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Fenamates block gap junction coupling and potentiate BK_{Ca} channels in guinea pig arteriolar cells

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Abstract

We determined the actions of the fenamates, flufenamic acid (FFA) and niflumic acid (NFA), on gap junction-mediated intercellular coupling between vascular smooth muscle cells (VSMC) *in situ* of acutely isolated arteriole segments from the three vascular beds: the spiral modiolar artery (SMA), anterior inferior cerebellar artery (AICA) and mesenteric artery (MA), and on non-junctional membrane channels in dispersed VSMCs. Conventional whole-cell recording methods were used. FFA reversibly suppressed the input conductance (G_{input}) or increased the input resistance (R_{input}) in a concentration dependent manner, with slightly different IC_{50} s for the SMA, AICA and MA segments (26, 33 and 56 μM respectively, $P>0.05$). Complete electrical isolation of the recorded VSMC was normally reached at 300 μM . NFA had a similar effect on gap junction among VSMCs with an IC_{50} of 40, 48 and 62 μM in SMA, AICA and MA segments, respectively. In dispersed VSMCs, FFA and NFA increased outward rectifier K^+ -current mediated by the big conductance calcium-activated potassium channel (BK_{Ca}) in a concentration-dependent manner, with a similar EC_{50} of ~300 μM for both FFA and NFA in the three vessels. Iberiotoxin, a selective blocker of the BK_{Ca}, suppressed the enhancement of the BK_{Ca} by FFA and NFA. The K_V blocker 4-AP had no effect on the fenamates-induced K^+ -current enhancement. We conclude that FFA and NFA blocked the vascular gap junction mediated electrical couplings uniformly in arterioles of the three vascular beds, and complete electrical isolation of the recorded VSMC is obtained at $\geq 300 \mu\text{M}$; FFA and NFA also activate BK_{Ca} channels in the arteriolar smooth muscle cells in addition to their known inhibitory effects on chloride channels.

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Keywords

flufenamic acid; niflumic acid; gap junction; potassium channel; arteriole; electrical coupling

1. Introduction

Fenamates, the derivatives of N-phenylanthranilic acid, are a family of nonsteroidal anti-inflammatory drugs including flufenamic acids (FFA), meclofenamic acid (MFA) and niflumic acids (NFA). The pharmacological knowledge of fenamates has expanded over the years but remains limited, which hinders a better understanding of the mechanisms for their clinic usefulness and side effects.

Fenamates have been demonstrated to block gap junctions in rat fibroblasts and neuroblastoma cells (Juszczak and Swiergiel, 2009), antagonize a subset of chloride channels (Duran et al., 2010; Hartzell et al., 2005), and exert variable effects on transient receptor potential (TRP) channels (Hill et al., 2004; Saleh et al., 2006). The present study is a detailed investigation of FFA- and NFA-induced gap junction blockade between arteriolar cells.

Gap junctions are specialized arrays of intercellular channels which connect adjacent cells in many tissues and provide intercellular chemical and electrical communications (McCracken and Roberts, 2006; Sohl et al., 2005). Gap junction channels comprise two hemi-channels or connexons, one contributed by each cell. Connexons are composed of six protein subunits called connexins (Cx). To date, more than 20 different connexin genes have been identified as expressed in many mammalian cell types and tissues (McCracken and Roberts, 2006; Tran and Welsh, 2009). Vascular gap junctions are assembled with various combinations of Cx37, Cx40, Cx43, and Cx45 (Tran and Welsh, 2009). Cx37, Cx40, and Cx43 are typically found in endothelial cells (ECs), and predominantly Cx43, but also Cx37 and Cx45, in vascular smooth muscle cells (VSMCs) (Figuroa et al., 2006).

Fenamates have been shown to inhibit gap junctions in a monolayer of normal rat kidney fibroblasts and SKHep1 cells which over-expresses connexin43 (Cx43) (Harks et al., 2001). FFA blocks gap junctions formed of Cx26, Cx32, Cx40, Cx43, Cx46, and Cx50 in N2A cells with low selectivity - the half inhibition concentration (IC₅₀) ranged between 20 – 60 μ M (Srinivas and Spray, 2003). FFA has also been found to block mouse connexin50 (Cx50) and rat connexin46 (Cx46) hemichannels expressed in *Xenopus laevis* oocytes (Eskandari et al., 2002). More recently, MFA, FFA, and NFA were reported to inhibit dye coupling in a retina network of A-type horizontal cells (Pan et al., 2007). However, analogous inhibition of gap junctions and other membrane effects in native vascular tissues are yet to be shown.

Gap junctions play a key role in the development, structure, physiology and pathology of many organs, particularly the vascular system (Figuroa et al., 2004; Figuroa et al., 2006; Griffith, 2004; Jiang et al., 2005; Sandow, 2004). For instance, vascular tone and conductive vasomotion rely on gap junction-mediated coupling and synchronization of the VSMCs and ECs (Figuroa et al., 2004; Juszczak and Swiergiel, 2009; Segal, 2005). Vaso-active agents such as ACh, substance P and bradykinin cause a primary hyperpolarization in ECs and a secondary hyperpolarization in VSMCs via so-called endothelium-derived hyperpolarizing factor (EDHF) (Busse et al., 2002; Juszczak and Swiergiel, 2009). Studies of various vascular preparations have implicated nitric oxide (NO), prostaglandins, and cytochrome P450 products epoxyeicosatrienoic acids (EETs) as the EDHF (Busse et al., 2002), but the gap junction appears to be the major and universal mechanism (Griffith, 2004; Sandow, 2004).

Using whole-cell voltage-clamp techniques, we studied fenamates actions on vascular gap junctions and non-junctional channels in VSMCs *in situ* of acutely isolated arteriole segments from the cochlear spiral modiolar artery (SMA), anterior inferior cerebellar artery (AICA) and mesenteric artery (MA), and in dispersed VSMCs. The present investigation should contribute to better understanding of fenamates effects/side effects in clinic as well as to a better data interpretation when they are used in basic research.

2. Material and Methods

2.1. Animals and preparations

Guinea-pigs (250–300g) were killed by exsanguination under deep general anesthesia by intramuscular injection of an anesthetic mixture (1 ml/kg) of ketamine (500 mg), xylazine (20 mg) and acepromazine (10 mg) in 8.5 ml water. The entire length of the SMA was harvested from the cochlea. Brain arteriolar segments were harvested from branches of the AICA in the pia. The MA and its branches were harvested from upper ileum mesentery. The animal use protocol was approved by the University Animal care and Use Committee of Oregon Health & Science University, USA and Shihezi University, China.

The arterioles were manually cleaned in a Petri dish filled with aerated normal external solution (NES) composed of (mM): NaCl 138, KCl 5, CaCl₂ 1.6, MgCl₂ 1.2, Na-HEPES 5, HEPES 6, Glucose 7.5, with pH 7.4 and osmolarity of 300 mOsm/L. A short segment of the vessel (~0.3 mm long, 30 – 50 µm OD) was secured at each end to the bottom of the dish by the weight of a platinum strip (~0.1×2 mm), and digested with collagenase A (1 mg/ml) dissolved in normal external solution at 37°C for 15 min. After completely washing out the enzyme, the vessel was further cleared of its adventitial tissue with fine tweezers under a microscope. The Petri dish was then placed on the stage of an inverted microscope equipped with micromanipulators. The arteriole segment and electrode pipette were visualized by DIC at 10 × 20 or 10 × 40 magnification.

Dissociated VSMCs were prepared from the SMA or arteriolar branches of the AICA or MA of guinea-pigs. The cleaned arterioles were incubated for 20 min in a low-Ca²⁺ buffer solution containing (mM): NaCl 142, KCl 5, CaCl₂ 0.05, MgCl₂ 1, Na-HEPES 5, HEPES 6 (pH 7.2), and glucose 7.5. The arterioles were cut into ~1 mm long segments and digested for 20 – 25 min at 37°C with this buffer solution containing 1.5 mg/ml papain, 2 mg/ml collagenase A, 3.75 mg/ml BSA, and 0.3 mg/ml dithiothreitol. After centrifuging (67 *g* for 5 min) and replacing the supernatant with enzyme-free buffer three times, the preparation was triturated with a Pasteur pipette. The cell-rich suspension was transferred to a Petri dish with a poly-L-lysine coated coverslip-bottom. Once the dispersed cells were attached to the glass bottom, the dish was mounted on an inverted microscope and perfused with normal extracellular solution for whole-cell recording. VSMCs were identified by their characteristic spindle-shape and robust delayed outward rectification (Guan et al., 2007; Ma et al., 2011).

2.2. Tight-seal whole-cell recording

The specimen was continuously superfused in normal external solution (0.2 ml/min) at room temperature (22–25°C). Conventional whole-cell recordings were performed using an Axon 700B amplifier (Molecular Device, USA). Recording pipettes were fabricated from borosilicate glass capillaries and filament with a Sutter Instruments P-97 puller. The pipette typically had a tip of ~1 µm OD and a resistance of ~5 MΩ after being filled with normal internal solution (NIS) containing (mM): K-gluconate 130, NaCl 10, CaCl₂ 2.0, MgCl₂ 1.2, HEPES 10, EGTA 5, and glucose 7.5, adjusted to pH 7.2 and osmolarity 290 mOsm/L. Pipette capacitance was well compensated when a giga Ohm seal with the cell was achieved. Membrane current or voltage signal was low-pass filtered at 5 kHz (–3 dB). Data were

recorded on a PC computer equipped with a Digidata 1440A AD-interface, running pClamp 10.2 software (Axon Instruments, Inc.), and sampling at intervals of 10, 20, or 100 μ s as needed. A Minidigi digitizer and Axoscope 10.2 software (Axon Instruments, Inc. USA) were used to carry out a gap-free recording at a sampling interval of 50 ms throughout the experiment.

The seal resistance usually reached 1 to 20 G Ω before rupture of the membrane. Membrane rupture was achieved by a buzz current and/or a suck pressure to the pipette. The transient current through the membrane input capacitance (C_{input}) was routinely uncompensated in order to monitor and calculate the access resistance (R_a), the membrane input resistance (R_{input}) and C_{input} online or off-line (de Roos et al., 1996; Lindau and Neher, 1988). The off-line calculation was done with exponential fit to the capacitive current transients and by the commonly used equations (Guan et al., 2007). The C_{input} for *in situ* cells were calculated according to $C=Q/V$, where the charge (Q) was obtained by 2 – 4 term exponential fit to the current transient elicited by a voltage step (Fig. 1). The voltage clamping error introduced by the current (I) passing the R_a 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, when appropriate, was also done offline.

2.3. Drug application & statistics

Drugs were applied by superfusion via an array of capillary inlets near the preparation in the dish. The solution flowing over the preparation could be switched to one that contained drugs by shifting the inlets without change of flow speed. Drugs used in this study included flufenamic acid (FFA), niflumic acid (NFA), iberitoxin (IBTX), 4-aminopyridine (4AP) (from Sigma-Research Biochemicals Inc.). FFA and NFA were dissolved in DMSO as stock solutions before being further diluted with normal external solution to final concentrations, the later containing DMSO 0.1%. DMSO at such low concentrations showed no membrane effects (Ma et al., 2011). Statistical values are expressed as means $\pm$ S.E.M. Significance of differences was determined using Student's *t*-test unless otherwise stated.

3. Results

3.1. Membrane property of cells in the arterioles

Whole-cell recordings were made on VSMCs *in situ* of and dispersed from the SMA, AICA and MA from 59 guinea pigs. Stable recording lasted from 5 min to 6 h on *in situ* cells, whereas only up to 2 h in dispersed VSMCs. Data of the measurement of input conductance (G_{input}) or R_{input} and C_{input} of the *in situ* and dispersed VSMCs from the three arteriole groups are summarized in Table 1. Note that G_{input} was 8, 8.5, and 11.5 times higher in *in situ* cells than in the dispersed cells of the SMA, AICA, and MA, respectively, consistent with electrical coupling in the three arterioles. Second, C_{input} was 14, 20, and 22 times higher in *in situ* cells than in dispersed cells of the SMA, AICA, and MA, also consistent with electrical coupling in the three vessels. Third, current transients recorded during voltage steps, showed a time course that fitted poorly to a single term exponential function in *in situ* cells (Fig. 1C & G), but did fit well to a four-term exponential function (not shown), indicating multiple membrane sources in the charging circuit, or electrical coupling of multiple cells in the arterioles. On the other hand, the dispersed VSMCs showed a fast step-induced capacitive current (Fig. 3) which fit well to a single term exponential function.

3.2. FFA and NFA block the electrical coupling of in situ smooth muscle cells

Application of 30–1000 μ M FFA and NFA caused a significant attenuation of the steady-state current amplitude induced by voltage steps from the holding potential (HP) –40 mV in

cells of the three arteriolar segments (Fig. 1), indicating an increase in R_{input} or reduction of G_{input} . The G_{input} inhibition was detectable within 1–2 min and reached steady state within ~5 min for 300 μM FFA and NFA. The inhibition was fully reversed by ~10 min wash with a drug-free NES (Fig. 1*I&J*). Figure 2 shows that, constructed with ramp voltage commands (–140 to 40 mV), the whole-cell I/V curve slope was reduced in the entire voltage range and the FFA-induced net current showed an approximately linear I/V relation with a reversal potential close to the resting potential of the recorded cell. These data suggest that the G_{input} reduction by FFA or NFA was mainly due to a blockade of the gap junction-mediated electrical coupling (Guan et al., 2007).

Figure 2 also summarizes the dose-response results for FFA and NFA and compares the potency of G_{input} blockage in the three arterioles. G_{input} inhibition by FFA or NFA was concentration-dependent, with the three arterioles not showing significantly different IC_{50}s (ANOVA). In the presence of 300 μM FFA or NFA, the R_{input} of VSMCs in the three arteriolar segments increased from several hundred mega ohms to several giga ohms, a value similar to that seen in dispersed VSMCs (Table 2 vs. Table 1). Furthermore, the voltage step-induced capacitive transients in cells superfused in 300 μM FFA or NFA were well fitted by a single term exponential function, with a time constant and capacitance similar to that seen in dispersed VSMCs. Therefore, a complete electrical isolation in VSMC is generally obtained with FFA or NFA at 300 μM .

3.3 The non-junctional effects of FFA or NFA: a study on dissociated VSMCs

Whole-cell voltage-clamp experiments were performed on dispersed VSMCs to determine the non-junctional effects of FFA and NFA on VSMCs. The dispersed VSMCs of the three vessel types show significantly different G_{input} (or R_{input}) and C_{input} , ordered as $\text{MA} > \text{AICA} > \text{SMA}$ (Table 1), consistent with the visual impression of cell size in the vessels. The whole-cell I/V relation of VSMCs of all the vessel types showed a robust outward rectification with a characteristic time delay at the beginning of the step command, thus classically called delayed rectifier potassium currents (K_{DR}) (Fig. 3). K_{DR} current was often visually more marked in the AICA and MA than in SMA cells. The current density of VSMCs of the SMA was indeed significantly smaller than those in AICA and MA at +20 and +40 mV (Fig. 4*E*).

The experiments confirm, as previously reported, that voltage-gated outward rectifier current contains two major components, one sensitive to the BK_{Ca} channel blocker IBTX, and the other suppressed by the K_{V} channel blocker 4-AP (Fig. 3) (Ma et al., 2011; Nelson and Quayle, 1995). The IBTX-sensitive BK_{Ca} current exhibits a time course that follows a total current trajectory (Fig. 3*A*), and the steady-state amplitude is approximately proportional to total outward current at voltages 0 to +40 mV (Fig. 3*B*). In contrast, the 4-AP-sensitive K_{V} current displays a dynamics quite different from that of total current, especially between +20 and +40 mV where it peaks in the beginning and then decays within a 500 ms step duration (Fig. 3*A*). 4-AP-sensitive K_{V} current is a major portion of total current at 0 mV, but a minor portion at 40 mV (Fig. 3*B*).

FFA or NFA enhanced the K_{DR} elicited by step voltage commands from –40 mV to –60 mV through +40 mV in 20 increments without significantly effecting the zero current (resting) membrane potential, G_{input} and I/V curves at potentials –40 mV in dispersed VSMCs (Fig. 4 and Fig. 5*A,B,E,F*). The enhanced net current exhibited a voltage-dependency and time course (dynamics) similar to those of the IBTX-sensitive net current but very different from those of 4-AP-sensitive net current (Fig. 4 vs. Fig. 3, and Fig. 5*B*).

The enhancement of the K_{DR} by FFA was concentration-dependent with an EC_{50} of 346, 270 and 293 μM in SMA, AICA and MA, respectively (Fig 4*C*). Although the difference in

EC₅₀s among the three arterioles is not statistically significant, the enhancement magnitude is. The latter is significantly smaller in the SMA than those in the MA and AICA cells. The order of enhancement percentage magnitude, MA>AICA>SMA, shows a trend similar to the K_{DR} current density of the three vessels (Fig. 4. *E&F*). NFA showed a similar action of FFA with an EC₅₀ of 371, 301 and 246 μ M in SMA, AICA and MA, respectively (Fig 4*D*).

More strikingly, the presence of 100 nM IBTX almost completely suppressed the enhancement of K_{DR} by FFA and NFA (Fig 5*A–D*). In contrast, in the presence of 1 mM 4-AP, FFA induced a net current not significantly different than without 4-AP (Fig. 5*E–H*).

4. Discussion

It was shown in previous studies that fenamates block gap junction mediated current spread and dye transfer in cultured kidney cells expressing Cx43 (Harks et al., 2001; Juszczak and Swiergiel, 2009), block dye transfer between N2A cells expressing defined connexins (Srinivas and Spray, 2003), and block Cx36-related electrical synapses in retinal amacrine and on-cone bipolar cells (Veruki and Hartveit, 2009). We extend these observations by examining the effect of FFA and NFA on coupling currents in the *in situ* cells of acutely dissected arteriolar segments. In addition, we analyzed the non-junctional membrane actions of FFA and NFA on K_{DR} in dissociated VSMCs. We demonstrated, first, FFA and NFA concentration-dependently blocked the gap junction coupling of *in situ* VSMCs in the SMA, MA and AICA with IC₅₀s of 26–62 μ M, with no significant difference between these two compounds and among the three arterioles. Closure of gap junctions by FFA and NFA was rapid and quickly reversible. Second, we showed that FFA and NFA caused an enhancement of BK_{Ca} channels in dispersed VSMCs of SMA, MA and AICA with potency about one order lower than that for junctional blockade. Third, we demonstrated again that whole-cell voltage-clamp recording from VSMCs embedded in intact arteriole segments is a suitable approach to study the pharmacology of arteriolar gap junctions. This method has several advantages for the study of ion channels in VSMCs, including avoidance of single-cell isolation by harsh enzymatic treatment thus permitting cells to be studied under a much better physiological condition (Guan et al., 2007; McGahon et al., 2005; Quinn and Beech, 1998; Yamazaki and Kitamura, 2003).

Our measurements of FFA and FNA concentrations for either half blockade or full blockade of vascular gap junctions are very close to the previous report on junctions composed of Cx43 in cultured kidney cells and SKHep1 cells (Harks et al., 2001). The potency similarity may be attributed to two factors. First, Cx43 are common connexin isoforms in both VSMCs and ECs (Figueroa et al., 2006). Second, fenamates are of low selectivity over the gap junctions composed of several different Cx isoforms (Srinivas and Spray, 2003).

Our previous study demonstrated that 18 β -glycyrrhetic acid (18 β GA) is more effective on VSMC gap junctions than on myoendothelial gap junctions (IC₅₀ ~2 μ M versus IC₅₀ ~4 μ M) (Guan et al., 2007). An analogous comparison was not attempted in the present study given the known low Cx isoform selectivity of fenamates, and consideration the two measurement modes would not be completely separable. IC₅₀ measurements on endothelial cell gap junctions for these drugs is now feasible in the endothelial tubule preparations we recently developed (Ma et al., 2011). However, due to the low Cx selectivity of fenamates, an IC₅₀ value similar to the present results might be expected if one would be interested in the measurement on EC tubules.

In addition to blockade of gap junctions, our data demonstrated that FFA and NFA facilitate BK_{Ca} channels in VSMCs. This is supported by following observations. First, the FFA and NFA-induced net current showed a voltage dependency and dynamics similar to 100 nM IBTX-sensitive net current but very different from 4AP-sensitive current (Fig. 3 and Fig. 4)

(Ma et al., 2011). Second, 100 nM IBTX, but not 4-AP, almost completely suppressed the net current induced by FFA and NFA (Fig. 5). In this regard, a previous study also reported that NFA and a structure-related Cl-channel blocker 5-Nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) potentiated depolarization-activated potassium currents in rat ventricular myocytes (Zhou et al., 2007). Wu *et al.* (2001) (Wu et al., 2001) also found that FFA activated BK_{Ca} channels in osteoblast-like MG-63 cells. It appears that fenamates potentiation of BK_{Ca} is a general effect across the mammalian tissues.

The magnitude of the augmentation of BK_{Ca} by FFA and NFA in the three arterioles is consistent with the order of current density exhibited in these vessels (MA > AICA > SMA). Considering that FFA and NFA show a similar EC₅₀ (~300 μ M) in these vessels, it is likely the difference in enhanced current magnitude is mainly, if not solely, due to a difference in density of channel expression in these arteriole cells.

The mechanism(s) underlying the fenamates enhancement of BK_{Ca} is beyond the scope of this study. Based on the literature available, however, it is pertinent to introduce some speculations as a future research direction. It is reported that FFA facilitates the current of α_1 -agonist-stimulated TRPC6, a channel highly Ca²⁺-permeable and expressed in vascular system (Hill et al., 2006; Inoue et al., 2001; Jung et al., 2002; Saleh et al., 2006). It would be interesting to identify whether FFA/NFA alone can stimulate TRPC6 channels, increase the sub-membrane Ca²⁺ concentration in the VSMCs and thus potentiate the BK_{Ca} conductance.

The present study did not include investigation of possible fenamates action on chloride and TRP channels. FFA and NFA have no significant effect in dispersed VSMCs on membrane potential, R_{input}, and I/V curve in the negative voltage domain (Fig. 5), suggesting these cells express little fenamates-sensitive Cl⁻ and TRP-conductances in the background experimental condition. It may be possible that the BK_{Ca} enhancement by FFA could mask its activated TRP-current at positive potentials and the TRP-induced depolarization. Cl⁻-channel blockers generally induce a hyperpolarization/relaxation and I/V slope reduction in VSMCs (but NFA, (Nelson et al., 1997)). The near complete elimination of FFA/NFA-induced current by IBTX indicates that, if any, Cl⁻- and TRP-channel currents play a minimal role in fenamate-induced net current. Although more vigorous tests are still needed to define the specifics of FFA/NFA action on Cl⁻ and TRP-channels, at this point it can be said that contamination of these two currents in FFA/NFA enhancement of K_{DR} observed is negligible.

The present results have clinic relevance as well as significance for basic science. A low dose of oral flufenamic acid (200 mg), blood levels of the drug are in the 20–30 μ M range (Glazko, 1967), which is quite close to those of IC₅₀s determined in this study (Fig. 2). Moreover, an activation of outward rectifier K⁺ current and hyperpolarization by 250 μ M FFA on the circular smooth muscle of human jejunum had been related to the dose-dependent FFA's common G–I side effects including diarrhea, nausea, vomiting, abdominal pain, and constipation (Farrugia et al., 1993). It is known that gap junction-mediated intercellular communications are also essential for G–I motility, Cx43 and Cx45 are highly expressed in both vessels and G–I tract, and the Cx43 channelopathy have been implicated in diarrhea (Guttman et al., 2010; Seki and Komuro, 2001). Considering that the fenamates are about one order more potent as gap junction inhibitors than as BK channel activators, G–I side effects are likely also the result of fenamate-induced gap junction inhibition.

Impaired gap junction function has been reported in several clinical conditions, including cardiovascular diseases (Figueroa et al., 2004). Saltman *et al.* reported that the gap junction blocker heptanol significantly reduced infarct size, and showed this effect is related to delayed electrical uncoupling during prolonged ischemia (Mao et al., 2005; Saltman et al.,

2002). Selective gap junction blockers/activators have potential for treatment of cardiovascular disease. While many groups of compounds have been found to block gap junctions, none of them so far has proved potent or selective enough to fit the demands of the clinic. Our characterization of the junctional and non-junctional effects of FFA and NFA laid a solid foundation for better interpretation of the past and future experimental results when these compounds are used.

For vascular gap junction blockade, we so far found that FFA and NFA are about 20 and 10 times less potent than 18β -glycyrrhetic acid (18β GA) and 2-aminoethoxydiphenyl borate (2-APB), respectively (Guan et al., 2007; Ma et al., 2011), and they all have non-junctional channel side effects. Considering the EC_{50} of FFA/NFA for BK_{Ca} facilitation is only one order higher than the IC_{50} for gap junction blockade and the possible actions on other channels, FFA/NFA appears to be the least useful gap junction blocker among the compounds tested in the lab, and 18β GA is the best. However, the fenamates and 18β GA exhibited opposite action on BK_{Ca} (Guan et al., 2007; Ma et al., 2011); there is a possibility that we can work out a proper combination of these two compounds which produces the needed gap junction blockade while effectively cancels the non-junctional action of BK_{Ca} .

In summary, our data demonstrated that FFA and NFA dose-dependently blocked the vascular gap junctions, achieving a complete electrical isolation of the recorded VSMC at $\geq 300 \mu M$; no significant difference was observed between these two compounds or among the three arterioles on the gap junction blockade potency. FFA and NFA also activate BK_{Ca} channel on dispersed VSMCs with a potency less than 10 times lower than its junctional inhibition.

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Fig. 1.

FFA and NFA inhibit the electrical coupling of smooth muscle cells *in situ* of an arteriole segment. (A–B) Whole-cell current traces induced by voltage steps before (A) and during (B) application of 300 μ M FFA. Note that 300 μ M FFA caused a substantial reduction in the steady-state current (I_{ss}), reflecting an increase R_{input} from 0.32 G Ω to 2.15 G Ω in this cell. (C–D) Time scale-expanded presentation of the initial part of A&B, respectively (some traces omitted for clarity), showing that a single term exponential function (gray lines between two cursors) fitted poorly ($r = 0.90$) with the current transients (black thin line) in the control (C) but well with 4 term exponential function (not shown). In the presence of FFA single term exponential fitted well with the current transient (D. $r > 0.98$). “ τ ”, time constant for the bottom trace. (E–H) Whole-cell currents show that 300 μ M NFA caused a cell an increase in R_{input} from 0.27 G Ω to 2.59 G Ω and a capacitive current transient becoming well fitted with a single exponential function. A and E were from different cells in AICA. (I–J) Gap-free traces show the time course of input conductance changes by FFA and NFA in two VSMCs of MA segments. The downward deflections are steady-state current induced by a 2 s step from HP -40 mV to -60 mV.

Fig. 2.

Concentration-dependent inhibition by FFA and NFA of the membrane input conductance of the three vessels. (A–C) Example ramp whole-cell I/V curves in the absence and presence of 300 μ M FFA in cells of the AICA, MA and SMA (top panels) and the respective FFA-sensitive net current I/V curves (lower panels). Note that FFA caused a reduction of slope conductance to a level (< 1 G Ω) of single cells in all three vessels. The net current reversed its polarity near the resting membrane potential (zero-current Potential) (Guan et al., 2007). (D–E) Plots of G_{input} against the concentrations of these two compounds. G_{input} was normalized to the control value of each cell. Each panel shows the action of one compound on cells of the three vessels. IC₅₀s are resulted from curve fit with the modified Hill equation: $Y = (1 - C) / (1 + (x/K)^h) + C$, where “C” is the theoretical residual conductance in supra maximal concentrations. All data points are from 6–12 cells.

Fig. 3.

The delayed rectifier current (K_{DR}) of dissociated VSMCs was distinctively inhibited by 4AP and IBTX. **(A)** Representative recordings of whole-cell currents elicited by step commands, showing that K_V blocker 4-AP and BK_{Ca} inhibited K_{DR} differently in time course: IBTX inhibited quite evenly over the step duration (*a*) whereas 4-AP caused stronger inhibition in the early component (*b*). The K_{DR} was additively inhibited by combination of 4AP and IBTX (*c*). "*a* – *c*" were from different cells in the MA. **(B)** Plots of leak-subtracted steady-state currents normalized to control values at +40 mV, showing that 1mM 4AP caused an inhibition similar to that of 100 nM IBTX at +40 mV, but a significantly more inhibition than IBTX at 0 and 20 mV. ** or ## $P < 0.01$; # or × $P < 0.05$. Data points were from 12–19 different cells.

Fig. 4.

FFA and NFA caused enhancement of the delayed rectifier K⁺ channels in dissociated VSMCs of the three arterioles. (A–B) Representative whole-cell current traces from two different dispersed VSMCs of the AICA in the absence (Control) and presence of 300 μ M FFA or NFA, showing an enhancement quite evenly over the step duration. (C–D) Collective data plots of steady-state amplitude enhancement of K_{DR} at +40 mV, showing that the EC₅₀s revealed by Hill equation fit are not significantly different among the three vessels ($P > 0.05$). Note that the enhancement ratio of SMA cells was significantly lower than those of the MA and AICA (* $P < 0.05$, ** $P < 0.05$ SMA vs MA; # $P < 0.05$ SMA vs AICA), whereas there were no significant difference between the MA and AICA. (E–F) The K_{DR} current density of the three vessels was approximately proportional to the enhancement ratio sequence (MA > AICA > SMA). The plots are based on data after leak-subtraction and normalized against the control value of each cell. Each data point was a mean from 9–13 cells.

Fig. 5. FFA facilitation of K_{DR} channels is sensitive to IBTX but not to 4-AP. (**A, E**) Representative whole-cell current traces from a dispersed VSMC of the AICA in the presence of 100 nM IBTX and 1 mM 4-AP, respectively. (**B, F**) I/V plots of leak-subtracted mean K_{DR} amplitudes, showing FFA enhanced outward rectification current at depolarizations beyond -40 mV. (**C–D, G–H**) Ramp-constructed I/V curves (**C** and **G**) or step-current I/V plots (**D** and **H**, leak-subtracted) depict that in the presence of IBTX, FFA induced very little net current. On the other hand, in 4-AP, adding FFA caused an enhanced net current not significantly different from that without 4-AP (**G** vs. **F** or **H**, $P > 0.05$ for net current at all data points). Each data point in **D** and **H** was from 6–9 cells of the AICA.

Membrane properties of smooth muscle cells *in situ* of a short arteriolar segments and dispersed status in whole-cell configuration.

Table 1

Membrane property	Cells <i>in situ</i> of vessel segment			Dispersed cells		
	SMA (n=26)	AICA (n=20)	MA (n=20)	SMA (n=25)	AICA (n=21)	MA (n=33)
^a R _{input} (MΩ)	523 ± 59	385 ± 44	300 ± 53 ^d	3523 ± 2225	2910 ± 198 ^f	2459 ± 115 ^{d,e}
^a G _{input} (nS)	2.48 ± 0.23	3.26 ± 0.40	5.16 ± 0.74 ^{d,e}	0.31 ± 0.02	0.38 ± 0.03	0.45 ± 0.03 ^d
^b C _{input} (pF)	97 ± 23	185 ± 27 ^f	312 ± 76 ^{c,e}	6.8 ± 0.26	8.94 ± 0.56 ^f	13.9 ± 0.54 ^{c,e}

^a Measured between -60 and -40 mV.

^b Measured by a step from -40 mV to -100 mV.

^c $P < 0.05$,

^d $P < 0.01$ vs SMA.

^e $P < 0.05$ vs AICA.

^f $P < 0.05$ vs SMA.

Table 2

Membrane actions of FFA and NFA on *in situ* VSMC.

Vessel	Membrane properties	FFA (300 μ M)		NFA (300 μ M)	
		Control	In treatment	Control	In treatment
SMA	^a R _{input} (M Ω)	340 $\pm$ 52	4248 $\pm$ 290 ^d (n=6)	463 $\pm$ 102	3571 $\pm$ 268 ^d (n=6)
	^a G _{input} (nS)	3.10 $\pm$ 0.53	0.24 $\pm$ 0.013 ^d (n=6)	2.47 $\pm$ 0.58	0.29 $\pm$ 0.043 ^c (n=6)
	^b C _{input} (pF)	79.8 $\pm$ 7.5	6.82 $\pm$ 1.29 ^d (n=6)	106 $\pm$ 12.5	8.01 $\pm$ 0.19 ^d (n=6)
AICA	^a R _{input} (M Ω)	379 $\pm$ 41	3910 $\pm$ 728 ^d (n=13)	436 $\pm$ 41	3248 $\pm$ 228 ^d (n=9)
	^a G _{input} (nS)	2.98 $\pm$ 0.56	0.29 $\pm$ 0.12 ^d (n=13)	3.11 $\pm$ 0.28	0.39 $\pm$ 0.047 ^c (n=9)
	^b C _{input} (pF)	185 $\pm$ 27	7.8 $\pm$ 0.49 ^d (n=13)	263 $\pm$ 47	8.8 $\pm$ 0.73 ^d (n=9)
MA	^a R _{input} (M Ω)	249 $\pm$ 31	3256 $\pm$ 616 ^d (n=9)	350 $\pm$ 68	2183 $\pm$ 338 ^d (n=6)
	^a G _{input} (nS)	4.78 $\pm$ 0.68	0.41 $\pm$ 0.082 ^d (n=9)	3.28 $\pm$ 0.57	0.52 $\pm$ 0.071 ^c (n=6)
	^b C _{input} (pF)	312 $\pm$ 76	11.36 $\pm$ 0.98 ^d (n=9)	293 $\pm$ 64	13.4 $\pm$ 1.14 ^d (n=6)

^a Measured between -60 and -40 mV.

^b measured by a step from -40 mV to -100 mV.

^c $P < 0.05$.

^d $P < 0.01$ vs control.