, these embodiments have been presented by way of example only, and are not intended to limit the scope of the inventions disclosed herein. Thus, nothing in the foregoing description is intended to imply that any particular feature, characteristic, step, module, or block is necessary or indispensable. Indeed, the novel methods and systems described herein may be embodied in a variety of other forms; furthermore, various omissions, substitutions and changes in the form of the methods and systems described herein may be made without departing from the spirit of the inventions disclosed herein. The accompanying claims and their equivalents are intended to cover such forms or modifications as would fall within the scope and spirit of certain of the inventions disclosed herein.

What is Claimed:

1. A method for treating a decrease in visual acuity associated with a disease or disorder that causes the accumulation of fluid in the subretinal space of a patient, comprising administering an amount of a prostone to the eye of the patient effective to decrease the amount of fluid present in the subretinal space of the patient.
2. The method of claim 1, wherein the disease or disorder is age-related macular degeneration, chronic macular edema, diabetic retinopathy, retinal detachment, or uveitis.
3. The method of claim 2, wherein the retinal detachment is a result of retinal injury or surgery.
4. The method of claim 3, wherein the disease or disorder is peripheral vitreoretinopathy.
5. The method of claim 1, wherein the disease or disorder is glaucoma.
6. The method of claim 1, wherein the disease or disorder is caused by exposure to a toxic compound or a drug.
7. The method of claim 1, wherein the prostone consists of a molecule selected from compounds of the structure



wherein A_1 and A_2 are the same or different halogen atoms and B is $-\text{COOH}$.

8. The method of claim 1, wherein the prostone consists of a molecule selected from the group consisting of 11-deoxy-13,14-dihydro-16,16-difluoro-PGE₁, 11-deoxy-13,14-dihydro-

15-keto-16,16-difluoro-PGE1 isopropyl ester, 2-decarboxy-2-(2-carboxyethyl)-11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 isopropyl ester, 2-decarboxy-2-(2-carboxyethyl)-11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-methyl-PGE1 isopropyl ester, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-methyl-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-ethyl-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 methyl ester, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-ethyl-PGE1 isopropyl ester, and 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGF1 α isopropyl ester.

9. The method of claim 1, wherein the prostone is co-administered with IFN γ .

10. The method of claim 1, wherein the prostone consists of a molecule selected from the group consisting of lubiprostone, unoprostone, and cobiprostone.

11. The method of claim 1, wherein the prostone is administered to the basolateral side of the retinal pigment epithelium.

12. The method of claim 11, wherein the prostone is administered to the basolateral side of the retinal pigment epithelium by administration to the anterior surface of the eye.

13. The method of claim 11, wherein the prostone is administered to the basolateral side of the retinal pigment epithelium by subtenon injection.

14. A method for treating age-related macular degeneration, chronic macular edema, diabetic retinopathy, retinal detachment, glaucoma, or uveitis comprising decreasing the amount of fluid present in the subretinal space of a patient suffering from such a disorder by administering an amount of a prostone to the eye of the patient effective to decrease the amount of fluid present in the subretinal space of the patient.

15. The method of claim 14, wherein the retinal detachment is a result of retinal injury or surgery.

16. The method of claim 14, wherein the patient suffers from peripheral vitreoretinopathy.

17. The method of claim 14, wherein the prostone is administered to the basolateral side of the retinal pigment epithelium.

18. The method of claim 17, wherein the prostone is administered to the basolateral side of the retinal pigment epithelium by administration to the anterior surface of the eye.

19. The method of claim 17, wherein the prostone is administered to the basolateral side of the retinal pigment epithelium by subtenon injection.

20. A method for decreasing the amount of fluid present in the subretinal space of a patient, comprising administering an amount of a prostone to the eye of the patient effective to decrease the amount of fluid present in the subretinal space of the patient.

21. The method of claim 21, wherein the prostone is administered to the basolateral side of the retinal pigment epithelium.

22. The method of claim 21, wherein the prostone is administered to the basolateral side of the retinal pigment epithelium by administration to the anterior surface of the eye.

23. The method of claim 21, wherein the prostone is administered to the basolateral side of the retinal pigment epithelium by subtenon injection.

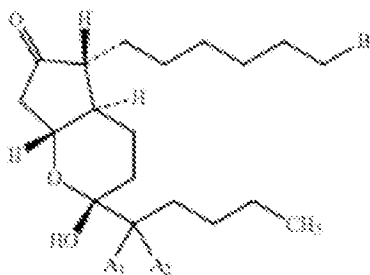
24. The method of claim 20, wherein the patient suffers from age-related macular degeneration, chronic macular edema, diabetic retinopathy, retinal detachment, or uveitis.

25. The method of claim 20, wherein the retinal detachment is a result of retinal injury or surgery.

26. The method of claim 20, wherein the patient suffers from peripheral vitreoretinopathy.

27. The method of claim 20, wherein the patient suffers from glaucoma.

28. The method of claim 20, wherein the prostone consists of a molecule selected from compounds of the structure



wherein A₁ and A₂ are the same or different halogen atoms and B is –COOH.

29. The method of claim 20, wherein the prostone consists of a molecule selected from the group consisting of 11-deoxy-13,14-dihydro-16,16-difluoro-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 isopropyl ester, 2-decarboxy-2-(2-carboxyethyl)-11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 isopropyl ester, 2-decarboxy-2-(2-carboxyethyl)-11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-methyl-PGE1 isopropyl ester, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-methyl-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-ethyl-PGE1, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGE1 methyl ester, 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-20-ethyl-PGE1 isopropyl ester, and 11-deoxy-13,14-dihydro-15-keto-16,16-difluoro-PGF1 α isopropyl ester.

30. The method of claim 20, wherein the prostone consists of a molecule selected from the group consisting of lubiprostone, unoprostone, and cobiprostone.

31. The method of claim 20, wherein the prostone is administered to the basolateral side of the retinal pigment epithelium.

32. The method of claim 20, wherein the prostone is co-administered with IFN γ .

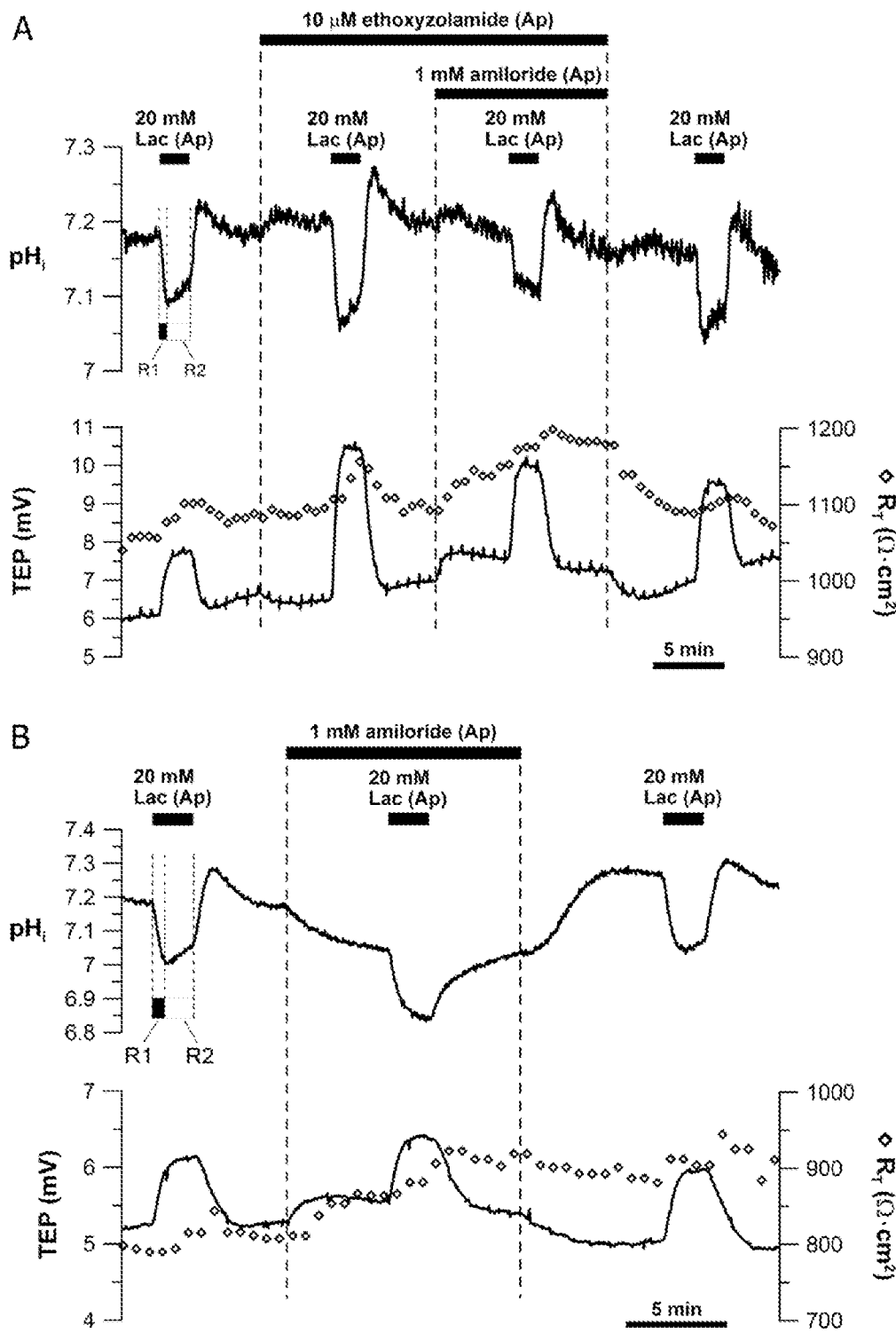


FIG. 1

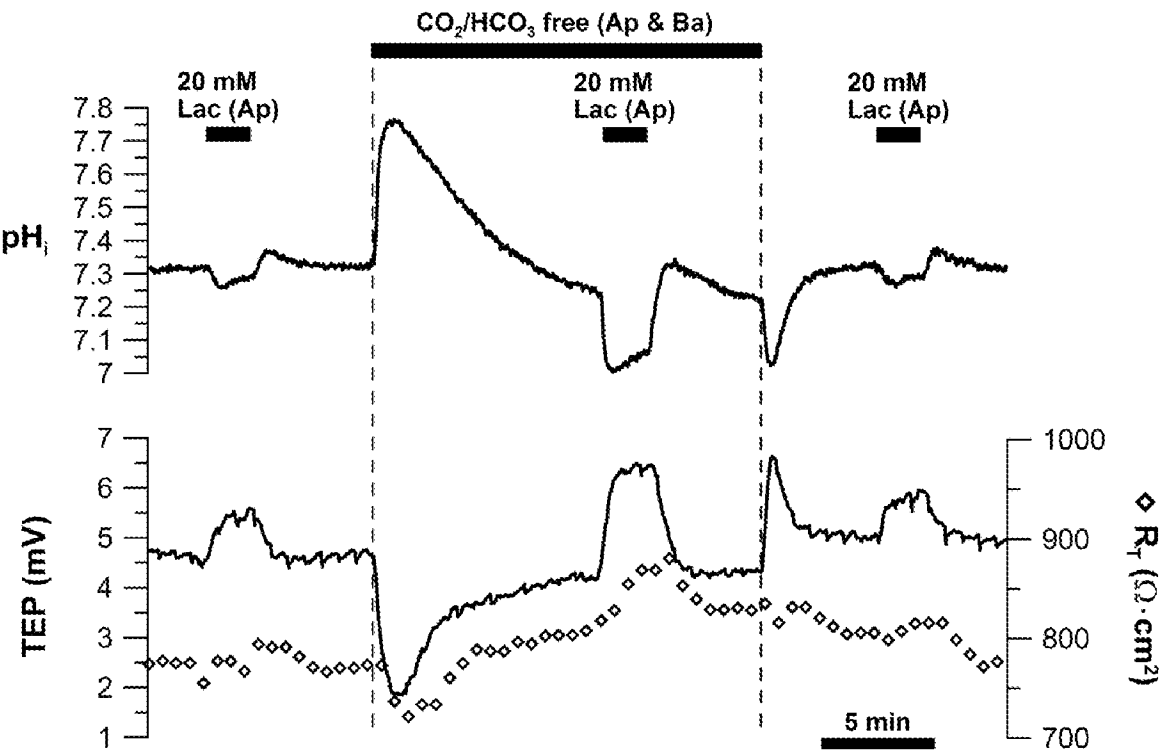


FIG. 2

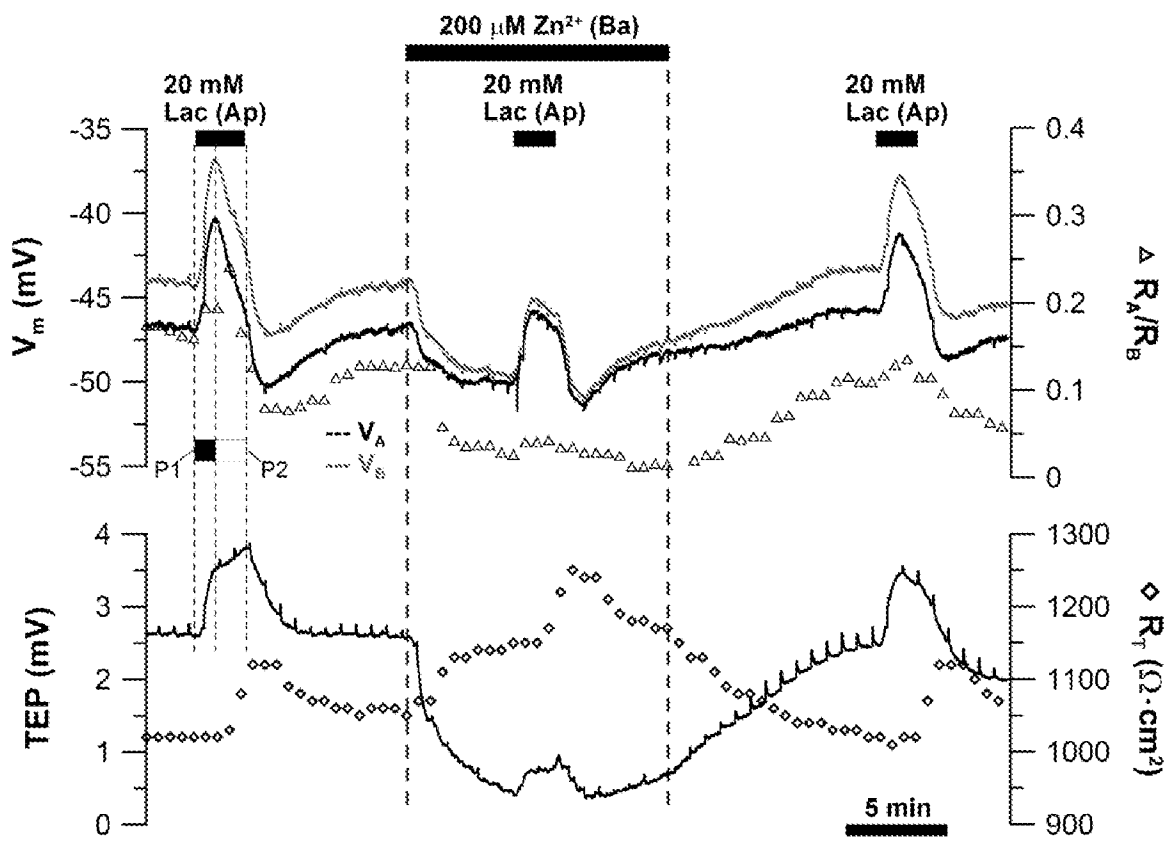


FIG. 3

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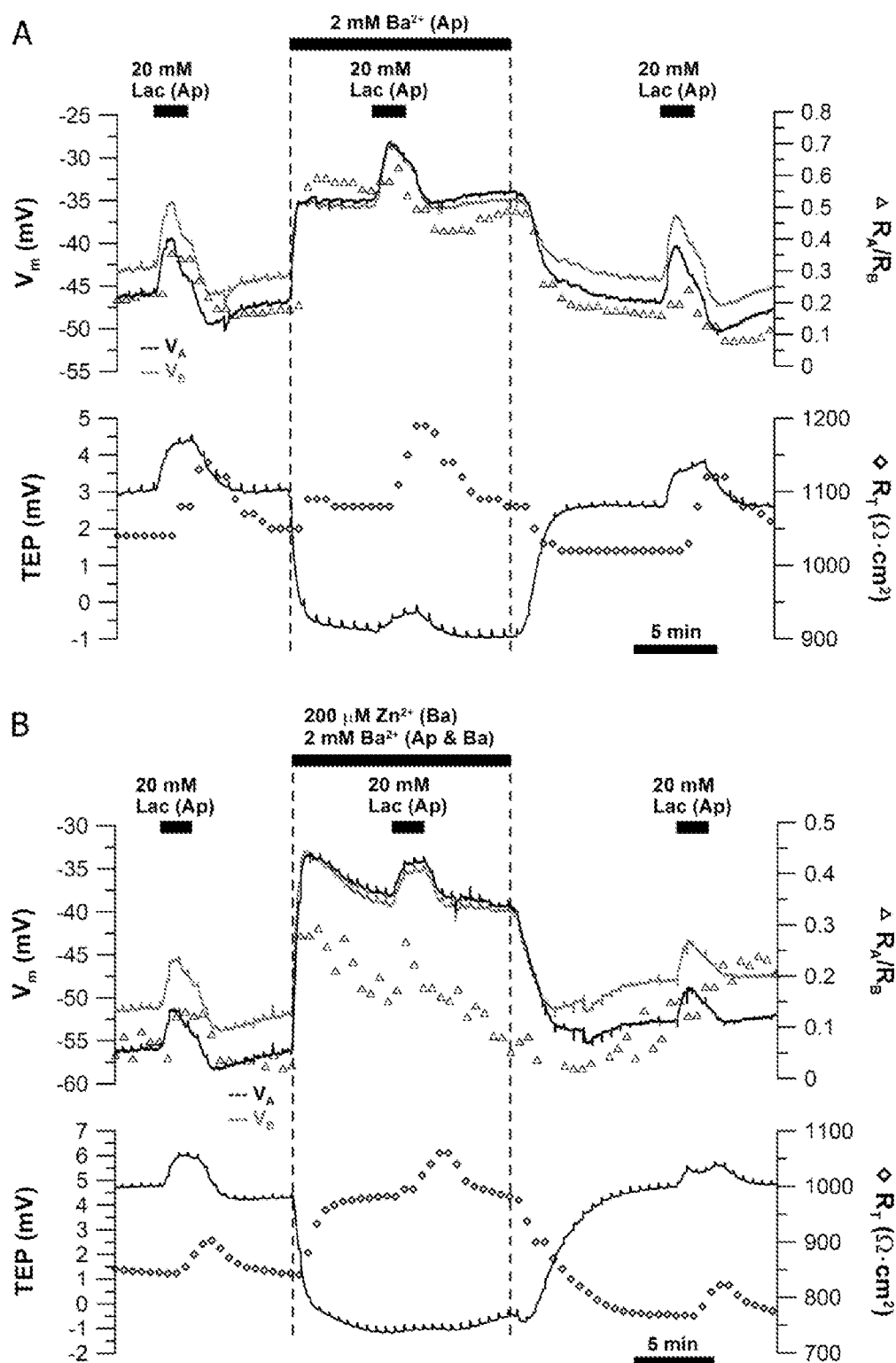


FIG. 4

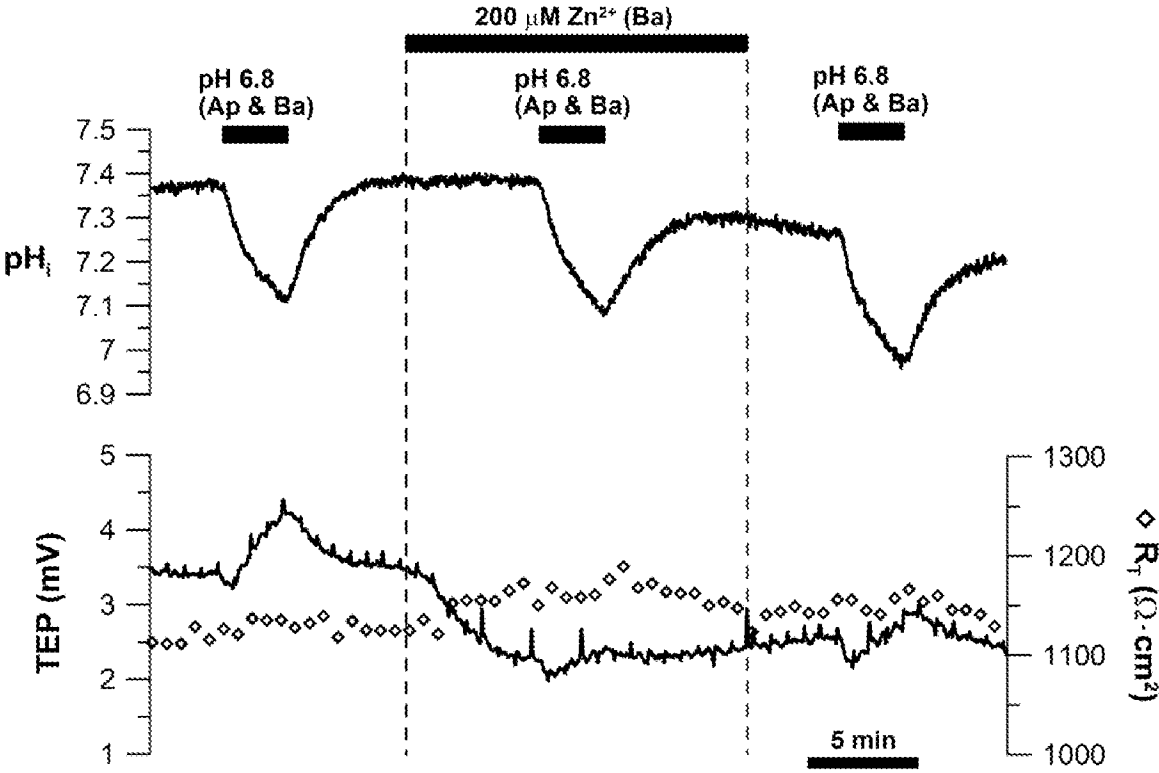


FIG. 5

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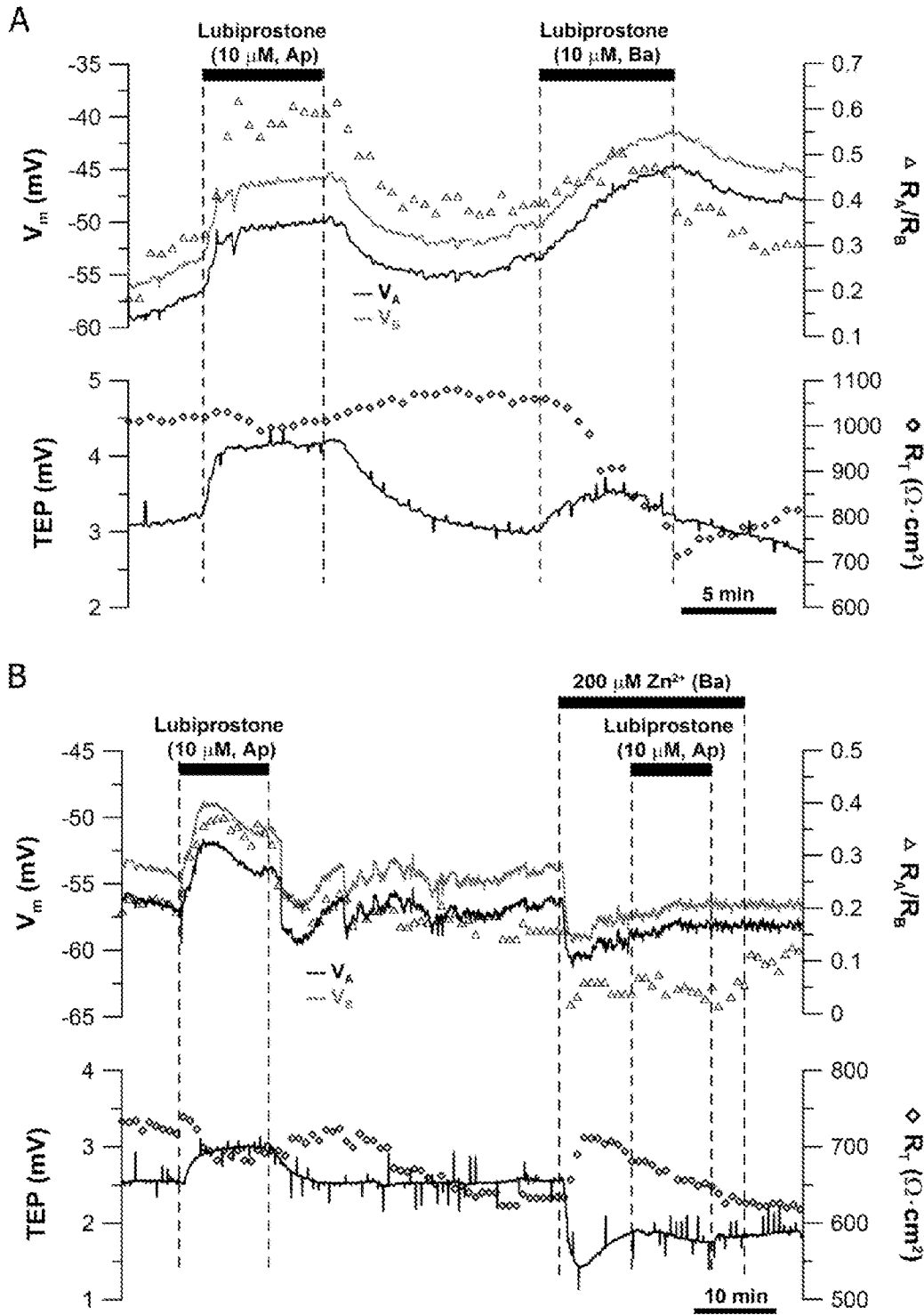


FIG. 6

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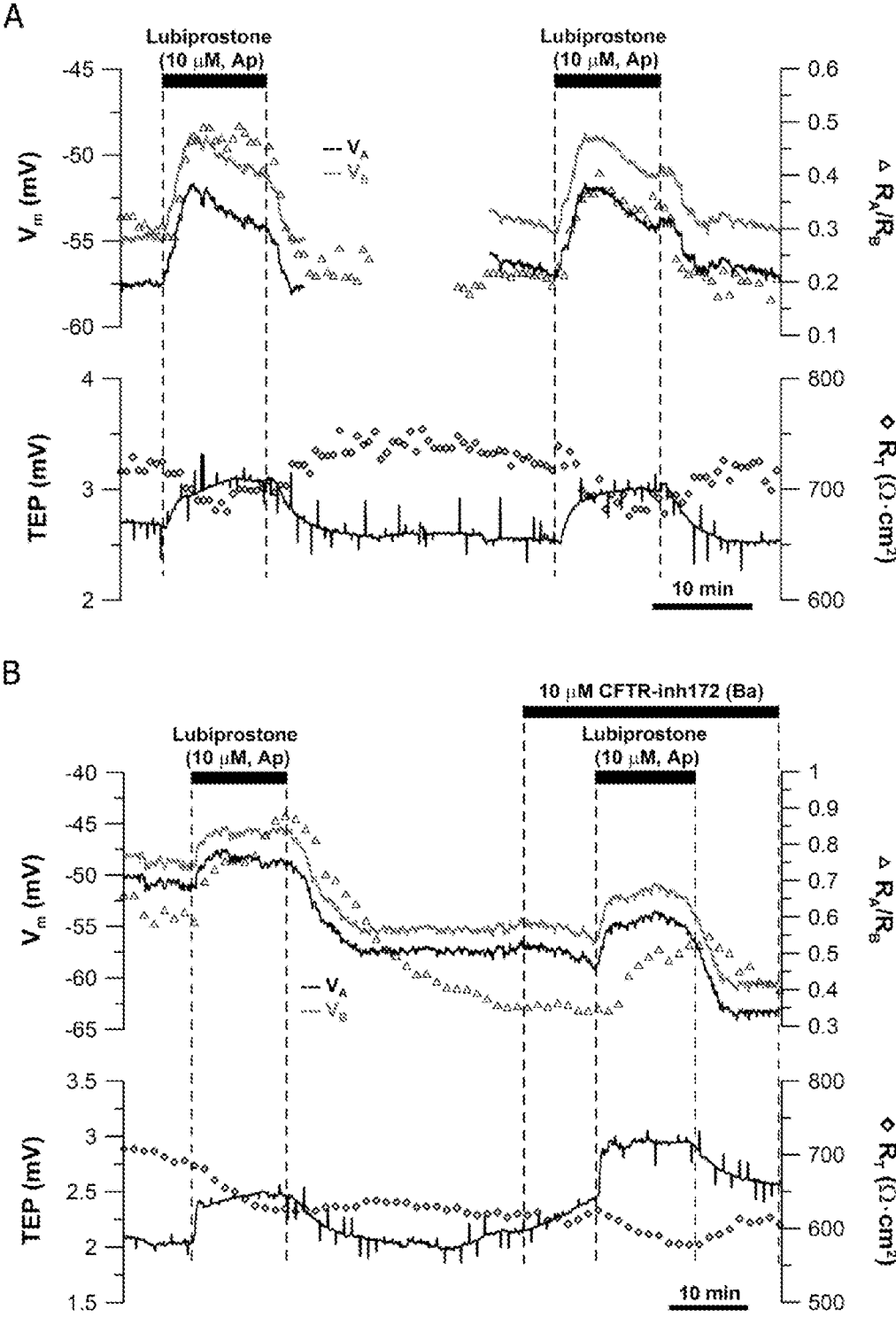


FIG. 7

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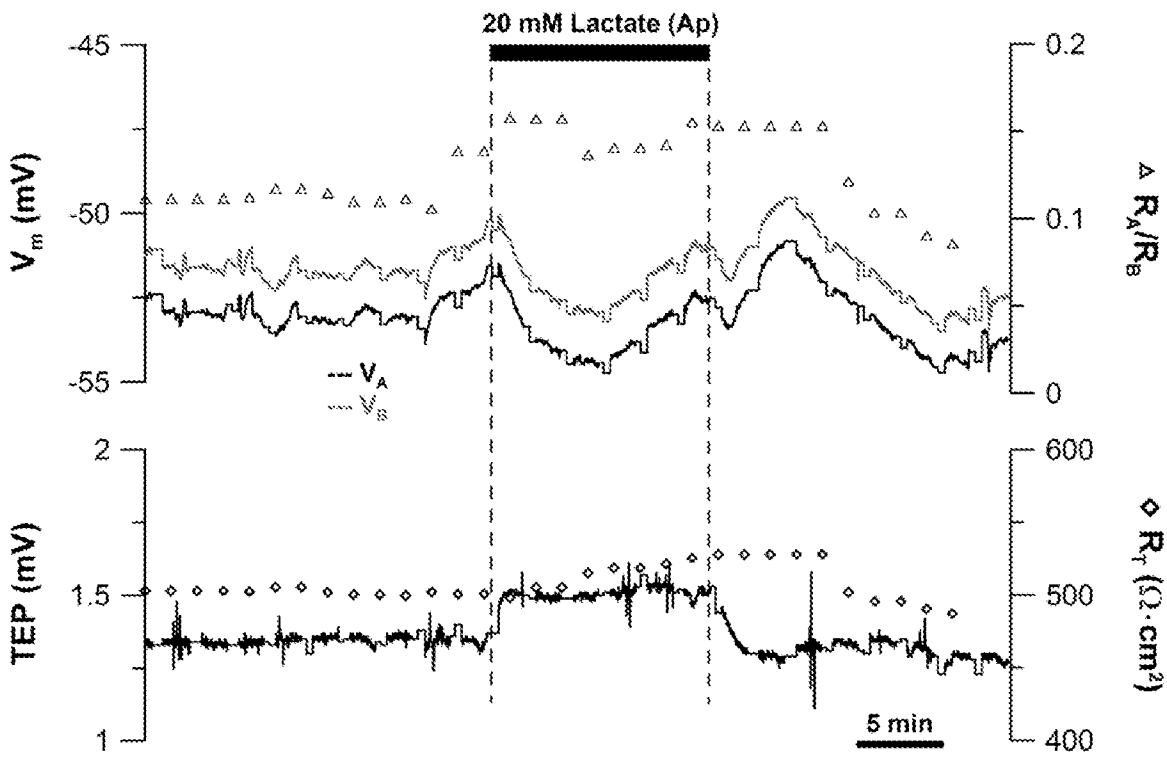


FIG. 8

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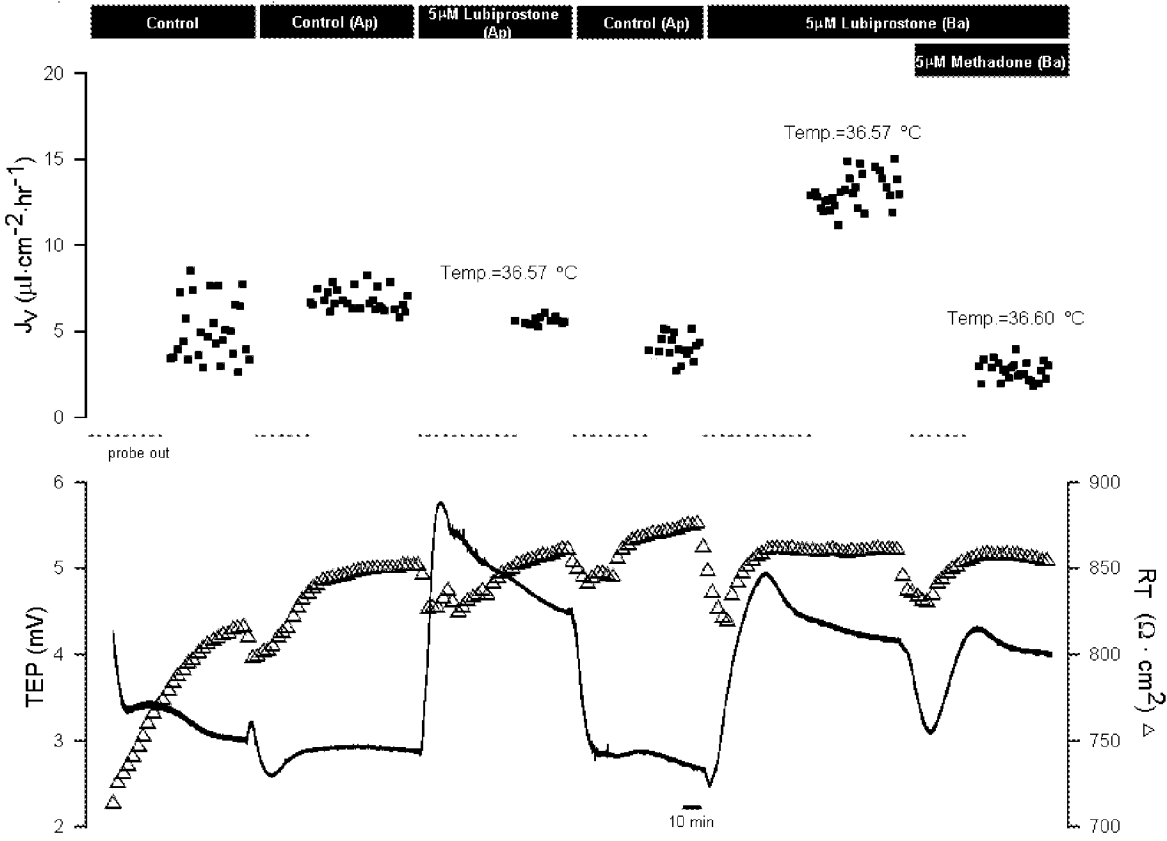


FIG. 9

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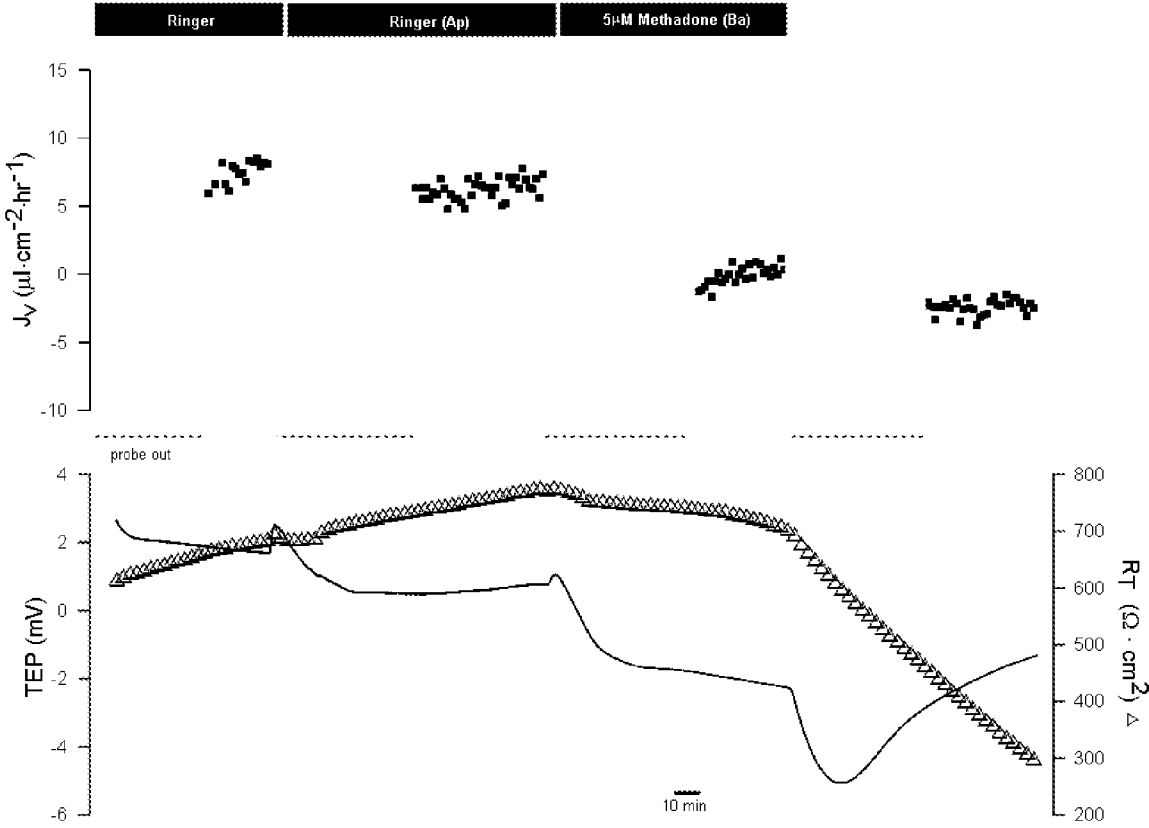


FIG. 10

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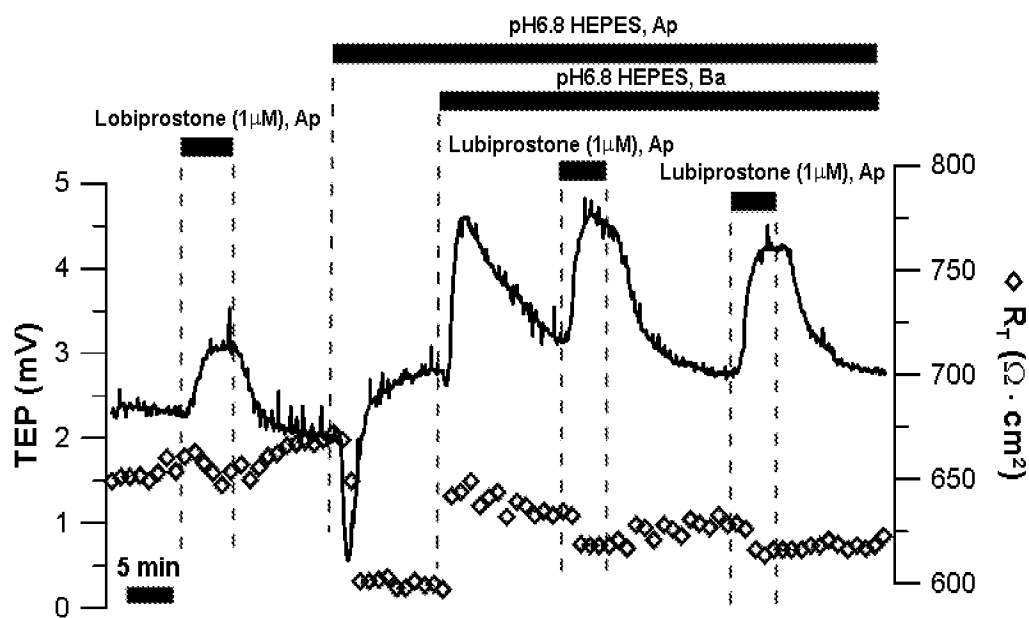


FIG. 11

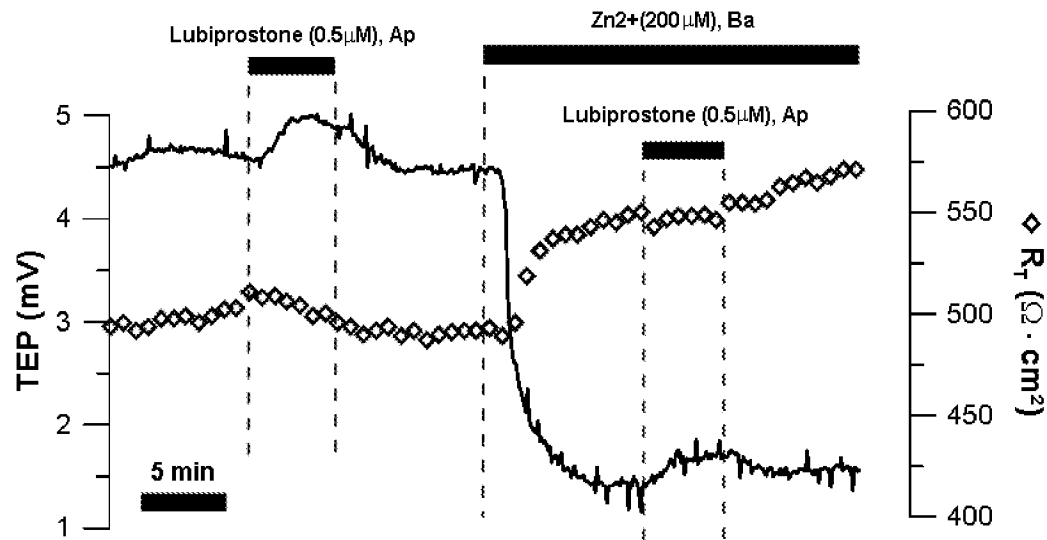


FIG. 12

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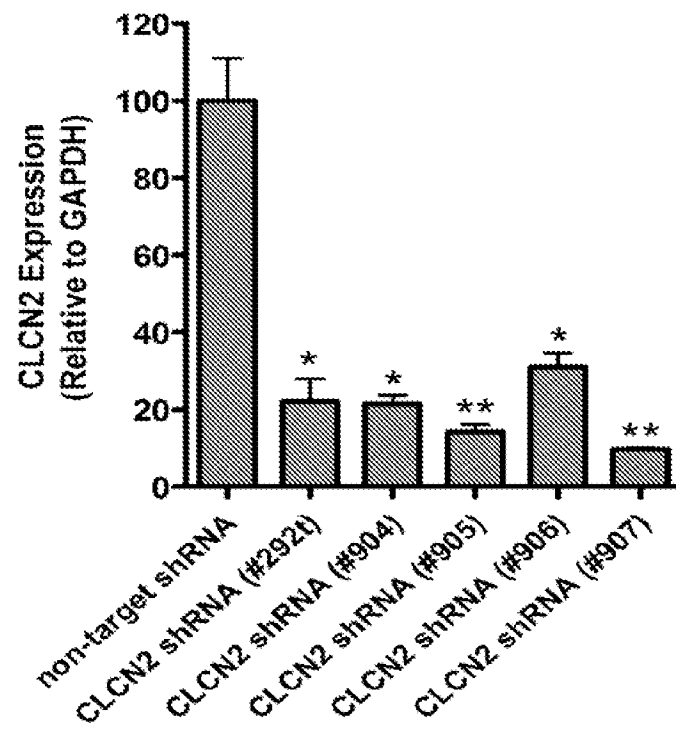


FIG. 13

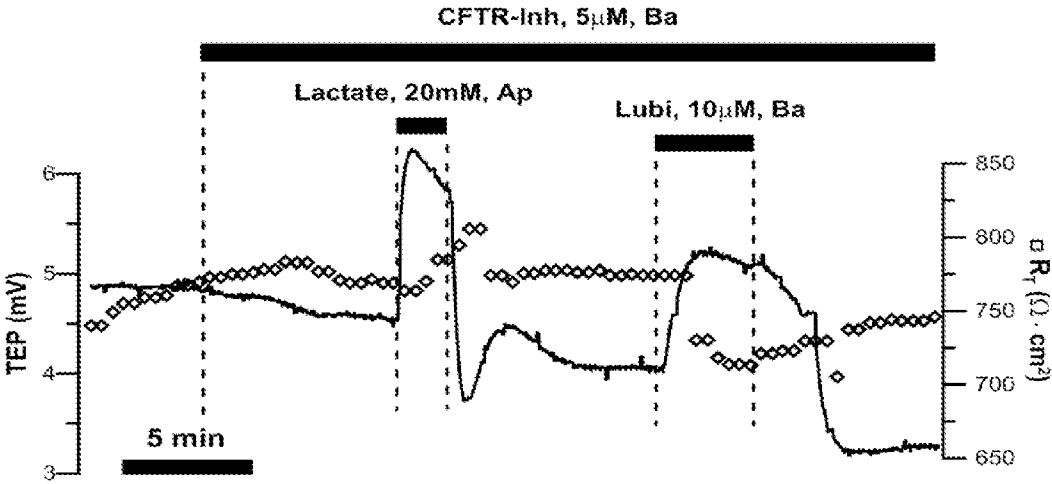


FIG. 14A

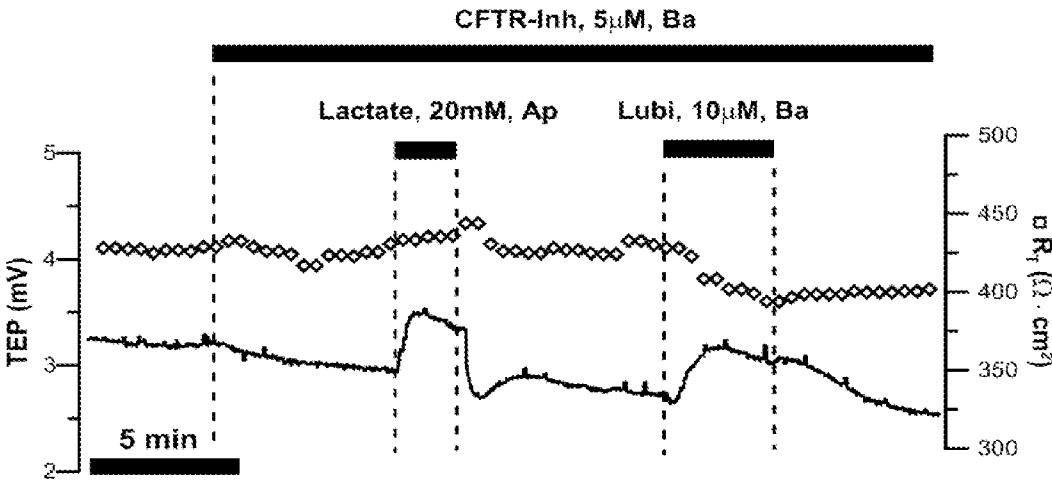


FIG. 14B

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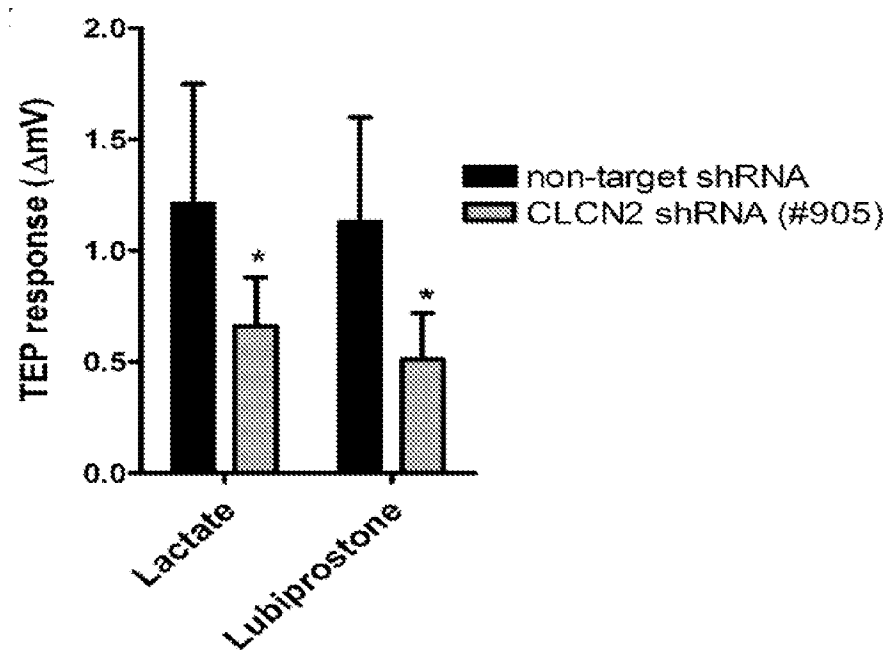


FIG. 15

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/024724

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/352 A61K31/5575 A61K31/558 A61P27/02 A61P27/06
A61K38/21 A61K45/06 A61K31/198

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/093348 A2 (SUCAMPO AG [CH]; UENO RYUJI [US]) 8 September 2006 (2006-09-08) paragraph [0126]; claims 1, 2 paragraph [0122] paragraph [0104] paragraphs [0026], [0123] -----	1-30, 32
X	US 2012/270945 A1 (UENO RYUJI [US]) 25 October 2012 (2012-10-25) cited in the application paragraph [0081] paragraph [0121] -----	1-32
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☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

26 May 2014

Date of mailing of the international search report

16/06/2014

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Baumgärtner, Heike

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/024724

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 2010/119986 A1 (SUCAMPO AG [CH]; UENO RYUJI [US]; CUPPOLETTI JOHN [US]) 21 October 2010 (2010-10-21) paragraph [0102] - paragraph [0103]; claim 10 paragraph [0068] -----	1-32
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International application No

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