Structure-activity relationship of fenamates as Slo2.1 channel activators

Priyanka Garg and Michael C. Sanguinetti

Nora Eccles Harrison Cardiovascular Research & Training Institute, Department of Pharmaceutics and Pharmaceutical Chemistry, Department of Physiology (P.G., M.C.S.) and Internal Medicine (M.C.S.), University of Utah, Salt Lake City, Utah
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Corresponding author:
Michael C. Sanguinetti, PhD
Nora Eccles Harrison Cardiovascular Research and Training Institute
Department of Physiology
University of Utah
95 South 2000 East
Salt Lake City, Utah 84112
Tele: 801-581-3058
Fax: 801-581-3128
Email: sanguinetti@cvrti.utah.edu

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ABBREVIATIONS: COX, cyclooxygenase; DFS, diclofenac sodium (2-[(2,6-dichloroanilino)phenyl]acetic acid); \( I_{K\text{Na}} \), intracellular Na\(^+\)-activated potassium current; \( I_{\text{Slo2.1}} \), Slo2.1 current; MCFA, meclofenamic acid (2-[(2,6-dichloro-3-methylphenyl)amino]benzoic acid); MFA, mfenamic acid (2-(2,3-dimethylanilino)benzoic acid); NFA, niflumic acid (2-[[3-(trifluoromethyl)phenyl]amino]pyridine-3-carboxylic acid); \( n_H \), Hill coefficient; NSAID, non-steroidal anti-inflammatory drug; PAA, N-phenylantranilic acid; TFA, tolfenamic acid (2-[(3-chloro-2-methylphenyl)amino]benzoic acid)
ABSTRACT

Niflumic acid, 2-{(3-(trifluoromethyl)phenyl)amino}pyridine-3-carboxylic acid (NFA), a nonsteroidal anti-inflammatory drug that blocks cyclooxygenase (COX), was previously shown to activate \([\text{Na}^+]_i\)-regulated Slo2.1 channels. Here we report that other fenamates, including flufenamic acid, mefenamic acid, tolfenamic acid, meclofenamic acid and a phenyl acetic acid derivative, diclofenac are also low potency \((\text{EC}_{50} = 80 \mu\text{M to } 2.1 \text{ mM})\), partial agonists of human Slo2.1 channels heterologously expressed in *Xenopus* oocytes. Substituent analysis determined that N-phenylanthranilic acid was the minimal pharmacophore for fenamate activation of Slo2.1 channels. The effects of fenamates were biphasic, with an initial rapid activation phase followed by a slow phase of current inhibition. Ibuprofen, a structurally dissimilar COX inhibitor, did not activate Slo2.1. Pre-incubation of oocytes with ibuprofen did not significantly alter the effects of NFA, suggesting that neither channel activation nor inhibition is associated with COX activity. A point mutation (A278R) in the pore-lining S6 segment of Slo2.1 increased the sensitivity to activation and reduced the inhibition induced by NFA. Together our results suggest that fenamates bind to two sites on Slo2.1 channels: an extracellular accessible site to activate, and a cytoplasmic accessible site in the pore to inhibit currents.
Introduction

Intracellular Na⁺-activated potassium currents (I_{K_{Na}}) were first identified in guinea pig cardiomyocytes 28 years ago (Kameyama et al., 1984) yet their physiological and pathophysiological roles are not well characterized. Closed under normal physiological and ionic conditions, K_{Na} channels are markedly activated upon elevation of [Na⁺]_{i} (Kameyama et al., 1984). In the heart, [Na⁺]_{i} is increased during ischemia and activation of K_{Na} currents may serve a cardioprotective function by shortening the action potential duration and preventing Ca^{2+} overload (Kameyama et al., 1984; Wang et al., 1991).

Two types of K_{Na} channels have been cloned, Slo2.1 (Bhattacharjee et al., 2003) and Slo2.2 (Yuan et al., 2003). Slo2.1 (aka K_{Ca}4.2 or Slick) channels are encoded by the KCNT2 gene in humans and are expressed in the nervous system and the heart. In addition to intracellular Na⁺, Slo2.1 channels are also activated by intracellular Cl⁻ and inhibited by intracellular ATP (Bhattacharjee et al., 2003). Low potency and non-specific blockers of Slo2.1 current (I_{Slo2.1}) include quinidine (90% block at 1 mM) and tetraethylammonium (60% block at 20 mM). The PKC activator phorbol 12-myristate13-acetate inhibits I_{Slo2.1} indirectly with an IC_{50} of 20 nM (Santi et al., 2006). We recently reported that the fenamates NFA and flufenamic acid (FFA, 2-[3-(trifluoromethyl)anilino]benzoic acid) can activate Slo2.1 with an EC_{50} of 2.1 mM and 1.4 mM, respectively, in the absence of a change in [Na⁺] (Dai et al., 2010). While NFA is the first compound shown to activate Slo2.1, its low potency and non-specificity severely limits its usefulness as a chemical probe in physiological studies.

Fenamates are N-substituted anthranilic acid derivatives used clinically as non-steroidal anti-inflammatory drugs (NSAID) for the treatment of fever, pain and inflammation (Jiang et al., 2012). These small molecules exert their action by inhibiting cyclooxygenase (COX) enzyme and thereby reduce the biosynthesis of prostaglandins (Flower et al., 1972; Sanger and Bennett, 1979). Fenamates are also low-potency modulators of a diversity of ion channels and enzymes, exhibiting either an activator or inhibitory effects. For example, fenamates inhibit Ca^{2+} activated Cl⁻ channels (Greenwood and Large, 1995; White and Aylwin, 1990), ATP-sensitive potassium channels (Grover et al., 1994) and non-selective cation channels (Gogelein et al., 1990), but activate large conductance Ca^{2+}-activated K⁺ (Slo1) channels
(Farrugia et al., 1993; Gribkoff et al., 1996; Ottolia and Toro, 1994), TRPA1 channels (Hu et al., 2010) and brain liver intestine Na⁺BLINaC channels (Wiemuth and Grunder, 2011). Fenamates have also been reported to enhance or alter the gating of Kv4.2 and Kv4.3 (Wang et al., 1997), KCNQ1/KCNE1 channels (Busch et al., 1994), KCNQ2/3 channels (Peretz et al., 2005) and hERG1-3 channels (Fernandez et al., 2008; Malykhina et al., 2002).

In the present study, we determined the effects of several fenamates on Slo2.1 channels heterologously expressed in *Xenopus* oocytes, including NFA, FFA, mefenamic acid (2-(2,3-dimethylanilino)benzoic acid), tolfenamic acid (2-[(3-chloro-2-methylphenyl)amino]benzoic acid), meclofenamic acid (2-[(2,6-dichloro-3-methylphenyl)amino]benzoic acid) and a phenyl acetic acid derivative, diclofenac (2-[2-(2,6-dichloroanilino)phenyl]acetic acid). We show that NFA and other fenamates exhibit a mixed agonist behavior. At high concentrations, NFA application causes a rapid activation of $I_{\text{Slo2.1}}$ followed by a partial inhibition. A limited structure activity relationship (SAR) study was performed to define the minimal pharmacophore requirement for fenamate-mediated activation of Slo2.1.

**Materials and Methods**

**Solutions and Drugs.** For two-microelectrode voltage clamp experiments, the extracellular solution (KCM 211) contained the following (in mM): 98 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, 5 HEPES (pH 7.6). NFA, FFA, mefenamic acid (MFA), tolfenamic acid (TFA), meclofenamic acid (MCFA) and diclofenac sodium (DFS), N-phenylanthranilic acid, diphenylamine, anthranilic acid, N-methylanthranilic acid, biphenyl-2-carboxylic acid and 2-benzyl benzoic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Ibuprofen was purchased from Cayman Chemicals (Ann Arbor, MI). Concentrated stock solutions (100 mM-1 M) of all compounds were prepared in DMSO, except MCFA and DFS that were dissolved in deionized water, and stored at -20°C until used. Solutions containing final [drug] were freshly prepared each day by diluting stock solutions and the pH was adjusted to 7.6 with 1 N NaOH.
**Molecular Biology.** KCNT2 cDNA (kindly provided by L. Kaczmarek, Yale University, New Haven, CT) was subcloned into the psGEM oocyte expression vector (Dai et al., 2010). A278R Slo2.1 was generated by using the Quikchange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and confirmed by DNA sequencing. KCNT1 (hSlo2.2) cDNA in the pCR-XL-TOPO vector was obtained from GenBank. A Mlu1 restriction site was introduced into the 5’ end, the cDNA was excised from the vector using Mlu1 and Xho1 and subcloned into the pUNIV vector (Addgene, Cambridge, MA). Finally, an Xba1 site was introduced into the vector region near the 3’ end of hSlo2.2. KCNMA1 (hSlo1) cDNA in pcDNA3.1 (+) vector was kindly provided by Jianmin Cui, Washington University, St. Louis, MO. Complementary RNA (cRNA) for all cDNAs were prepared by in vitro transcription with mMessage mMachine T7 (Life Technologies, Grand Island, NY) after linearization of the plasmid with SfiI (KCNT2), Xba1 (KCNT1) or Pvu1 (KCNMA1). The concentrations of cRNA were determined using the Ribogreen assay (Life Technologies).

**Oocyte isolation and cRNA injection.** Protocols for oocyte isolation from *Xenopus laevis* were approved by the Institutional Animal Care and Use Committee, University of Utah. Frogs were anaesthetized with a 0.2% tricaine methane sulfonate solution before a small surgical incision was made to remove ovarian lobes. Oocytes were manually separated from the lobes using tweezers and digested with 1 mg/ml of type II collagenase (Worthington Biochemical Corp., Lakewood, NJ) for 60 minutes to remove the follicle cell layer. Collagenase solution was prepared using ND96-Ca²⁺ free solution (pH 7.6) that contained (in mM): 96 NaCl, 2 KCl, 1 MgCl₂ and 5 HEPES..

For characterization of $I_{\text{Slo2.1}}$, stage IV and V oocytes were injected with 0.5 - 1 ng WT cRNA or 0.2 - 0.5 ng A278R mutant KCNT2 cRNA and incubated for 1-2 days at 18°C in Barth’s saline solution (pH 7.4) that contained in mM: 88 NaCl, 1 KCl, 0.41 CaCl₂, 0.33 Ca(NO₃)₂, 1 MgSO₄, 2.4 NaHCO₃, 10 HEPES, 1 pyruvate, 50 mg/L gentamycin. To record $I_{\text{Slo1}}$, oocytes were injected
with .04 - 1.0 ng \( KCNMA1 \) cRNA and currents were recorded 1-3 days later. Slo2.2 channels express poorly in oocytes. Therefore, to record \( I_{\text{Slo2.2}} \), oocytes were injected with 32 ng \( KCNT1 \) cRNA and currents were recorded after 4-7 days.

**Voltage Clamp.** Whole-cell currents were recorded from oocytes using a standard two-microelectrode voltage-clamp technique (Dai et al., 2010; Stühmer, 1992). Pipettes were pulled from borosilicate glass and filled with 1% agarose dissolved in 3 M KCl and then back-filled with 3 M KCl to fabricate agarose-cushion microelectrodes (Schreibmayer et al., 1994). All voltage clamp recordings were performed at room temperature (23-25°C) and the recording chamber was perfused with the drug solutions at a rate of 1 ml/min. For time-course and drug concentration-response studies, the holding potential was \(-80\) mV and step pulses of 300 ms duration were applied to 0 mV with an interval of 30 s until a steady state change in current magnitude was achieved. To determine current-voltage (\( I-V \)) relationships, test pulses to voltages between \(-120\) and \(+80\) mV were applied in 20 mV increments.

**Data analysis and statistics.** Currents were analyzed using pClamp 8.2 (Molecular Devices, Inc., Sunnyvale, CA), Origin 8.5 (Originlab, Northampton, MA) and Excel (Microsoft Corp, Redmond, WA) software. Results are expressed as mean ± SEM (\( n \) = number of oocytes). For concentration-response curves, currents were normalized to the maximum response produced by each test compound. These data were fitted by non-linear curve fitting (Origin 8.5) to the logistic equation to estimate EC\(_{50}\) and Hill coefficient, \( n_H \). Statistical significance was evaluated by Student’s t-test (\( p \leq 0.05 \) was considered significant). Chemical structures were drawn using ChemSketch (Advanced Chemistry Development, Toronto, ON).

**Results**

**Biphasic action of NFA on Slo2.1 channels.** As previously reported (Dai et al., 2010), negligible currents were observed in oocytes injected with low amounts of \( KCNT2 \) cRNA under control conditions...
amplitude (Fig. 1A, lower panel). The I-V relationship for NFA-activated \( I_{\text{Slo2.1}} \) exhibited outward rectification with a reversal potential of \(-95\) mV (Fig. 1B). Thus, when relatively small amounts of cRNA (<0.5 ng/oocyte) are injected, Slo2.1 channel activity is small or barely detectable, but can be rapidly and robustly activated with NFA.

The time course of NFA on \( I_{\text{Slo2.1}} \) was characterized by applying repetitive test pulses to 0 mV once every 30 s. The onset of \( I_{\text{Slo2.1}} \) activation by 1 mM NFA was rapid and reached maximal effect in 4-5 min. The effects of NFA were rapidly and completely reversible upon washout. However, after ~5 min in the continued presence of NFA, currents slowly decreased in magnitude over the next 35 min until they decayed to 30% of its peak value (Fig. 2A). As described for CLC-K chloride channels (Liantonio et al., 2006), the biphasic effects of NFA on Slo2.1 could result from drug binding to distinct activator and inhibitory sites on the channel. Activation of Slo2.1 channels by extracellular NFA in inside-out patches (Dai et al., 2010) and the more potent activity of external NFA on Slo1 channels expressed in lipid bilayers (Ottolia and Toro, 1994) indicates that the activator effect is mediated by interaction with an extracellular domain of the channel. The delayed and slow decay of current magnitude that follows the initial channel activation could result from the time required for the acidic drug to cross the cell membrane and inhibit channels by a second mechanism, either causing a direct pore block or a consequence of inhibition of cytosolic COX activity (e.g., inhibition by elevated levels of arachidonic acid). To test for the later possibility, oocytes expressing WT Slo2.1 were pre-incubated with 1 mM ibuprofen for 40-120 min prior to co-application of 1 mM NFA and 1 mM ibuprofen. Ibuprofen is structurally dissimilar to the fenamates and does not activate \( I_{\text{Slo2.1}} \) (not shown), but should completely inhibit COX as it inhibits human recombinant COX-1 and COX-2 with an IC\(_{50}\) of 2.6 µM and 1.53 µM, respectively (Barnett et al., 1994). Pretreatment of oocytes with ibuprofen did not alter the magnitude or
time course of NFA-mediated current activation and inhibition (Fig. 2B). Thus, the NFA mediated inhibition of Slo2.1 is independent of the cyclooxygenase-prostaglandin pathway.

**A278R mutant channels are more sensitive to the activator effect, but less sensitive to inhibitory effect of NFA.** Mutations in Slo2.1 can alter constitutive channel activity and response to NFA (Dai et al., 2010). In the S6 segment, we found that mutation of Ala278 to Arg increased basal activity of Slo2.1 and greatly increased its sensitivity to the activator action of NFA. The effect of 1 mM NFA on A278R channel currents is illustrated in Fig. 3A. Outward currents in the presence of NFA were nearly instantaneous and mostly time-independent during the 300 ms test pulse, except at the most positive test potentials. This differs from WT channels, where the time-dependent component was more prominent (compare Figs. 1A and 3A). I-V relationships measured before and after 1 mM NFA are plotted in Fig. 3B. The magnitude of NFA-activated inward currents of A278R channels was larger than for WT channels, indicating a negative shift in the conductance-voltage relationship. Similar to our previous report (Dai et al., 2010), activation of WT channels by NFA was concentration dependent over the range of 1 to 10 mM with an EC₅₀ of 2.1 ± 0.1 mM and nᵢ of 2.4 ± 0.1 (n = 9). A278R channels were 19-fold more sensitive to NFA with an EC₅₀ of 0.11 ± 0.01 mM and nᵢ of 2.0 ± 0.2 (n = 13) (Fig. 3C).

At the same concentration used to characterize WT channels, 1 mM NFA only reduced the peak $I_{\text{Slo2.1}}$ by 20% after 40 min (Fig. 3D and E). No time-dependent reduction in constitutively active channel activity was observed in the absence of NFA (Fig. 3E), confirming that current inhibition was caused by NFA and was not due to channel run-down.

**Structure-activity relationship for fenamates.** The effects of several other fenamates, including FFA, MFA, TFA and MCFA and a phenyl-acetic acid derivative, diclofenac (Fig. 4A) on WT Slo2.1 channels were determined. The concentration-response relationships for all the drugs are shown in Fig. 4B. MCFA was the most potent activator with an EC₅₀ of 0.08 ± 0.01 mM (n = 5). The rank order of potency of fenamates for the activation of Slo2.1 was MCFA > TFA > MFA > DFS > FFA > NFA (Table 1). Similar to NFA, the onset of current activation for all other fenamates was rapid and completely reversible after 6-9 min washout of the drug.
A278R mutant channels were used to estimate the activator effects of other fenamates in the relative absence of inhibitory activity. As summarized in Fig. 4C, A278R Slo2.1 channels were more sensitive than WT channels to fenamates. Identical to NFA, A278R channels were 19-times more sensitive to FFA (EC$_{50}$ = 0.06 ± 0.01 mM). The leftward shifts of the concentration-response relationships were less for MFA, TFA, MCFA and DFS, exhibiting approximately 3-7 fold reduction in EC$_{50}$. The $n_H$ varied from 1.7 to 2.3 indicating similar positive cooperativity for A278R and WT channels. Evidently, A278R Slo2.1 channels are primed for gating modification by fenamates, presumably because of a reduced energy of activation induced by the mutation.

We further probed the SAR of fenamates to determine the minimum structural requirement for activation of Slo2.1. The effects of a series of commercially available compounds, including N-phenylanthranilic acid (PAA), diphenylamine, anthranilic acid, N-methylantranilic acid, biphenyl-2-carboxylic acid and 2-benzyl benzoic acid (Fig. 5A) were determined. PAA is a planar molecule with a carboxylic acid, two six-membered aromatic rings (I and II) and a bridging imino group. The carboxylic group is attached to ring I at the ortho position to the imino N-atom. Coplanarity between these groups is stabilized by resonance interactions and an internal hydrogen bonding between the carboxylic and the imino group (Dhanaraj and Vijayan, 1988). PAA is the parent moiety for all fenamates and lacks substituents on ring II. As shown in Figs. 5B-D, PAA activates $I_{\text{Slo2.1}}$ with an EC$_{50}$ of 0.79 ± 0.03 mM ($n_H$ = 1.5 ± 0.03; $n$ = 7) indicating that the presence of substituents on ring II is not essential for activity.

We dissected the requirement for each functional group in the PAA moiety by testing compounds lacking a specific functional group at their highest soluble concentration. For compounds that showed no response, 1 mM NFA was applied to confirm that the oocytes tested had adequate channel expression. Diphenylamine, lacking the carboxylic acid of ring I did not activate $I_{\text{Slo2.1}}$ at 1 mM (Fig. 5E). Elimination of ring II yields anthranilic acid which also did not activate currents at a concentration of 3 mM (Fig. 5F). We reasoned that ring II may endow essential hydrophobicity to the compounds; therefore, we tested N-methylantranilic acid (10 mM) wherein ring II is replaced by a methyl group. This compound also failed to activate $I_{\text{Slo2.1}}$ (Fig. 5G) indicating that this second aromatic group is indispensable for activity.
Biphenyl-2-carboxylic acid lacks the ring-bridging imino group. The application of 3 mM biphenyl-2-carboxylic acid did not activate $I_{\text{Slo2.1}}$ (Fig. 5H). Finally, replacement of the anilinic N with carbon in 2-benzyl benzoic acid, also failed to activate Slo2.1 at 10 mM (Fig. 5I), indicating the possible importance of internal H-bonding between the imino N atom and the carboxylic O atom.

**Relative efficacy of fenamates.** We evaluated the apparent efficacy of all fenamates on WT channels by comparing the peak outward $I_{\text{Slo2.1}}$ measured at 0 mV for each compound with NFA at their previously determined EC$_{90}$. The same batch of oocytes was used for each comparative experiment. The rank order of efficacy was NFA = TFA $\geq$ FFA $>$ MCFA = MFA = DFS = PAA (Fig. 6). Even though NFA was the least potent, it exhibited greater maximal efficacy. In contrast, MCFA was the most potent activator of Slo2.1, but showed lower efficacy than NFA ($p = 0.025$). Thus, fenamates have variable efficacy that differs from the rank order of their potencies. The difference in potency vs. efficacy of fenamate-mediated activation might be due to simultaneous activation and inhibition mediated by separate binding sites, or an intrinsic property of a single receptor; i.e., the compounds are partial agonists.

**PAA does not inhibit Slo2.1.** In contrast to what we observed for NFA, 1 mM PAA inhibited WT channel currents $< 10\%$ after 20 min before exhibiting a 2nd slower phase of continuing activation (Fig. 7A), suggesting that the fenamate induced inhibition is a characteristic of its more hydrophobic derivatives and not the parent moiety itself. The time course of MCFA, the most potent activator of Slo2.1 was also investigated to determine if its decreased efficacy could be explained by a faster than normal onset of inhibition. For this experiment, the approximate EC$_{20}$ for MCFA (0.03 mM) was used to match the EC$_{20}$ of NFA (1 mM) previously studied for its inhibitory effect. However, as shown in Fig. 7B, the onset of time dependent inhibition exerted by MCFA was similar to that induced by NFA, suggesting that it is a partial agonist of the Slo2.1 channel.

**Fenamates also activate Slo2.2 and Slo1 channels.** Slo2.2 is closely related to Slo2.1 and is also activated by intracellular Na$^+$. Slo2.2 channels express poorly in oocytes, requiring injection of larger amounts of cRNA and longer periods of incubation as compared to Slo2.1 to achieve functional expression. As expected, Slo2.2 was also activated by NFA (Fig. 8A and 8B) with an EC$_{50}$ (2.7 ± 0.19
mM; Fig. 8C) similar to that determined for Slo2.1. Slo1 channels are large conductance K+ channels activated by intracellular Ca\(^{2+}\) that have previously been reported to be activated by fenamates (Farrugia et al., 1993; Gribkoff et al., 1996; Ottolia and Toro, 1994). However, as shown in Fig. 9, fenamates are less potent activators of Slo1 channels than Slo2 channels. The effects of 1 mM NFA, MCFA and PAA on \(I_{\text{Slo1}}\) are illustrated in Fig. 9A-C. \(I-V\) relationships for \(I_{\text{Slo1}}\) under control conditions and after activation of channels by several concentrations of these fenamates are plotted in Fig. 9D-F. The EC\(_{50}\) for \(I_{\text{Slo1}}\) activation was 0.68 ± 0.19 mM (\(n = 11\)) for MCFA (Fig. 9G), ~9-fold less sensitive than Slo2.1 channels. Slo1 was also less sensitive to NFA and PAA, as only partial activation was achieved with a concentration of 10 mM (Fig. 9G). The EC\(_{50}\) for these two compounds, 10.0 ± 0.9 mM (\(n = 5\)) for NFA and 13.4 ± 0.7 mM (\(n = 7\)) for PAA, was determined by extrapolation of the concentration-response relationships (Fig. 9G) with the assumption that \(n_H\) for these compounds was the same as determined for Slo2.1 channels. Finally, we compared the efficacy of NFA to MCFA (Fig. 9H). Similar to our finding with Slo2.1 channels, MCFA was more potent, but less efficacious than NFA in activation of Slo1 channels.

**Discussion**

In the present study, we have shown that fenamate NSAIDs including NFA, FFA, MFA, TFA, MCFA and a phenyl-acetic acid derivative, diclofenac are low potency modulators of Slo2.1 channels heterologously expressed in *Xenopus* oocytes. These agents exhibit a biphasic action on Slo2.1 with a rapid onset of activation and a relatively slow and delayed onset of inhibition. Fenamates are widely used clinically to inhibit COX activity. However, their activation and inhibitory effects on Slo2.1 are not associated with COX inhibition as pretreatment of oocytes with 1mM ibuprofen did not alter the magnitude or time-course of \(I_{\text{Slo2.1}}\) modification by 1mM NFA. The potency of NFA for activation of intracellular Na\(^{+}\)-modulated Slo2.2 channels (EC\(_{50}\) = 2.7 mM) was similar to that determined for Slo2.1 (EC\(_{50}\) = 2.1 mM); however, intracellular Ca\(^{2+}\)-modulated Slo1 channels were 5-fold less sensitive to NFA (EC\(_{50}\) ~ 10 mM). Slo1 channels were also ~9-fold and ~17-fold less sensitive to activation by MCFA and
PAA, respectively. Thus, while fenamates are known to block, or modulate the gating of a plethora of ion channels, these compounds are more potent modulators of Slo2 than Slo1 channels.

The dual action of fenamates has been previously reported for CIC-KCl channels (Liantonio et al., 2006; Liantonio et al., 2008). In our study, the activation effect of NSAIDs was rapid in onset and completely and quickly reversible on washout, suggesting these drugs bind to a readily accessible extracellular domain of the channel protein as previously demonstrated for Slo1 channels. NFA was shown to be 5-times more potent in activation of Slo1 channels reconstituted into lipid bilayers when added to the extracellular side compared to the internal side of the channel (Ottolia and Toro, 1994). In contrast, the onset of fenamate induced inhibition of $I_{\text{Slo2.1}}$ was delayed and much slower to develop. Slow inhibition can be explained by the longer time required for these acidic compounds to cross the cell membrane and bind to an inhibitory site accessible only from the cytoplasm. Current inhibition could result from a direct occlusion of the Slo2.1 channel pore. Consistent with a pore block mechanism was our finding that inhibition of A278R channels was much reduced compared to WT channels. Ala278 residues are located in each of the four S6 segments that line the central cavity of Slo2.1. Reduced sensitivity to inhibition suggests that substitution of Ala with Arg reduces binding of fenamates to a pore-occluding binding site. A278R channels also exhibited an increased basal activity and enhanced sensitivity to activation by fenamates. Together, these mutant channel properties were useful for quantifying the activator effects of fenamates in the relative absence of inhibitory activity.

The fenamates examined here exhibited a range of potencies and apparent efficacies for activation of Slo2.1. For example, NFA exhibited high efficacy but low potency, whereas MCFA exhibited low efficacy but high potency. These attributes could result from differences in intrinsic activity (partial agonism) and/or the balance between binding to two distinct receptor sites that mediate activation and inhibitory effects on gating and/or ion permeation. A possible explanation for the low potency of NFA as compared to other fenamates is that it has a nearly coplanar conformation (Dhanaraj and Vijayan, 1988). Other fenamates have non-planar orientations of the two aromatic rings.
With the exception of NFA, all the other fenamates are N-aryl-substituted derivatives of anthranilic acid with different substituents on the phenyl ring. Starting from the parent structure, N-phenylanthranilic acid as the lead compound and dissecting all its functional moieties thereafter, the minimal structural requisite for activation of Slo2.1 was established. Together, the acidic carboxylic group, two aromatic rings and an imino moiety bridging the two rings, constitute the minimal fenamate pharmacophore for Slo2.1 activation. Since fenamates are amphipathic molecules (Dhanaraj and Vijayan, 1988), the binding site of these drugs may comprise both hydrophilic and hydrophobic regions. The carboxylic group may interact with a polar region while ring II could interact with a hydrophobic site on the protein. However, replacement of ring II with –CH₃ (N-methylanthranilic acid) resulted in loss of activity, indicating the requirement for either an aryl or hydrophobic substituent larger than a methyl group at this position.

In summary, we have shown that fenamates are both partial agonists and antagonists of Slo2.1 and that these activities are likely mediated by distinct binding sites. PAA was established as the minimum structural requirement for activation of Slo2.1 channels. Although the mechanism of activation of the examined set of fenamates appear to be similar, relatively minor perturbations in the basic structure can lead to marked changes in their potency and efficacy. These studies represent the first step in the search for selective and potent modulators of Slo2.1 channels that will be needed to pharmacologically probe the physiological and pathophysiological roles of these channels.
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Authorship Contributions:

Participated in research design: Garg, Sanguinetti.

Conducted experiments: Garg, Sanguinetti.

Performed data analysis: Garg, Sanguinetti

Wrote or contributed to the writing of the manuscript: Garg, Sanguinetti.
References


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Reprint requests: M.C. Sanguinetti, Nora Eccles Harrison Cardiovascular Research & Training Institute, Department of Physiology, University of Utah, 95 South 2000 East, Salt Lake City, UT 84112; E-mail: sanguinetti@cvrti.utah.edu
Figure legends

Fig. 1. Effect of NFA on WT Slo2.1 channels. (A) Voltage clamp protocol (upper panel) and currents recorded from an oocyte expressing WT Slo2.1 channels before (middle panel, Control) and after treatment with 1 mM NFA (lower panel). Oocytes were recorded after 3 days of injection with 0.4 ng WT Slo2.1 cRNA. Arrows indicate 0 current. (B) Average I-V relationships for WT $I_{Slo2.1}$ recorded before (Control) and after treatment with 1 mM NFA ($n = 4$).

Fig. 2. Biphasic action of NFA on Slo2.1. (A) Left panel: Effects of 1 mM NFA on $I_{Slo2.1}$ recorded at a test pulse of 0 mV. $a$, current before application of NFA. $b$, current at peak of activation response. $c$, current after 40 min exposure to NFA. Right panel: Time-dependent effect of 1 mM NFA on $I_{Slo2.1}$. Currents were normalized to peak activation response for each oocyte ($n = 11$). (B) Left panel: Time-dependent activity of 1 mM NFA with co-application of the non-selective COX inhibitor ibuprofen (IBP, 1 mM) on $I_{Slo2.1}$. $a$, current at 0 mV before application of drugs. $b$, current at peak of activation response. $c$, current after 40 min exposure to NFA + IBP. Right panel: Time-dependent effect of NFA + IBP (red square) and NFA alone (open circle). Currents were normalized to peak activation response for each oocyte. Average peak current for NFA treatment alone: $4.0 \pm 0.4 \, \mu A$ ($n = 6$) for NFA + IBP: $4.9 \pm 0.7 \, \mu A$ ($n = 8$). Data summarized in panel B were obtained from a single batch of oocytes.

Fig. 3. A278R Slo2.1 channels are more sensitive to NFA. (A) Currents recorded from an oocyte expressing A278R mutant channels before (upper panel, control) and after treatment with 1 mM NFA (lower panel). Oocytes were recorded 1 day after injection with 0.2 ng cRNA. $V_t$ was varied from $-140$ to $+80$ mV and applied in 20 mV increments from a holding potential of $-80$ mV. Arrows indicate 0 current. (B) Average I-V relationships for A278R $I_{Slo2.1}$ recorded before (Control) and after treatment with 1 mM NFA ($n = 10$). (C) [NFA]-response relationships for WT ($n = 9$) and A278R ($n = 13$) $I_{Slo2.1}$ measured at 0 mV. Data was fitted with a logistic equation (smooth curve). For WT channels: $EC_{50} = 2.1 \pm 0.1$, $n_H = 2.4$.
± 0.06. For A278R channels: EC50 = 0.11 ± 0.01, nH = 2.0 ± 0.2. (D) Effects of 1 mM NFA on A278R 
I_{Slo2.1} recorded at a test pulse of 0 mV. a, current recorded at a test pulse of 0 mV before application of 
NFA. b, current at peak of activation response. c, current after 40 min exposure to NFA. (E) Constitutively 
active A278R I_{Slo2.1} is stable over 30 min of recording (Control, n = 7), while NFA-activated A278R I_{Slo2.1} 
exhibits a 20% decline over 40 min (n = 11).

Fig. 4. Concentration-response relationships for fenamates and diclofenac. (A) Chemical structures of 
fenamates and diclofenac. (B, C) Concentration-response relationships for compounds on WT (B) and 
A278R (C) I_{Slo2.1}. For each compound, I_{Slo2.1} was measured at 0 mV and normalized to the peak response. 
Data were fitted with a logistic equation (smooth curve) to determine the EC50 and nH as presented in 
Table 1.

Fig. 5. PAA is the minimal structural requirement for activation of Slo2.1 channels. (A) Chemical 
structures of PAA (rings I and II are indicated), DPA, ANA, MAA, BCA and BBA. (B) Average I-V 
relationship for WT I_{Slo2.1} recorded before (Control) and after treatment with 3 mM PAA. (C) Traces of 
WT currents recorded at 0 mV before (Control) and after exposure of oocytes to indicated concentrations 
of PAA. (D) Concentration-response relationship for PAA. EC50 = 0.79 ± 0.03 mM, nH = 1.5 ± 0.03 (n = 
7). (E-I) I-V relationships determined before (Control), after treatment of oocytes with indicated test 
compound, then finally with 1 mM NFA (n = 3 - 5).

Fig. 6. Apparent efficacy of several fenamates compared to NFA. An equieffective concentration (~EC90) 
of each fenamate was applied to oocytes expressing WT Slo2.1 (NFA = 5.4 mM, FFA = 2.9 mM, MFA = 
1.65 mM, TFA = 0.98 mM, MCFA = 0.32 mM, PAA = 3.14 mM, DFS = 1.86 mM). The maximal 
response (I_{peak} at 0 mV) for each compound (n = 6 - 7) was normalized relative to the activation measured 
with NFA from the same batch of oocytes. The efficacy of MFA, MCFA, PAA and DFS was less than 
NFA (*p < 0.05, **p < 0.01).
Fig. 7. Time-dependent effects of PAA and MCFA on $I_{Slo2.1}$. (A) Left panel: Effects of 1 mM PAA on $I_{Slo2.1}$ recorded at a test pulse of 0 mV. $a$, current before application of PAA. $b$, peak current response. $c$, current after 40 min of PAA. Right panel: Time-dependent effect of 1 mM PAA on $I_{Slo2.1}$. Currents were normalized to peak activation response for each oocyte ($n = 10$). (B) Left panel: Effects of 0.03 mM MCFA on $I_{Slo2.1}$ recorded at a test pulse of 0 mV. $a$, current before application of MCFA. $b$, peak current response. $c$, current after 40 min of MCFA. Right panel: Time-dependent effect of 0.03 mM MCFA on $I_{Slo2.1}$. Currents were normalized to peak activation response for each oocyte ($n = 9$).

Fig. 8. NFA activates Slo2.2 channels. (A) Currents recorded from an oocyte expressing Slo2.2 channels before (upper panel) and after treatment with 1 mM NFA (lower panel). Oocytes were recorded after 6 days of injection with 32 ng WT Slo2.2 cRNA. Arrows indicate 0 current. (B) Average I-V relationships for WT $I_{Slo2.2}$ recorded before (Control) and after treatment with indicated concentrations of NFA ($n = 7$). (C) Concentration-response relationships for NFA on $I_{Slo2.2}$ measured at 0 mV and normalized to the peak response ($n = 7$). Data were fitted with a logistic equation to determine the EC$_{50}$ (2.7 ± 0.19 mM) and n$_H$ (2.0 ± 0.14).

Fig. 9. Effect of fenamates on Slo1 channels. (A – C) Currents recorded from oocytes expressing Slo1 channels before (upper panels) and after treatment with 1 mM of indicated fenamate (lower panels). Arrows indicate 0 current. (D – F) Concentration-dependent effects of NFA ($n = 5$), MCFA ($n = 11$) and PAA ($n = 7$) on averaged I-V relationships. Oocytes were recorded after 1-3 days of injection with .04 - 1.0 ng WT Slo1 cRNA. (G) Concentration-response relationships for indicated fenamate. Data were fitted with a logistic equation (smooth curves) to estimate EC$_{50}$. For MCFA, EC$_{50}$ was 0.68 ± 0.19 (n$_H$ = 2.6 ± 0.4; $n = 11$). For NFA and PAA, n$_H$ was fixed at 2.4 and 1.5 respectively to estimate EC$_{50}$: 10.0 ± 0.9 mM ($n = 5$) for NFA, 13.4 ± 0.7 mM ($n = 7$) for PAA. For NFA, $I_{Slo1}$ was measured at 0 mV and responses in uninjected oocytes were used to correct for activation of endogenous currents. For MCFA and PAA, $I_{Slo1}$
was measured at +60 mV without correction as endogenous currents evoked by these compounds was very small. 

(\textit{H}) Apparent efficacy of Slo2.2 channel activation for MCFA compared to NFA. The maximal response ($I_{\text{peak}}$ at 0 mV) for each compound ($n = 5-11$) was normalized relative to the activation measured with NFA from the same batch of oocytes. The efficacy of MCFA was less than NFA ($*p < 0.001$).
Table 1. Comparison of activation effect (EC50 values) of fenamates on WT and A278R Slo2.1 channels.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>WT hSlo2.1</th>
<th></th>
<th>A278R hSlo2.1</th>
<th></th>
<th>Ratio (EC50-WT/EC50-A278R)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (mM)</td>
<td>nH</td>
<td>n</td>
<td>EC50 (mM)</td>
<td>nH</td>
</tr>
<tr>
<td>Niflumic acid</td>
<td>2.09 ± 0.14</td>
<td>2.4 ± 0.06</td>
<td>9</td>
<td>0.11 ± 0.01</td>
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<td>0.059 ± 0.008</td>
<td>2.0 ± 0.1</td>
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<tr>
<td>Mefenamic acid</td>
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<td>1.5 ± 0.1</td>
<td>13</td>
<td>0.051 ± 0.006</td>
<td>1.7 ± 0.06</td>
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<tr>
<td>Tolfenamic acid</td>
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<td>1.9 ± 0.09</td>
<td>8</td>
<td>0.047 ± 0.004</td>
<td>2.0 ± 0.09</td>
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<tr>
<td>Meclofenamic acid</td>
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<td>1.7 ± 0.07</td>
<td>5</td>
<td>0.017 ± 0.005</td>
<td>1.7 ± 0.2</td>
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<tr>
<td>Diclofenac</td>
<td>0.72 ± 0.07</td>
<td>2.4 ± 0.2</td>
<td>5</td>
<td>0.31 ± 0.02</td>
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<tr>
<td>N-phenylanthranilic acid</td>
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<td>1.5 ± 0.03</td>
<td>7</td>
<td>0.29 ± 0.02</td>
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<td>Benzyl-benzoic acid</td>
<td>No activation</td>
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</tbody>
</table>
Figure 2

A

B

Normalized $I_{S02,1}$

Time (min)

0 10 20 30 40

Normalized $I_{S02,1}$

Time (min)

0 10 20 30 40

0.5 μA

50 ms

0.5 μA

50 ms

1 mM NFA + 1 mM IBP

1 mM NFA
Figure 3

A

Control

2 μA

50 ms

1 mM NFA

B

Control

-○- 1 mM NFA

C

Relative current

[0.0, 0.2, 0.4, 0.6, 0.8, 1.0]

[1E-4, 0.01, 1, 100]

WT

A278R

D

E

Normalized ISo2.1

0.0

0.2

0.4

0.6

0.8

1.0

0

10

20

30

40

Time (min)

1 mM NFA

Control

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Figure 4

A

Niflumic acid (NFA)

Flufenamic acid (FFA)

Mefenamic acid (MFA)

Tolfenamic acid (TFA)

Meclofenamic acid (MCFA)

Diclofenac sodium (DFS)

B

C

Relative current

1.0

0.8

0.6

0.4

0.2

0.0

1E-3

0.1

10

NSAID, [mM]

1E-4

1E-3

0.01

0.1

1

NSAID, [mM]
Figure 6
Figure 7

A) PAA

B) MCFA

Normalized / Time (min)

0.5 µA
50 ms

0.2 µA
50 ms
Figure 8

A

Control

1 mM NFA

2 μA

50 ms

B

C

- Control
- 0.3 mM NFA
- 1 mM NFA
- 3 mM NFA
- 6 mM NFA

Relative current

NFA, [mM]

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Figure 9

A

Control

1 mM NFA

B

Control

1 mM MCFA

C

Control

1 mM PAA

D

- Control
- 1 mM NFA
- 3 mM NFA
- 6 mM NFA

\[ \mu A \]

\( -120 \rightarrow -80 \rightarrow -40 \rightarrow 40 \rightarrow 80 \)

E

- Control
- 0.1 mM MCFA
- 0.3 mM MCFA
- 0.5 mM MCFA
- 1 mM MCFA

\[ \mu A \]

\( -120 \rightarrow -80 \rightarrow -40 \rightarrow 40 \rightarrow 80 \)

F

- Control
- 1 mM PAA
- 3 mM PAA
- 6 mM PAA
- 10 mM PAA

\[ \mu A \]

\( -120 \rightarrow -80 \rightarrow -40 \rightarrow 40 \rightarrow 80 \)

G

- NFA
- MCFA
- PAA

Relative current

\[ 0.1 \rightarrow 0.2 \rightarrow 0.4 \rightarrow 0.8 \rightarrow 1.0 \]

\text{Fenamate, [mM]}

H

Normalized peak response

\[ 0.2 \rightarrow 0.4 \rightarrow 0.8 \rightarrow 1.2 \]

NFA

MCFA

*