

(51) International Patent Classification:
A61K 31/455 (2006.01)(21) International Application Number:
PCT/US2012/030201(22) International Filing Date:
22 March 2012 (22.03.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/467,739 25 March 2011 (25.03.2011) US(71) Applicant (for all designated States except US): **THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK** [US/US]; Office of the General Counsel, 412 Low Library, Mail Code 4308, 535 West 116th Street, New York, NY 10027 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **EMALA, Charles William** [US/US]; 1 Evan Ct., Woodcliff Lake, New Jersey 07677 (US). **YIM, Peter** [US/US]; 2022 Central Ave. Apt. 301, Fort Lee, New Jersey 07024 (US). **GALLOS, George**

[US/US]; 139 Hudson Ave., Tenafly, New Jersey 07670 (US).

(74) Agent: **HOOPER, Kevin C.**; Bryan Cave LLP, 1290 Avenue of the Americas, New York, NY 10104 (US).

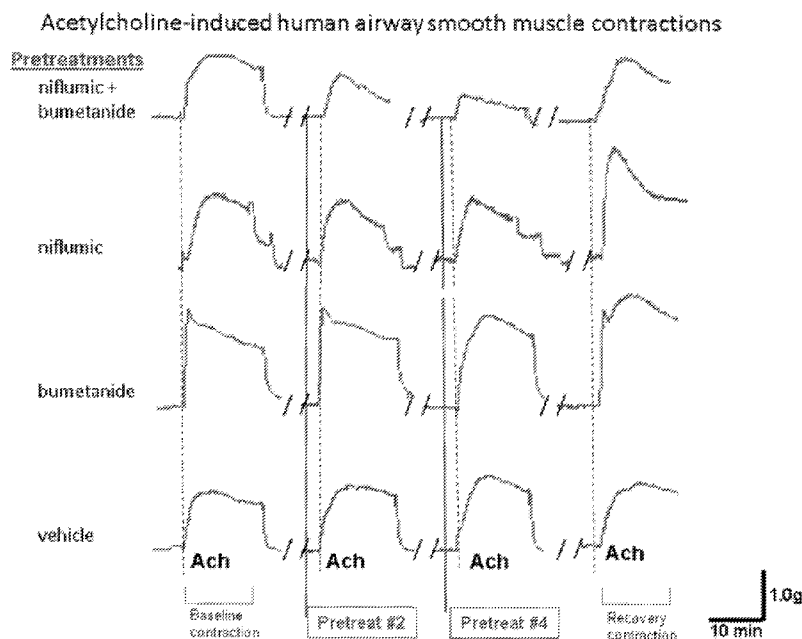
(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,

[Continued on next page]

(54) Title: CHLORIDE CHANNEL AND CHLORIDE TRANSPORTER MODULATORS FOR THERAPY IN SMOOTH MUSCLE DISEASES

Fig. 1



(57) Abstract: The present invention provides, inter alia, methods and pharmaceutical compositions for treating or ameliorating the effects of a disease characterized by altered smooth muscle contractility, such as e.g., asthma and chronic obstructive pulmonary disease. It has been found that depolarization of the plasma membrane and intracellular concentrations of chloride are decreased by simultaneous blockade of CaCCs and the NKCCs.

WO 2012/134965 A1



SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, **Published:**
GW, ML, MR, NE, SN, TD, TG).

— *with international search report (Art. 21(3))*

**CHLORIDE CHANNEL AND CHLORIDE TRANSPORTER MODULATORS FOR
THERAPY IN SMOOTH MUSCLE DISEASES**

GOVERNMENT FUNDING

[0001] This invention was made with government support under GM065281 awarded by the National Institutes of Health. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims priority to United States Provisional Patent Application No. 61/467,739, filed March 25, 2011, the entire content of which is hereby incorporated by reference as if recited in full herein.

FIELD OF THE INVENTION

[0003] The present invention relates to, *inter alia*, pharmaceutical compositions, and methods for modulating calcium-activated chloride channel (CaCC) and sodium-potassium-chloride co-transporter (NKCC) activity.

BACKGROUND OF THE INVENTION

[0004] The National Heart, Lung and Blood Institute of the National Institutes of Health estimate that over 22 million adults and 6 million children in the United States have asthma. It is the leading medical cause for school absenteeism in children and the world-wide incidence of asthma is increasing (Duan, 2009). The

incidence of asthma continues to increase globally with an associated increase in the morbidity and mortality associated with this common disease (Duan, 2009). Despite this pandemic, the pharmacologic approach to asthma has changed little in several decades, relying primarily on inhaled β_2 -adrenoceptor agonists and anti-inflammatory therapies. Although acutely effective, chronic therapy with long-acting β_2 -agonists (LABA) is associated with an increased death rate from asthma (Kazani *et al.*, 2010). Thus, there is an urgent need for alternative therapies due to patients who are refractory to this therapy and the increased mortality associated with the use of long-acting β_2 -adrenoceptor agonists (Kazani *et al.*, 2010). Furthermore, treatment options for patients with bronchospasm and other hyperreactive airway are limited. For example, bronchospasm in association with intubation and airway suctioning are common clinical problems for anesthesiologists and intensivists. β -adrenoceptor agonists have been in the mainstay of therapy for several decades with little progress in identifying improved novel therapies.

[0005] Asthma involves a complex interplay with many cell types including nerves, inflammatory cells, airway epithelium and airway smooth muscle (ASM). ASM is recognized as one important regulator of airway tone in asthma. Recent interest has re-focused on the role of ASM in asthma as airway remodeling, including increased ASM mass, which is now recognized as an important component of chronic asthma. Moreover, one of the more recent innovative therapies for asthma is directly related to the reduction of ASM mass from moderate sized airways through thermoablation techniques (Rubin, 2010). Although current anti-inflammatory, anti-cholinergic, and β_2 -agonist therapies all target different phenotypic aspects of ASM function, no new approaches directed at ASM have been identified in many decades.

[0006] Although the requirement for calcium in smooth muscle contraction has been recognized for a long time, elevation of calcium alone does not dictate the magnitude of smooth muscle contraction: calcium sensitivity of contractile proteins is modulated by the phosphorylation state of contractile proteins and their regulatory kinases/phosphatases. This may explain the long recognized finding that bradykinin receptors greatly increase calcium in ASM but cause rather weak contractions and the very recent discovery of bitter taste receptors in ASM that actually increase calcium but induce smooth muscle relaxation (Deshpande *et al.*, 2010). Emerging evidence in ASM research suggests that calcium entry following membrane depolarization activates RhoA (Liu *et al.*, 2005), a classic upstream modulator of the phosphorylation state of myosin phosphatase and in turn myosin light chain, a final determinant of contractile sensitivity to calcium. There is even evidence in other cell types that a change in membrane potential alone may directly activate RhoA (independent of calcium entry), a fundamental paradigm shift in the understanding of ASM contraction.

[0007] Contractile agonists classically couple via Gq proteins to the synthesis of inositol phosphates and the release of calcium from intracellular sarcoplasmic reticulum stores, but the sensitivity of the contractile apparatus to this calcium (Liu *et al.*, 2006) is another important level of contractile regulation. The importance of calcium sensitivity relative to cytosolic calcium levels is highlighted by the observation that elevation of intracellular calcium alone (*e.g.* bradykinin or bitter taste receptor activation) is insufficient to induce contraction. Traditionally the importance of extracellular calcium and membrane potential in ASM has been thought to be less important than in other smooth muscle subtypes (*e.g.* vascular smooth muscle). However, the recent identification of the role of voltage-sensitive transient receptor

potential (TRP) channels which contribute to the refilling of intracellular calcium stores (White *et al.*, 2006) and the demonstration that membrane potential can modulate calcium sensitivity via the small G protein rho A (Liu *et al.*, 2005; Janssen *et al.*, 2004), has reaffirmed an important role for both extracellular calcium and membrane potential in the control of ASM contraction.

[0008] The efflux of chloride via calcium-activated chloride channels (CaCCs) is known to contribute to the depolarization of the plasma membrane following exposure of contractile agonists (Hirota *et al.*, 2006; Janssen *et al.*, 1995), such as acetylcholine and caffeine. Although the efflux of chloride through the plasma membrane has been identified as the major contributor to plasma membrane depolarization, the fundamental importance of ASM membrane potential in contractile tone has been questioned. Previous attempts to relax ASM via individual blockade of only these channels have shown limited effects. For example, unlike vascular smooth muscle, dihydropyridine therapies directed against voltage dependent L-type calcium channels, one of the earliest discovered voltage-dependent channels in ASM (Kotlikoff *et al.*, 1992), were ineffective at treating asthma (Gupta *et al.*, 1993, Talwar *et al.*, 1993). It was recognized that the range of membrane potential required for activation of this channel was not commonly achieved during depolarization of ASM.

[0009] However, at least six findings continue to support a mechanistic importance for membrane potential in the control of ASM tone: (1) a component of β -adrenoceptor agonist relaxation of ASM involves potassium efflux through calcium activated potassium channels (K_{Ca}) inducing relative plasma membrane hyperpolarization; (2) refilling of sarcoplasmic reticulum with calcium following a classic contractile agonist exposure involves opening of voltage-sensitive plasma

membrane calcium channels (stored operated calcium entry (SOCE)) likely including non-selective cation channels of the transient receptor potential (TRP) family of which both TRP-C (Corteling *et al.*, 2004) and TRP-V (Jia *et al.*, 2004) members are recently identified on ASM; (3) the recent discovery of the expression of both GABA (Gallos *et al.*, 2008) and glycine-modulated (Yim *et al.*, 2011) chloride channels on ASM that modulate contractile tone; (4) the recent discovery that bitter taste receptors relax ASM despite an increase in intracellular calcium through a hypothesized opening of K_{Ca} (a membrane hyperpolarizing event) (Deshpande *et al.*, 2010); (5) changes in membrane potential have been shown to activate both M3 muscarinic receptors (Gq-coupled) in ASM cells (Billups *et al.*, 2006) and M2 muscarinic receptors (Gi-coupled) (Ben-Chaim *et al.*, 2006) independent of receptor occupancy by ligand; and (6) the characterization of T-type calcium channels in ASM (Yamakage *et al.*, 2001) which are activated within membrane potential ranges achieved during agonist-induced ASM contraction and depolarization (Janssen, 1997). Moreover, evidence that inositol phosphates can in turn regulate membrane potential (Zhang *et al.*, 1993; Gromada, 1996) suggests that second messenger regulation of intracellular SR calcium release and plasma membrane potential do not operate in isolation.

SUMMARY OF THE INVENTION

[0010] In view of the foregoing, improved compositions and methods for modulating CaCC and NKCC activity *in vivo* are needed. The present invention is directed to meeting these, and other, needs.

[0011] In the present invention, it has been found that depolarization of the plasma membrane and intracellular concentrations of chloride are decreased by simultaneous blockade of CaCCs and the NKCCs. This results in (1) impaired refilling of sarcoplasmic reticulum calcium stores due to inadequate intracellular chloride available to influx into the SR to balance charge generation (Janssen, 2002) during calcium refilling and (2) a decrease in membrane depolarizing-dependent activation of rhoA (Janssen *et al.*, 2004), a key modulator of smooth muscle calcium sensitivity. The simultaneous blockade of CaCCs and the NKCCs interrupts the plasma membrane's ability to effectively cycle chloride in and out of the cell, leading to direct relaxation of human ASM. Additionally, in the present invention, it has been found that blockade of CaCC causes membrane hyperpolarization, and that the simultaneous blockade of NKCC shifts the equilibrium potential of chloride, thereby attenuating depolarization.

[0012] In the present invention, it has also been found that combining two pharmacologic therapies, originally evaluated separately, have profound effects on the ability of human ASM to constrict to acetylcholine, a classic endogenous or exogenous constrictor of ASM. Both of these pharmacologic tools target different aspects of chloride handling in ASM cells which then modulate both sarcoplasmic reticulum refilling of calcium as well as the sensitivity of the contractile apparatus to calcium: two fundamental cellular aspects of smooth muscle contraction.

[0013] An especially exciting aspect of these findings is that members of both drug classes (*e.g.*, talnifumate as a pro-drug of niflumic acid (CaCC inhibitor) (Walker *et al.*, 2006) used for anti-inflammatory properties and *e.g.*, bumetanide and furosemide (NKCC inhibitors) as a diuretic, for example) have been previously used clinically for other therapies, encouraging rapid clinical translation of these studies.

Thus, the present invention includes a novel therapeutic approach to the treatment of bronchospastic airway diseases that may circumvent the current limitations of β_2 -adrenoceptor agonist therapy.

[0014] For example, one embodiment of the present invention is a method of treating or ameliorating the effects of a disease characterized by altered smooth muscle contractility. This method comprises administering to a patient suffering from such a disease an effective amount of a calcium-activated chloride channel (CaCC) modulator and a sodium-potassium-chloride co-transporter (NKCC) modulator.

[0015] Another embodiment of the present invention is a pharmaceutical composition for treating or ameliorating the effects of a disease characterized by altered smooth muscle contractility. This composition comprises a pharmaceutically acceptable carrier, a CaCC modulator, and a NKCC modulator.

[0016] Yet another embodiment of the present invention is a method of relaxing airway smooth muscle. This method comprises administering to a patient in need thereof an effective amount of a CaCC modulator and a NKCC modulator.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Fig. 1 is a graph showing that simultaneous blockade of the CaCC and NKCC with 100 μ M each of niflumic acid and bumetanide blocks repetitive acetylcholine (ACh)-induced contractions in human airway smooth muscle. Note the progressive decrease in the magnitude of the acetylcholine-induced contractions following 4 repetitive pretreatments with niflumic + bumetanide (top tracings) but not with other pretreatments. Progressive decrease with repetitive contractions is consistent with depletion of calcium from intracellular stores. Tracings from only

pretreatments #2, #4 are shown for figure clarity. The tracings shown are representative of 8 airways from 4 patients.

[0018] Fig. 2. is a bar graph showing acetylcholine-induced contractions in human airway smooth muscle strips following pretreatment with 100 μ M niflumic acid, 100 μ M bumetanide, or both. Asterisk (*) indicates $p < 0.05$. N = 3-5.

[0019] Fig. 3A is a bar graph showing organ bath muscle force studies using guinea pig tracheal rings. Tetraethylammonium contractions were induced and muscle force was measured after treatments with 100 μ M niflumic acid (CaCC blocker), 10 μ M nifedipine, or 300 μ M furosemide (NKCC blocker). N = 4-5. Asterisk (*) indicates $p < 0.001$ compared to basal. Double asterisks (**) indicate $p < 0.001$ compared to basal. Triple asterisks (***) indicate $p < 0.001$ compared to basal. Fig. 3B is a bar graph showing organ bath muscle force studies using guinea pig tracheal rings. Tetraethylammonium contraction (% acetylcholine contraction control) comparing normal to decreased bath chloride concentration. Sodium gluconate was used to correct osmolarity. Muscle forces were proportional to the extracellular chloride concentration. N = 4-6. Asterisk (*) indicates $p < 0.05$. Double asterisks (**) indicate $p < 0.001$ compared to 124 mM [Cl].

[0020] Fig. 4 shows *in vivo* airway pressure measurements in guinea pig. Representative pulmonary inflation in guinea pigs pretreated 12 minutes before administration of acetylcholine with either vehicle (100 μ l DMSO, intravenous (i.v.)) or niflumic acid (5 mg, i.v.) + furosemide (5 mg, i.v.). Acetylcholine (14 μ g/kg, i.v.) was then injected 6 times at 30 second intervals. Airway responses to injections 1, 3, and 5 are shown for clarity. Note the sequential decrease in the magnitude of airway constriction in response to acetylcholine following pretreatment with niflumic acid + furosemide (representative of 2 animals).

[0021] Fig. 5 shows representative membrane potential measurements in human airway smooth muscle cells by (A) relative fluorescent unit (RFU) changes of FLIPR potentiometric probe or (B) electrophysiologic recordings of voltage changes under current clamp in whole cell configuration. Niflumic acid (100 μ M) hyperpolarized the cell membrane while bumetanide (10 μ M) was without effect. The measurements shown are representative of 4 independent recordings.

[0022] Fig. 6 shows representative tracings of intracellular chloride in human airway smooth muscle cells following blockade of CaCC (100 μ M niflumic acid) (Fig. 6A) or blockade of NKCC (10 μ M bumetanide) (Fig. 6B). Blockade of CaCC increases intracellular chloride quenching MQAE fluorescence while blockade of NKCC blocks chloride refilling unquenching MQAE fluorescence. Tracings shown are representative of 3 measurements in separate cell populations.

[0023] Fig. 7 are bar graphs showing membrane potential (current clamp, whole cell) (Fig. 7A) and intracellular calcium (fluo4-AM fluorescence) (Fig. 7B) in human airway smooth muscle cells in response to 10mM tetraethylammonium (TEA)-Cl or 60-75 mM potassium (K)-gluconate. TEA and K gluconate depolarized the cell, but only K gluconate increased calcium. Asterisk (*) indicates $p < 0.05$ compared to control. $N = 3-6$.

[0024] Fig. 8 is a bar graph showing RhoA activation in human airway smooth muscle cells. Primary cultures of cells were treated for 2 minutes with 10 mM tetraethylammonium (TEA)-acetate (Ac), or for various times with 60 mM potassium (K)-gluconate before cell solubilization and isolation of activated (GTP-bound) rhoA by rhotekin-binding pull down assay and detection by immunoblot.

[0025] Fig. 9 shows representative tracings of force measurements on human airway smooth muscle strips. Control contractions were performed with an EC_{50}

concentration of acetylcholine. Each strip was either treated with 100 μM niflumic acid and 10 μM bumex or 0.1% ethanol control and contracted with an EC_{50} of acetylcholine. The strips were then thoroughly washed and recontracted.

[0026] Fig. 10 is a bar graph showing an analysis of force measurements on human airway smooth muscle strips. All measurements were normalized as percentages of the control EC_{50} acetylcholine contraction. Control vs. the bumex/niflumic treatment group shows significant blockade of a control EC_{50} acetylcholine contraction. Asterisk (*) indicates $p < 0.05$. $N = 3$.

[0027] Fig. 11A shows a representative intracellular whole cell tracing of a single guinea pig airway smooth muscle cell, treated with 10 mM TEA (K channel blocker). The cell was voltage clamped in a step protocol, with voltages ranging from about 40 to 100 mV recorded at intervals of 10 mV. The equilibrium potential was recorded at 2.2 mV. The recoding is linear around 0 mV with an exponential rise in the positive mV range consistent with a Cl current. Figure 11B shows whole cell intracellular voltage clamp recordings of a single guinea pig smooth muscle at a holding potential of about 60 mV. After treatments with TEA, spontaneous transient inward currents (STIC's) were enhanced, ranging from 30-50 pA. Subsequent bath application of about 100 μM niflumic acid (calcium activated chloride channel blocker) abolishes STIC's. Insert in Figure 11B shows an enlarged STIC signal before niflumic acid.

[0028] Fig. 12 is a graph showing intracellular quenching of MQAE fluorescence by chloride in human airway smooth muscle cells. Bumetanide (10 μM) alone decreases $[\text{Cl}^-]_i$. Niflumic acid (100 μM) added with bumetanide reduces the bumetanide effect but the net effect is still reduced $[\text{Cl}^-]_i$ concentration from baseline levels. Results shown are representative of 5 trials.

[0029] Fig. 13 is a graph showing intracellular calcium concentrations in human airway smooth muscle cells. Cells were treated with thapsigargin to block SR Ca^{2+} -ATPase mediated refilling in the absence of extracellular calcium. The re-introduction of 2 mM CaCl_2 induces a rapid increase in $[\text{Ca}^{2+}]_i$, indicative of store-operated calcium entry (SOCE). Reduced concentrations of extracellular Cl^- concentrations (replaced with gluconate), resulting in depletion of intracellular Cl^- concentrations, decreased the magnitude of SOCE. Measurements shown are representative of 4 trials.

[0030] Fig. 14 is a graph showing intracellular quenching of MQAE fluorescence by chloride in human airway smooth muscle cells. Depolarization induced by tetraethylammonium (TEA)-acetate is accompanied by an efflux of chloride causing reduced quenching of MQAE fluorescence. The tracings shown are representative of 5 trials.

[0031] Fig. 15 is a cartoon showing the mechanistic hypotheses (dashed lines) of airway smooth muscle relaxation by simultaneous blockade of calcium activated chloride channel (CaCC) and Na^+ - K^+ - Cl^- transporter (NKCC). Bumetanide blockade of NKCC blocks intracellular Cl^- refilling resulting in reduced extracellular efflux through niflumic-acid insensitive Cl^- channels which decreases membrane depolarization impairing RhoA activation and reduced intracellular Cl^- available to balance charge generation during Ca^{2+} refilling of SR. Niflumic acid blockade of CaCC induces hyperpolarization which impairs RhoA activation. SR= sarcoplasmic reticulum; MLC = myosin light chain; CaM = Ca^{2+} calmodulin dependent protein; MLCK = myosin light chain kinase; ROCK = Rho associated protein kinase.

[0032] Fig. 16 shows airway lumen area measured in peripheral small airways in rat lung slices. Fig. 16A shows representative light micrographs of the same

peripheral airway under baseline (Rest) and following contraction induced by acetylcholine (ACh), potassium chloride (KCl), or TEA. Fig. 16B shows a real-time measurement of lumen area from images in Fig. 16A. Arrows indicate time that each image from Fig. 16A was captured. Fig. 16C is a bar graph showing percent decrease in lumen area after treatment with ACh, KCl, or TEA as compared to the sample at rest. All three contractile agonists result in a significant decrease in lumen area. Asterisk (*) indicates $p < 0.01$. $n = 6$. Fig. 16D shows a real-time measurement of lumen area after treatment with KCl, TEA, and niflumic acid (NFA). Fig. 16E is a bar graph showing the percent decrease in lumen area as a result of TEA-induced contraction and with NFA treatment. NFA at 100 μM significantly relaxes the peripheral airway contracted with TEA. Asterisk (*) indicates $p < 0.01$. $n = 6$.

[0033] Fig. 17A is a line graph showing membrane potential changes in the potentiometric probe FLIPR Blue in human airway smooth muscle cells. Fig. 17B is a bar graph showing the changes in membrane potential. The chloride channel blocker NFA hyperpolarizes the airway smooth muscle cell plasma membrane, favoring cellular relaxation. The K^+ channel opener NS1619 was used as a positive control for hyperpolarization while TEA chloride and KCl were used to demonstrate depolarization.

[0034] Fig. 18 shows a representative tracing of a whole cell electrophysiologic recording of a human airway smooth muscle cell under current clamp. Following depolarization of the plasma membrane with TEA chloride (favoring contraction), NFA reverses membrane potential (favoring smooth muscle cell relaxation).

[0035] Fig. 19 shows the structure of the water-soluble sodium salt form of niflumic acid (NFA).

[0036] Fig. 20A shows representative muscle force tracings in an organ bath of guinea pig airway smooth muscle relaxed to a greater extent with low concentration (10 μ M) of the water soluble form of NFA compared to the hydrophobic form of the parent compound. Fig. 20B is a bar graph showing muscle force at 60 minutes as a percent of initial force after TEA treatment. The water soluble form demonstrates enhanced potency at relaxing *ex vivo* guinea pig airway smooth muscle.

[0037] Fig. 21 shows that chemically modifying niflumic acid (NFA) as a sodium salt to increase water-solubility retains its ability to inhibit acetylcholine-induced contractions in human airway smooth muscle. Representative muscle force tracings of *ex vivo* human airway smooth muscle airway strips are shown. In the upper traces, human airway smooth muscle strips were pretreated with 100 μ M water-soluble NFA + 10 μ M bumetanide before repetitive contractions #2 and #4. In the lower traces, human airway smooth muscle strips were pretreated with a vehicle control. In the upper traces, note the loss of magnitude of acetylcholine-induced contraction following pretreatments that recovers following washout of pretreatments, while the magnitude in the lower traces is unaffected. W = washout of buffer in organ baths between contractile challenges and pretreatments.

DETAILED DESCRIPTION OF THE INVENTION

[0038] One embodiment of the present invention is a method of treating or ameliorating the effects of a disease characterized by altered smooth muscle contractility. This method comprises administering to a patient suffering from such a disease an effective amount of a calcium-activated chloride channel (CaCC) modulator and a sodium-potassium-chloride co-transporter (NKCC) modulator.

[0039] As used herein, in relation to a disease, the term “characterized by” means one of the characteristics or one of the symptoms of the disease. The term “altered” means different from the norm (*i.e.* the population at large or an individual not suffering from such a disease). The term “smooth muscle” refers to a group of non-striated muscles, generally found in the walls of hollow organs of the body (except the heart), including but not limited to the blood vessels, the respiratory tract, the gastrointestinal tract, the bladder, or the uterus. Preferably, the smooth muscle is airway smooth muscle. The term “contractility” refers to properties associated with the contraction (*e.g.*, of smooth muscle), such as contraction and relaxation of smooth muscles. The contraction and relaxation of smooth muscles is usually not under voluntary control.

[0040] As used herein, a “CaCC modulator” is a substance that changes the activity or the opening or the closing of a calcium-activated chloride channel. Preferably, the CaCC modulator of the present invention is a CaCC inhibitor. As used herein, “a CaCC inhibitor” means a substance that acts directly or indirectly on the CaCC to reduce or completely arrest its function, such as, *e.g.*, to close the channel. The CaCC inhibitor may be selected from any known or to be discovered compound or composition having the above described function. Preferably, the CaCC inhibitor is selected from the group consisting of niflumic acid, 5-nitro-2-(3-

phenylpropylamino)-benzoate (NPPB), talnifumate, flufenamic acid, 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS), indanyloxyacetic acid 94 (IAA-94), tamoxifen, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), anthracene-9-carboxylic acid (A9C), diphenylamine-2-carboxyl acid (DPC), 6-*t*-butyl-2-(furan-2-carboxamido)-4,5,6,7-tetrahydrobenzo[*b*] thiophene-3-carboxylic acid (CaCC_{inh}-A01), 2-hydroxy-4-(4-*p*-tolylthiazol-2-ylaminobenzoic acid (CaCC_{inh}-B01), morniflumate (Sanofi-Aventis, France), calcium-sensitive chloride channel antagonist (Takeda Pharmaceutical Co. Ltd., Japan), pharmaceutically acceptable salts thereof, and combinations thereof. Other forms of these drugs, including pro-drug forms, whether or not specifically identified herein, are also contemplated. Preferably, the CaCC inhibitor is niflumic acid, or a pharmaceutically acceptable salt thereof. As used herein, a "pharmaceutically acceptable salt" means a salt of the compounds of the present invention which are pharmaceutically acceptable, as defined herein, and which possess the desired pharmacological activity. Such salts include acid addition salts formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or with organic acids such as acetic acid, propionic acid, hexanoic acid, heptanoic acid, cyclopentanepropionic acid, glycolic acid, pyruvic acid, lactic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, *o*-(4-hydroxybenzoyl)benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, 1,2-ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, *p*-chlorobenzenesulfonic acid, 2-naphthalenesulfonic acid, *p*-toluenesulfonic acid, camphorsulfonic acid, 4-methylbicyclo[2.2.2]oct-2-ene-1-carboxylic acid, glucoheptonic acid, 4,4'-methylenebis(3-hydroxy-2-ene-1-carboxylic acid), 3-phenylpropionic acid, trimethylacetic acid, tertiary butylacetic acid, lauryl

sulfuric acid, gluconic acid, glutamic acid, hydroxynaphthoic acid, salicylic acid, stearic acid, muconic acid and the like. Pharmaceutically acceptable salts also include base addition salts which may be formed when acidic protons present are capable of reacting with inorganic or organic bases. Acceptable inorganic bases include sodium hydroxide, sodium carbonate, potassium hydroxide, aluminum hydroxide and calcium hydroxide. Acceptable organic bases include ethanolamine, diethanolamine, triethanolamine, tromethamine, N-methylglucamine and the like. Thus, in one preferred embodiment, the CaCC inhibitor is the sodium salt of niflumic acid shown in Fig. 19.

[0041] As used herein, a “NKCC modulator” is a substance that changes the activity of the NKCC. Preferably, the NKCC modulator is a NKCC inhibitor. A “NKCC inhibitor” is a substance that acts directly or indirectly to abolish or decrease the activity of the NKCC. The NKCC inhibitor may be selected from any known or to be discovered compound or composition having the above-described function. Preferably, the CaCC inhibitor is selected the group consisting of bumetanide, furosemide, torasemide, azosemide, piretanide, tripamide, etozoline and its metabolite ozolinone, cicletanine, ethacrynic acid, muzolimine, LR-14-890 (Menarini, Italy), lemidosul (Sanofi-Aventis, France), M-12285 (Mochida, Japan), alilusem (Mochida, Japan), sulosemide sodium (Sano-Aventis, France), BTS-39542 (Abbott Laboratories, Abbott Park, Illinois), AY-31906 (Pfizer, New York, New York), brocrinat (Sanofi-Aventis), SA-9000 (Santen, Japan), A-52773 (Abbott Laboratories), A-53385 (Abbott Laboratories), CL-301 (Chlorion Pharma, Canada), Abbott-49816 (Abbott Laboratories), ethacrynic acid (Telor Ophthalmic Pharmaceuticals, Wilmington, Massachusetts), pharmaceutically acceptable salts thereof, and combinations thereof. Other forms of these drugs, including pro-drug forms, whether

or not specifically identified herein, are also contemplated. In another preferred embodiment, the NKCC inhibitor is selected from the group consisting of bumetanide, furosemide, pharmaceutically acceptable salts thereof, and combinations thereof. More preferably, the NKCC inhibitor is bumetanide.

[0042] In one aspect of this embodiment, the disease characterized by altered smooth muscle contractility is selected from the group consisting of asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, adult respiratory distress syndrome, and bronchospasm. Other non-limiting examples of diseases characterized by altered smooth muscle contractility include hypertension, bladder spasms, and pre-term labor. Preferably, the disease is asthma or COPD, and the patient is human.

[0043] In another aspect of this embodiment, the CaCC modulator and the NKCC modulator are administered as part of a pharmaceutical composition. In the pharmaceutical composition, one or more CaCC modulator(s) are present together with one or more NKCC modulator(s). The exact physical form of the pharmaceutical composition is not critical. Thus, the CaCC and NKCC modulator(s) may be intermixed, physically separated, or otherwise formulated to achieve the desired clinical outcome. Preferably, the pharmaceutical composition is in a unit dosage form.

[0044] In this embodiment, the pharmaceutical composition may be co-administered with a β -agonist. In the present invention, "co-administration" includes administration of a pharmaceutical composition comprising a CaCC modulator and a NKCC modulator along with one or more β -agonist(s) together in the same composition, simultaneously in separate compositions, or as separate compositions administered at different times, as deemed most appropriate by a physician.

[0045] Non-limiting examples of a β -agonist according the present invention include albuterol, levalbuterol, salmeterol, formoterol, isoproterenol, pirbuterol, and combinations thereof. Co-administration of the pharmaceutical composition comprising a CaCC modulator and a NKCC modulator with a β -agonist leads to synergism (*i.e.*, greater than additive effects). In view of this, lower doses of β -agonist(s) may be used in conjunction with a composition comprising a CaCC modulator and a NKCC modulator, which may result in lower overall side effects.

[0046] Another embodiment of the present invention is a pharmaceutical composition for treating or ameliorating the effects of a disease characterized by altered smooth muscle contractility. This composition comprises a pharmaceutically acceptable carrier, a CaCC modulator, and a NKCC modulator. Suitable CaCC modulators and NKCC modulators are as described above. In this embodiment, more than one CaCC and/or NKCC modulator(s) are also contemplated.

[0047] Preferably, the disease is selected from the group consisting of asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, adult respiratory distress syndrome, and bronchospasm. More preferably, the disease is asthma or COPD. The pharmaceutical composition may be in a unit dosage form. Furthermore, the pharmaceutical composition may be co-administered with a β -agonist, as described above.

[0048] Yet another embodiment of the present invention is a method of relaxing airway smooth muscle. This method comprises administering to a patient in need thereof an effective amount of a CaCC modulator and a NKCC modulator. Preferably, the CaCC modulator is a CaCC inhibitor, and the NKCC modulator is a NKCC inhibitor. Suitable CaCC inhibitors and NKCC inhibitors are as exemplified above.

[0049] As used herein, "relaxing airway smooth muscle" means reducing the force, tension, or contraction of the smooth muscles related to the portion of the respiratory system through which air flows.

[0050] In the present invention, an "effective amount" is an amount sufficient to effect beneficial or desired clinical results. An effective amount can be administered in one or more doses. In terms of treatment, an "effective amount" of a CaCC modulator or a NKCC modulator is an amount sufficient to treat or ameliorate the effects of a disease characterized by altered smooth muscle contractility. Detection and measurement of these indicators of efficacy are disclosed below.

[0051] An effective amount is generally determined by a physician on a case-by-case basis and is within the skill of one in the art. Several factors are typically taken into account when determining an appropriate dosage. These factors include age, sex and weight of the patient, the condition being treated, the severity of the condition and the form of the drug being administered.

[0052] Effective dosage forms, modes of administration, and dosage amounts may be determined empirically, and making such determinations is within the skill of the art. It is understood by those skilled in the art that the dosage amount will vary with the route of administration, the rate of excretion, the duration of the treatment, the identity of any other drugs being administered, the age, size, and species of animal, and like factors well known in the arts of medicine and veterinary medicine. In general, a suitable dose of a CaCC modulator or a NKCC modulator according to the invention will be that amount of the compound, which is the lowest dose effective to produce the desired effect. The effective dose of a CaCC modulator or a NKCC modulator may be administered as two, three, four, five, six or more sub-doses, administered separately at appropriate intervals throughout the day, with the proviso

that the doses of the CaCC and NKCC modulator simultaneously block the CaCC and NKCC.

[0053] A compound or pharmaceutical composition of the present invention may be administered in any desired and effective manner. Preferably, the compound or pharmaceutical composition of the present invention is administered to a patient in need thereof through a mucosal lining, by, *e.g.*, a nasal or pulmonary spray.

[0054] Thus, compounds and pharmaceutical compositions according to the present invention may be administered in an aqueous solution as a nasal or pulmonary spray and may be dispensed in spray form by a variety of methods known to those skilled in the art. Exemplary systems for dispensing liquids as a nasal spray are disclosed in U.S. Pat. No. 4,511,069. The formulations may be presented in multi-dose containers, for example in the sealed dispensing system disclosed in U.S. Pat. No. 4,511,069. Additional aerosol delivery forms may include, *e.g.*, compressed air-, jet-, ultrasonic-, and piezoelectric nebulizers, which deliver the compound or pharmaceutical composition according to the present invention dissolved or suspended in a pharmaceutical solvent, *e.g.*, water, ethanol, or a mixture thereof.

[0055] For example, a nebulizer may be selected on the basis of allowing the formation of an aerosol of a modulator disclosed herein. The delivered amount of a modulator provides a therapeutic effect for the diseases disclosed herein. The nebulizer may deliver an aerosol comprising a mass median aerodynamic diameter from about 2 microns to about 5 microns with a geometric standard deviation less than or equal to about 2.5 microns, a mass median aerodynamic diameter from about 2.5 microns to about 4.5 microns with a geometric standard deviation less than or equal to about 1.8 microns, and a mass median aerodynamic diameter from about

2.8 microns to about 4.3 microns with a geometric standard deviation less than or equal to about 2 microns. In other instances, the aerosol can be produced using a vibrating mesh nebulizer. An example of a vibrating mesh nebulizer includes the PARI E-FLOW™ nebulizer or a nebulizer using PARI eFlow technology. More examples of nebulizers are provided in U.S. Pat. Nos. 4,268,460; 4,253,468; 4,046,146; 3,826,255; 4,649,911; 4,510,929; 4,624,251; 5,164,740; 5,586,550; 5,758,637; 6,644,304; 6,338,443; 5,906,202; 5,934,272; 5,960,792; 5,971,951; 6,070,575; 6,192,876; 6,230,706; 6,349,719; 6,367,470; 6,543,442; 6,584,971; 6,601,581; 4,263,907; 5,709,202; 5,823,179; 6,192,876; 6,644,304; 5,549,102; 6,083,922; 6,161,536; 6,264,922; 6,557,549; and 6,612,303; all of which are hereby incorporated by reference in their entireties. More commercial examples of nebulizers that can be used with the CaCC modulators and the NKCC modulators described herein include Respirgard II™, Aeroneb™, Aeroneb™ Pro, and Aeroneb™ Go produced by Aerogen; AERx™ and AERx Essence™ produced by Aradigm; Porta-Neb™, Freeway Freedom™, Sidestream, Ventstream and I-neb produced by Respirationics, Inc. (Murrysville, PA); and PARI LC-Plus™, PARI LC-Start, produced by PARI Respiratory Equipment Inc. (Midlothian, VA). By further non-limiting example, U.S. Pat. No. 6,196,219, is hereby incorporated by reference in its entirety.

[0056] Suitable, non-limiting examples of dosages of a CaCC modulator and/or a NKCC modulator according to the present invention administered, *e.g.*, via a nebulizer to an adult human may be from about 0.1 mg/m²/day to 100 mg/m²/day, such as from about 0.5 mg/m²/day to about 80 mg/m²/day, including from about 1 mg/m²/day to about 50 mg/m²/day, about 1 mg/m²/day to about 20 mg/m²/day, about 1 mg/m²/day to about 10 mg/m²/day, about 1 mg/m²/day to about 7 mg/m²/day, or about 3 mg/m²/day to about 7 mg/m²/day. Other representative dosages of a

modulator of the present invention include about 0.1 mg/m²/day, 0.2 mg/m²/day, 0.3 mg/m²/day, 0.4 mg/m²/day, 0.5 mg/m²/day, 0.6 mg/m²/day, 0.7 mg/m²/day, 0.8 mg/m²/day, 0.9 mg/m²/day, 1 mg/m²/day, 2 mg/m²/day, 3 mg/m²/day, 4 mg/m²/day, 5 mg/m²/day, 6 mg/m²/day, 7 mg/m²/day, 8 mg/m²/day, 9 mg/m²/day, 10 mg/m²/day, 11 mg/m²/day, 12 mg/m²/day, 13 mg/m²/day, 14 mg/m²/day, 15 mg/m²/day, 16 mg/m²/day, 17 mg/m²/day, 18 mg/m²/day, 19 mg/m²/day, 20 mg/m²/day, 25 mg/m²/day, 30 mg/m²/day, 35 mg/m²/day, 40 mg/m²/day, 45 mg/m²/day, 50 mg/m²/day, 55 mg/m²/day, 60 mg/m²/day, 65 mg/m²/day, 70 mg/m²/day, 75 mg/m²/day, 80 mg/m²/day, 85 mg/m²/day, 90 mg/m²/day, 95 mg/m²/day, or 100 mg/m²/day. Dosages may be reduced in a child. As noted above, the effective dose of a modulator may be administered as two, three, four, five, six or more sub-doses, administered separately at appropriate intervals throughout the day, with the proviso that the doses of the CaCC and NKCC modulators simultaneously block the CaCC and NKCC.

[0057] Nasal and pulmonary spray solutions of the present invention typically comprise the modulators or pharmaceutical composition to be delivered, optionally formulated with a surface-active agent, such as a nonionic surfactant (e.g., polysorbate-80), and one or more buffers. In some embodiments of the present invention, the nasal spray solution further comprises a propellant. The pH of the nasal spray solution is optionally between about pH 3.0 and 6.0, such as 5.0 +/- 0.3. Suitable buffers for use with the modulators or pharmaceutical compositions are as described herein or as otherwise known in the art. Other components may be added to enhance or maintain chemical stability, including preservatives, surfactants, dispersants, or gases. Suitable preservatives include, but are not limited to, phenol, methyl paraben, paraben, m-cresol, thiomersal, chlorobutanol, benzylalkonium

chloride, and the like. Suitable surfactants include, but are not limited to, oleic acid, sorbitan trioleate, polysorbates, lecithin, phosphatidyl cholines, and various long chain diglycerides and phospholipids. Suitable dispersants include, but are not limited to, ethylenediaminetetraacetic acid, and the like. Suitable gases include, but are not limited to, nitrogen, helium, chlorofluorocarbons (CFCs), hydrofluorocarbons (HFCs), carbon dioxide, air, and the like.

[0058] Within alternate embodiments, mucosal formulations of the present invention may be administered as dry powder formulations comprising the CaCC and NKCC modulators or pharmaceutical compositions according to the present invention in a dry, usually lyophilized, form of an appropriate particle size, or within an appropriate particle size range, for intranasal delivery. Minimum particle size appropriate for deposition within the nasal or pulmonary passages is often about 0.5 μm mass median equivalent aerodynamic diameter (MMEAD), commonly about 1 μm MMEAD, and more typically about 2 μm MMEAD. Maximum particle size appropriate for deposition within the nasal passages is often about 10 μm MMEAD, commonly about 8 μm MMEAD, and more typically about 4 μm MMEAD. Intranasally respirable powders within these size ranges can be produced by a variety of conventional techniques, such as jet milling, spray drying, solvent precipitation, supercritical fluid condensation, and the like. These dry powders of appropriate MMEAD can be administered to a patient via a conventional dry powder inhaler (DPI), which rely on the patient's breath, upon pulmonary or nasal inhalation, to disperse the powder into an aerosolized amount. Alternatively, the dry powder may be administered via air-assisted devices that use an external power source to disperse the powder into an aerosolized amount, *e.g.*, a piston pump.

[0059] Dry powder devices typically require a powder mass in the range from about 1 mg to 20 mg to produce a single aerosolized dose ("puff"). If the required or desired dose of the compound or pharmaceutical composition according to the present invention is lower than this amount, the powdered active agent will typically be combined with a pharmaceutical dry bulking powder to provide the required total powder mass. Preferred dry bulking powders include sucrose, lactose, dextrose, mannitol, glycine, trehalose, human serum albumin (HSA), and starch. Other suitable dry bulking powders include cellobiose, dextrans, maltotriose, pectin, sodium citrate, sodium ascorbate, and the like.

[0060] To formulate compositions for mucosal delivery within the present invention, the compound or pharmaceutical composition according to the present invention can be combined with various pharmaceutically acceptable additives, as well as a base or carrier for dispersion of the active agent(s). Desired additives include, but are not limited to, pH control agents, such as arginine, sodium hydroxide, glycine, hydrochloric acid, citric acid, etc. In addition, local anesthetics (e.g., benzyl alcohol), isotonicizing agents (e.g., sodium chloride, mannitol, sorbitol), adsorption inhibitors (e.g., Tween 80), solubility enhancing agents (e.g., cyclodextrins and derivatives thereof), stabilizers (e.g., serum albumin), and reducing agents (e.g., glutathione) can be included. When the composition for mucosal delivery is a liquid, the tonicity of the formulation, as measured with reference to the tonicity of 0.9% (w/v) physiological saline solution taken as unity, is typically adjusted to a value at which no substantial, irreversible tissue damage will be induced in the nasal mucosa at the site of administration. Generally, the tonicity of the solution is adjusted to a value of about 1/3 to 3, more typically 1/2 to 2, and most often 3/4 to 1.7.

[0061] The CaCC and NKCC modulators or pharmaceutical compositions of the present invention may be dispersed in a base or vehicle, which may comprise a hydrophilic compound having a capacity to disperse the compounds or compositions of the present invention and any desired additives. The base may be selected from a wide range of suitable carriers, including but not limited to, copolymers of polycarboxylic acids or salts thereof, carboxylic anhydrides (*e.g.* maleic anhydride) with other monomers (*e.g.* methyl (meth)acrylate, acrylic acid, etc.), hydrophilic vinyl polymers such as polyvinyl acetate, polyvinyl alcohol, polyvinylpyrrolidone, cellulose derivatives such as hydroxymethylcellulose, hydroxypropylcellulose, etc., and natural polymers such as chitosan, collagen, sodium alginate, gelatin, hyaluronic acid, and nontoxic metal salts thereof. Often, a biodegradable polymer is selected as a base or carrier, for example, polylactic acid, poly(lactic acid-glycolic acid) copolymer, polyhydroxybutyric acid, poly(hydroxybutyric acid-glycolic acid) copolymer and mixtures thereof. Alternatively or additionally, synthetic fatty acid esters such as polyglycerin fatty acid esters, sucrose fatty acid esters, etc. can be employed as carriers. Hydrophilic polymers and other carriers can be used alone or in combination, and enhanced structural integrity can be imparted to the carrier by partial crystallization, ionic bonding, crosslinking and the like. The carrier can be provided in a variety of forms, including, fluid or viscous solutions, gels, pastes, powders, microspheres and films for direct application to the nasal mucosa. The use of a selected carrier in this context may result in promotion of absorption of the compound or composition according to the present invention.

[0062] The CaCC and NKCC modulators or pharmaceutical compositions of the present invention can be combined with the base or carrier according to a variety of methods, and release of the CaCC and NKCC modulators or pharmaceutical

compositions of the present invention may be by diffusion, disintegration of the carrier, or associated formulation of water channels. In some circumstances, the active agent(s) is/are dispersed in microcapsules (microspheres) or nanocapsules (nanospheres) prepared from a suitable polymer, *e.g.*, isobutyl 2-cyanoacrylate and dispersed in a biocompatible dispersing medium applied to the nasal mucosa, which yields sustained delivery and biological activity over a protracted time.

[0063] To further enhance mucosal delivery of CaCC and NKCC modulators or pharmaceutical compositions of the present invention, formulations comprising such agents may also contain a hydrophilic low molecular weight compound as a base or excipient. Such hydrophilic low molecular weight compounds provide a passage medium through which a water-soluble active agent, such as a physiologically active peptide or protein, may diffuse through the base to the body surface where the active agent is absorbed. The hydrophilic low molecular weight compound optionally absorbs moisture from the mucosa or the administration atmosphere and dissolves the water-soluble active peptide. The molecular weight of the hydrophilic low molecular weight compound is generally not more than 10,000 and preferably not more than 3,000. Exemplary hydrophilic low molecular weight compounds include polyol compounds, such as oligo-, di- and monosaccharides such as sucrose, mannitol, sorbitol, lactose, L-arabinose, D-erythrose, D-ribose, D-xylose, D-mannose, trehalose, D-galactose, lactulose, cellobiose, gentibiose, glycerin and polyethylene glycol. Other examples of hydrophilic low molecular weight compounds useful as carriers within the invention include N-methylpyrrolidone, and alcohols (*e.g.* oligovinyl alcohol, ethanol, ethylene glycol, propylene glycol, etc.) These hydrophilic low molecular weight compounds can be used alone or in combination with one another or with other active or inactive components of the intranasal formulation.

[0064] In sum, mucosal administration according to the invention allows effective self-administration of treatment by patients, provided that sufficient safeguards are in place to control and monitor dosing and side effects. Mucosal administration also overcomes certain drawbacks of other administration forms, such as injections, that are painful and expose the patient to possible infections and may present drug bioavailability problems. For nasal and pulmonary delivery, systems for controlled aerosol dispensing of therapeutic liquids as a spray are well known. For example, metered doses of CaCC and NKCC modulators or pharmaceutical compositions of the present invention are delivered by means of a specially constructed mechanical pump valve, U.S. Pat. No. 4,511,069.

[0065] In the present invention, other methods of delivery may also be used. Such methods include, for example, administration by oral ingestion, or as an ointment or drop for local administration to the eyes, or for parenteral or other administration in any appropriate manner such as intraperitoneal, subcutaneous, topical, intradermal, rectal, vaginal, sublingual, intramuscular, intravenous, intraarterial, intrathecal, or intralymphatic. Further, a pharmaceutical composition of the present invention may be administered in conjunction with other treatments. A pharmaceutical composition of the present invention may be encapsulated or otherwise protected against gastric or other secretions, if desired.

[0066] Suitable, non-limiting examples of dosages of a CaCC modulator according to the present invention administered, *e.g.*, via oral ingestion or via injection, to an adult human may be from about 0.05 mg/day to 20 mg/day, such as from about 0.1 mg/day to about 10 mg/day, including from about 0.5 mg/day to about 2 mg/day. Other representative dosages of a modulator of the present invention include about 0.1 mg/day, 0.2 mg/day, 0.3 mg/day, 0.4 mg/day 0.5 mg/day, 0.6

mg/day, 0.7 mg/day, 0.8 mg/day, 0.9 mg/day, 1 mg/day, 2 mg/day, 3 mg/day, 4 mg/day, 5 mg/day, 6 mg/day, 7 mg/day, 8 mg/day, 9 mg/day, 10 mg/day, 11 mg/day, 12 mg/day, 13 mg/day, 14 mg/day, 15 mg/day, 16 mg/day, 17 mg/day, 18 mg/day, 19 mg/day, or 20 mg/day. Suitable, non-limiting examples of dosages of a NKCC modulator according to the present invention administered, *e.g.*, via oral ingestion or via topical application, to an adult human may be from about 0.1 g/day to 5 g/day, such as from about 0.25 g/day to about 2 g/day, including from about 0.75 g/day to about 1.5 g/day. Other representative dosages of a modulator of the present invention include about 0.1 g/day, 0.2 g/day, 0.3 g/day, 0.4 g/day, 0.5 g/day, 0.6 g/day, 0.7 g/day, 0.8 g/day, 0.9 g/day, 1 g/day, 2 g/day, 3 g/day, 4 g/day, or 5 g/day. Dosages may be reduced in a child. As noted above, the effective dose of a modulator may be administered as two, three, four, five, six or more sub-doses, administered separately at appropriate intervals throughout the day, with the proviso that the doses of the CaCC and NKCC modulators simultaneously block the CaCC and NKCC.

[0067] The pharmaceutical compositions of the invention comprise one or more active ingredients, *e.g.*, CaCC and NKCC modulators, in admixture with one or more pharmaceutically-acceptable carriers and, optionally, one or more other compounds, drugs, ingredients and/or materials, including, *e.g.*, β -agonists. Regardless of the route of administration selected, the modulators/pharmaceutical compositions of the present invention are formulated into pharmaceutically-acceptable dosage forms, including unit dosage forms, by conventional methods known to those of skill in the art. See, *e.g.*, Remington, The Science and Practice of Pharmacy (21st Edition, Lippincott Williams and Wilkins, Philadelphia, PA.).

[0068] Pharmaceutically acceptable carriers are well known in the art (see, e.g., Remington, The Science and Practice of Pharmacy (21st Edition, Lippincott Williams and Wilkins, Philadelphia, PA.) and The National Formulary (American Pharmaceutical Association, Washington, D.C.)) and include sugars (e.g., lactose, sucrose, mannitol, and sorbitol), starches, cellulose preparations, calcium phosphates (e.g., dicalcium phosphate, tricalcium phosphate and calcium hydrogen phosphate), sodium citrate, water, aqueous solutions (e.g., saline, sodium chloride injection, Ringer's injection, dextrose injection, dextrose and sodium chloride injection, lactated Ringer's injection), alcohols (e.g., ethyl alcohol, propyl alcohol, and benzyl alcohol), polyols (e.g., glycerol, propylene glycol, and polyethylene glycol), organic esters (e.g., ethyl oleate and tryglycerides), biodegradable polymers (e.g., polylactide-polyglycolide, poly(orthoesters), and poly(anhydrides)), elastomeric matrices, liposomes, microspheres, oils (e.g., corn, germ, olive, castor, sesame, cottonseed, and groundnut), cocoa butter, waxes (e.g., suppository waxes), paraffins, silicones, talc, silicylate, etc. Each pharmaceutically acceptable carrier used in a pharmaceutical composition of the invention must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject. Carriers suitable for a selected dosage form and intended route of administration are well known in the art, and acceptable carriers for a chosen dosage form and method of administration can be determined using ordinary skill in the art. More generally, "pharmaceutically acceptable" means that which is useful in preparing a pharmaceutical composition that is generally safe, non-toxic, and neither biologically nor otherwise undesirable and includes that which is acceptable for veterinary use as well as human pharmaceutical use.

[0069] The pharmaceutical compositions of the invention may, optionally, contain additional ingredients and/or materials commonly used in such pharmaceutical compositions. These ingredients and materials are well known in the art and include (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and silicic acid; (2) binders, such as carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, hydroxypropylmethyl cellulose, sucrose and acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, sodium starch glycolate, cross-linked sodium carboxymethyl cellulose and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, and sodium lauryl sulfate; (10) suspending agents, such as ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth; (11) buffering agents; (12) excipients, such as lactose, milk sugars, polyethylene glycols, animal and vegetable fats, oils, waxes, paraffins, cocoa butter, starches, tragacanth, cellulose derivatives, polyethylene glycol, silicones, bentonites, silicic acid, talc, salicylate, zinc oxide, aluminum hydroxide, calcium silicates, and polyamide powder; (13) inert diluents, such as water or other solvents; (14) preservatives; (15) surface-active agents; (16) dispersing agents; (17) control-release or absorption-delaying agents, such as hydroxypropylmethyl cellulose, other polymer matrices, biodegradable polymers, liposomes, microspheres, aluminum monostearate, gelatin, and waxes; (18) opacifying agents; (19) adjuvants; (20) wetting agents; (21)

emulsifying and suspending agents; (22), solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan; (23) propellants as disclosed above, such as hydrofluoroalkane, particularly 1,1,1,2-tetrafluoroethane, heptafluoroalkane (HFA) such as 1,1,1,2,3,3,3-heptafluoro-n-propane or mixtures thereof, as well as other chlorofluorohydrocarbons and other volatile unsubstituted hydrocarbons, such as butane and propane; (24) antioxidants; (25) agents which render the formulation isotonic with the blood of the intended recipient, such as sugars and sodium chloride; (26) thickening agents; (27) coating materials, such as lecithin; and (28) sweetening, flavoring, coloring, perfuming and preservative agents. Each such ingredient or material must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject. Ingredients and materials suitable for a selected dosage form and intended route of administration are well known in the art, and acceptable ingredients and materials for a chosen dosage form and method of administration may be determined using ordinary skill in the art.

[0070] Pharmaceutical compositions suitable for oral administration may be in the form of capsules, cachets, pills, tablets, powders, granules, a solution or a suspension in an aqueous or non-aqueous liquid, an oil-in-water or water-in-oil liquid emulsion, an elixir or syrup, a pastille, a bolus, an electuary or a paste. These formulations may be prepared by methods known in the art, e.g., by means of conventional pan-coating, mixing, granulation or lyophilization processes.

[0071] Solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules and the like) may be prepared, e.g., by mixing the active ingredient(s) with one or more pharmaceutically-acceptable carriers and, optionally, one or more fillers, extenders, binders, humectants, disintegrating agents, solution retarding agents, absorption accelerators, wetting agents, absorbents, lubricants, and/or coloring agents. Solid compositions of a similar type may be employed as fillers in soft and hard-filled gelatin capsules using a suitable excipient. A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using a suitable binder, lubricant, inert diluent, preservative, disintegrant, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine. The tablets, and other solid dosage forms, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein. They may be sterilized by, for example, filtration through a bacteria-retaining filter. These compositions may also optionally contain opacifying agents and may be of a composition such that they release the active ingredient only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. The active ingredient can also be in microencapsulated form.

[0072] Liquid dosage forms for oral administration include pharmaceutically-acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. The liquid dosage forms may contain suitable inert diluents commonly used in the art. Besides inert diluents, the oral compositions may also include adjuvants, such as

wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents. Suspensions may contain suspending agents.

[0073] Pharmaceutical compositions for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more active ingredient(s) with one or more suitable nonirritating carriers which are solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound. Pharmaceutical compositions which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such pharmaceutically-acceptable carriers as are known in the art to be appropriate.

[0074] Dosage forms for the topical or transdermal administration include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches, drops and inhalants as previously disclosed. The modulators/pharmaceutical compositions may be mixed under sterile conditions with a suitable pharmaceutically-acceptable carrier. The ointments, pastes, creams and gels may contain excipients. Powders and sprays may contain excipients and propellants as previously disclosed.

[0075] Pharmaceutical compositions suitable for parenteral administrations comprise one or more of each kind of CaCC and NKCC modulators in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or non-aqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain suitable antioxidants, buffers, solutes which render the formulation isotonic with the blood of the intended recipient, or suspending or thickening agents. Proper fluidity can be maintained, for example, by the use of coating materials, by the maintenance of the required particle size in the case of

dispersions, and by the use of surfactants. These compositions may also contain suitable adjuvants, such as wetting agents, emulsifying agents and dispersing agents. It may also be desirable to include isotonic agents. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption.

[0076] In some cases, in order to prolong the effect of a drug (*e.g.*, pharmaceutical formulation), it is desirable to slow its absorption from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility.

[0077] The rate of absorption of the CaCC and NKCC modulators then depends upon their rates of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered CaCC and NKCC modulators may be accomplished by dissolving or suspending the CaCC and NKCC modulators in an oil vehicle. Injectable depot forms may be made by forming microencapsule matrices of the CaCC and NKCC modulators in biodegradable polymers. Depending on the ratio of the CaCC and NKCC modulators to polymer, and the nature of the particular polymer employed, the rate of CaCC and NKCC modulator release can be controlled. Depot injectable formulations are also prepared by entrapping the modulators in liposomes or microemulsions which are compatible with body tissue. The injectable materials can be sterilized for example, by filtration through a bacterial-retaining filter.

[0078] The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampules and vials, and may be stored in a lyophilized condition requiring only the addition of the sterile liquid carrier, for example water for injection, immediately prior to use. Extemporaneous injection solutions and

suspensions may be prepared from sterile powders, granules and tablets of the type described above.

[0079] The following examples are provided to further illustrate the methods and compositions of the present invention. These examples are illustrative only and are not intended to limit the scope of the invention in any way.

EXAMPLES

Example 1

[0080] Epithelial denuded guinea pig tracheal rings were suspended in organ baths under 1g resting tension with continuous digital recordings of muscle force. In separate studies, guinea pig airway smooth muscle cells were enzymatically dispersed and cultured.

[0081] Induced electrophysiological changes in membrane potential and current were measured using traditional whole cell patch clamp methods. Immortalized human airway smooth muscle cells were grown to confluence on collagen-treated T25 flasks. Collagenase type IV in SmBM2 medium (Lonza, Walkersville, MD) was used to release cells adherent to the collagen matrix in the flask. Medium with cells in suspension was then harvested in a 10-ml conical tube and centrifuged at 300 x g. Supernatant was removed, and the pellet was resuspended in SmBM2 medium and transferred into collagen-treated glass bottom 1-cm Petri dishes at about 10% confluence. Each dish was then incubated at 37°C and 5% CO₂ for 1–4 hours for reattachment of cells to glass-bottom Petri dishes.

[0082] Glass-bottom dishes served as a disposable recording chamber. ALA VM-8, an 8-chamber pressure-driven drug application system, was used in a still bath of extracellular salt solution. Whole-cell intracellular voltage recordings under

current clamp conditions were performed with a 2-kHz Bessel filter, recording at 10 kHz using an Axopatch 200b amplifier (Axon Instruments, Foster City, CA, USA). Electrodes were pulled using a P-97 micropipette puller from 1.5-mm OD borosilicate capillary glass (Sutter Instruments, Novato, CA, USA). Glass electrode resistances ranged from 5 to 10 M Ω with intracellular solution. Whole-cell intracellular current recordings under voltage-clamp conditions were performed with a 2-kHz Bessel filter, recording at 10 kHz using an Axopatch 200b amplifier. For current measurements (Fig. 11) intracellular solutions consisted of (in mM) 140 TEA-Cl, 5 MgATP, 5 EGTA, 1 MgCl₂, 10 HEPES, and 5 CaCl₂ (pH 7.2). Extracellular solution consisted of (in mM) 134 NaCl, 1.4 KCl, 10 HEPES, 1 MgCl₂, 1.8 CaCl₂, and 10 glucose (pH 7.4). This induced the classic spontaneously transient inward currents (STICs) and spontaneously transient outward currents (STOCs) well described in this cell type. STOCs are believed to represent activation of calcium activated potassium (K_{Ca}) channels while STICs are believed to represent activation of CaCCs. TEA-Cl was included in the buffer to enhance STICs which were then recorded during the addition of 100 μ M niflumic acid. When recording TEA- and niflumic acid-induced responses on membrane potential (Δ mV), the intracellular solutions contained (in mM) 140 KCl, 5 MgATP, 5 EGTA, 1 MgCl₂, 10 HEPES, and 5 CaCl₂ (pH 7.2), and the extracellular solutions consisted of (in mM) 134 NaCl, 5 MgATP, 5 EGTA, 1 MgCl₂, 10 HEPES, and 5 CaCl₂ (pH 7.2). All recordings were analyzed on Clampfit 8.0 software (Molecular Devices).

[0083] Guinea pig airway smooth muscle contractions induced by membrane depolarization by K channel blockade with 10 mM tetraethylammonium chloride (TEA) were relaxed by the L-type calcium channel blocker nifedipine (111%; $p < 0.001$, $n = 5$), the NKCC blocker furosemide (77.6%, $p < 0.001$, $n = 4$), and the CaCC

channel blocker niflumic acid (82.5%, $p < 0.001$ $n=5$) (Fig. 3A) directly. TEA-induced increase in muscle force was proportional to external buffer chloride concentrations (6.30567 mM, 65.3 mM, 124 mM), an electrochemical gradient in favor of inward chloride movement (7.9 ± 5.5 , 48.1 ± 6.9 , 71.7 ± 6.3 % of control acetylcholine contraction, respectively ($N = 4-6$) (Fig 3B). In contrast, electrophysiological recordings in isolated airway smooth muscle cells in the presence of TEA revealed a CaCC dependent outward anion flow, with a current/voltage relationship consistent with chloride (Fig. 11).

[0084] These functional and electrophysiologic results suggested that a chloride cycle comprised of outward Cl flow through CaCC channels and re-uptake via NKCC is required for maintenance of a depolarized membrane potential and the maintenance of airway smooth muscle tone. Blockade of these chloride regulatory proteins offers potential therapeutic targets.

Example 2

[0085] Human muscle tissue was acquired from excess lung airways trimmed during surgery from healthy lung transplant donors. Acquired tissue was stored overnight at 20°C. Airway smooth muscle contractions measured *ex vivo* in organ baths were performed as previously described (Gallos *et al.*, 2008; Gallos *et al.*, 2009, Gallos *et al.*, 2011; Gleason *et al.*, 2010; Yim *et al.*, 2011; Mitzuta *et al.*, 2008). Closed guinea pig tracheal rings or strips of human airway smooth muscle (tracheal or main stem bronchus) were suspended in organ baths had 95% oxygen constantly perfusing Dulbecco's Modified Eagle Medium. Rings were cut on the cartilaginous borders of the smooth muscle. The epithelial layer was dissected under microscopic assistance. Briefly, tissues were suspended in a water-jacketed (37°C) 2-ml organ

bath (Radnoti Glass Technology, Monrovia, CA) and attached to a Grass FT03 force transducer (Grass Telefactor, West Warwick, RI) coupled to a computer via BioPac hardware and Acqknowledge 7.3.3 software (Biopac Systems, Goleta, CA). Krebs-Henseleit (KH) buffer was continuously bubbled with 95% oxygen and 5% carbon dioxide and tissues were allowed to equilibrate at 1 g (guinea pig) or 1.5 g (human) isotonic force for 1 hour with fresh KH buffer changes every 15 minutes.

[0086] Following equilibration, in guinea pig experiments, the capsaicin analog *N*-vanillylnonanamide (10 μ M final) was added to the organ baths to first activate and then deplete nonadrenergic, noncholinergic nerves. After *N*-vanillylnonanamide induced force had returned to baseline (about 50 minutes), the tracheal rings were washed and then subjected to two cycles of increasing cumulative concentrations of acetylcholine (0.1 μ M to 0.1 mM) to determine the EC₅₀ concentrations of acetylcholine required for each individual ring. In experiments with human tissue, no vanillylnonanamide pretreatment was done. To avoid bias between treatment groups, tissues were contracted to individually calculated EC₅₀ values for acetylcholine and tissues with similar E_{max} values were randomly assigned to treatments within individual experiments. Following extensive KH buffer changes (8–9 times) tissues were allowed to stabilize at isotonic resting tension (about 1.0 g). To remove confounding effects of other procontractile pathways, each bath received a complement of antagonists 20 minutes prior to subsequent contractile challenge. The antagonists included pyrilamine (10 μ M; H₁ histamine receptor antagonist), and tetrodotoxin (1 μ M; blocker of endogenous cholinergic or C-fiber neuronal effects) in guinea pig experiments or pyrilamine and 10 μ M MK571 (leukotriene receptor antagonist) in human tissue experiments.

[0087] Following these preliminary contractile challenges and pretreatments, one of two paradigms was utilized; a single contractile challenge (e.g. TEA in Fig. 20) or repetitive challenges with an EC₅₀ concentration of acetylcholine, interspersed with buffer changes and redosing of pyrilamine/tetrodotoxin/MK571. For the repetitive challenges control responses were first established. After three control challenges with an EC₅₀ of acetylcholine, tissues were pretreated with either the chloride channel blocker niflumic acid (10-100 μ M) alone, the NKCC blockers bumetanide (10 μ M) or furosemide (100 μ M) alone or a combination of niflumic acid with bumetanide. Following three repetitive acetylcholine challenges in the presence of these blockers, three recovery acetylcholine challenges were performed to determine the reversibility of the blocker effect and confirm functional recovery of smooth muscle contractile function (See, Fig. 9).

[0088] The difference was insignificant between control contractions and the vehicle treatment. Treatments of niflumic acid and bumex showed significant attenuation by $51 \pm 5.976\%$ SEM compared to control and was statistically significant ($n=3$, $p<0.05$). Recovery contractions, after thorough washing, showed a loss of significant difference as compared to control (See, Fig. 10).

[0089] These experiments showed that the combination of calcium activated chloride channel blockade and sodium potassium chloride co-transporter blockade have significant contractile antagonistic effects on human airway smooth muscle *in vitro*. These data suggest that this combination of drugs deactivates contractile mechanisms involving membrane depolarization and/or membrane potential dependent calcium entry. This combination can flourish as a viable treatment option for airway hyperresponsiveness and can be a viable alternative or an adjunct to β -agonist therapy.

Example 3

[0090] The following experiments further demonstrate that blockade of the plasma membrane chloride cycle relaxes human airway smooth muscle. The experiments show, *inter alia*, that simultaneous blockade of CaCC and NKCC is necessary and sufficient to attenuate contraction of airway smooth muscle in response to classic contractile agonists (acetylcholine, histamine, substance P) both in isolated human and guinea pig airways *in vitro* and in intact guinea pig airways *in vivo*.

[0091] A model of bronchoconstriction in intact guinea pigs and isolated ASM from large airways of both humans and guinea pigs will be used to demonstrate that simultaneous blockade of the CaCC (for example, by using niflumic acid, 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) and talnifumate) and NKCC (for example, by using bumetanide, furosemide) are necessary and sufficient to attenuate ASM contraction or airway constriction in response to diverse contractile agonists (acetylcholine, histamine, tachykinins, vagal nerve stimulation).

[0092] Complementary *in vivo* and *in vitro* approaches will be used. For *in vivo* experiments, guinea pigs will be used because the inventors have shown complementary data in guinea pigs and humans in isolated airway studies, and because both intravenous or aerosolized delivery of chloride channel/transporter blockers can be performed before bronchoconstriction is induced with intravenous challenges (*e.g.* acetylcholine, histamine, tachykinins) or vagal nerve stimulation in guinea pigs. Although *in vivo* studies have distinct advantages including high translational potential, complex physiological interactions may limit mechanistic studies. In contrast, *in vitro* studies eliminate complex external physiological influences. *In vitro*, freshly isolated airways from human (excess tracheal and

bronchial smooth muscle from lung donors) and guinea pig will be used. Thus, guinea pig ASM will be used for exploratory experiments and definitive experiments will be performed on human ASM. Additionally, mechanistic electrophysiologic and ion flux studies will be performed in both freshly isolated and cultured human ASM cells. By utilizing both *in vitro* and *in vivo* experimental approaches, the limitations incurred by each system are minimized.

Example 4

[0093] Natural native ligand acetylcholine and salts (K-gluconate and TEA-acetate) will be used for contracting/depolarizing airway smooth muscle. Acetylcholine is a common ligand used in *in vitro* contraction assays as it is a natural endogenous constrictor of ASM. However, cell signaling events following acetylcholine are very complex including the activation of both Gi (via M2 muscarinic receptors) and Gq (via M3 muscarinic receptors) which in turn activate calcium release from the SR following inositol triphosphate (IP₃) synthesis, inhibition of synthesis of cyclic AMP, activation of the small G proteins including RhoA (modulating calcium sensitivity) and depolarization of the membrane potential. Therefore, dissecting cellular mechanisms using acetylcholine-induced contractions are difficult. To study the isolated effects of membrane potential and increases in intracellular calcium on SR calcium refilling and activation of RhoA, two contractile agonists that are devoid of direct G protein activation will also be used. KCl induces membrane depolarization and increases intracellular calcium while tetraethylammonium (TEA) chloride depolarizes the plasma membrane without an increase in intracellular calcium (Fig. 7). Potassium gluconate and TEA-acetate will be used to retain the depolarizing effects of the cations of these reagents without the confounding effects of adding large concentrations of chloride while studying chloride

handling. Thus, K-gluconate and TEA-acetate will be useful to separate out the mechanistic effects of membrane potential and increased intracellular calcium in SR calcium refilling and RhoA activation studies in both isolated native airways and isolated ASM cells.

Example 5

[0094] Simultaneous blockade of the CaCC and NKCC is required to block acetylcholine-induced contraction of airway smooth muscle.

[0095] In native ASM strips from human lung transplants, simultaneous pretreatment with blockers of CaCC and NKCC reduces the magnitude of an acetylcholine induced contraction (Fig. 1, top tracings). In contrast, separate blockade of CaCC with niflumic acid or separate blockade of NKCC with bumetanide does not significantly reduce contraction (Fig. 1 and Fig. 2). These results explain why separate blockade of these chloride-handling pathways in ASM cells did not result in significant attenuation of contractions. These results are important because these are functional contractions in native human ASM and these contractions are in response to a classic endogenous constrictor of ASM (acetylcholine).

[0096] Contractile studies were then performed in guinea pig tracheal rings using 10 mM tetraethylammonium (TEA) chloride to induce a contraction dependent initially solely on membrane depolarization. It was reasoned that if the hypotheses that niflumic acid hyperpolarized the plasma membrane while another NKCC blocker, furosemide, stabilized membrane potential (resisting depolarization) that pretreatment with either of these drugs alone should attenuate a TEA-induced depolarizing contraction. These hypotheses were confirmed as demonstrated in Fig. 3A.

[0097] In vivo airway pressure in guinea pigs was measured as follows. Male Hartley guinea pigs (about 400 g) were anesthetized and instrumented in this established model of airway inflation pressure measurements in protocols previously described (Sunaga *et al.*, 2010, Gleason *et al.*, 2009; Jooste *et al.*, 2007) and approved by the Columbia University Institutional Animal Care and Use Committee. Anesthesia was induced by intraperitoneal injection of urethane (1.5 g/kg) and increased by 0.5 g i.p. until lack of foot pinch response before the start of the procedure. Urethane was chosen for its long duration of action (about 10 hours) and lack of influence on respiratory nerve function. Animals received a tracheostomy with a 1-inch 14-g angiocatheter attached to a microventilator (model 683; Harvard Apparatus, South Natick, MA; IMV, volume control, tidal volume 2.6 ml, 66 breaths/min). The ventilator circuit was connected via side ports to two separate pressure monitors with different sensitivities (TSD160B 0–12.5 cmH₂O and TSD160C 0–25 cmH₂O; Biopac Systems, Goleta, CA) using rigid pressure tubing and was continuously monitored and recorded using Acqknowledge software. Animals then received bilateral external jugular catheters by using PE-50 tubing for a continuous succinylcholine infusion (5 mg·kg⁻¹·h⁻¹), started after a bolus of 1.5 mg/kg to remove any influence of chest wall or diaphragm muscle tone on airway pressures, and an independent catheter for delivery of study drugs. A carotid arterial line was placed to monitor blood pressure and heart rate and to ensure adequate depth of anesthesia by monitoring hemodynamic responses. After preparation, each animal received increasing intravenous acetylcholine (4–28 µg/kg, i.v.) until consistent increases in peak pulmonary inflation pressures (P_{pi}; 50–100% above baseline) were achieved. Animals were then pretreated 12 minutes before repetitive acetylcholine challenges with vehicle (100 µl DMSO, i.v.) or 5 mg niflumic acid i.v. +

5 mg furosemide i.v. The optimized acetylcholine dose for each animal was then injected 6 times at 30 second intervals while continuously measuring airway pressure responses (peak pulmonary inflation pressure) and hemodynamics.

[0098] Whether simultaneous blockade of CaCC (with intravenous (i.v.) niflumic acid) and NKCC (with i.v. furosemide) could attenuate *in vivo* bronchoconstriction was questioned. These studies were performed in a well-characterized *in vivo* guinea pig model of bronchoconstriction measured by an increase in pulmonary inflation pressures following intravenous-, aerosolized- or vagal nerve-induced bronchoconstriction. As shown in Fig. 4, pretreatment with blockers of both CaCC and NKCC resulted in an attenuation of bronchoconstriction during repetitive challenges with i.v. acetylcholine at 30 second intervals. These results agree with *in vitro* data demonstrated in isolated airway rings in Fig. 1 and are consistent with the mechanistic hypothesis that blockade of these Cl⁻ pathways interrupts refilling of intracellular Ca²⁺ stores resulting in sequential decreases in contractile forces.

Example 6

[0099] The Example 5 findings will be confirmed by using additional blockers of CaCC (alone and in combination with NKCC blockade) in organ bath force measurements in human and guinea pig ASM strips contracted with acetylcholine, K gluconate or TEA. Specifically, NPPB, a CaCC blocker with a broader selectivity for CaCC subtypes, will be compared to niflumic acid. NPPB is believed to block CaCC on both plasma membrane and sarcoplasmic reticulum (SR) membrane while niflumic acid blockade is thought to be limited to plasma membrane CaCC. The results of this comparison will be important in the mechanistic studies to determine a potential role for CaCC on the SR in modulating Cl⁻ influx to balance charge

generation during Ca^{2+} refilling of the SR. Additionally, CaCC blockers previously used clinically as anti-inflammatory (diclofenac) or anti-mucus (talnifumate (niflumic acid pro-drug) therapies may also be used.

Example 7

[0100] Bumetanide is clinically used as a diuretic due to blockade of NKCC. To confirm that blockade of NKCC is the mechanism of action responsible for the results disclosed herein, NKCC will be inhibited using furosemide, another clinically used but chemically distinct NKCC blocker in organ bath force measurements (alone and in combination with CaCC blockade) in native ASM from humans and guinea pigs contracted with acetylcholine, K gluconate or TEA.

[0101] Although acetylcholine is a classic constrictor of ASM contraction *in vivo*, additional contractile agonists contribute to *in vivo* contraction in humans including histamine, tachykinins and leukotrienes. One effective combination of CaCC and NKCC inhibitor against these alternative contractile mediators will be used in native ASM of human and guinea pig to determine whether simultaneous blockade of CaCC and NKCC block ASM contraction induced by a wide range of contractile mediators.

[0102] ASM rings will be flash frozen from experimental paradigms outlined above for the measurement of RhoA activation via a rhotekin pull-down assay and myosin light chain phosphorylation via immunoblotting.

[0103] Guided by the results in isolated native guinea pig airway rings, the effects of separate and simultaneous blockade of CaCC and NKCC against contractile mediators will be measured in guinea pigs *in vivo*. A well-established model of pulmonary inflation measurements will be used in urethane-anesthetized

guinea pigs in response to intravenous, aerosolized or vagal nerve induced smooth muscle constriction. Following the establishment of a repetitive baseline response to intravenous or aerosolized acetylcholine or histamine or vagal nerve stimulation, blockers of CaCC (e.g. niflumic acid, diclofenac, talnifumate) plus blockers of NKCC (furosemide, bumetanide) will be given as pretreatments (intravenously or by aerosol) before subsequent re-challenge with the contractile mediators.

Example 8

[0104] The following experiments demonstrate that the mechanism(s) of relaxation induced by modulation of the chloride cycle at the plasma membrane is mediated by changes in membrane potential and intracellular chloride concentrations, which in turn impair refilling of the sarcoplasmic reticulum with calcium and calcium sensitization of the contractile apparatus of human airway smooth muscle. The experiments show that hyperpolarization of the plasma membrane by blockade of CaCCs results in reduced depolarization-induced activation of rhoA resulting in reduced phosphorylation of myosin light chain in both native human airway smooth muscle and cultured human airway smooth muscle. They also show that reduced intracellular concentrations of chloride due to blockade of NKCCs results in impaired refilling of the sarcoplasmic reticulum with calcium due to insufficient balance of charge generation normally accomplished by chloride influx from the cytosol to SR.

[0105] The mechanistic underpinnings of the effects of separate and simultaneous blockade of CaCC and NKCC on membrane potential, chloride flux, store-operated calcium entry (SOCE) and RhoA activation/calcium sensitivity will be

studied in freshly isolated native ASM from human and guinea pig and freshly isolated and cultured human ASM cells.

[0106] Freshly isolated native and cultured human ASM cells will be used for the measurement of membrane potential (by both classic current clamp whole cell recordings and the potentiometric fluorescent probe FLIPR), calcium activation assays (fluorescent fluo4-AM), chloride flux assays (fluorescent MQAE assays) and RhoA activation assays (rhotekin-bead binding of GTP-bound RhoA). The mechanism to be addressed in this Example is summarized in Fig. 15.

Example 9

[0107] It is believed that the mechanisms of blockade of acetylcholine contraction in human ASM by simultaneous blockade of CaCC and NKCC is due to effects on cellular chloride and membrane potential. These effects on membrane potential in turn modulate both SR calcium refilling and activation of RhoA, which modulates calcium sensitivity.

[0108] To determine whether blockade of CaCCs or NKCCs induce membrane potential changes in cultured human airway smooth muscle cells, the FLIPR in vitro fluorescent dye assay (Molecular Devices) was used as described by Wafford *et al.* (2009). Briefly, human airway smooth muscle cells were grown to 100% confluence in 96-well black-walled plates and were washed with warmed (37°C) normal-chloride buffer [consisting of (in mM) 130 sodium chloride, 2 CaCl₂, 1 MgCl₂, 10 D-glucose, and 10 HEPES, pH 7.4] four times. A stock solution (100% dye) of FLIPR blue dye (Molecular Devices) was prepared by reconstitution of 1 vial (125 mg) with 100 ml of the normal-chloride buffer (assay buffer). A 50% working stock was prepared by further diluting the reconstituted blue dye 1:1 with assay

buffer and was used to load cells (90 μ l/well) over 20 minutes at 37°C. All reagents were dissolved in assay buffer. Baseline fluorescence was measured for 3 minutes prior to the first control additions (assay buffer). Three minutes later, airway smooth muscle cells were exposed to 100 μ M niflumic acid, 10 μ M bumetanide, 10 mM tetraethylammonium (TEA) chloride (K^+ channel blocker), 40 mM KCl (depolarization stimulus), 10 μ M NS1619 (K^+ channel opener), 60 mM potassium gluconate (depolarization stimulus), or appropriate vehicles (0.1% ethanol for niflumic acid and bumetanide). The fluorescence produced by membrane potential change following solution additions was quantified after subtracting changes induced by assay buffer alone.

[0109] To determine whether blockade of CaCCs or NKCCs induce changes in intracellular chloride concentrations in human airway smooth muscle cells, the MQAE in vitro fluorescent dye assay (Invitrogen) was used. Briefly, human airway smooth muscle cells were grown to 100% confluence in 96-well black-walled plates. Cells were loaded overnight with 10 mM MQAE in serum-free basal cell culture medium (M199, Invitrogen) and were washed once with warmed (37°C) normal-chloride buffer [consisting of (in mM) 130 sodium chloride, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, and 10 HEPES, pH 7.4]. Fluorescence was measured at 2 second intervals (excitation/emission 350/460 nm) before and after the addition of 10 mM TEA-acetate, 100 μ M niflumic acid, 10 μ M bumetanide, or a combination of niflumic acid/bumetanide or appropriate vehicles. An increase in fluorescence represents decreased intracellular chloride (removal of halide quenching of MQAE) while decreased fluorescence represents increased intracellular chloride (enhanced halide quenching of MQAE).

[0110] To demonstrate that not all depolarizing stimuli result in an increase in intracellular calcium and that extracellular chloride concentrations influence the magnitude of store-operated calcium entry (SOCE), human airway smooth muscle cells plated on 96-well plates were incubated in 100 μ l Hanks' balanced salt solution (HBSS) (in mM: NaCl 138, KCl 5, CaCl_2 2.5, MgSO_4 0.4, MgCl_2 0.5, Na_2HPO_4 0.34, NaHCO_3 4.2, KH_2PO_4 0.44, D-glucose 5.5, HEPES 20, pH 7.4) containing 5 μ M Fluo-4 AM (DMSO vehicle final concentration of 0.5 %), 0.05 % Pluronic[®] F-127 (DMSO vehicle final concentration of 0.25 %) and 2.5 mM probenecid for 30 minutes at 37°C. Cells were washed twice with HBSS containing 2.5 mM probenecid and left for further 30 minutes at room temperature to allow complete de-esterification of the intracellular AM esters. This buffer was exchanged at 100 μ l/well just before starting the measurement of fluorescence. The fluorescence was then continuously recorded every 5 seconds at wavelengths of 485 nm excitation and 528 nm emission using a microplate reader (FlexStation III, Molecular Devices). Fluorescence was measured before and after 10 mM TEA-acetate or 40 mM potassium gluconate to compare different depolarizing stimuli. In separate experiments the effect of extracellular chloride concentrations on SOCE was measured by the predepletion of extracellular calcium in low or high chloride buffer (55 mM versus 110 mM NaCl, osmotically balanced with Na gluconate) in the presence of thapsigargin to deplete intracellular calcium stores. Calcium induced fluorescence changes were measured with the reintroduction of 2 mM extracellular CaCl_2 .

[0111] Measurement of activated rhoA in cultured human airway smooth muscle cells were conducted as follows. Confluent cultured human airway smooth muscle cells in 10 cm^2 dishes were washed once with warm Hanks balanced salt solution (HBSS) and then stimulated for various time points with 10 mM TEA-acetate

or 60 mM potassium gluconate. Reaction was stopped by solubilizing cells in buffer. Following clearance of non-solubilized cell material, the solubilized cells were reacted with Rhotekin bound to beads, which isolated activated RhoA from total RhoA. Fractions were then analyzed on a commercially available ELISA plate that quantitates activated versus total RhoA (Cytoskeleton, Inc.)

[0112] In this example, it has been demonstrated in human ASM cells that blockade of CaCC with niflumic acid hyperpolarizes the plasma membrane (demonstrated by both whole cell current clamp and FLIPR potentiometric fluorescent probe assays) (Fig. 5) while blocking efflux of cellular chloride (demonstrated by enhanced quenching of the chloride sensitive fluorescent probe, MQAE) (Fig. 6A). Furthermore, demonstrated herein is that treatment of human ASM cells with bumetanide stabilizes membrane potential (Fig. 5A) and impairs chloride entry (Fig. 6B). These opposing effects of CaCC blockade and NKCC blockade on intracellular chloride concentrations (Fig. 6) is consistent with the hypothesis but raises an additional question: if the mechanism of NKCC blockade favoring muscle relaxation involves a reduced intracellular chloride concentration impairing charge balance in the SR during Ca^{2+} refilling, then the combined treatment with niflumic acid and bumetanide should still yield a reduced intracellular chloride concentration. This indeed occurred as demonstrated in Fig. 12.

[0113] It is believed that this reduced intracellular concentration of chloride reduces the amount of cytosolic chloride available to influx into the SR to balance charge generation during Ca^{2+} refilling (Janssen, 2002). Thus, store-operated calcium entry (SOCE) was induced by pretreating human ASM cells with thapsigargin in Ca^{2+} -free buffer. In parallel wells, 50% of the external chloride was replaced with gluconate. This resulted in a decrease in the magnitude of SOCE

consistent with the hypothesis that reduced extracellular chloride reduces intracellular chloride, which in turn reduces charge balance and the ability of the SR to refill with Ca^{2+} (Fig. 13).

[0114] The results shown in Fig. 7A demonstrated that K-gluconate and TEA depolarized ASM cells as measured in a whole cell configuration under current clamp. However, K-gluconate but not TEA elevated intracellular Ca^{2+} as measured by fluo4-AM (Fig 7B). Furthermore, TEA-acetate results in decreased intracellular chloride concentrations (Fig. 14), consistent with the hypothesis that depolarization includes efflux of Cl^- . It is believed that this is due to the degree of membrane depolarization induced by high concentrations of K gluconate (60-75 mM) as opposed to low concentrations of TEA (10 mM). This suggests that the threshold for membrane depolarization is lower than the threshold for increases in intracellular calcium. This will be further clarified in dose response studies that will evaluate the dose responses of KCl, K-gluconate and TEA-acetate for membrane potential and intracellular calcium effects. The ability of low concentrations of TEA to depolarize without increasing Ca^{2+} will be exploited to address the hypothesis that activation of rhoA signaling (and thus enhanced Ca^{2+} sensitivity) can occur independent of Ca^{2+} .

[0115] The results shown in Fig. 8 demonstrated that this low depolarizing concentration of TEA which does not increase intracellular Ca^{2+} , activates rhoA.

Example 10

[0116] Further experiments are directed at demonstrating that blockade of CaCC hyperpolarizes the cell membrane, and that blockade of NKCC resists depolarization of the cell membrane. In turn, this combined resistance to

depolarization impairs activation of rhoA which classically increases the sensitivity of the contractile proteins to calcium.

[0117] Membrane potential and intracellular Cl^- concentrations $[\text{Cl}^-]_i$ will be measured in cultured human ASM cells in response to varying concentrations (1 nM - 1 mM) of inhibitors of the CaCC (niflumic acid, flufenamic acid, 4,4'-Diisothiocyantostilbene-2,2'-disulfonate (DIDS), indanyloxyacetic acid 94 (IAA-94), NPPB) using both fluorescent potentiometric probes (FLIPR) and classical whole cell recordings under current clamp conditions. These studies are expected to confirm that CaCC blockade hyperpolarizes the plasma membrane and increases $[\text{Cl}^-]_i$. Furthermore, two of these inhibitors (NPPB and DIDS) block more members of the CaCC family than niflumic acid and will address the hypothesis that the additive effect of NKCC blockade is the removal of internal chloride that continues to efflux through niflumic-insensitive channels.

[0118] Membrane potential and $[\text{Cl}^-]_i$ will be measured in cultured human ASM cells pretreated with varying concentrations (1 nM - 1 mM) of two clinically utilized inhibitors of the NKCC (furosemide and bumetanide) before attempting to depolarize the cell with 10 mM TEA using both fluorescent potentiometric probes (FLIPR) and classical whole cell recordings under current clamp conditions. These studies are expected to confirm that NKCC blockade decreases $[\text{Cl}^-]_i$ and resists depolarization of the plasma membrane.

[0119] $[\text{Cl}^-]_i$ will be measured in cultured human ASM cells while reducing $[\text{Cl}^-]_o$ (replaced equimolar with gluconate). These experiments are expected to confirm that reducing $[\text{Cl}^-]_o$ reduces $[\text{Cl}^-]_i$, an important control for the following experiment.

[0120] Store-operated calcium entry (SOCE) will be measured by standard methods in human ASM cells (thapsigargin treatment in Ca^{2+} -free external buffer with reintroduction of external Ca^{2+}). SOCE will be measured with either blockade of NKCC or by varying the concentration of $[\text{Cl}^-]_o$ (replaced with equimolar gluconate). These experiments are expected to confirm that reducing $[\text{Cl}^-]_i$ by either reductions in $[\text{Cl}^-]_o$ or NKCC blockade result in reductions in SOCE.

[0121] A second and not mutually exclusive mechanism to explain the smooth muscle relaxation effect of membrane potential, is a direct link to rhoA activation and a change in the Ca^{2+} sensitivity of the ASM cell. rhoA activation will be measured via a rhotekin binding assay in both native and cultured human ASM cells and native guinea pig airways. The same samples will also be assayed for myosin light chain phosphorylation (a distal signaling event of rhoA activation) by immunoblotting. Native or cultured ASM will be subjected to depolarization without elevating intracellular Ca^{2+} (10 mM TEA-acetate) or depolarization with elevation of intracellular Ca^{2+} (60 mM K gluconate). These studies are expected to establish (1) a link between membrane depolarization and rhoA activation (and thus calcium sensitivity), and (2) the dependence of increases in intracellular Ca^{2+} for rhoA activation.

Example 11

[0122] Airway smooth muscle is distributed along the branching bronchioles of the lung down to the level of the respiratory bronchioles. It is believed that the airway smooth muscle of the small airways is most important in bronchoconstriction in asthma. Relaxant effects of niflumic acid were confirmed in small peripheral airways in lung slices (Fig. 16D and E). The results shown in Figure 16

demonstrated that blockade of chloride channels in small airways, directly visualized in lung slices, reverses constriction induced by a depolarizing stimulus, such as tetraethylammonium chloride. These findings further augment the therapeutic feasibility of this class of drugs in relaxing the small airways thought most important in asthma.

Example 12

[0123] The measurement of airway contraction of a luminal area of small lung airways using the *ex vivo* rat lung slice method was performed as described (Perez-Zoghbi *et al.*, 2007). The trachea was cannulated with an intravenous catheter, and after the chest cavity was opened, the collapsed lungs were reinflated with 1.3 ± 0.1 ml of 2 % agarose (low-gelling temperature) in Sigma-Hanks' Balanced Salt solution (sHBSS), followed by the injection of 0.2 ± 0.1 of air to flush the agarose-sHBSS out of the airways and into the distal alveolar space. Subsequently, a warm (37°C) solution of gelatin (type A, porcine skin, 300 bloom, 6% in sHBSS) was perfused through the intrapulmonary blood vessels via the pulmonary artery by injecting about 0.3 ml into the right ventricle. The warm agar and gelatin were gelled with cold sHBSS. A single lung lobe was removed and cut into serial sections of about 130 μm thick with a vibratome at about 4°C, starting at the lung periphery. Slices were maintained in DMEM (Invitrogen) at 37°C and 10% CO₂ for up to 3 days. At 37°C, the gelatin in the blood vessel lumen dissolved, leaving the blood vessel lumen empty. Lung slices were mounted in a custom-made perfusion chamber and held in place with a small sheet of nylon mesh. A second cover glass edged with silicone grease was placed over the lung slice. Perfusion of the lung slice was performed using a gravity-fed perfusion system. The volume of the chamber was about 100 μl

with a perfusion rate of about 800 $\mu\text{l}/\text{min}$. For phase-contrast microscopy, the lung slice was observed with an inverted microscope with a 10X objective, and images were recorded using a charge-coupled device camera and image acquisition software (Video Savant, IO Industries Inc., Ontario, Canada). Digital images were recorded in time lapse (30 frames/min). The area of the bronchiole and arteriole lumen was calculated, from each image, by pixel summing using custom-written software. Experiments were performed at room temperature.

[0124] The functional relaxation of airway smooth muscle by chloride channel blockade is thought to include a change in membrane potential (hyperpolarization) which reverses depolarization induced by contractile agonists. Figure 17 demonstrates that the chloride channel blocker niflumic acid (NFA) hyperpolarizes cell plasma membrane potential using a fluorescent dye that detects membrane potential. Figure 18 shows this hyperpolarizing effect another way by using traditional whole cell, current clamp electrophysiology methods in human airway smooth muscle cells.

Example 13

[0125] Traditional therapeutic delivery of effective compounds for bronchoconstriction in asthma occurs via nebulized or dry powder inhalation to the lungs. A limitation of the parent niflumic acid (NFA) compound used in previous examples was its poor water solubility, making it sub-optimal for formulations used in inhalation therapy. To remedy this, a water-soluble form of NFA was synthesized, the structure of which is given in Figure 19.

[0126] To synthesize this water-soluble form of NFA, the parent compound (niflumic acid, Sigma-Aldrich N0630, St. Louis, MO) was exposed to 10% methanolic

potassium hydroxide for 16 hours, followed by thin layer chromatography to confirm salt conversion. Sample was then processed via nuclear magnetic resonance (NMR) to confirm the absence of any unanticipated byproduct formation.

[0127] Airway smooth muscle contractions measured *ex vivo* in organ baths were performed as set forth above. Muscle force measurements taken from organ baths of airway smooth muscle relaxed with water-soluble or hydrophobic NFA demonstrated enhanced potency of water-soluble NFA at relaxing airway smooth muscle (Figure 20).

[0128] To determine if adding water solubility to NFA allowed it to retain its ability to inhibit acetylcholine-induced contractions in human airway smooth muscle, human airway smooth muscle strips were pretreated with either water-soluble NFA + bumetanide or a vehicle control in organ baths. Contraction magnitude was then recorded. Water-soluble NFA + bumetanide caused a reduction in magnitude of acetylcholine-induced contractions compared to controls. Thus, the water-soluble formulation of niflumic acid also impairs the repetitive acetylcholine-induced contractions in *ex vivo* human airway smooth muscle in a reversible manner (Figure 21).

[0129] The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

DOCUMENTS

[0130] All documents cited are incorporated by reference as if recited in full herein.

Ben-Chaim Y, Chanda B, Dascal N, Bezanilla F, Parnas I, Parnas H: Movement of 'gating charge' is coupled to ligand binding in a G-protein-coupled receptor. *Nature* 2006; 444: 106-9

Billups D, Billups B, Challiss RA, Nahorski SR: Modulation of Gq-protein-coupled inositol trisphosphate and Ca²⁺ signaling by the membrane potential. *Journal of Neuroscience* 2006; 26: 9983-95

Corteling RL, Li S, Giddings J, Westwick J, Poll C, Hall IP: Expression of transient receptor potential C6 and related transient receptor potential family members in human airway smooth muscle and lung tissue. *American Journal of Respiratory Cell and Molecular Biology* 2004; 30: 145-54

Deshpande DA, Wang WC, McIlmoyle EL, Robinett KS, Schillinger RM, An SS, Sham JS, Liggett SB: Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction. *Nat.Med.* 2010; 16: 1299-304

Duan QL, Tantisira KG: Pharmacogenetics of asthma therapy. *Curr.Pharm.Des* 2009; 15: 3742-53

Engelhardt B, Elliott S, Hazinski TA. Short- And Long-Term Effects Of Furosemide On Lung Function In Infants With Bronchopulmonary Dysplasia. *J Pediatr.* 1986;109(6):1034-9.

Gallos G, Gleason NR, Zhang Y, Pak SW, Sonett JR, Yang J, Emala CW: Activation of endogenous GABAA channels on airway smooth muscle potentiates isoproterenol mediated relaxation. *Am.J.Physiol Lung Cell Mol.Physiol* 2008

Gallos G, Gleason NR, Virag L, Zhang Y, Mizuta K, Whittington RA, Emala CW: Endogenous gamma-aminobutyric acid modulates tonic guinea pig airway tone and propofol-induced airway smooth muscle relaxation. *Anesthesiology* 2009; 110: 748-58

Gallos G, Yim P, Chang S, Zhang Y, Xu D, Cook JM, Gerthoffer WT, Emala CW, Sr.: Targeting the restricted alpha subunit repertoire of airway smooth muscle GABAA receptors augments airway smooth muscle relaxation. *Am.JPhysiol Lung Cell Mol.Physiol* 2011;

Gleason NR, Gallos G, Zhang Y, Emala CW: Propofol preferentially relaxes neurokinin receptor-2-induced airway smooth muscle contraction in guinea pig trachea. *Anesthesiology* 2010; 112: 1335-44

Gleason NR, Gallos G, Zhang Y, Emala CW: The GABAA agonist muscimol attenuates induced airway constriction in guinea pigs in vivo. *J.Appl.Physiol* 2009; 106: 1257-63

Gromada J, Dissing S: Membrane potential and cytosolic free calcium levels modulate acetylcholine-induced inositol phosphate production in insulin-secreting BTC3 cells. *Biochimica et Biophysica Acta* 1996; 1310: 145-8

Grubbe RE, *et al.*, Effect Of Inhaled Furosemide On The Bronchial Response To Methacholine And Cold-Air Hyperventilation Challenges. *J Allergy Clin Immunol.* 1990;85(5):881-4.

Gupta S, Meena HS, Chopra R, Bidani R: Effect of aerosolised verapamil and nifedipine in bronchial asthma. J.Assoc.Physicians India 1993; 41: 425-7

Hirota S, Trimble N, Pertens E, Janssen LJ: Intracellular Cl^- fluxes play a novel role in Ca^{2+} handling in airway smooth muscle. Am.J.Physiol Lung Cell Mol.Physiol 2006; 290: L1146-L1153

Janssen LJ, Sims SM: Ca^{2+} -dependent Cl^- current in canine tracheal smooth muscle cells. Am.J.Physiol 1995; 269: C163-C169

Janssen LJ: T-type and L-type Ca^{2+} currents in canine bronchial smooth muscle: characterization and physiological roles. Am.J.Physiol 1997; 272: C1757-C1765

Janssen LJ: Ionic mechanisms and Ca^{2+} regulation in airway smooth muscle contraction: do the data contradict dogma? Am.J.Physiol Lung Cell Mol.Physiol 2002; 282: L1161-L1178

Janssen LJ, Tazzeo T, Zuo J, Pertens E, Keshavjee S: KCl evokes contraction of airway smooth muscle via activation of RhoA and Rho-kinase. Am.J.Physiol Lung Cell Mol.Physiol 2004; 287: L852-L858

Jia Y, Wang X, Varty L, Rizzo CA, Yang R, Correll CC, Phelps PT, Egan RW, Hey JA: Functional TRPV4 channels are expressed in human airway smooth muscle cells. Am.J.Physiol Lung Cell Mol.Physiol 2004; 287: L272-L278

Jooste E, Zhang Y, Emala CW: Neuromuscular blocking agents' differential bronchoconstrictive potential in Guinea pig airways. Anesthesiology 2007; 106: 763-72

Kao LC *et al.*, Furosemide acutely decreases airways resistance in chronic bronchopulmonary dysplasia. J Pediatr. 1983, 103(4):624-9.

Kazani S, Ware JH, Drazen JM, Taylor DR, Sears MR: The safety of long-acting beta-agonists: more evidence is needed. Respirology. 2010; 15: 881-5

Kotlikoff MI, Kume H, Tomasic M: Muscarinic regulation of membrane ion channels in airway smooth muscle cells. Biochemical Pharmacology 1992; 43: 5-10

Liu C, Zuo J, Pertens E, Helli PB, Janssen LJ: Regulation of Rho/ROCK signaling in airway smooth muscle by membrane potential and $[Ca^{2+}]_i$. Am.J.Physiol Lung Cell Mol.Physiol 2005; 289: L574-L582

Liu C, Zuo J, Janssen LJ: Regulation of airway smooth muscle RhoA/ROCK activities by cholinergic and bronchodilator stimuli. European Respiratory Journal 2006; 28: 703-11

Mizuta K, Xu D, Pan Y, Comas G, Sonett JR, Zhang Y, Panettieri RA, Jr., Yang J, Emala CW, Sr.: GABAA receptors are expressed and facilitate relaxation in airway smooth muscle. Am.J.Physiol Lung Cell Mol.Physiol 2008; 294: L1206-L1216

Perez-Zoghbi JF, Sanderson MJ: Endothelin-induced contraction of bronchiole and pulmonary arteriole smooth muscle cells is regulated by intracellular Ca^{2+} oscillations and Ca^{2+} sensitization. Am.J. Physiol Lung Cell Mol. Physiol 2007; 293: L1000-L1011

Prabhu VG, Keszler M, Dhanireddy R. Pulmonary Function Changes After Nebulised And Intravenous Frusemide In Ventilated Premature Infants. Arch Dis Child Fetal Neonatal Ed. 1997;77(1):F32-5.

Prabhu VG, Keszler M, Dhanireddy R. Dose-Dependent Evaluation Of The Effects Of Nebulized Furosemide On Pulmonary Function In Ventilated Preterm Infants. J Perinatol. 1998; 18(5):357-60.

Prandota J., Furosemide: progress in understanding its diuretic, anti-inflammatory, and bronchodilating mechanism of action, and use in the treatment of respiratory tract diseases. Am J Ther. 2002;9(4):317-28.

Rastogi A *et al.*, Nebulized Furosemide In Infants With Bronchopulmonary Dysplasia. J Pediatr. 1994;125:976-9.

Rubin AS, Cardoso PF: Bronchial thermoplasty in asthma. J.Bras.Pneumol. 2010; 36: 506-12

Sunaga H, Zhang Y, Savarese JJ, Emala CW: Gantacurium and CW002 do not potentiate muscarinic receptor-mediated airway smooth muscle constriction in guinea pigs. Anesthesiology 2010; 112: 892-9

Talwar D, Jindal SK: Effect of calcium channel antagonists on cholinergic bronchial responsiveness in asthma. J.Assoc.Physicians India 1993; 41: 279-80

Wafford KA, van Niel MB, Ma QP, Horridge E, Herd MB, Peden DR, Belelli D, Lambert JJ: Novel compounds selectively enhance delta subunit containing GABA A receptors and increase tonic currents in thalamus. Neuropharmacology 2009; 56: 182-9

Walker NM, Simpson JE, Levitt RC, Boyle KT, Clarke LL: Talniflumate increases survival in a cystic fibrosis mouse model of distal intestinal obstructive syndrome. *Journal of Pharmacology and Experimental Therapeutics* 2006; 317: 275-83

White TA, Xue A, Chini EN, Thompson M, Sieck GC, Wylam ME: Role of transient receptor potential C3 in TNF-alpha-enhanced calcium influx in human airway myocytes. *American Journal of Respiratory Cell and Molecular Biology* 2006; 35: 243-51

Yim PD, Gallos G, Xu D, Zhang Y, Emala CW: Novel expression of a functional glycine receptor chloride channel that attenuates contraction in airway smooth muscle. *FASEB Journal* 2011

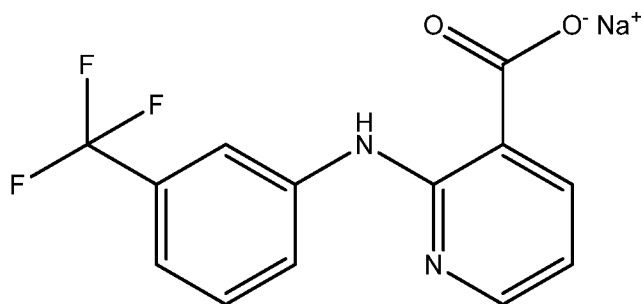
Yamakage M, Chen X, Tsujiguchi N, Kamada Y, Namiki A: Different inhibitory effects of volatile anesthetics on T- and L-type voltage-dependent Ca^{2+} channels in porcine tracheal and bronchial smooth muscles. *Anesthesiology* 2001; 94: 683-93

Zhang GH, Melvin JE: Membrane potential regulates Ca^{2+} uptake and inositol phosphate generation in rat sublingual mucous acini. *Cell Calcium* 1993; 14: 551-62

WHAT IS CLAIMED IS:

1. A method of treating or ameliorating the effects of a disease characterized by altered smooth muscle contractility comprising administering to a patient suffering from such a disease an effective amount of a calcium-activated chloride channel (CaCC) modulator and a sodium-potassium-chloride co-transporter (NKCC) modulator.
2. The method according to claim 1, wherein the CaCC modulator is a CaCC inhibitor.
3. The method according to claim 2, wherein the CaCC inhibitor is selected from the group consisting of niflumic acid, 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), talnifumate, flufenamic acid, 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS), indanyloxyacetic acid 94 (IAA-94), tamoxifen, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), anthracene-9-carboxylic acid (A9C), diphenylamine-2-carboxyl acid (DPC), 6-*t*-butyl-2-(furan-2-carboxamido)-4,5,6,7-tetrahydrobenzo[*b*] thiophene-3-carboxylic acid (CaCC_{inh}-A01), 2-hydroxy-4-(4-*p*-tolylthiazol-2-ylaminobenzoic acid (CaCC_{inh}-B01), morniflumate (Sanofi-Aventis, France), calcium-sensitive chloride channel antagonist (Takeda Pharmaceutical Co. Ltd., Japan), a pharmaceutically acceptable salt thereof, and combinations thereof.
4. The method according to claim 3, wherein the CaCC inhibitor is niflumic acid or a pharmaceutically acceptable salt thereof.

5. The method according to claim 3, wherein the CaCC inhibitor is



6. The method according to claim 1, wherein the NKCC modulator is a NKCC inhibitor.

7. The method according to claim 6, wherein the NKCC inhibitor is selected from the group consisting of bumetanide, furosemide, torasemide, azosemide, piretanide, triparamide, etozoline and its metabolite ozolinone, cicletanine, ethacrynic acid, muzolimine, LR-14-890 (Menarini, Italy), lemidosul (Sanofi-Aventis, France), M-12285 (Mochida, Japan), alilusem (Mochida, Japan), sulosemide sodium (Sano-Aventis, France), BTS-39542 (Abbott Laboratories, Abbott Park, Illinois), AY-31906 (Pfizer, New York, New York), brocrinat (Sanofi-Aventis), SA-9000 (Santen, Japan), A-52773 (Abbott Laboratories), A-53385 (Abbott Laboratories), CL-301 (Chlorion Pharma, Canada), Abbott-49816 (Abbott Laboratories), ethacrynic acid (Telor Ophthalmic Pharmaceuticals, Wilmington, Massachusetts), a pharmaceutically acceptable salt thereof, and combinations thereof.

8. The method according to claim 7, wherein the NKCC inhibitor is selected from the group consisting of bumetanide, furosemide, a pharmaceutically acceptable salt thereof, and combinations thereof.
9. The method according to claim 8, wherein the NKCC inhibitor is bumetanide.
10. The method according to claim 1, wherein the disease is selected from the group consisting of asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, adult respiratory distress syndrome, and bronchospasm.
11. The method according to claim 1, wherein the disease is asthma or COPD.
12. The method according to claim 1, wherein the CaCC modulator and the NKCC modulator are administered as part of a pharmaceutical composition.
13. The method according to claim 12, wherein the pharmaceutical composition is in a unit dosage form.
14. The method according to claim 12, wherein the pharmaceutical composition is co-administered with a β -agonist.

15. A pharmaceutical composition for treating or ameliorating the effects of a disease characterized by altered smooth muscle contractility, the composition comprising a pharmaceutically acceptable carrier, a CaCC modulator, and a NKCC modulator.
16. The pharmaceutical composition according to claim 15, wherein the disease is selected from the group consisting of asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, adult respiratory distress syndrome, and bronchospasm.
17. The pharmaceutical composition according to claim 15, wherein the disease is asthma or COPD.
18. The pharmaceutical composition according to claim 15, which is in a unit dosage form.
19. The pharmaceutical composition according to claim 15, which is co-administered with a β -agonist.
20. A method of relaxing airway smooth muscle comprising administering to a patient in need thereof an effective amount of a CaCC modulator and a NKCC modulator.

21. The method according to claim 20, wherein the CaCC modulator is a CaCC inhibitor, and the NKCC modulator is a NKCC inhibitor.

Fig. 1

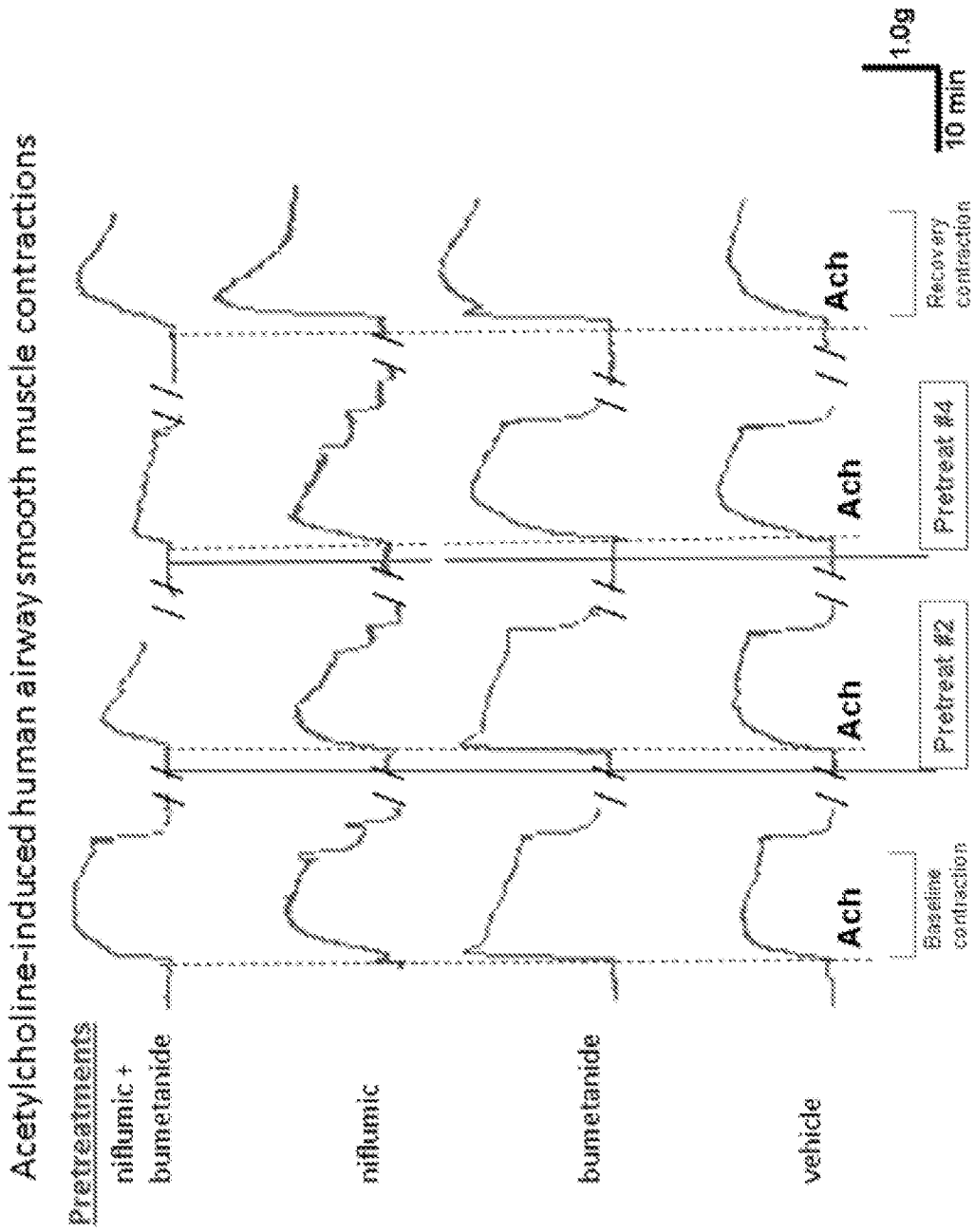


Fig. 2

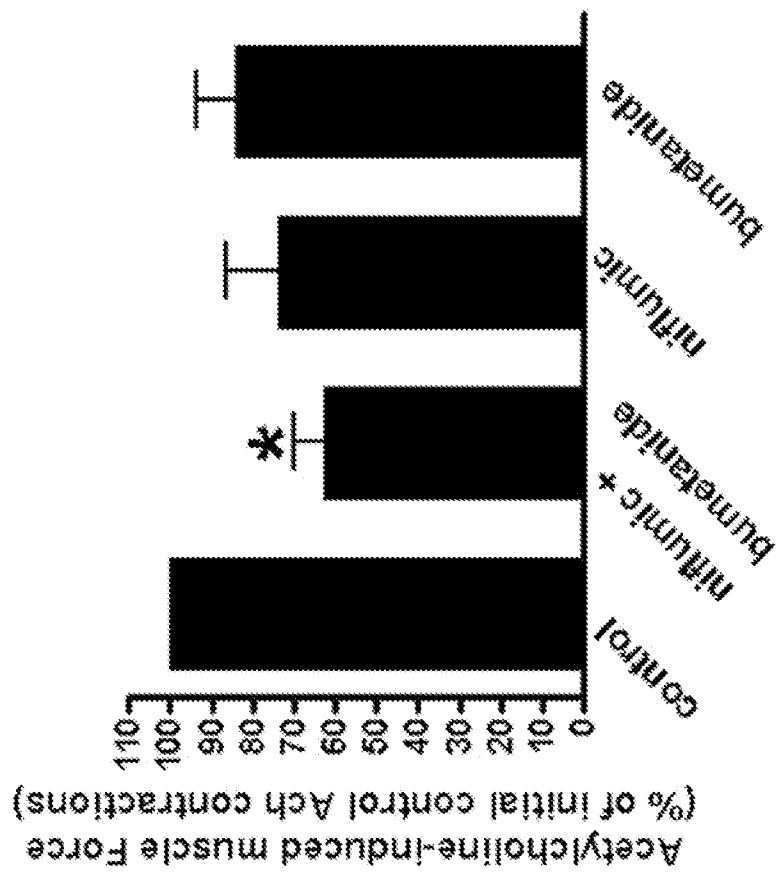


Fig. 3

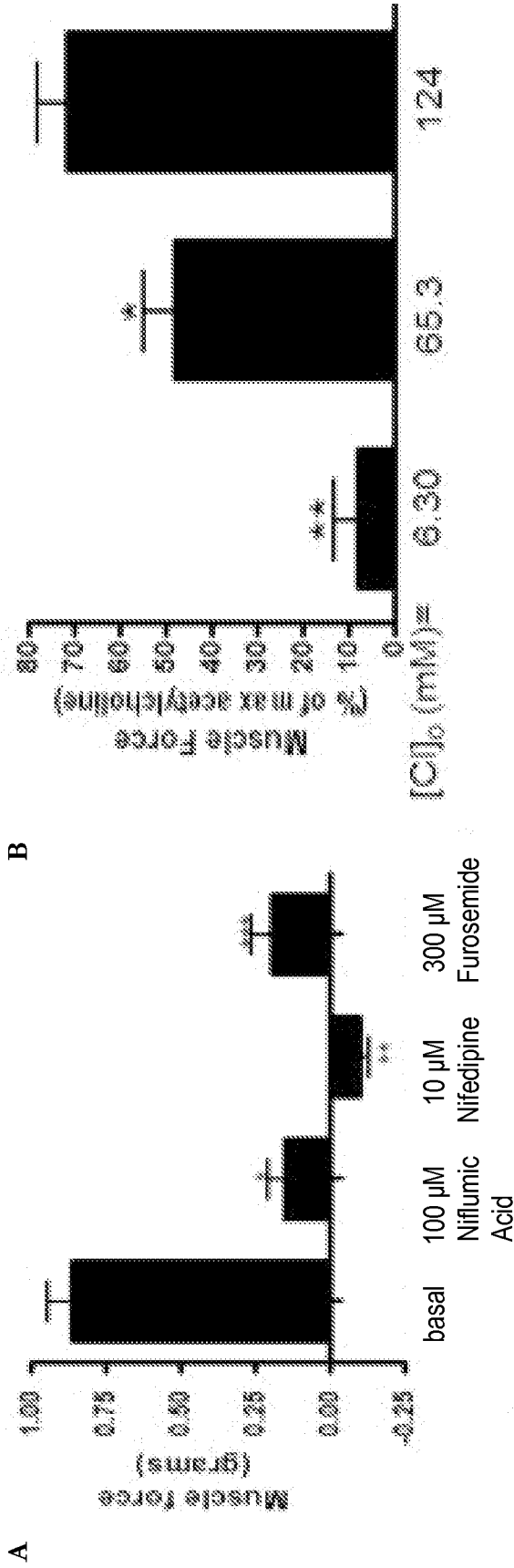


Fig. 4

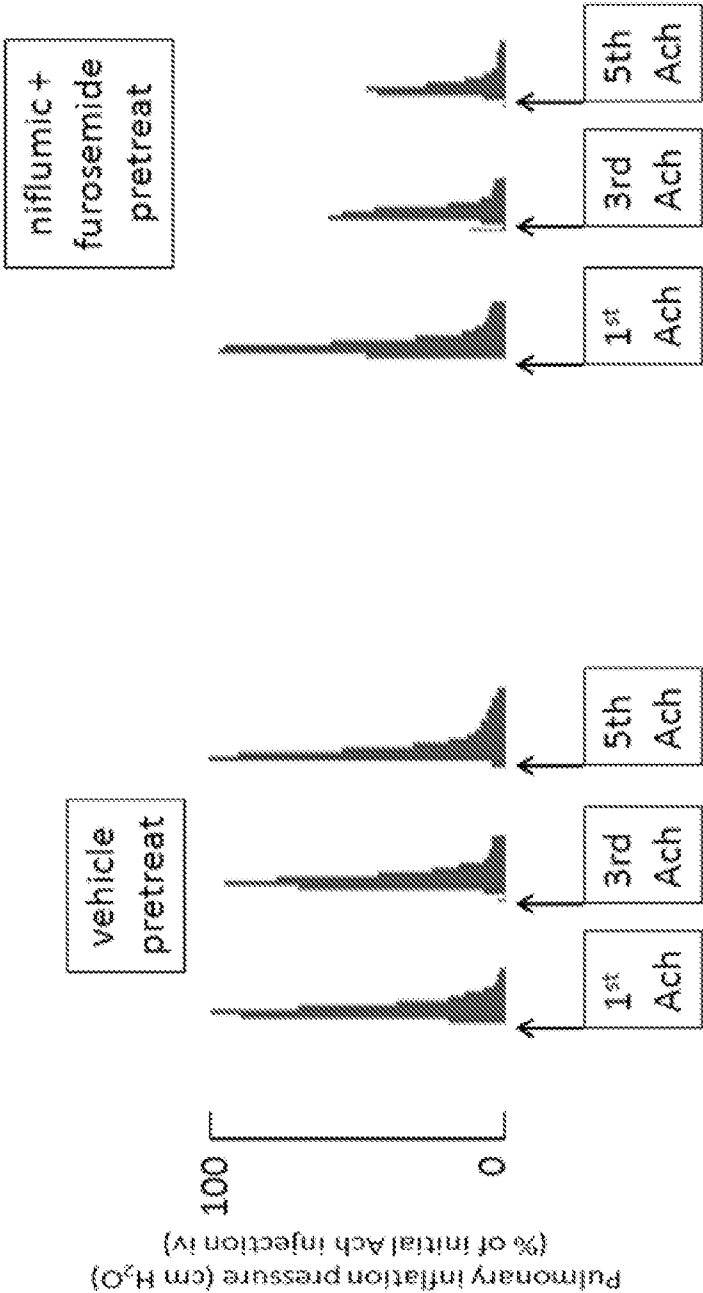


Fig. 5

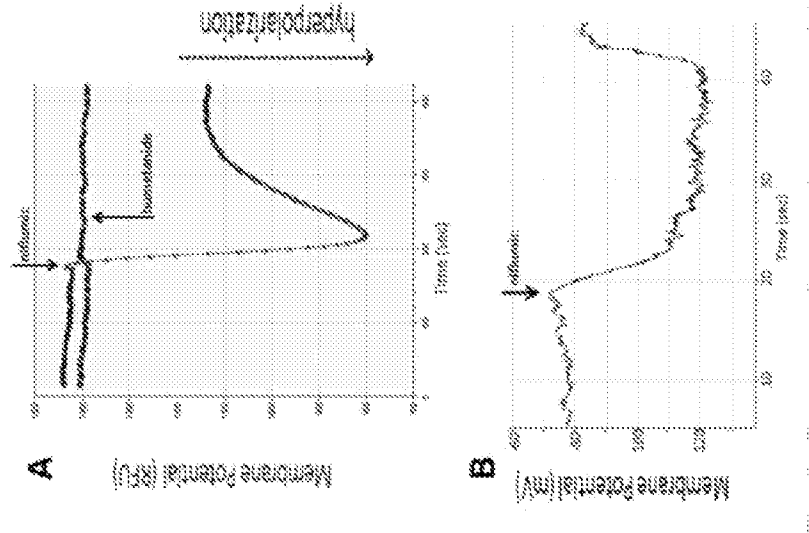
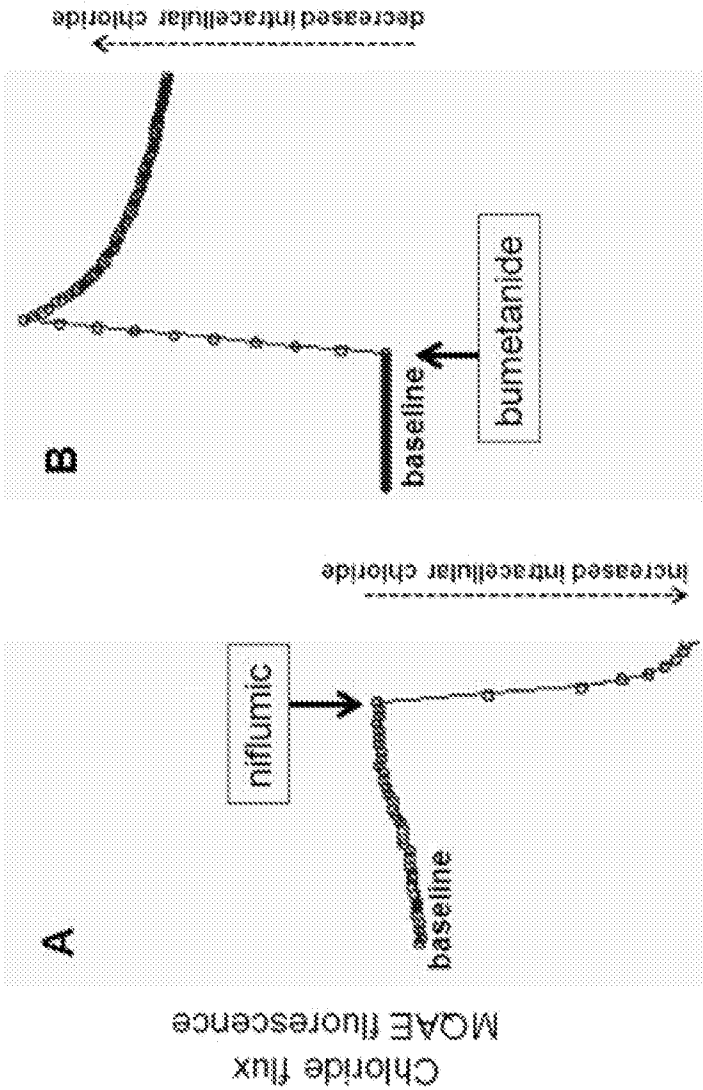


Fig. 6



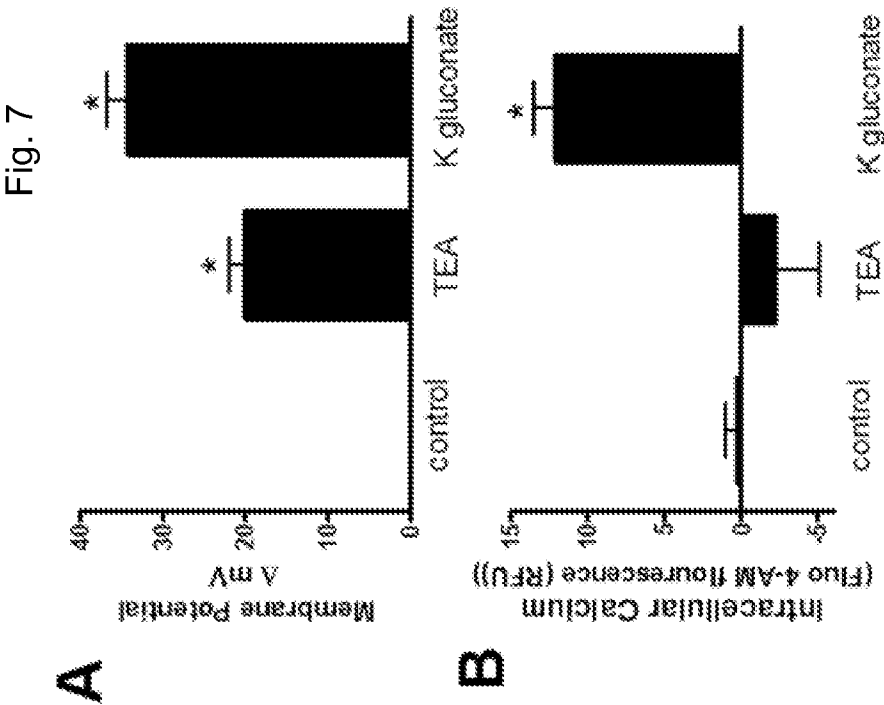


Fig. 8

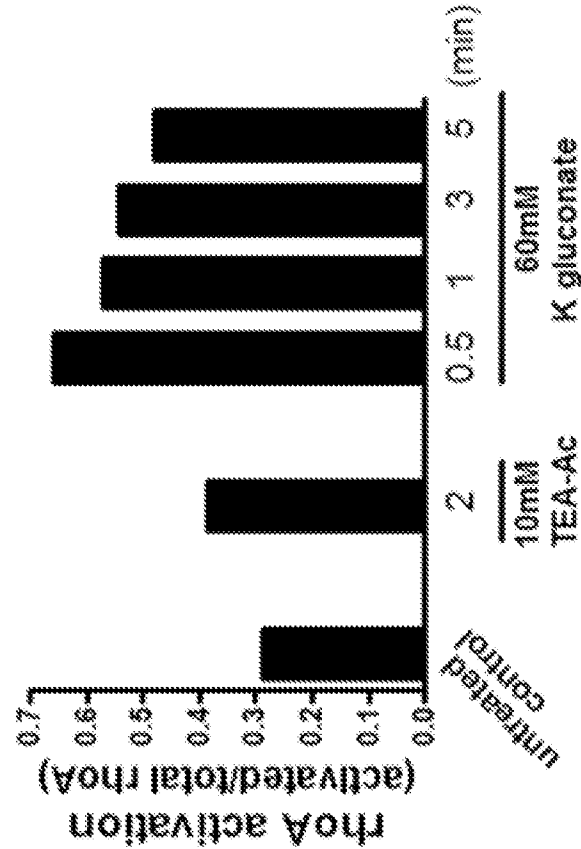


Fig. 9

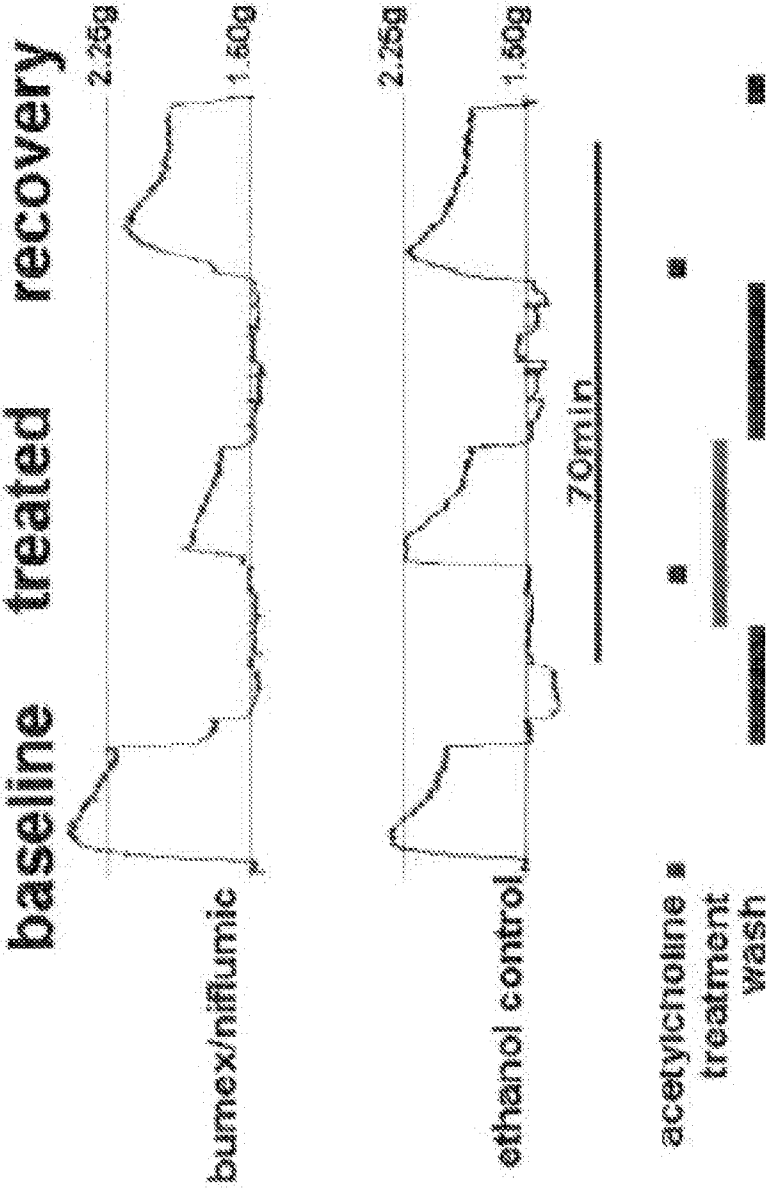


Fig. 10

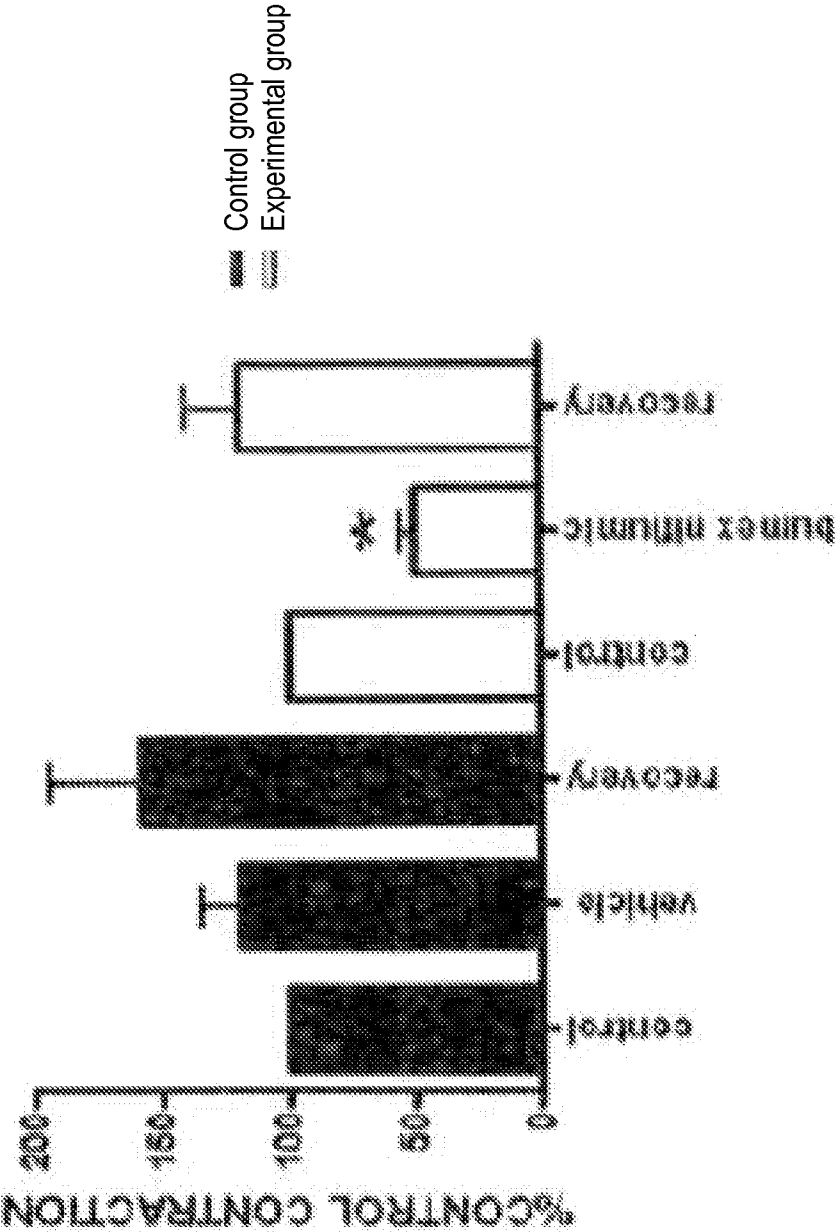


Fig. 11

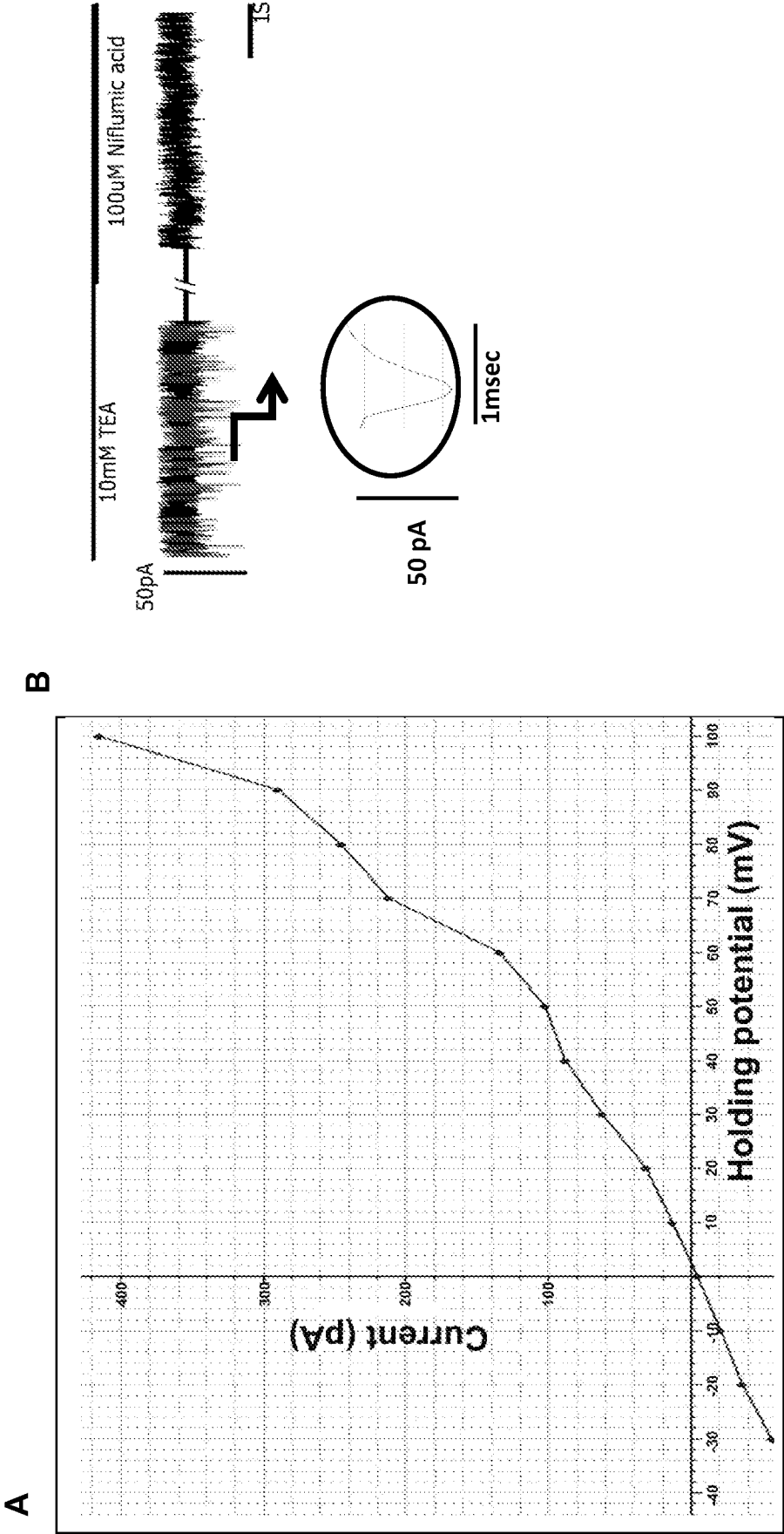


Fig. 12

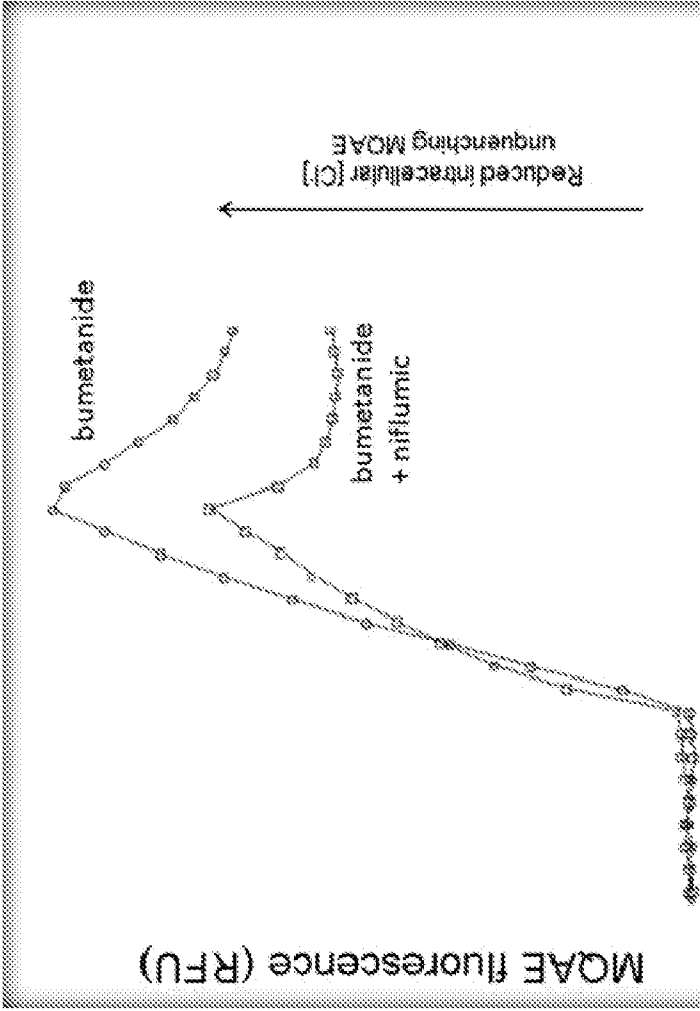


Fig. 13

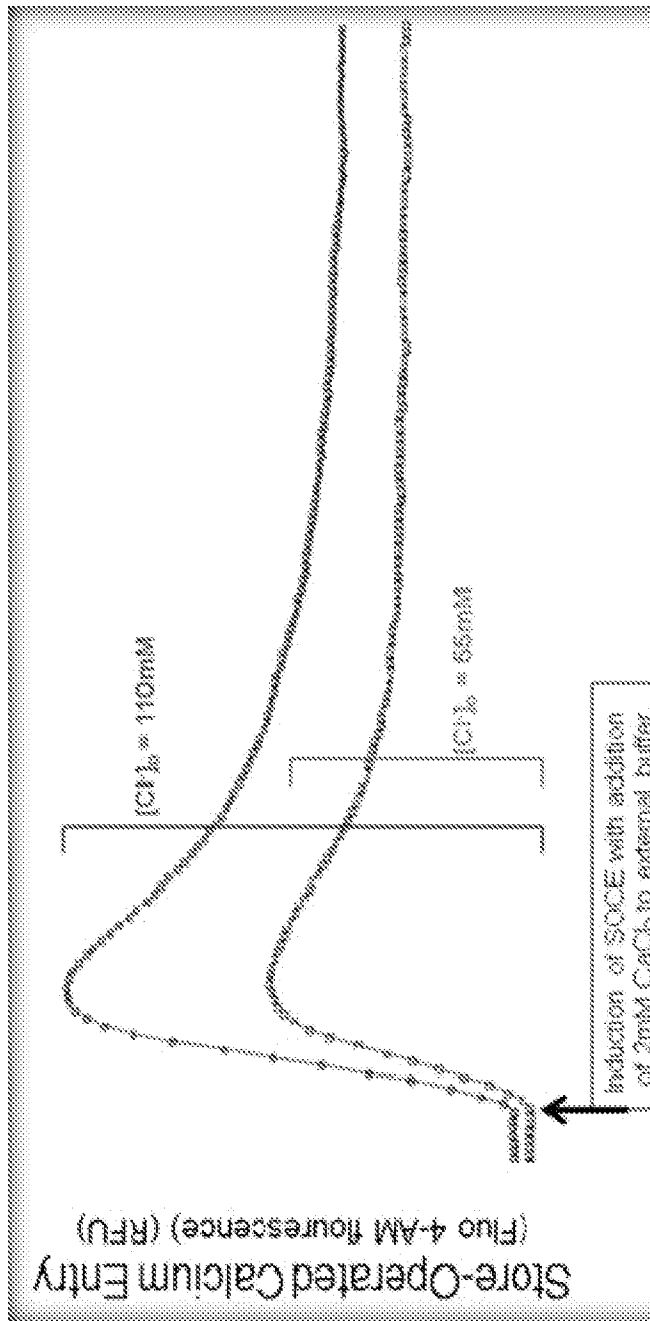


Fig. 14

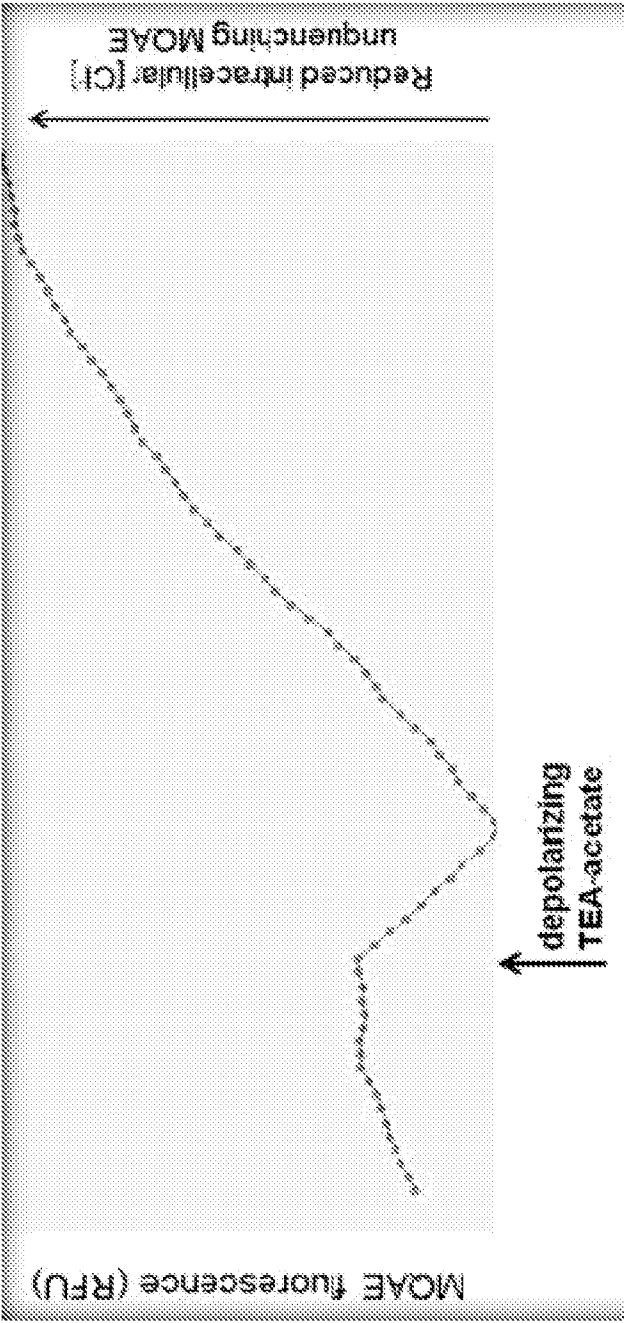
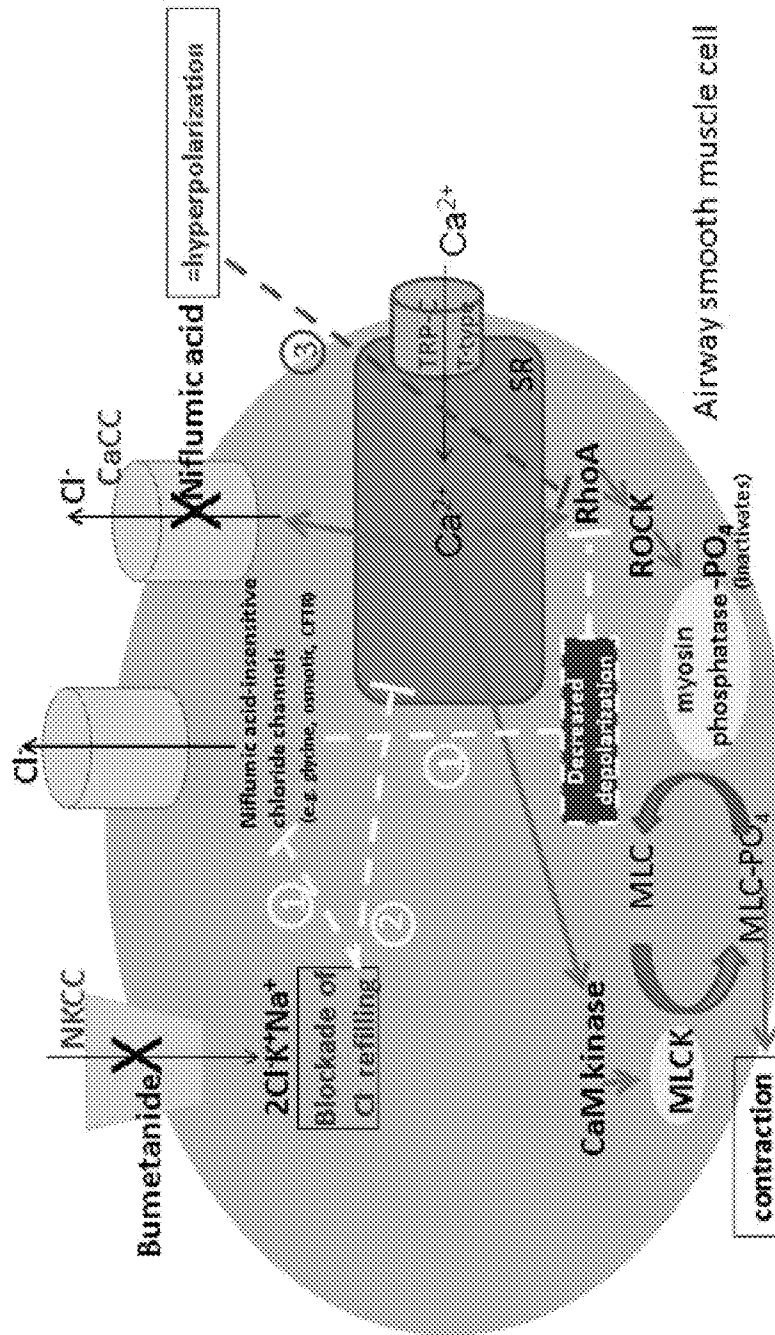


Fig. 15



Mechanistic hypotheses (dashed lines) of airway smooth muscle relaxation by simultaneous blockade of calcium activated chloride channel (CaCC) and Na⁺-K⁺-Cl⁻ transporter (NKCC). Bumetanide blockade of NKCC blocks intracellular Cl⁻ refilling resulting in (1) reduced extracellular efflux through niflumic acid insensitive Cl⁻ channels which decreases membrane depolarization impairing RhoA activation, and (2) reduced intracellular Cl⁻ available to balance charge generation during Ca²⁺ refilling of SR. Niflumic acid blockade of CaCC induces hyperpolarization which (3) impairs RhoA activation. SR= sarcoplasmic reticulum; MLC= myosin light chain; CaM=Ca²⁺ calmodulin dependent protein; MLCK= myosin light chain kinase; ROCK=Rho associated protein kinase.

16/20

Fig. 16

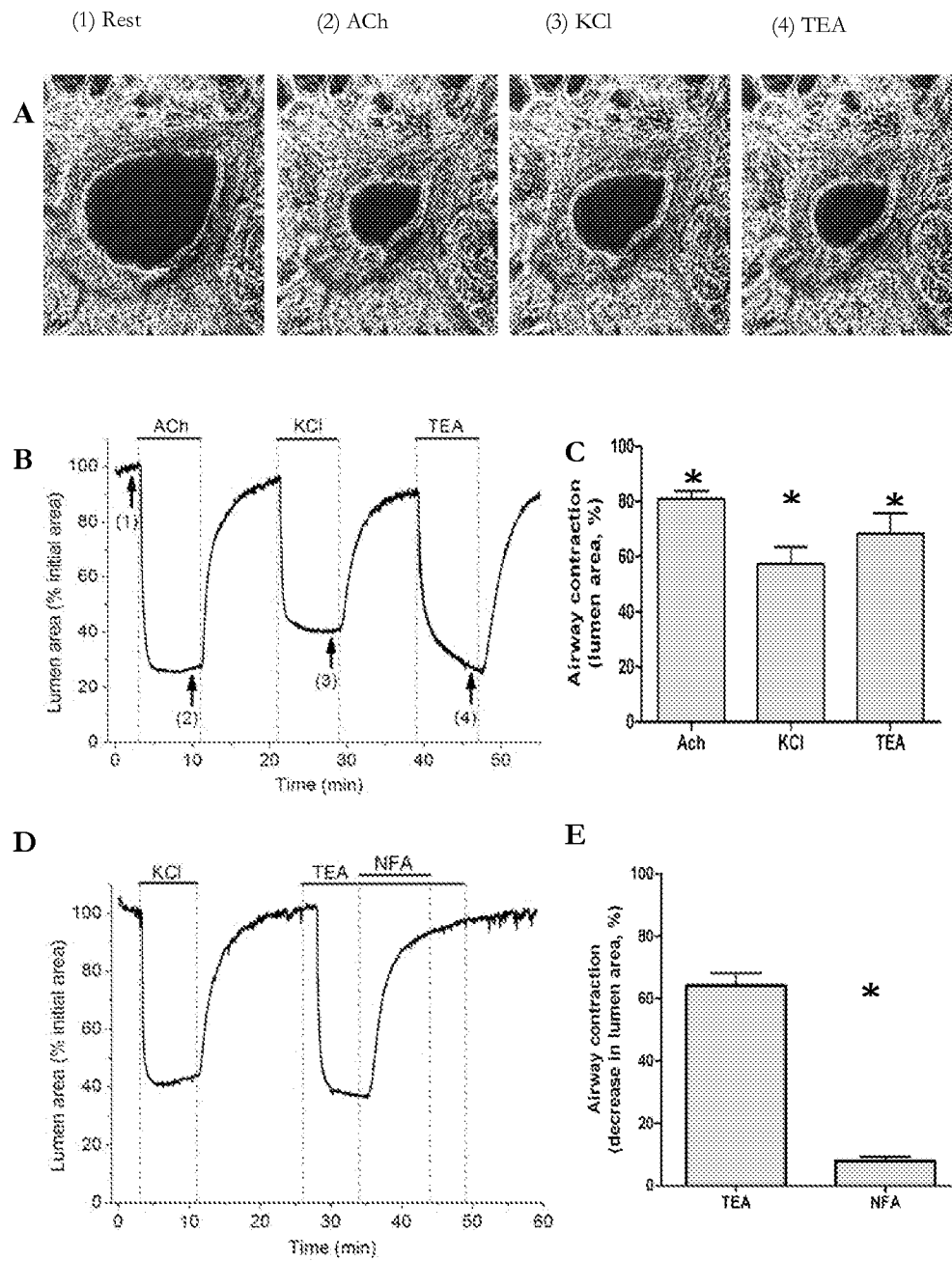


Fig. 17

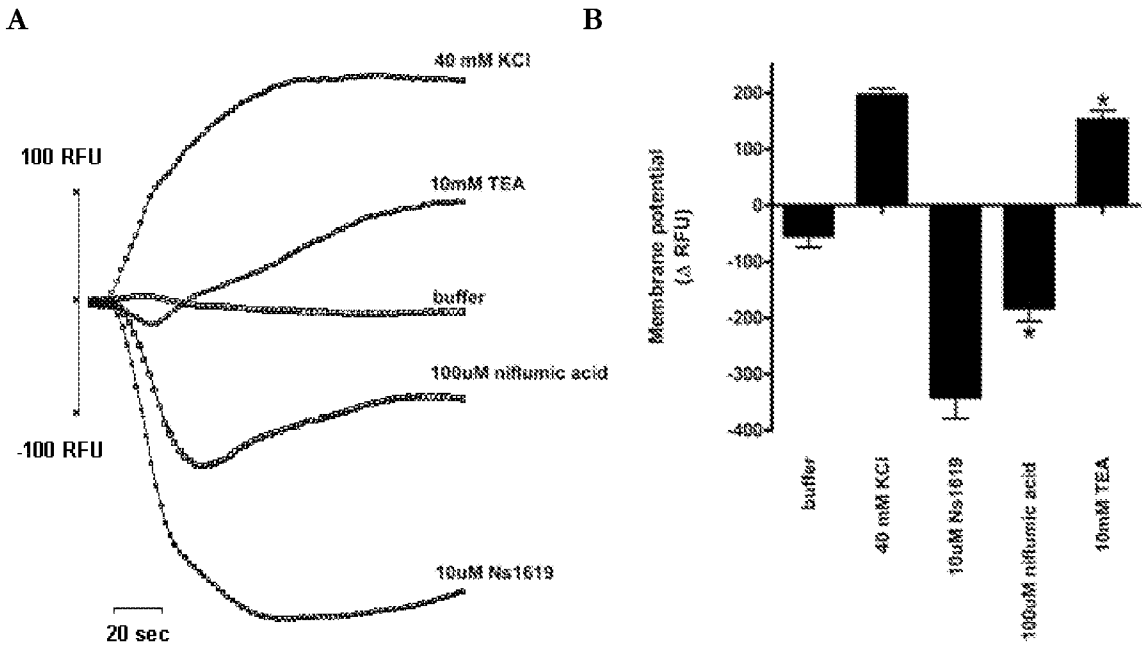


Fig. 18

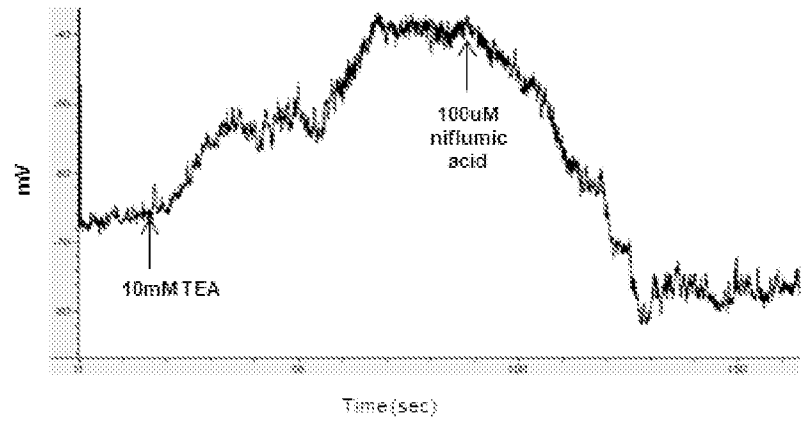


Fig. 19

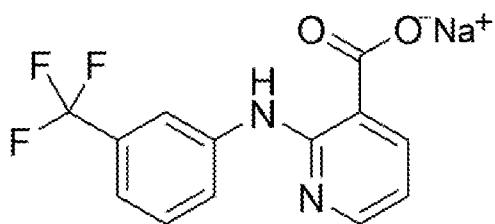


Fig. 20

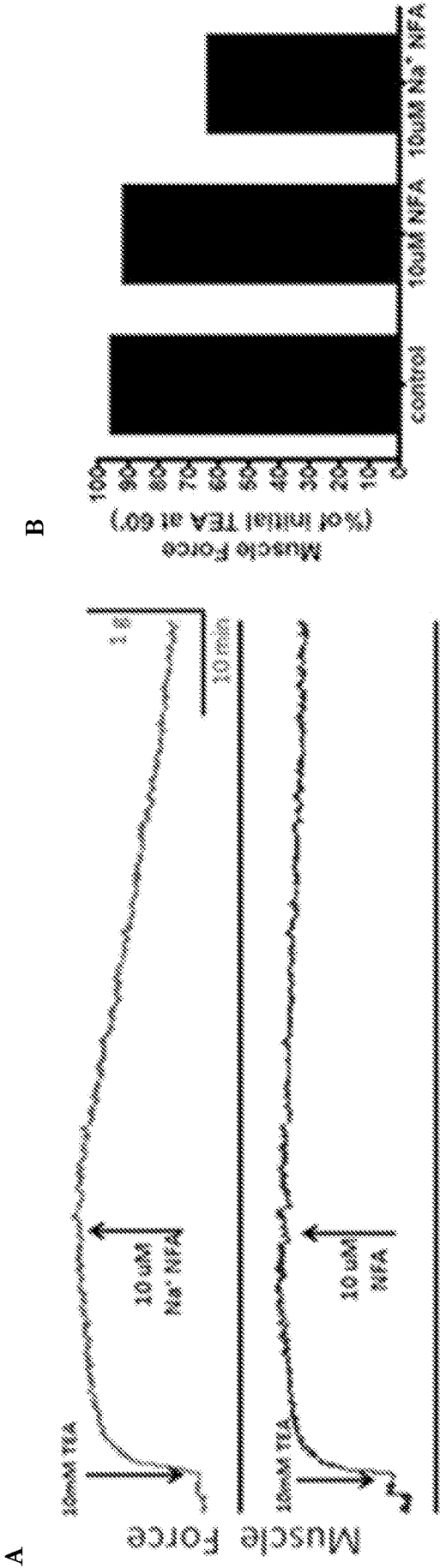
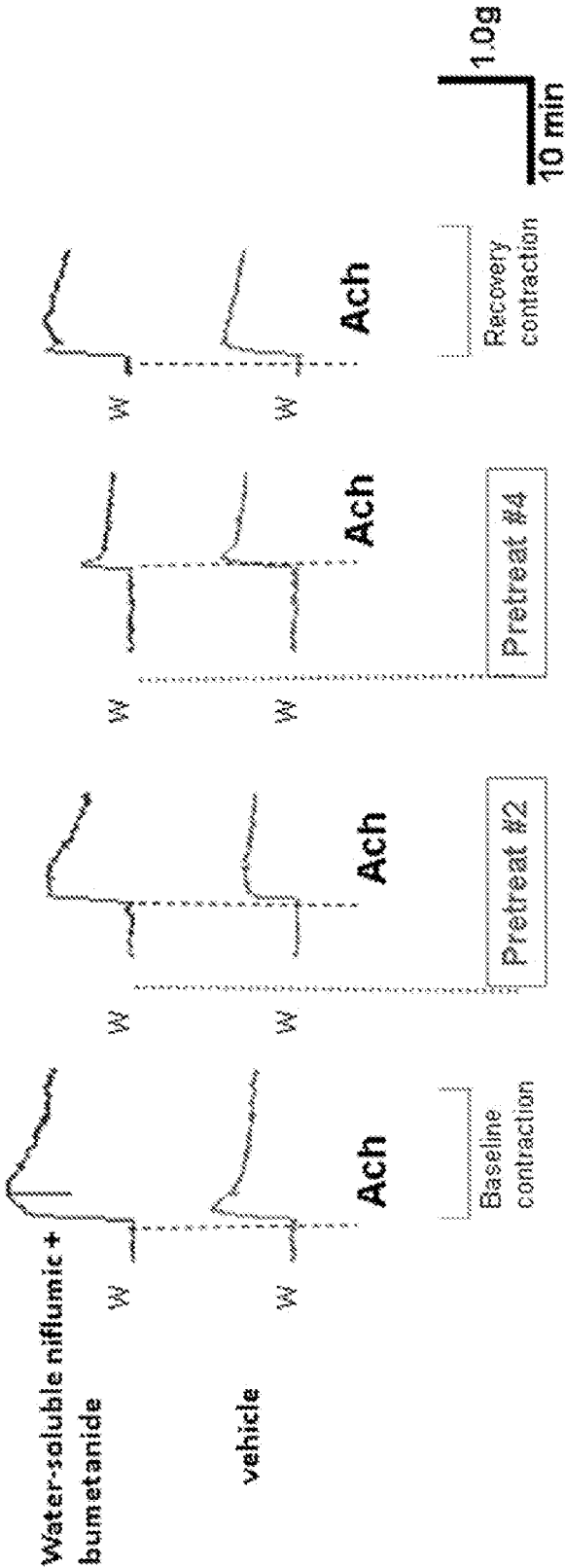


Fig. 21



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2012/030201

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/455 (2012.01)

USPC - 514/356

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)

IPC(8) - A01N 43/40; A61K 31/455, 31/56; A61P 9/08, 11/00, 11/02, 11/08, 21/02; C07D 213/72; C07J 1/00 (2012.01)

USPC - 514/171, 172, 277, 356; 546/304, 310; 552/502

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)

Patbase, Google Patents, Google, Proquest

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,908,382 A (BIANCO) 13 March 1990 (13.03.1990) entire document	1-4, 6-21
Y	US 2011/0015239 A1 (VERKMAN et al) 20 January 2011 (20.01.2011) entire document	1-4, 6-21
Y	US 7,345,051 B2 (ZHOU et al) 18 March 2008 (18.03.2008) entire document	4
Y	US 2007/0043034 A1 (STALEY et al) 22 February 2007 (22.02.2007) entire document	9
Y	US 2007/0293460 A1 (RAY, II et al) 20 December 2007 (20.12.2007) entire document	14, 19
A	US 3,946,035 A (JACQUET et al) 23 March 1976 (23.03.1976) entire document	1-21
A	US 4,168,313 A (BAGO) 18 September 1979 (18.09.1979) entire document	1-21
A	US 2006/0089350 A1 (HOCHMAN et al) 27 April 2006 (27.04.2006) entire document	1-21
A	US 2010/0048732 A1 (EMALA, SR) 25 February 2010 (25.02.2010) entire document	1-21

☐ Further documents are listed in the continuation of Box C.


* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 June 2012

Date of mailing of the international search report

22 JUN 2012

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774