Niflumic acid hyperpolarizes the smooth muscle cells by opening BK$_{Ca}$ channels through ryanodine-sensitive Ca$^{2+}$ release in spiral modiolar artery

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Abstract: The mechanism by which niflumic acid (NFA), a Cl$^{-}$ channel antagonist, hyperpolarizes the smooth muscle cells (SMCs) of cochlear spiral modiolar artery (SMA) was explored. Guinea pigs were used as subjects and perforated patch clamp and intracellular recording technique were used to observe NFA-induced response of SMC in the acutely isolated SMA preparation. The results showed that bath application of NFA, indanyloxyacetic acid 94 (IAA-94) and disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS) caused hyperpolarization and evoked outward currents in all cells at low resting potential (RP), but had no effects in cells at high RP. In the low RP SMCs, the average RP was about (-42.47±1.38) mV ($n=24$). Application of NFA (100 $\mu$mol/L), IAA-94 (10 $\mu$mol/L) and DIDS (200 $\mu$mol/L) shifted the RP to (13.7±4.3) mV ($n=9$, $P<0.01$), (11.4±4.2) mV ($n=7$, $P<0.01$) and (12.3±3.7) mV ($n=8$, $P<0.01$), respectively. These drug-induced responses were in a concentration-dependent manner. NFA-induced hyperpolarization and outward current were almost blocked by charybdotoxin (100 nmol/L), iberiotoxin (100 nmol/L), tetraethylammonium (10 mmol/L), BAPTA-AM (50 $\mu$mol/L), ryanodine (10 $\mu$mol/L) and caffeine (0.1–10 mmol/L), respectively, but not by nifedipine (100 $\mu$mol/L), CdCl$_2$ (100 $\mu$mol/L) and Ca$^{2+}$-free medium. It is concluded that NFA induces a release of intracellular calcium from the Ca$^{2+}$ stores and the released intracellular calcium in turn causes concentration-dependent and reversible hyperpolarization and evokes outward currents in the SMCs of the cochlear SMA via activation of the Ca$^{2+}$-activated potassium channels.

Key words: spiral modiolar artery; smooth muscle cell; niflumic acid; hyperpolarization; Ca$^{2+}$ stores
Many chloride channel inhibitors, including members of the non-steroidal anti-inflammatory drug family such as niflumic acid (NFA), indanyloxyacetic acid 94 (IAA-94) and disodium 4,4’-disothiocyanatostibene-2,2’-disulfonate (DIDS), not only inhibit Cl- conductance[1], but also stimulate large conductance calcium-activated potassium channels (BKCa) in vascular smooth muscle cells (SMCs) of rabbit portal vein[2], pig coronary[3]. NFA may activate the BKCa that is sensitive or not sensitive to charybdotoxin (ChTX)[3,4]. In our preliminary study, we found that calcium-activated potassium channels (KCa) might exist in the membrane of SMCs and the endothelial cells (ECs) of the cochlear spiral modiolar artery (SMA)[5]. Moreover, chloride channel blockers could inhibit excitatory junction potentials in SMCs of the cochlear SMA in guinea pigs[6]. BKCa channels are present in a variety of cell types[5,7,8]. In neurons they may regulate cell firing[9], and in smooth muscle they seem to play an important role in maintaining visceral and vascular tone[10-12].

In the SMCs within the wall of small pressurized cerebral arteries, elevation of intravascular pressure causes a membrane depolarization and increases arterial wall [Ca2+] through opening[13] voltage-dependent Ca2+ channel which leads to increase in Ca2+ entry[14,15]. As cerebral arteries develop myogenic tone, KCa channels are activated to cause a tonic hyperpolarization to oppose the depolarization in response to higher pressure[10]. KCa channels appear to be activated by local calcium release events (‘calcium sparks’) through ryanodine-sensitive Ca2+ release channels, which are also referred to as RyR channels or ryanodine receptors, in the sarcoplasmic reticulum (SR) of arterial SMCs[16,17]. Therefore, Ca2+ sparks via KCa channels appear to represent a negative feedback pathway to oppose SMCs’ membrane potential[10,16]. On the other hand, cAMP and cGMP can increase KCa channel activity by increasing Ca2+ spark frequency and by directly increasing the open probability of the KCa channel. Together these actions lead to increased Ca2+ spark frequency and higher amplitude of KCa channels, which could cause membrane potential hyperpolarization[18].

The aim of the present study is to use direct intracellular recordings of membrane potential and conventional whole-cell recordings to investigate the intracellular mechanisms by which NFA induces hyperpolarization and evokes outward currents in the SMCs in the cochlear SMA of guinea pigs. The results suggest that NFA hyperpolarizes SMCs by activating BKCa channels through increase in intracellular calcium. The calcium comes from the Ca2+ stores within the cells and does not come from an influx through Ca2+ channel in the cell membrane from the extracellular fluid.

1 MATERIALS AND METHODS

1.1 Animals and SMA preparation

Guinea pigs (300–500 g) were anesthetized and then killed by exsanguination[19]. The anesthesia was accomplished by intramuscular injection of anesthetic mixture (1 mL/kg) of ketamine (500 mg), xylazine (20 mg) and acepromazine (10 mg) in 8.5 mL H2O. Both bullae of each animal were rapidly removed and transferred to a Petri dish filled with a physiological solution (Krebs) composed of (in mmol/L): NaCl 125, KCl 5, CaCl2 1.6, MgCl2 1.2, NaH2PO4 1.2, NaHCO3 18, glucose 8.2, and this solution was continuously bubbled with 95% O2 and 5% CO2 at 35 °C (pH 7.4). The SMA and some associated radiating arterioles were further dissected out 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 segment of the SMA of 2–5 mm long was cleaned free of spongy connective tissues and pinned with minimum nails to the silicon rubber layer (Sylgard 184, Dow Corning) in the bottom of a 0.5 mL organ bath and was continuously perfused with a 35 °C Krebs solution. When needed, a high potassium Krebs solution was made with additional KCl and accordingly reduced NaCl. The role of Ca2+ channels in the cell membrane in activation of the outward K+ current was investigated by extracellular application of a Ca2+-free solution containing 0.5 mmol/L EGTA, in which MgCl2 was increased to 3.9 mmol/L to keep membrane charge screening approximately constant.

1.2 Intracellular recording

Intracellular recording was made from a segment of the SMA in the base and the second turn of the cochlea as described previously[6,20]. Briefly, the micro-electrode was
filled with 2 mol/L KCl, having a tip resistance of 60–150 MΩ. Intracellular penetration was obtained by advancing the electrode into adventitial surface of the vessel. 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 PC computer equipped with Axoscope8 and pClamp6 software (Axon Instruments, USA) using sampling intervals of 0.1, 0.5 or 10 ms. The resting potential (RP) 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[19,20].

### 1.3 Perforated patch clamp technique

Using an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA, USA), perforated patch clamp recording with 200 μg/mL Nystatin was performed on SMCs in situ from the SMA[21]. The specimen was continuously superfused with the normal external solution (0.2 mL/min) 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 Sutter Instruments P-80 puller (Sutter Instruments, USA). The pipette was filled with an internal solution containing (mmol/L): K-glucuronate 130, NaCl 10, MgCl2 1.2, HEPES 10, EGTA 0.5 and glucose 7.5, adjusted to pH 7.2 and to osmolarity of 290 mOsm/L. The recording pipettes had a tip of ~1 μm OD and a resistance of ~5 MΩ. Pipette capacitance was well compensated, while membrane input capacitance (Cm) was uncompensated to monitor access resistance (Ra) and membrane parameters online. The voltage clamping error introduced by the current (I) passing the access resistance was corrected offline according to the equation \( V_m = V_c - IR_a \) (in which \( V_m \) 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. Membrane current or voltage signal was low-pass filtered at 5 or 10 kHz (-3 dB); data were recorded on a PC computer equipped with a Digidata 1322A AD-interface and pClamp 9.2 software (Axon Instruments Inc., USA) at a sampling interval of 10, 20 or 100 ms. A gap-free recording was simultaneously carried out by a Minidigiti...
perpolarizations to (13.7±4.3) mV (n=9, P<0.01), (11.4±4.2) mV (n=7, P<0.01) and (12.3±3.7) mV (n=8, P<0.01), respectively, from initial low RP of (-42.47±1.38) mV (n=24). The NFA, IAA-94 and DIDS-induced responses were in a concentration-dependent manner (data not shown). On the other hand, NFA, IAA-94 and DIDS had little effect on the membrane potential of SMCs that had an initial high RP (Fig. 1).

2.3 The effect of K⁺ channel on NFA-induced hyperpolarizations
To test the contribution of K⁺ channels to NFA-induced hyperpolarization in the SMCs, the effect of K⁺ channel antagonists was observed. NFA-induced hyperpolarization was almost completely inhibited by IbTX (100 nmol/L, n=7, P<0.05), a specific blocker of BKCa channels (Fig. 2A), ChTX (50–100 nmol/L, n=8, P<0.05), a non-selective blocker of KCa channels (Fig. 2B) and TEA (10 mmol/L, n=6, P<0.05), a general blocker of a variety of potassium channels including the KCa channels (Fig. 2C). We also used conventional whole cell patch clamp to testify which kind of channels could be activated by NFA. I/V relation of the NFA-induced net current showed that the reversal potential was at -77 mV, which was very close to calculated E_k (-83 mV) (data not shown).

Fig. 1. Cl⁻ channel antagonists evoked membrane hyperpolarization in all of the low RP cells, but had little effect on high RP cells. A: NFA (100 μmol/L) induced different responses in the low and high RP cells. B: IAA-94 (10 μmol/L) evoked different responses in the low and high RP cells. C: DIDS (200 μmol/L) induced different responses in the low and high RP cells. Scale bars apply to all traces.

Fig. 2. NFA-evoked hyperpolarization was mediated by KCa channel. A: NFA-evoked hyperpolarization was reversibly blocked by 100 nmol/L IbTX, a specific blocker of large-conductance Ca²⁺-activated potassium channel. B: NFA-evoked hyperpolarization was completely inhibited by a non-selective Ca²⁺-activated potassium channel inhibitor ChTX (100 nmol/L). C: NFA-evoked hyperpolarization was almost completely blocked by a general Ca²⁺-activated potassium channel blocker TEA (10 mmol/L).
We also studied the effects of 4-AP [a selective voltage-activated potassium channels (Kv) blocker] (0.5–1 mmol/L, n=7), barium [a selective inwardly-rectifying potassium channels (Kir) blocker] (20–100 μmol/L, n=25), glipizide [a selective ATP-sensitive potassium channels (KATP) blocker] (3–5 μmol/L, n=7), apamin [a selective small conductance Ca²⁺-activated potassium channels (SKCa) blocker] (50–100 nmol/L, n=7) and ouabain (a Na⁺-K⁺ pump current inhibitor) (100 μmol/L, n=19), but they had little effect on NFA-induced hyperpolarization in SMCs.

2.4 The effect of intracellular calcium on NFA-induced hyperpolarization

Our experiment found that NFA-induced hyperpolarization could be almost completely inhibited by BAPTA-AM (a membrane-permeant Ca²⁺ chelator, 50 μmol/L, n=7, P<0.05) (Fig. 3A) and ryanodine (an inhibitor of calcium release channels in SR, 10 μmol/L, n=5, P<0.05) (Fig. 3B). Moreover, our experiment found that NFA-induced outward currents could also be almost completely inhibited by caffeine (a Ca²⁺ consuming drug of store, 0.1–10 mmol/L, n=17, P<0.05) (Fig. 4), when the bath time of caffeine was more than 10 min. The column plots of data statistics exhibited that 0.1–10 mmol/L caffeine had significant inhibitory effect on the NFA-induced outward currents (P<0.05, P<0.01, paired t test). But CdCl₂ (a blocker of non-selective Ca²⁺ channel, 100 μmol/L, n=6), nifedipine

Fig. 3. NFA-evoked hyperpolarization was blocked by BAPTA-AM and ryanodine. A: NFA-induced hyperpolarization was almost completely inhibited by 50 μmol/L BAPTA-AM, a membrane-permeant Ca²⁺ chelator. B: NFA-evoked response was nearly completely blocked by 10 μmol/L ryanodine, an inhibitor of calcium release channels in sarcoplasmic reticulum.

Fig. 4. NFA-evoked outward currents were mediated by caffeine. A: NFA-evoked outward currents were inhibited by different concentrations of caffeine (0.1, 1, 10 mmol/L), a Ca²⁺ consuming drug of store. B: Column plots of data statistics showing a significant inhibition by 0.1–10 mmol/L caffeine. *P<0.05, **P<0.01 vs control, paired t test. The number of cells used at test is indicated above each column.

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(a blocker of voltage-dependent L-type calcium channel, 100 μmol/L, n=7) and Ca2+-free medium (bathing solution containing 0.5 mmol/L EGTA and 3.9 mmol/L MgCl2, n=5) had little effect on NFA-induced hyperpolarization in SMCs (Fig. 5).

3 DISCUSSION

The study provides the first direct evidence that NFA induces hyperpolarization in low RP SMCs via activation of ryanodine-sensitive Ca2+ release channels and KCa channels, and that the intracellular calcium comes from the Ca2+ stores and does not come from an influx through Ca2+ channel in the cell membrane.

Our data support the notion that the NFA-induced hyperpolarization or outward currents in SMCs of SMA was generated by opening of BKCa and that KCa channels were involved in the NFA-induced hyperpolarization. This conclusion comes from the evidence that the hyperpolarization or outward current were blocked by ChTX, IbTX, TEA, and the reversal potential of NFA-induced net current near EK, but not by blockers selective for other K+ channels including Ba2+, glipizide, 4-AP, apamin and ouabain[5,20,22]. On the other hand, NFA-induced responses were blocked by BAPTA-AM, ryanodine and caffeine, not by CdCl2, Ca2+-free medium and nifedipine.

Ottolia et al.[3] indicated that BKCa channels possessed a specific NFA receptor. Furthermore, the opening of BKCa channels induced by NFA is caused by an increase in the sensitivity of channel gating to calcium. NFA binding site is not the same as the one for ChTX or TEA. Taken together, the receptor for NFA is not located at or near the pore of the BKCa channel and the binding of NFA to its receptor does not alter the functional properties of TEA or ChTX receptors located in the external vestibule of the channel pore[3]. Jury et al. reported that among the K+ channels that NFA could open, some were TEA-sensitive and some not. BKCa channels are sensitive to both Ca2+ and voltage[23].

Multiple types of KCa channels have been identified in different systems and in the same tissue including vascular smooth muscle; they are differentiated by several parameters such as conductance, pharmacology, and kinetics[7,24]. Large, intermediate, and small conductance calcium-activated potassium channels have all been identified in the ECs[20]. The BKCa channel 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 bound to sites on the cytoplasmic face of the channel, and in the presence of calcium, channel opening extent is increased by membrane depolarization. It is conceivable reason that NFA, IAA-94 and DIDS had little effect on the membrane potential of SMCs that had an initial high RP.

The research of Nelson’s group had demonstrated a close functional coupling between Ca2+ release through ryanodine-sensitive Ca2+ release channels in the SR and hyperpolarization activated by BKCa channels in SMCs from rat cerebral arteries[25,26]. Three possible explanations for observed ryanodine-sensitive Ca2+ release in the SR are as follows: (1) ryanodine-sensitive Ca2+ release in the SR through
membrane potential depolarization activates voltage-dependent Ca\(^{2+}\) channels leading to increased cytosolic Ca\(^{2+}\); (2) cAMP and cGMP increase the frequency of local Ca\(^{2+}\) release events from the SR\(^{[18]}\); (3) depletion of SR calcium content leads to the activation of sarcoplasmic calcium reentry pathway that is independent of the voltage-dependent calcium channel, i.e. "store depletion activated calcium entry" observed in many non-excitable cells\(^{[25-29]}\). But our data not support the first and third explanations, because our preparation is not non-excitable cells and NFA-induced hyperpolarization can not be inhibited by nimodipine, CdCl\(_2\) and Ca\(^{2+}\) free medium. However, K\(_{ca}\) channels can be activated by cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG)\(^{[30,31]}\). cAMP and cGMP can increase Ca\(^{2+}\) spark frequency 2–3 folds as well as have a small, direct effect on K\(_{ca}\) channel open probability\(^{[30-32]}\). Increasing frequency and amplitude of Ca\(^{2+}\)-sensitive K\(^+\) currents should cause membrane potential hyperpolarization. Furthermore, NFA enhanced the cardiac steady-state K\(^+\) current, and enhancing effect of Cl\(^-\) channel to smooth muscle function. We think that drugs of NFA are unreliable pharmacological tool to evaluate contributions of Cl\(^-\) channel to smooth muscle function.

In summary, through intracellular and perforated whole-cell recording methods, we suggest that NFA induces concentration-dependent reversible hyperpolarizations or outward currents in the SMCs in the cochlear SMA via activation of the BK\(_{ca}\) channels through ryanodine-sensitive Ca\(^{2+}\) release.

**REFERENCES**


