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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_{50s} 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 ≧300 μM; FFA and NFA also activate BK_{Ca} channels in the arteriolar smooth muscle cells in addition to their known inhibitory effects on chloride channels.

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

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predominantly Cx43, but also Cx37 and Cx45, in vascular smooth muscle cells (VSMCs) (Figueroa 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 (IC_{50}) ranged between 20 and 60 μM (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 (Figueroa et al., 2004, 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 (Figueroa 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 (250–300 g) 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 and 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, $CaCl_2$ 1.6, $MgCl_2$ 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-Ca^{2+} buffer solution containing (mM): NaCl 142, KCl 5, $CaCl_2$ 0.05, $MgCl_2$ 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, $CaCl_2$ 2.0, $MgCl_2$ 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 μs 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 1–20 GΩ 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 and 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), iberiotoxin (IBTX), 4-aminopyridine (4AP) (from Sigma-Research Biochemicals Inc.). FFA