Acta Pharmacol Sin 2008 Jul; 29 (7): 789–799

Full-length article

# Niflumic acid hyperpolarizes smooth muscle cells via calcium-activated potassium channel in spiral modiolar artery of guinea pigs[1]

Li LI[2,3], Ke-tao MA[3,4], Lei ZHAO[3], Jun-qiang SI[3,4,5], Zhong-shuang ZHANG[3], He ZHU[3], Jing LI[3]

*[2]Departmeng of Pharmacology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430030, China; [3]Laboratory of Xinjiang Endemic and Ethnic Diseases, Shihezi University Medical College, Xinjiang 832002, China; [4]Fundamental Medical School of Wuhan University, Wuhan 430071, China*

## Key words

spiral modiolar artery; smooth muscle cells; niflumic acid; hyperpolarization; $Ca^{2+}$-activated potassium channels; cochlea

[1]This work was supported by the National Natural Science Foundation of China (No 30460043); the Scientific and Technological Program for Overseas Personnel, the Ministry of Personnel, China (2006); and the Key Program of Scientific and Technological Research, the Ministry of Education, China (Local Universities; No 207134).
[5]Correspondence to Prof Jun-qiang SI.
Phn 86-993-205-7851.
Fax 86-993-201-5620.
E-mail sijunqiang11@hotmail.com

Received 2008-01-02
Accepted 2008-03-18

doi: 10.1111/j.1745-7254.2008.00803.x

## Abstract

**Aim:** The influence of niflumic acid (NFA), a $Cl^-$ channel antagonist, on the membrane potentials in smooth muscle cells (SMC) of the cochlear spiral modiolar artery (SMA) in guinea pigs was examined. **Methods:** The intracellular recording and whole-cell recording technique were used to record the NFA-induced response on the acutely-isolated SMA preparation. **Results:** The SMC had 2 stable but mutually convertible levels of resting potentials (RP), that is, one was near –45 mV and the other was approximately –75 mV, termed as low and high RP, respectively. The bath application of NFA could cause a hyperpolarization in all the low RP cells, but had little effect on high RP cells. The induced responses were concentration-dependent. Large concentrations of NFA (≥100 μmol/L) often induced a shift of a low RP to high RP in cells with an initial RP at low level, and NFA (up to 100 μmol/L) had little effect on the membrane potentials of the high RP cells. However, when the high RP cells were depolarized to a level beyond –45 mV by barium and ouabain, NFA hyperpolarized these cells with the similar effect on those cells initially being the low RP. The NFA-induced response was almost completely blocked by charybdotoxin, iberiotoxin, tetraethylammonium, 1,2-*bis*(2-aminophenoxy) ethane-*N*,*N*,*N*',*N*'-tetraacetic acid tetrakis acetoxymethyl ester, but not by 4-aminopyridine, barium, glipizide, apamin, ouabain, and $CdCl_2$. **Conclusion:** NFA induces a concentration-dependent reversible hyperpolarization in SMC in the cochlear SMA via activation of the $Ca^{2+}$-activated potassium channels.

## Introduction

Changing blood circulation in the inner ear is implicated in many physiological and pathological conditions of hearing function. For example, the stimulation of loud sound can cause a significant reduction in cochlear blood flow[1], while anoxia or interruption of cochlear blood flow causes a drastic reduction of cochlear function[2]. Some forms of sudden deafness may also be due to inner ear circulation problems[3,4]. In addition, disturbances of inner ear circulation are associated with increased sensitivity to ototoxic drugs and noise-induced trauma[5,6]. Thus it is very important to study the characteristics of inner ear microcirculation to reveal the physiological function of the smooth muscle cells (SMC) in the cochlear spiral modiolar artery (SMA), which is only an arteriole into the inner ear.

Large-conductance calcium-activated potassium ($K_{Ca}$) channels are present in a variety of cell types[7-12]. In neurons, they may regulate cell firing[13,14], and in smooth muscle, they seem to play an important role in maintaining visceral and vascular tone[15-18]. Many chloride channel inhibitors, including members of the non-steroidal anti-inflammatory drug family, such as niflumic acid (NFA), not only inhibit $Cl^-$ conductance[19-21], but also stimulate large-conductance $K_{Ca}$ channels in vascular smooth muscle of the rabbit portal vein[22] and pig coronary[23]. In our preliminary study, we found that $K_{Ca}$ channels might exist in the SMC and endothelial cells (EC) of the cochlear SMA[12]. Moreover, chloride channel blockers could inhibit excitatory junction potentials in the SMC of the

cochlear SMA in guinea pigs[24].

The aim of the present work was to use direct intracellular recordings of membrane potential and conventional whole-cell recordings to investigate the effect of NFA (a chloride channel blocker) on the SMC in the cochlear SMA of guinea pigs. The results suggest that NFA hyperpolarizes SMC by activating $K_{Ca}$ channels in the SMA of guinea pigs.

## Materials and methods

**Animals and SMA preparation** Guinea pigs (300–500 g) were anesthetized and then killed by exsanguination[25]. The anesthesia was accomplished by an intramuscular injection of an anesthetic mixture (1 mL/kg) of 500 mg ketamine, 20 mg xylazine, and 10 mg acepromazine in 8.5 mL $H_2O$. Both bullae were rapidly removed and transferred to a Petri dish filled with a physiological solution (Krebs) composed of (in mmol/L): 125 NaCl, 5 KCl, 1.6 $CaCl_2$, 1.2 $MgCl_2$, 1.2 $NaH_2PO_4$, 18 $NaHCO_3$, and 8.2 glucose, and saturated with 95% $O_2$ and 5% $CO_2$ at 35 °C (pH 7.4). The SMA and some related radiating arterioles were further dissected from the cochlea under a dissecting microscope. The vascular preparation was incubated for 0.5–24 h in the physiological solution and then transferred to a recording bath. A 2-5 mm long segment of the SMA was cleaned free of spongy connective tissues and pinned with minimum nails to the silicon rubber layer (Sylgard 184, Dow Corning, USA) in the bottom of an organ bath (volume 0.5 mL) and continuously perfused with a 35 °C Krebs solution. When needed, a high potassium Krebs solution was made by additional KCl and accordingly-reduced NaCl.

**Intracellular recording** Intracellular recording was made from a segment of the SMA in the basal and the second turn of the cochlea, as described previously[25,26]. Briefly, the micro-electrode was filled with 2 mol/L KCl, with a tip resistance of 60–150 MΩ. Intracellular penetration was obtained by advancing the electrode into the adventitial surface of the vessel. The transmembrane potentials and current were simultaneously monitored by an Axoclamp 2B preamplifier (Axon Instruments, Burlingame, CA, USA).

The electrical signals were recorded on a pen recorder and a personal computer equipped with Axoscope8 and pClamp6 software (Axon Instruments, USA) using sampling intervals of 0.1, 0.5, or 10 ms. The resting potential was usually determined 5 min after the initial voltage jump at penetration, and checked by the voltage jump at the withdrawal of the electrode. The input resistance was measured by applying 0.5 nA 0.5–1 s current pulses via the recording electrode with the bridge balance well adjusted on the preamplifier[26].

**Tight-seal whole-cell recording** Using the Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA), conventional whole-cell recordings were performed on smooth muscle cells *in situ* from the SMA[27]. The specimen was continuously superfused with the normal external solution (0.2 mL·min$^{-1}$) at room temperature (22–25 °C). Recording pipettes were made of borosilicate glass capillaries with filament (OD 1.5 mm, ID 0.84 mm; World Precision Instruments, Sarasota, FL, USA) and pulled by a P-80 puller (Sutter Instruments, USA). The pipette was filled with an internal solution containing (in mmol/L): 130 KCl, 10 NaCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 5 EGTA (118 nmol/L free $Ca^{2+}$), and 7.5 glucose, and adjusted to pH 7.2 and to osmolarity 290 mOsm/L. The recording pipettes had a tip of ~1 μmol/L OD and a resistance of ~5 MΩ. Pipette capacitance was well compensated, while membrane input capacitance uncompensated to monitor access resistance and membrane parameters online. The voltage clamping error introduced by the current passing the access resistance was corrected offline according to the equation $V_m=V_c-IR_a$ (where $V_m$ is the actual clamping membrane voltage and $V_c$ is the apparent command voltage), except where noted otherwise. Leak subtraction was done offline when appropriate. The membrane current or voltage signal was low-pass filtered at 5 or 10 kHz (–3 dB); data were recorded on a personal computer equipped with a Digidata 1322A AD-interface and pClamp 9.2 software (Axon Instruments, USA) at a sampling interval of 10, 20, or 100 ms. A gap-free recording was simultaneously carried out by a Minidigi digitizer and Axoscope 9.2 software (Axon Instruments, USA) at a sampling interval of 50 ms.

**Drugs application** Drugs in known concentrations were applied via the bathing solution. The solution passing the recording chamber could be switched, without change in the flow rate or temperature, to one that contained a drug or one that was of different ionic composition. The drugs used in this study were: NFA, indanyloxyacetic acid 94 (IAA-94), disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS), charybdotoxin (ChTX), iberiotoxin (IbTX), 1,2-*bis*(2-aminophenoxy) ethane-*N*,*N*,*N*',*N*'-tetraacetic acid tetrakis acetoxymethyl ester (BAPTA-AM), tetraethylammonium (TEA), 4-aminopyridine (4-AP), barium, glipizide, apamin, $CdCl_2$, and ouabain (all from Sigma Research Biochemicals, St Louis, MO, USA).

**Statistical analysis** The values of NFA-induced responses were expressed as mean±SEM and compared with *t*-test.

## Results

**Effects of NFA, IAA-94, and DIDS on SMC in SMA** The resting potentials (RP) of SMC in the SMA were measured in

a normal Krebs solution (5 mmol/L potassium) with 5–10 min duration after the cell was penetrated and the membrane potential level became stabilized. We previously reported that SMC have 2 stable but mutually-convertible (Figure 1B, 2A,4A), levels of RP, that is, 1 was near -45 mV and the other was approximately –75 mV, termed as low and high RP, respectively[26]. So under the same condition, stable intracellular recordings were successfully made in more than 210 cells randomly penetrated along the segments of the proximal half of 77 SMA from either side. The recording lasted from 5 min to 6.5 h. The mean RP were –75.46±0.59 mV ($n$=87) and –40.66±0.41 mV ($n$=123) in the high and low RP cells, respectively. The mean RP showed no difference from our preliminary study result (–74±0.46 mV and –41±0.52 mV)[26].

Direct intracellular recordings of SMC membrane potential indicated that membrane potential responses to bath application of the $Cl^-$ channel antagonists (10–1000 μmol/L NFA, 0.1–10 μmol/L IAA-94 and 200 μmol/L DIDS) made striking difference between the low RP and high RP types of SMC (Figure 1,2A,4A). In low RP SMC, NFA (100 μmol/L) caused a hyperpolarization of 13.9±3.4 mV (mean±SEM, $n$=27, $P$<0.01) from an initial low resting membrane potential of –43.59±1.47 mV (Figure 1A,1C). The amplitude of NFA (100 μmol/L)-induced hyperpolarization ranged from 9.8 mV to 21.6 mV. The NFA-induced responses were concentration - dependent. Figure 2A shows the records of membrane hyperpolarization in response to different concentrations of NFA (10-300 μmol/L) obtained from 1 cell. Figure 2B reveals the concentration–response curve of NFA (3–1000 μmol/L)-induced hyperpolarization. The curve was a good fit for the

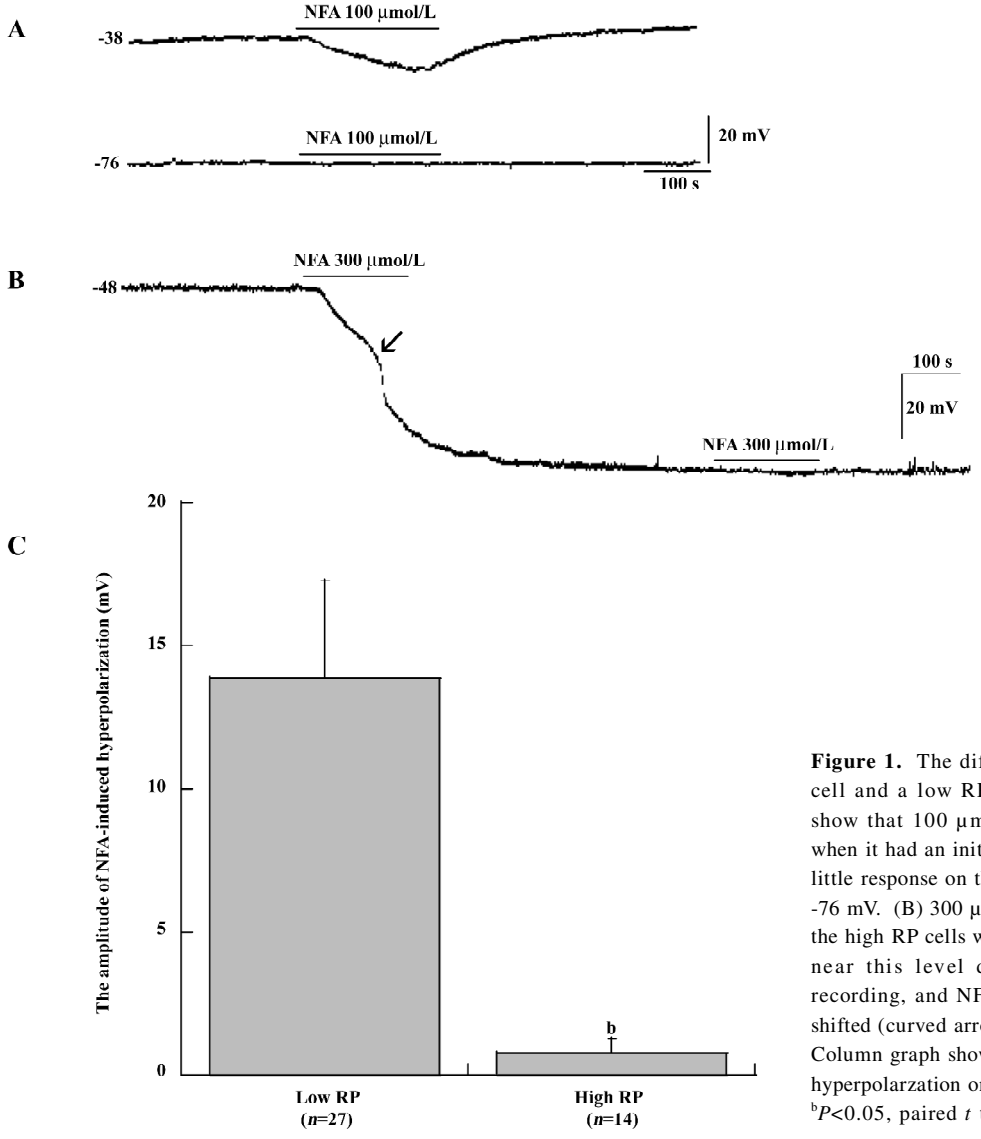



**Figure 1.** The different effect of NFA on a high RP cell and a low RP cell. (A) Representative traces show that 100 μmol/L NFA hyperpolarized the cell when it had an initial RP of -38 mV whereas NFA has little response on the cell when it had an initial RP of -76 mV. (B) 300 μmol/L NFA shift the low RP cells to the high RP cells with an initial RP of -48 mV, stayed near this level during the remaining period of recording, and NFA also has little effect on the cell shifted (curved arrow) to a high resting potential. (C) Column graph showing the amplitude of NFA-induced hyperpolarzation on low or high RP cells, respectively. $^bP$<0.05, paired $t$ test.

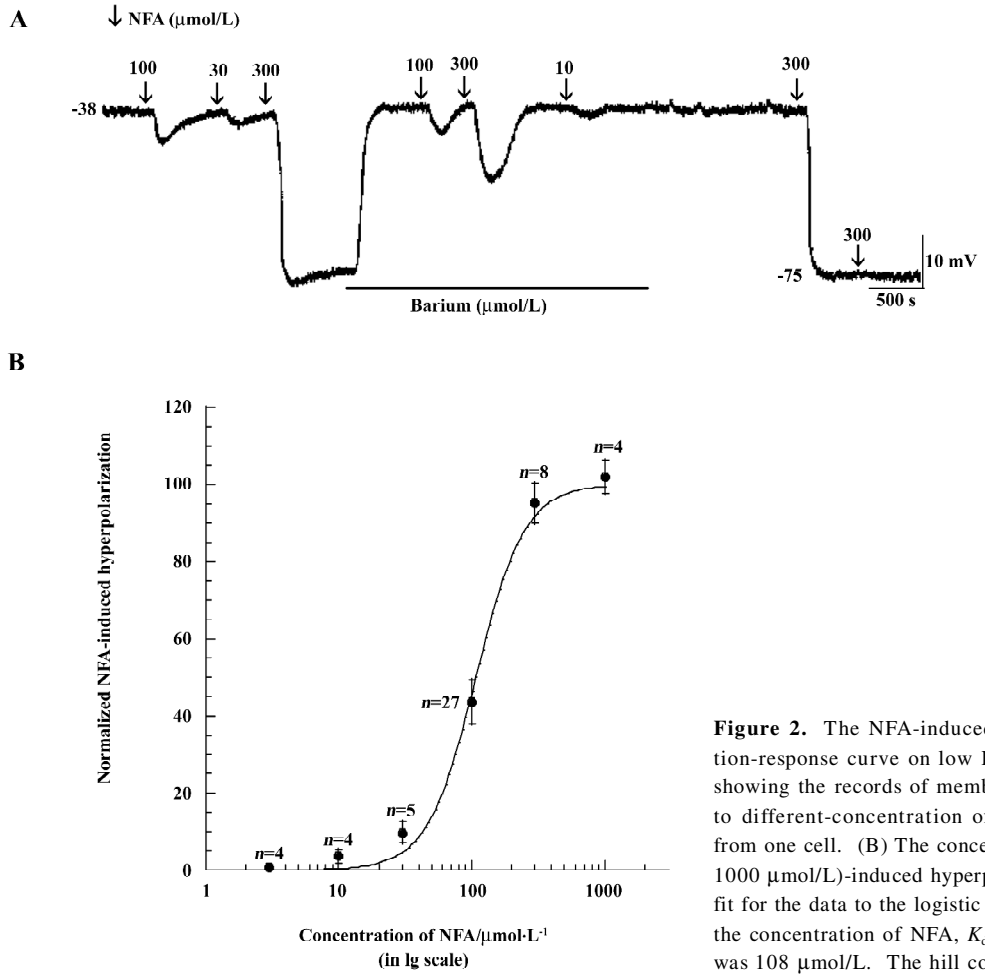



**Figure 2.** The NFA-induced hperpolarizations and concentration-response curve on low RP cells. (A) Representative trace showing the records of membrane hyperpolarization in response to different-concentration of NFA (10–300 μmol/L) obtained from one cell. (B) The concentration-response curve of NFA (3–1000 μmol/L)-induced hyperpolarization. The curve was a good fit for the data to the logistic equation $Y=E_{max}/[1+(K_d/C)^n]$, C was the concentration of NFA, $K_d$, the dissociation constant of NFA, was 108 μmol/L. The hill coefficient ($n$) was 2.38.

data to the logistic equation $Y=E_{max}/(1+[K_d/C]^n)$ (where $C$ was the concentration of NFA, and $K_d$, the dissociation constant of NFA, was 108 μmol/L). The Hill coefficient ($n$) was 2.38. (In some data, this figure was reached with the pre-application of 50–100 μmol/L barium.) However, NFA (up to 100 μmol/L) had little effect on the membrane potential of cells that had an initial high RP level (Figure 1A) or that had a high RP shifted from a low RP level (Figure 1B).

**NFA shifts membrane potential** A shift of low RP SMC to a permanent high RP level (approximately –65 mV to –90 mV) was frequently observed. The hyperpolarization shift was triggered by a 1–3 min application of high extracellular potassium (10 mmol/L), acetylcholine (ACh; 3–10 μmol/L), DPTA–NONOate (10 μmol/L, a nitric oxide donor), pinacidil (≥100 μmol/L, an activator of the ATP-sensitive potassium channel), or an unknown spontaneous reason. In these cases, the washout recovery of the membrane potential was aborted[25, 26]. In the present experiment, NFA (≥100 μmol/L), IAA-94 (≥10 μmol/L), and DIDS (≥200 μmol/L) also shifted the low RP cells to the high RP cells with an initial RP at a low RP level, the RP further shifted to a level near –75 mV and stayed at this level during the remaining period of recording from 15 min to 2 h (Figure 1B, 2A, 3A, 4A).

**Effects of barium and ouabain on NFA-induced hyperpolarization** Barium (1–500 μmol/L) caused a robust depolarization in high RP cells to a low level from –50 to –25 mV [26]. The depolarization induced by 1–50 μmol/L barium was usually completely reversible after a 5–10 min washout. Moreover, in some cells, the RP shifted from a high level (–71 to –85 mV) and remained at a level of approximately -40 mV in the remaining 25–45 min recording time after the 50 μmol/L barium washout. Figure 3A shows that barium (100 μmol/L) could cause depolarization of the high RP cell. Barium (100 μmol/L) and NFA (300 μmol/L) did not shift the RP from a high level to a low level. On the contrary, NFA (300 μmol/L) did not shift the RP from a low level to a high level with simultaneously-incubating barium (100 μmol/L). We noted that NFA (300 μmol/L) maintained the membrane po-

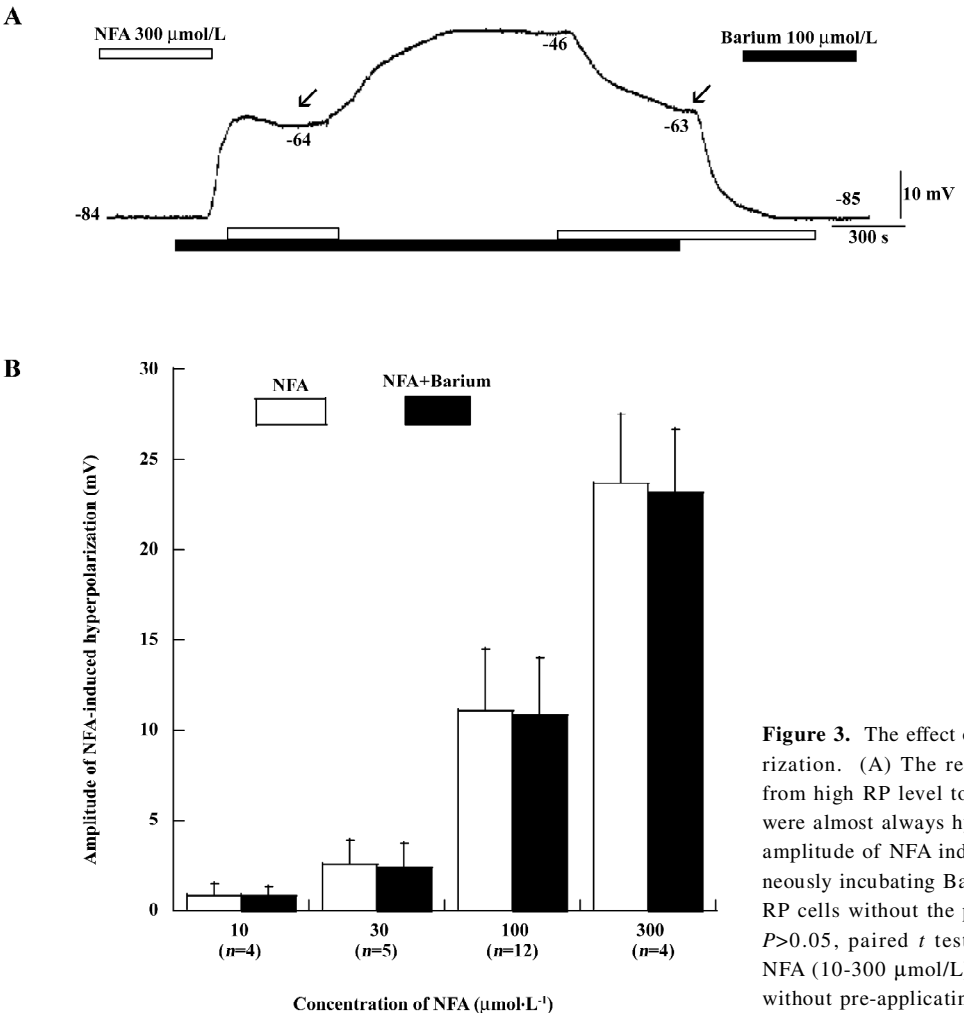



**Figure 3.** The effect of barium on NFA-induced hyperpolarization. (A) The responses of the cells that had shifted from high RP level to low RP level, with or without $Ba^{2+}$, were almost always hyperpolarized by NFA. However, the amplitude of NFA induced hyperpolarization with simultaneously incubating $Ba^{2+}$ were lower than those seen in low-RP cells without the presence of $Ba^{2+}$ (100 μmol/L) ($n$=7, $P$>0.05, paired $t$ test). (B) The different-concentration NFA (10-300 μmol/L)-induced hyperpolarization with and without pre-applicating barium (100 μmol/L).

tential around –63 to –64 mV with incubating barium (see arrows of Figure 3A). Ouabain (a $Na^+$–potassium pump current inhibitor, 100 μmol/L)-induced depolarization in the high RP cells was often largely (32.8±1.8 mV, $n$=10) or fully reversible after 15 min washout. Figure 4A shows that NFA (100 μmol/L) could cause hyperpolarization in low RP cells and shift the membrane potential from a low to high RP level. Ouabain (100 μmol/L) could shift the membrane potential from a high to low RP level again. Moreover, NFA (100 μmol/L) did not shift from a low to high RP level with simultaneously-incubating ouabain (100 μmol/L). Note that the membrane potential of cells automatically shifted from a low to high RP level when ouabain was removed (Figure 4A).

The responses of the cells that had shifted from a high to low RP level with or without barium and ouabain were almost always hyperpolarized by NFA (Figure 2A, 3A, 4A). However, the amplitude of NFA-induced hyperpolarization with simultaneously-incubating barium and ouabain was lower than those seen in the low RP cells without the presence of barium (100 μmol/L) and ouabain (100 μmol/L) ($n$=7, $P$>0.05, paired $t$-test ). Figures 3 and 4 reveal the different concentrations of NFA (10–300 μmol/L)-induced hyperpolarization with and without the pre-application of barium (100 μmol/L; Figure 3B) and ouabain (100 μmol/L; Figure 4B).

Moreover, 4-AP (1–10 mmol/L), glipizide (1–10 μmol/L), ChTX (50–100 nmol/L), apamin (50–100 nmol/L), IbTX (100 nmol/L), and TEA (10 mmol/L) also caused either a small depolarization (1–5 mV) or no membrane potential change in high RP cells ($n$≥4), but never caused a shift to the low RP level. These compounds each caused a 3–10 mV depolarization in the low RP cells ($n$≥4, data not shown).

**Mechanism of NFA-induced responses** To test the mechanism of NFA-induced response in the SMC of the SMA, the effect of antagonists of the potassium channels was obtained.

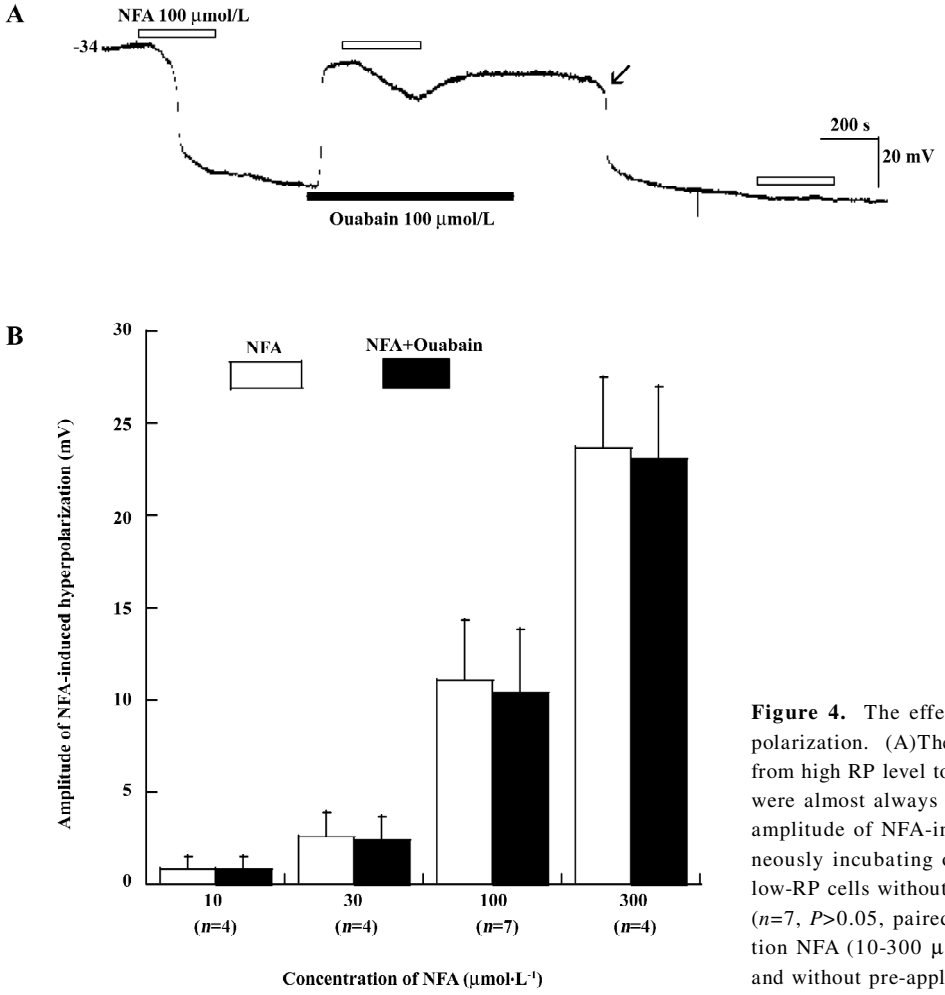



**Figure 4.** The effect of ouabain on NFA-induced hyperpolarization. (A)The responses of the cells that had shifted from high RP level to low RP level, with or without ouabain, were almost always hyperpolarized by NFA. However, the amplitude of NFA-induced hyperpolarization with simultaneously incubating ouabain were lower than those seen in low-RP cells without the presence of ouabain (100 μmol/L) ($n$=7, $P$>0.05, paired $t$ test ). (B) The different-concentration NFA (10-300 μmol/L)-induced hyperpolarization with and without pre-applicating ouabain (100 μmol/L).

NFA-induced hyperpolarization was almost completely inhibited by IbTX (100 nmol/L, $n$=6, $P$<0.05, paired $t$-test), a specific blocker of large-conductance $Ca^{2+}$-activated potassium channels (Figure 7C), ChTX (50-100 nmol/L, $n$=8, $P$<0.05, paired $t$-test), a non selective blocker of $Ca^{2+}$-activated potassium channels (Figure 7A), and TEA (1-10 mmol/L, $n$=10, $P$<0.05, paired $t$-test), a general blocker of a variety of potassium channels, including the $Ca^{2+}$-activated potassium current (Figure 5A). Besides this, we also used conventional whole-cell patch-clamp to determine what kind of channel was activated by NFA. Figure 6A shows that the gap-free trace represents the trace of the NFA-induced outward current. The 2 deflections (a and b) were whole-cell currents caused by ramp voltage commands applied before (a) and during (b) the NFA-induced outward current. Figure 6B shows the *I/V* curves (a and b) constructed by the ramp commands (a and b) in Figure 6A. Figure 6C shows the *I/V* relation of the NFA-induced net current (b–a), which had a reversal potential at –77 mV (after correction for $R_a$). The reversal potential was very close to the calculated $E_k$ (–83 mV).

We also studied the effects of 4-AP (a selective voltage-activated potassium channel [$K_V$] blocker), barium (a selective inwardly-rectifying potassium channel [$K_{ir}$] blocker), glipizide (a selective ATP-sensitive potassium channel [$K_{ATP}$] blocker), apamin (a selective small conductance $Ca^{2+}$-activated potassium channel [$SK_{Ca}$] blocker) and ouabain (a $Na^+$–potassium pump current inhibitor) on SMC in order to test whether other types of potassium channels take part in the response induced by NFA. However, they had little effect on NFA-induced hyperpolarization in SMC in spite of the concentration of 4-AP (0.5-1 mmol/L, $n$=7), barium (20-100 μmol/L, $n$=25), glipizide (3–5 μmol/L, $n$=7), apamin (50-100 nmol/L, $n$=7), and ouabain (100 μmol/L, $n$=19), which were many times the half-block constant when corresponding to potassium channels (Figure 2A,3,4,5B,5C,8)[28,29].

In addition, our experiment found that NFA-induced hyperpolarization could be almost completely inhibited by BAPTA-AM (a membrane-permeant $Ca^{2+}$ chelator, 50-100

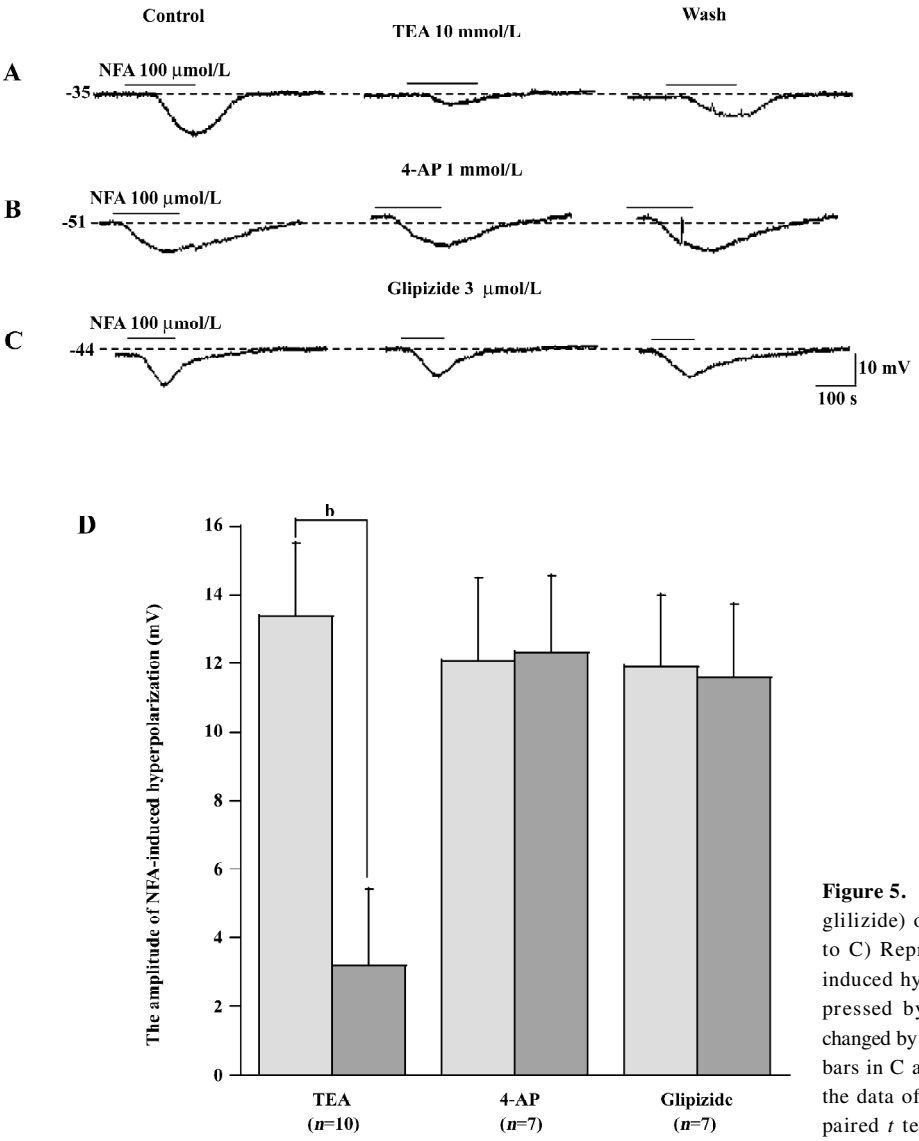



**Figure 5.** Effect of K+ channel antagonists (TEA, 4-AP, glilizide) on the NFA-induced hyperpolarization. (A to C) Representative traces show 100 μmol/L NFA-induced hyperpolarization was almost completely suppressed by 10 mmol/L TEA, but not significantly changed by 1 mmol/L 4-AP and 3 μmol/L glipizide. Scale bars in C apply to all traces. (D) Column graph shows the data of mean±SEM from the cell groups. $^{b}P<0.05$, paired *t* test.

μmol/L, $n=9$, $P<0.05$, paired *t*-test; Figure 7D). After more than 20 min, the response induced by BAPTA-AM largely recovered if the bath time of BAPTA-AM was less than 7 min. However, $CdCl_2$ (a blocker of the non-selective $Ca^{2+}$ channel, 100 μmol/L, $n=6$) had little effect on NFA-induced hyperpolarization in SMC (Figure 7E).

The column plots of data statistics exhibited that 100 nmol/L ChTX, 100 nmol/L IbTX, 10 mmol/L TEA, and 50 μmol/L BAPTA-AM had a significant ($P<0.05$, $P<0.01$, paired *t*-test) inhibition, but 1 mmol/L 4-AP, 3 μmol/L glipizide, 50 nmol/L apamin, 10–300 μmol/L barium, 10-300 μmol/L ouabain, and 100 μmol/L $CdCl_2$ had no significant (paired *t*-test) effects on NFA hyperpolarization.

NFA-induced hyperpolarizations could be blocked by ChTX, IbTX, TEA, and BAPTA-AM, and the reversal potential of the NFA-induced net current was near the $E_k$. This suggests that the $Ca^{2+}$-activated potassium channels were involved in the hyperpolarization in SMC.

## Discussion

The main findings of this study include: (1) NFA, IAA-94, and DIDS caused concentration-dependent hyperpolarizations in low RP level cells, but not in high RP level cells of the SMA; (2) the NFA-induced hyperpolarization was specifically blocked by a non-selective $Ca^{2+}$-activated potassium channel blocker (ChTX), a specific large-conductance $Ca^{2+}$-activated potassium channel blocker (IbTX), a variety of potassium channel general blockers (TEA), and membrane-permeant $Ca^{2+}$ chelator (BAPTA-AM), where it was not affected by other potassium channel blockers; (3) these triggers

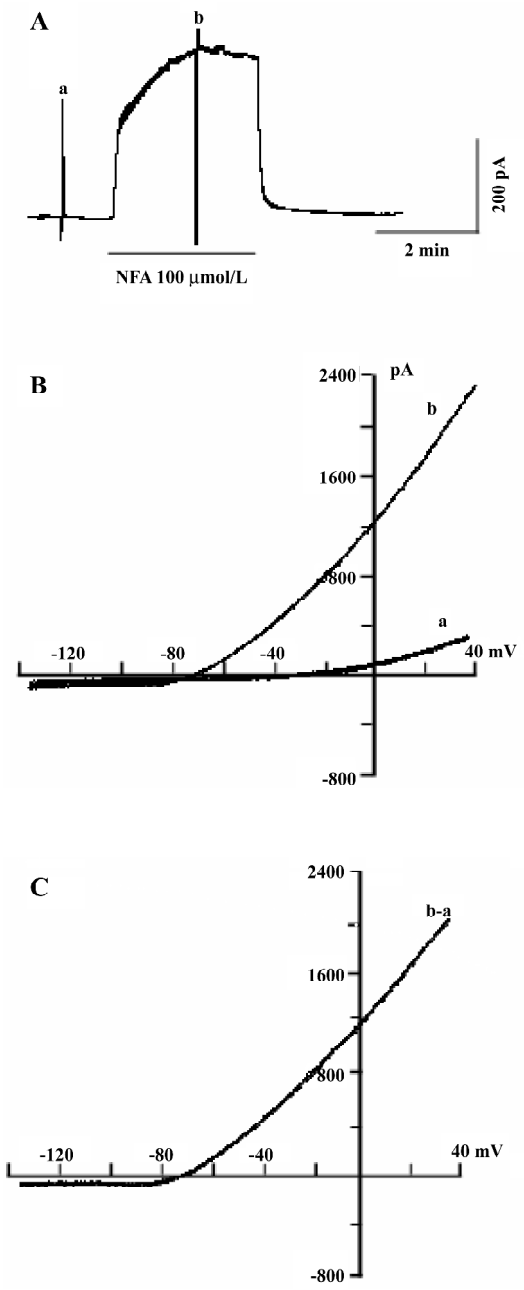
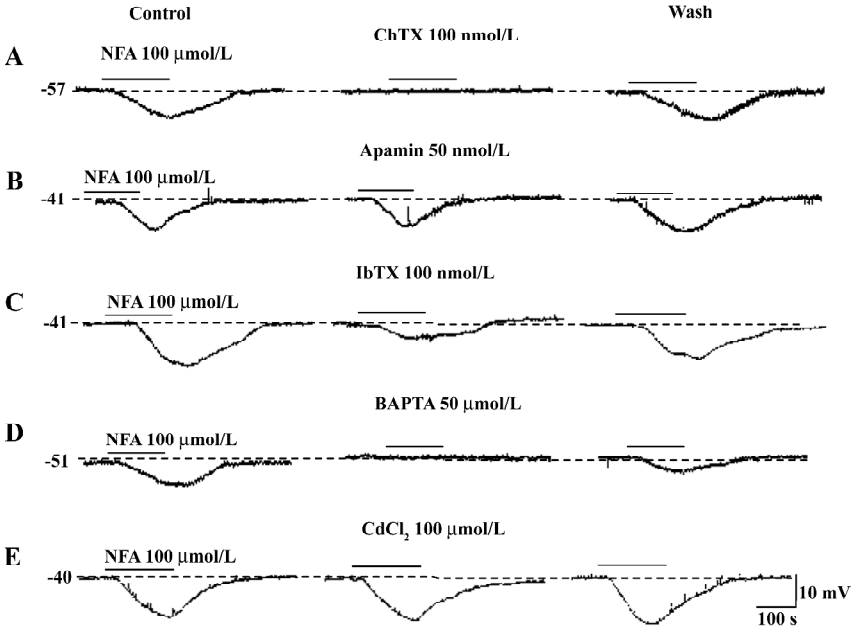



**Figure 6.** The NFA-induced outward current from a situ smooth muscle cell. (A) Representative trace of NFA-induced outward current. The two deflections (a and b) were whole-cell currents caused by ramp voltage commands applied before (a) and during (b) the NFA-induced outward current. (B) *I/V* curves (a and b) constructed by the ramp commands at (a and b) in (A). (C) *I/V* relation of the NFA-induced net current (b-a), which had a reversal potential at -77 mV (after correction for $R_a$). The reversal potential was very close to calculated $E_k$ (-83 mV).

(including NFA) had a large membrane hyperpolarization in common, which was a key factor to invoke the maximal $K_{ir}$ and $Na^+$–potassium pump activation and keep it activated in the SMC. Taken together, the results suggest that NFA, a $Cl^-$ channel antagonist, hyperpolarizes the vascular cells when the cells are at the low RP level. Our findings extended the observations from jejunum SMC, corneal epithelium cells, and coronary SMC, where NFA and flufenamic acids (a $Cl^-$ channel antagonist) increased a calcium-dependent or independent potassium current[23,30-32].

Moreover, the recorded cells were composed of both SMC and EC roughly at a 2:1 ratio (data not shown). Both types of cells showed electrical coupling within the same type of cells and between the 2 types of cells, thus they generally had similar electrical membrane properties; for example, both had 2 RP levels and responded the same way to the application of high potassium and ACh[26], so we refer to both SMC and EC by "SMC" or "cells" unless specified.

Our data support the notion that the NFA-induced hyperpolarization or the outward current in SMC of the SMA is generated by opening the $K_{Ca}$ channel. Evidence shows that the hyperpolarization was blocked by ChTX for $K_{Ca}$, IbTX for $BK_{Ca}$[33], BAPTA-AM for chelate intracellular $Ca^{2+}$, TEA for a variety of potassium channels[34], and the reversal potential of the NFA-induced net current near the $E_k$, but not by selectively blockers for other potassium channels, including barium for $K_{ir}$, glipizide for $K_{ATP}$, 4-AP for $K_V$, apamin for $SK_{Ca}$ and ouabain for the $Na^+$–potassium pump[12,25,26,35-37]. Multiple types of $K_{Ca}$ channels have been described in different systems and in the same tissue, including vascular smooth muscle, and they are differentiated by several parameters ,such as conductance, pharmacology, and kinetics[8,38-40]. Large, intermediate, and small conductance $K_{Ca}$ channels have all been identified in EC[26,35]. The large-conductance $K_{Ca}$ is a special member of the family of ligand-gated potassium channels because its gating is both ligand- and voltage-dependent. Channel opening requires calcium binding to sites on the cytoplasmic face of the channel, and in the presence of calcium, channel opening is increased by membrane depolarization. NFA may activate the $BK_{Ca}$ that is sensitive or not to ChTX[23,41]. Ottolia and Toro[23] indicate that $BK_{Ca}$ channels possess a specific NFA receptor. Furthermore, the opening of $BK_{Ca}$ channels by NFA is caused by an increase in the sensitivity of channel gating to calcium. The NFA binding site is not the same as the one for ChTX and TEA. Generally, the receptor for NFA is not located at or near the pore of the $BK_{Ca}$ channel, and NFA association to its receptor does not alter the functional properties of TEA and ChTX receptors located in the external vestibule of the channel pore[23]. Jury *et al* reported that NFA acts by opening potassium channels, some of them TEA sensitive and some not.



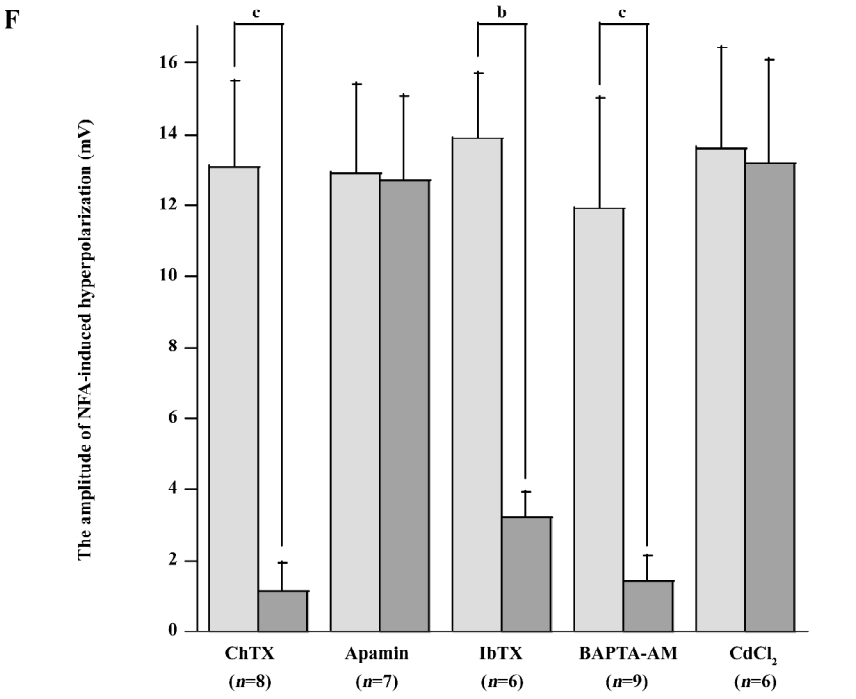




**Figure 7.** Hyperpolarization by NFA were blocked by ChTX, IbTX and BAPTA-AM, but not by apamin and $CdCl_2$. (A to E) Representative traces show 100 μmol/L NFA-induced hyperpolarization was almost completely suppressed by 100 nmol/L ChTX, 100 nmol/L IbTX, 50 μmol/L BAPTA-AM, but not significantly changed by 50 nmol/L apamin and 100 μmol/L $CdCl_2$. Scale bars in E apply to all traces. (F) Column graph shows the data of means±SEM from the cell groups. $^bP<0.05$, $^cP<0.01$, paired *t* test.

$BK_{Ca}$ channels are sensitive to both $Ca^{2+}$ and voltage. It has been postulated that these channels might be involved in the membrane repolarization that follows depolarization and the associated increase in intracellular free calcium during an action potential[8]. Poronnik *et al*'s work has demonstrated that flufenamic acid can raise intracellular calcium in $ST_{885}$ cells, a neonatal mouse mandibular line[43]. As the outward potassium current reported in Farrugia *et al*'s study was not calcium sensitive, changes in intracellular calcium should not be a factor in modulating this current in jejunal SMC[31].

The application of BAPTA-AM indicates that NFA-induced hyperpolarization was dependent on the increase of the intracellular calcium concentration. Recently, it has found that there is overlapping pharmacology of calcium-dependent potassium and calcium-dependent $Cl^-$ channels[44].

However, in the present study, NFA-induced hyperpolarization could be blocked by ChTX , IbTX, and TEA. Furthermore, the reversal potential of the NFA-induced outward current was near the $E_k$ (approximately –77 mV; Figure 7D). Also, NFA-induced hyperpolarization was blocked by BAPTA-AM (Figure 8D), not by $CdCl_2$ (Figure 8E). This evidence suggests that the $K_{Ca}$ channel is involved in NFA-induced hyperpolarization. However, a lot of questions still remain: for example, where does cytosolic calcium come from, and which receptor can be activated by NFA? This study will be the basis of our significant work in the future.

The elevation of extracellular potassium can activate the $K_{ir}$ and the electrogenic $Na^+$–potassium pump that causes hyperpolarization[45-47]. NFA indirectly hyperpolarizes the SMC through potassium release by activating the $K_{Ca}$, then the increased extracellular potassium activates the $K_{ir}$ and $Na^+$–potassium pump current in SMC. A permanent shift from a low to high RP level can be triggered by high potassium ($K_{ir}$ and $Na^+$–potassium pump activator), ACh[23,26], NFA[23,41] (activating $BK_{Ca}$), pinacidil, and nitric oxide ($K_{ATP}$ activators)[25]. Since these triggers (including NFA) have a large membrane hyperpolarization in common, it is suggested that the hyperpolarization itself is a key factor to invoke the maximal $K_{ir}$ and $Na^+$–potassium pump activation and keep it activated.

In summary, using intracellular and tight-seal whole-cell recording methods, we suggest that NFA induces concentration-dependent, reversible hyperpolarization in SMC in the cochlear SMA via the activation of the $Ca^{2+}$-activated potassium channels. Thus we think NFA is an unreliable pharmacological tool to evaluate $Cl^-$ channel contributions to smooth muscle function[42].

## Author contribution

Jun-qiang SI designed research; Li LI, Ke-tao MA performed research; Lei ZHAO contributed new analytical reagents and tools; Zhong-shuang ZHANG, He ZHU, Jing LI analyzed data; Li LI, Jun-qiang SI wrote the paper.

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