Molecular Pharmacology Fast Forward. Published on February 7, 2014 as doi:10.1124/mol.113.091199

MOL #91199

Influence of the N-terminus on the Biophysical Properties and Pharmacology of TREK1 Potassium Channels

By

Emma L Veale, Ehab Al-Moubarak, Naina Bajaria, Kiyoyuki Omoto, Lishuang Cao, Stephen J Tucker, Edward B Stevens, Alistair Mathie

Medway School of Pharmacy, University of Kent, Central Avenue, Chatham Maritime, Kent, ME4 4TB, UK (ELV, E Al-M, NB, AM). Pfizer Neusentis, The Portway Building, Granta Park, Great Abington, Cambridge CB21 6GS, UK (KO, LC, EBS), Clarendon Laboratory, Department of Physics, University of Oxford, Oxford, OX1 3PU, UK (SJT).

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: N-terminus modulates TREK1 channel pharmacology

[4]To whom correspondence should be addressed: Alistair Mathie, Medway School of Pharmacy, University of Kent, Central Avenue, Chatham Maritime, Kent, ME4 4TB, UK. Phone: 01634 202955 Fax: 01634 883927 Email: a.a.mathie@kent.ac.uk

Text Pages: 21
Tables: 0
Figures: 8
References: 52
Abstract: 190 words
Introduction: 680 words
Discussion: 1056 words

Abbreviations:

BL-1249	(5,6,7,8-Tetrahydro-naphthalen-1-yl)-[2-(1H-tetrazol-5-yl)-phenyl]-amine
CTD	C terminus domain
Diclofenac	2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid
FFA	flufenamic acid, 2-{[3-(Trifluoromethyl)phenyl]amino}benzoic acid
K2P	two pore domain potassium
MFA	mefenamic acid, 2-(2,3-dimethylphenyl)aminobenzoic acid
NFA	niflumic acid, 2-{[3-(trifluoromethyl)phenyl]amino}nicotinic acid
TREK	TWIK-related K

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

TREK1 potassium channels are members of the two pore domain (K2P) potassium channel family and contribute to background potassium conductances in many cell types where their activity can be regulated by a variety of physiological and pharmacological mediators. Fenamates such as FFA, MFA, NFA and diclofenac and the related experimental drug, BL-1249 enhance the activity of TREK1 currents and we show that BL-1249 is the most potent of these compounds. Alternative translation initiation produces a shorter, N-terminus truncated form of TREK1 with a much reduced open probability and a proposed increased permeability to sodium compared to the longer form. We show that both forms of TREK1 can be activated by fenamates and that a number of mutations which affect TREK1 channel gating occlude the action of fenamates but only in the longer form of TREK1. Furthermore, fenamates produce a marked enhancement of current through the shorter, truncated form of TREK1 and reveal a K^+-selective channel, like the long form. These results provide insight into the mechanism of TREK1 channel activation by fenamates and, given the role of TREK1 channels in pain, they suggest a novel analgesic mechanism for these compounds.

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

TREK1 (TWIK-related K, also $K_{2P}2.1$, *KCNK2*) potassium channels are members of the K2P channel family (Enyedi and Czirják, 2010) and contribute to background potassium conductances in many cell types where they show activity over a wide range of voltages. Their activity can be up or down regulated by a variety of physiological and pharmacological mediators (Honore *et al,* 2007, Noel *et al,* 2011). These regulatory mechanisms include membrane stretch, membrane depolarisation, heat, intracellular acidosis as well as changes in extracellular pH, arachidonic acid and other polyunsaturated fatty acids (PUFAs). Activation of Gαq- and Gαo- coupled receptors and protein kinases such as PKC and PKA inhibit the activity of TREK1 channels (Enyedi and Czirjak, 2010). However, there is an enhancement of TREK1 activity in response to the activation of Gαi-coupled receptors including $GABA_B$ receptors (Cain *et al,* 2008, Sandoz *et al,* 2012). A number of clinically important drugs also affect the activity of TREK1 including the neuroprotective agent riluzole (Duprat *et al,* 2000), the antipsychotic agent chlorpromazine (Patel *et al,* 1998) and the antidepressant agent fluoxetine (Kennard *et al,* 2005).

TREK1 channels are expressed in sensory neurons, particularly in nociceptors (Alloui *et al,* 2006, Marsh *et al,* 2012), and they are also broadly distributed in the central nervous system (Fink *et al,* 1996, Talley *et al,* 2001, Aller and Wisden, 2008). A role for TREK1 in depression (Heurteaux *et al,* 2006), polymodal pain perception (Alloui *et al,* 2006, Noel *et al,* 2009) and diseases related to blood brain barrier dysfunction (Bittner *et al,* 2013) has been proposed. A number of gaseous general anesthetic agents such as halothane (Patel *et al,* 1999), nitrous oxide, xenon and cyclopropane which are effective in the clinically relevant range (Gruss *et al,* 2004) also enhance the activity of TREK1 channels. Furthermore, in TREK1-deficient animals, the anesthetic efficiency of chloroform, halothane, sevoflurane, and desoflurane was significantly reduced (Heurteaux *et al,* 2004) suggestive of the importance of TREK1 channels in mediating, at least in part, the effect of gaseous general anesthetic agents.

TREK1 knockout (KO) mice are more sensitive to painful heat sensations and a variety of other painful stimuli including mechanical and inflammatory stimuli compared to control animals (Alloui *et al,* 2006). This change in pain sensitivity of TREK1 KO mice and the presence of TREK1 in sensory neurons, including DRG neurons, suggests that TREK1 channels may be a promising target for the development of new analgesics which act to enhance the activity of these channels (Alloui *et al,* 2006, Woolf and Ma, 2007, Mathie, 2010).

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

Intriguingly, alternative translation initiation (ATI) of *KCNK2* (TREK1) transcripts has been shown to produce a shorter form of TREK1 that is truncated at the N-terminus. Both short and long forms appear to be expressed as proteins in both neurons (Thomas *et al,* 2008) and recombinant expression systems (Eckert *et al,* 2011). The alternative translation initiation codon is a methionine that immediately precedes the first transmambrane helix (M1) and results in a complete deletion of the intracellular N-terminus of the channel. The truncated form gives rise to a current with a much reduced open probability and a proposed measurable permeability to sodium (Thomas *et al,* 2008).

Fenamate compounds such as diclofenac (Voltaren®), flufenamic acid (FFA, Mobilisin®), mefenamic acid (MFA, Ponstel®, Dolfenal®) and niflumic acid (NFA), are non-steroidal anti-inflammatory drugs (NSAIDs), already used clinically in the treatment of pain. They have been shown to up or down regulate the activity of a number of ion channels, including TREK1 where they act to enhance current (Takahira *et al*, 2005).

The aim of this study was to characterise the mechanism of fenamate action on TREK1 channels. Our results reveal that the activity of both short and longer forms of TREK1 are enhanced by fenamates, but that their action on the truncated form also reveals a K^+ selective current. Their activatory effect is also enhanced in the truncated forms of TREK1. Furthermore, we show that gating mutations can occlude the effect of fenamates, but only in the the longer form of TREK1. The results provide an important insight into the mechanism of action of these compounds.

Materials and Methods

Cell culture

tsA201 cells (ECACC, Sigma-Aldrich, Gillingham, Dorset, UK), modified human embryonic kidney 293 cells, were grown in a monolayer tissue culture flask maintained in a growth medium which was composed of 88% minimum essential media with Earle's salts and 2mM L-glutamine, 10% of heat-inactivated fetal bovine serum, 1% penicillin (10,000 units ml^{-1}) and streptomycin (10 mg ml^{-1}), and 1% non-essential amino acids. The cells were placed in an incubator at 37^{o}C with a humidified atmosphere of 95% oxygen and 5% carbon dioxide. After 2 or 3 days, when the cells were 70 to 90% confluent, they were split and resuspended in a 4 well plate containing 13 mm diameter cover slips (poly-D-lysine coated) in 0.5 ml of media, ready to be transfected the next day.

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

Transfection

For the electrophysiological experiments, pcDNA3.1 vector was cloned with the gene of interest (hTREK1 or hTRAAK wild-type or mutated). This and a similar vector containing GFP were incorporated into the cells (0.5 µg per well for each plasmid) using the calcium phosphate method. The cells were incubated for 6 - 12 hours at 37^{o}C in 95% oxygen and 5% carbon dioxide. Then, cells were washed using a phosphate buffered saline solution (PBS), and new media was added to each well. The cells were used for experiments after 24 hours.

Mutations and truncations

Point mutations were introduced by site-directed mutagenesis into the TREK1 or TRAAK using the Quikchange kit (Stratagene, Amsterdam, The Netherlands) and all mutations were confirmed by direct sequencing.

Whole cell patch clamp electrophysiology

Currents were recorded using the whole cell patch clamp in a voltage clamp configuration in tsA201 cells transiently transfected with the channel of interest. The cover slip with the cells was placed in a recording chamber filled with an external medium composed of 145 mM, NaCl, 2.5 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM HEPES (pH to 7.4, using NaOH). The internal medium used in the glass pipette comprised 150 mM KCl, 3 mM $MgCl_2$, 5 mM EGTA and 10 mM HEPES (pH to 7.4, using KOH). Modulatory compounds were applied by bath perfusion at a rate of 4-5 ml min ml^{-1}. Complete exchange of bath solution occurred within 100-120s. All data were collected at room temperature (19-22°C). The transfected cells were detected using a fluorescent microscope with UV light. Cells were voltage-clamped using an Axopatch 1D or Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and low pass filtered at 5 kHz before sampling (2-10 kHz) and online capture.

In order to study the potassium leak current, a ”step-ramp” voltage protocol was used. For the step component of the protocol, cells were hyperpolarised from a holding voltage of -60 mV to -80 mV for 100ms then stepped to -40 mV for 500 ms. For the ramp, cells were then stepped to -120 mV for 100 ms, followed by a 500 ms voltage ramp to +20 mV and a step back to -80 mV for another 100 ms, before being returned to the holding voltage of -60 mV. This protocol was composed of sweeps lasted 1.5 seconds (including sampling at the holding voltage) and was repeated once every 5 seconds. An example of the typical current response seen for WT TREK1 channels to this protocol is illustrated in Fig. 1B. For analysis of outward current, we measured the current difference between the - 80 mV and - 40 mV steps. The current-voltage graphs were obtained from the ramp change in voltage between -

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

120 mV and + 20 mV. The currents obtained with the imposed voltage protocol were recorded and analysed using pCLAMP 10.2 software and Microsoft Excel. For each cell, the current amplitude (pA) was normalised to the cell capacitance (pF).

Data analysis

Data were expressed as mean ± standard error of the mean (SEM) and 'n' represents the number of cells used for the experiment. The statistical analyses used either the Student t-test or a one way ANOVA with the post-hoc Dunnett's multiple comparisons test, using GraphPad Prism 6.02. For the t-test, the differences between means were considered as significant for $p < 0.05$. For the Dunnett's test, data were considered significantly different at the < 0.05 level (confidence interval > 95% for the difference between the two compared means).

Chemicals

All fine chemicals were purchased from Sigma-Aldrich, Gillingham, Dorset, UK.

Homology Modelling

The homology model of hTREK1 (UniProtKB/Swiss-Prot ID: O95069-2, isoform 2) was created using Modeller 9v8 (Sali and Blundell, 1993) using the human TRAAK structure (PDB ID: 4I9W) as template. ClustalW (Higgins *et al,* 1996) was used to align the TRAAK and TREK1 sequences.

Results

Enhancement of TREK1 current by fenamates.

A number of fenamate compounds, such as FFA, NFA, MFA and diclofenac, enhance the activity of wild type TREK1-channels (see also Takahira *et al,* 2005). Figure 1 shows the percentage enhancement observed following bath application of 100 µM of each compound. The degree of enhancement is greatest with FFA (250 ± 35 %, n = 17) and least with diclofenac (37 ± 6%, n = 11). BL-1249, another fenamate-like structure and a putative activator of TREK1-like currents in human bladder myocytes (Tertyshnikova *et al,* 2005) also activated recombinantly expressed TREK1. Interestingly, BL-1249 was around 30-100 times more potent than FFA with 1 µM producing a 130 ± 17% (n = 8) enhancement (figure 1) and 3 µM producing a 414 ± 110% (n = 3) enhancement.

BL-1249 and each of the fenamates are non-selective COX (cyclo-oxygenase) inhibitors. COX inhibition can lead to an increase in intracellular arachidonic acid. Since external application of arachidonic acid is also known to stimulate TREK1 channels (Patel *et al,*

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

1998), it was therefore important to determine whether the enhancement of current is mediated through an increased concentration of arachidonic acid. Ibuprofen is a non-selective COX inhibitor but had little effect on WT TREK1 current, with 100 μM producing only a 10 ± 4% (n = 9) enhancement of current under the same conditions that BL-1249 and the other fenamates enhanced TREK1 current (figure 2). Furthermore, the COX-1 selective NSAID, indomethacin (100 μM) caused a small, but significant decrease in TREK1 current with a reversible inhibition of 24 ± 1% (*n* = 3) (figure 2).

Gating mutations interfere with fenamate enhancement of TREK1 current

A number of amino acids in TREK1 have been identified as being important for the regulation of channel gating. For example, E306 in the intracellular C terminus of TREK1 is a key amino acid in transducing channel gating following the action of agents such as pH(i), polyunsaturated fatty acids, arachidonic acid, anesthetic gases and heat, which modulate the activity of TREK1 (Maingret *et al,* 2000a, b, Honore *et al,* 2002, Gruss *et al,* 2004, Sandoz *et al,* 2006). Mutation of this amino acid (e.g. E306A, E306G) produces a gain of function phenotype which mimics intracellular acidosis (Honore *et al,* 2002, Kennard *et al,* 2005) and it is difficult to further up or down regulate the channel.

More recently, several amino acids at the extracellular end of transmembrane domain 4 (M4), close to the selectivity filter, have also been shown to give rise to a gain of function and interfere with channel gating. In particular mutation W275S, blunts regulation by both external and internal regulators such as extracellular and intracellular pH changes, heat and arachidonic acid (Bragiantsev *et al,* 2011, 2012).

Figure 3 shows the effect of both of these mutations on activation of TREK1 by FFA (100 μM) and BL-1249 (1 μM). For both mutations the degree of enhancement by FFA and BL-1249 was significantly reduced compared to wild type TREK1. This suggests that these compounds interfere with this gating pathway, or, alternatively, that this gating pathway, when fully activated, occludes the action of fenamates.

M1, M2 and M4 mutations in TREK1 affect fenamate activation

The recent experiments of Bagriantsev *et al,* (2011 and 2012), Piechotta *et al,* (2011) and Rapedius *et al,* (2012), suggest that TREK1 channels may not gate directly at the lower bundle-crossing like many other classical tetrameric K^+ channels (Cohen *et al,* 2009, Mathie *et al,* 2010). Instead, it appears that most, if not all, regulators of TREK1 activity produce their effect by altering gating at the selectivity filter of the channel, regardless of where in the channel they interact. Thus the effect of a number of regulators which act via the intracellular

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

C terminus (C-terminus domain, CTD) of the channel, such as heat, intracellular acidification, stretch, arachidonic acid and activation of G*α*i coupled receptors, must somehow be transduced to the selectivity filter and the pore-lining M4 helix is thought to play an important role in this transduction due to its direct attachment to the regulatory CTD.

In several other K^+ channels, the equivalent region to M4 has been suggested to be important for the binding of regulatory molecules. For example, in hERG channels, this region appears to be involved in the binding of both channel activators and inhibitors (Hosaka *et al,* 2007, Perry *et al,* 2010, Garg *et al,* 2011). We therefore sought to investigate whether this region of TREK1 might be important for binding of fenamates and/or gating of the channel.

Using a previous structural model of TREK1 originally based upon KvAP (Piechotta *et al,* 2011) we identified a number of mutations in M1/M2/M4 that had a profound effect upon fenamate activation of the longer form of TREK1 and which initially suggested a putative binding site (not shown). Three mutations in particular, W44A, L174A and Y284A substantially reduced activation by both FFA (figure 4) and BL-1249 (supplemental figure 1) whereas mutation of several adjacent amino acids had no effect. However, a subsequent homology model based on the more recent crystal structure of TRAAK (Brohawn *et al,* 2013) suggests that these residues do not cluster together (figure 4). The influence of these mutations on current density is shown in supplemental figure 2. It is therefore more likely that these mutations act in a similar way to the E306A mutation by influencing TREK1 gating and thereby the efficacy of fenamate activation.

<u>TRAAK isoforms and their regulation by fenamates.</u>

To investigate this further, we considered the regulation of a related K2P channel (TRAAK) by FFA. Two isoforms of human TRAAK were studied: a short form (393 aa) and longer form (419 aa) where the differences in length are due to differences in the N-terminus that precedes M1. Transcripts for both isoforms have been reported but their relative abundance and/or importance is not known (Ozaita *et al,* 2002). For both isoforms the basal current is small, but substantial enhancement of current by FFA is seen in both cases. M4 is highly conserved in TRAAK and TREK1 but in direct contrast to mutation of Y284 in TREK1, mutation of the equivalent residue in either the long (figure 5) or short isoform of TRAAK did not influence enhancement by FFA.

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

TREK1 isoforms and their regulation by fenamates

ATI produces an isoform of TREK1 truncated at a similar position the N-terminus to the short form of TRAAK. To study these different versions of TREK1 in isolation, a point mutation (isoleucine for methionine) was introduced (M42I) which blocks ATI to give only the long form of the channel. To isolate the shorter truncated form of the channel the sequence encoding the first 41 amino acids were physically removed from the expression construct (Veale *et al,* 2010).

All compounds which were shown to enhance the activity of WT TREK1 (BL-1249, FFA, NFA, MFA and diclofenac), also enhanced the activity of the long form of the channel (TREK1_M42I), as shown in figure 6. There were no significant differences in the degree of enhancement observed.

By contrast, the current seen in control solutions through the N-terminus deleted form of TREK1 (TREK1ΔN) was very small in normal external K^+ (2.5 mM), to the extent that it was not clear whether there was any significant expression of the channel (see supplementary figure 2). Current through TREK1ΔN channels had an amplitude of just 4 ± 1 pA/pF (n = 12) compared to 48 ± 4 pA/pF (n = 14) for WT TREK1 and a measured reversal potential of -47 ± 5 mV (n = 14) compared to -85 ± 1 mV (n = 27), for WT TREK1 under the same conditions. However, changing the external K^+ concentration to 25 and 147.5 mM gave measurable current (supplementary figure 4), particularly when compared with untransfected cells, allowing confidence that although the current was small, it was present and detectable.

Application of FFA, MFA, NFA and BL-1249 (though not diclofenac) gave rise to a large increase in current through the short form of the channel (figure 7). These fenamate-enhanced currents had a reversal potential close to WT TREK1 currents, showing that in the presence of these compounds the current was K^+ selective. The huge percentage increase in current in normal external K^+ was not simply a result of there being negligible current to begin with. In 147.5 external K^+, there is significant current through TREK1ΔN (219 ± 32 pA, n = 13, at -80 mV) and this was enhanced by over 5,000 % (5,566 ± 1,633%, n = 8) in the presence of 100 µM FFA (supplemental figure 3). Thus it is likely that the large percentage increase seen is due to the initial low open probability of TREK1ΔN.

In direct contrast, truncation of the TREK1 C-terminus (TREK1ΔC, deletion of all amino acids after T322) also reduces the basal current to levels similar to that seen with TREK1ΔN (6 ± 1 pA/pF, n = 11, see also Kennard *et al,* 2005), but this is enhanced by FFA proportionally similar to WT TREK1 (259 ± 29%, n = 11, see figure 4). Since TREK1ΔC has

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

been proposed to represent the C terminus of the channel in its dephosphorylated form, compared to a mixture of phosphorylated and dephosphorylated channels present in WT TREK1 (Honore *et al,* 2002, Kennard *et al,* 2005), this suggests that the action of FFA does not depend on the phosphorylation state of the C terminus.

Mutations of short form alter both current density and regulation by fenamates

The gain of function mutation E306A, also enhanced current through TREK1ΔN (figure 8). Interestingly, the reversal potential of this enhanced current was exactly the same as the reversal potential for WT TREK1 (-85 ± 1 mV, n = 16), and FFA was able to further enhance the current further through TREK1ΔN(E306A) (figure 8).

A number of the other mutations which altered the effectiveness of FFA on the longer form WT TREK1 (L174A, Y284A, W275S) also altered both the current density and the K^+ selectivity of the shorter TREK-1ΔN, whilst notably, one other mutation (W44A), close to the methionine start codon in TREK-1ΔN did not. These data are illustrated in figure 8. However, for all mutations, the current could still be enhanced further by FFA.

The data are summarised as absolute current density measurements for the mutated channels in the presence and absence of FFA (figure 8). In this way, it can be seen that both mutations and fenamates increase the size of the current, but the size of the effect they produce depends, to a large extent, on the initial current levels (and most likely channel open probability). Thus, the fenamates can induce a massive percentage increase in current through TREK1ΔN, especially where the initial current is very low. By contrast, the fenamates have little further enhancement of the gain of function, E306A mutation in WT TREK1 where current (and open probability) is already large (figure 3). Thus the different manipulations that enhance TREK1 current density appear to act synergistically and reach saturation (figure 8). It is of particular interest that the effects of N-terminus truncation are largely overcome either by gain of function mutations (E306A, W275S, L174A and Y284A) or fenamate-mediated enhancement, or both.

Discussion

The fenamate group of compounds enhance the activity of TREK1 and TRAAK channels, with flufenamic acid being the most potent and diclofenac the least potent among the four clinically used compounds tested. By contrast, FFA has relatively little effect on the related K2P channel, TASK3 (Veale *et al,* 2014). The relative effectiveness of FFA, MFA and NFA on TREK1 is consistent with that found previously by Takahira *et al* (2005). However, the

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

experimental fenamate, BL-1249, is around 30 - 100 times more potent than FFA. Fenamates are known to enhance current through a variety of K^+ channels including hERG (Fernandez *et al,* 2008) and K_{Ca} channels (Farrugia *et al,* 1993). They also enhance current through KCNQ/Kv7, but for these channels, the potency sequence is rather different to to that found for TREK1 channels with diclofenac being more potent than FFA, MFA or NFA (Peretz *et al,* 2005, 2007) suggesting either differences in the binding site between the two channels or the mechanism of fenamate activation. Fenamates also both activate and inhibit the Na-dependent Slo2.1 ($K_{Ca}4.2$) channels through binding to distinct sites on the channel (Garg and Sanguinetti, 2012). In these channels, MFA and diclofenac were more potent activators than FFA and NFA. The lack of effect of indomethacin and ibuprofen on TREK1 suggests that the fenamates produce their effect by binding directly to the channel rather than by an indirect action through alterations in arachidonic acid levels following inhibition of COX enzymes.

Our initial studies based upon an earlier model of TREK1 which used KvAP as a structural template proposed a number of amino acids which may contribute to a binding site for FFA and BL-1249 (Cao *et al,* 2010). However, more accurate templates indicated that these residues (W44, L174 and Y284) were too far apart to comprise a binding site and that these mutations may simply interfere with channel gating and thereby affect ligand efficacy, as appears to be the case for the other gain of function mutations E306A (Maingret *et al,* 2000a, b, Honore *et al,* 2002, Sandoz *et al,* 2006) and W275S (Bragiantsev *et al,* 2011, 2012). Our on-going experiments suggest that mutation of Y284 also occludes the action of other regulators of TREK1 gating, such as alterations in extracellular pH. To directly address this issue we have studied a related K2P channel (TRAAK), which is also enhanced by fenamates, and by the use of the N terminus truncated form of TREK1 (TREK1ΔN), which has a very small basal current. For TRAAK channels, the mutations equivalent to Y284 in TREK-1 did not alter the effectiveness of FFA or BL1249 showing these compounds must still bind to TRAAK. However, it also shows that there must be subtle differences in the gating mechanisms between TREK1 and TRAAK since despite their homology, they are differentially affected by this mutation.

Furthermore, channels with equivalent mutations in TREK1ΔN (see below) were also still enhanced by FFA and other fenamates, showing that binding of these compounds is still able to occur in these mutant channels. Thus although we cannot completely discount the contribution of the mutated residues to the binding site of fenamates, these mutations are more likely to be explained by alterations in channel gating than drug binding. Therefore TREK1ΔN may be helpful in future studies aimed at identification of agonist binding sites on

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

TREK1 as well as understanding the mechanisms by which these drugs influence gating at the selectivity filter.

TREK1 can exist in two forms following alternative translation initiation. Each of these forms are expressed as proteins in both neurons (Thomas *et al,* 2008) and recombinant expression systems (Eckert *et al,* 2011). Thomas *et al,* (2008) describe the N terminus truncated form of TREK-1 (TREK1ΔN) as being regionally and developmentally regulated in a number of different regions of the rat CNS. Both forms are expressed in COS-7 cells (Thomas *et al,* 2008) but predominantly the long form in HEK-293 cells (Ma *et al,* 2011). For the related K2P channel, TREK2, which also undergoes ATI (Simkin *et al,* 2008), the degree of ATI has been shown to be tissue specific (Staudacher *et al,* 2011) through differential regulation of mRNA translation. In addition to a number of splice variants that have recently been identified in TREK1 (Veale *et al,* 2010; Rinné *et al,* 2013), these ATI variants may also play an important role in those tissues in which they are expressed.

TREK1ΔN has been described as a constituitively “non conductive” variant with a “collapsed selectivity filter” (Ma *et al,* 2011) that is permeable to sodium under normal physiological conditions, leading to membrane depolarisation when it is active in neurons (Thomas *et al,* 2008). Furthermore, TREK1ΔN has been shown to co-purify with the longer form (Thomas *et al,* 2008) and therefore heteromers may also exist.

In this study, we have shown current through TREK1ΔN is markedy enhanced by fenamates. Interestingly, measurements of the reversal potential in the presence of these compounds, reveals that these enhanced currents are highly K^+ selective. Similarly, gain of function mutations which influence channel gating (E306A, W275S, L174A, Y284A) not only increase channel current but also reveal a K^+ selective current.

Given an equal level of expression of both forms of the channel, the long form will dominate under control conditions since its open probablility appears far greater than TREK1ΔN. However, this would not be the case following fenamate activation (or following gain of function mutations) which produce a substantial increase in the apparent open probablility of TREK1ΔN. Since the action of fenamates (and gain of function mutations) reveal a K^+ selective conductance, fenamate action on TREK1ΔN would amplify its effect on any coexpressed longer forms of TREK1 to produce an increased hyperpolarisation and decreased excitability of the cell membrane. Heteromeric combinations of the longer and short forms of the channel also would behave in the same manner in the presence of fenamates.

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

The involvement of TREK1 in pain (Alloui *et al,* 2006, Noel *et al,* 2009) suggests that compounds which enhance their activity, such as the fenamates describe here, would be of considerable value as lead compounds for potential new analgesics targeting this channel. Activation of postsynaptic TREK1 channels would hyperpolarise the membrane of central neurons and depress neuronal activity in the pain pathway thus countering excitatory stimulation by increased neurotransmission. Furthermore, enhanced activity of TREK1 channels located presynaptically will limit excitatory neurotransmitter release.

Authorship Contributions

Participated in research design: Mathie, Stevens, Tucker, Veale, Cao, Omoto
Conducted experiments: Veale, Bajaria, Al-Moubarak
Performed data analysis: Veale, Bajaria, Al-Moubarak, Mathie
Wrote or contributed to the writing of the manuscript: Mathie, Veale, Tucker, Stevens.

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

References

Aller MI and Wisden W (2008) Changes in expression of some two-pore domain potassium channel genes (KCNK) in selected brain regions of developing mice. *Neuroscience* **151(4)**: 1154-1172.

Alloui A, Zimmermann K, Mamet J, Duprat F, Noël J, Chemin J, Guy N, Blondeau N, Voilley N, Rubat-Coudert C, Borsotto M, Romey G, Heurteaux C, Reeh P, Eschalier A and Lazdunski M (2006) TREK1, a K+ channel involved in polymodal pain perception. *EMBO J.* **25(11):** 2368-2376.

Bagriantsev SN, Clark KA, and Minor DL Jr (2012) Metabolic and thermal stimuli control K(2P)2.1 (TREK1) through modular sensory and gating domains. *EMBO J* **31:** 3297-3308.

Bagriantsev SN, Peyronnet R, Clark KA, Honore E, and Minor DL Jr (2011) Multiple modalities converge on a common gate to control K2P channel function. *EMBO J* **30:** 3594-3606.

Bittner S, Ruck T, Schuhmann MK, Herrmann AM, Moha ou Maati H, Bobak N, Göbel K, Langhauser F, Stegner D, Ehling P, Borsotto M, Pape HC, Nieswandt B, Kleinschnitz C, Heurteaux C, Galla HJ, Budde T, Wiendl H, and Meuth SG (2013) Endothelial TWIK-related potassium channel-1 (TREK1) regulates immune-cell trafficking into the CNS. *Nat Med* **19(9):** 1161-1165.

Brohawn SG, Campbell EB, and MacKinnon R (2013) Domain-swapped chain connectivity and gated membrane access in a Fab-mediated crystal of the human TRAAK K+ channel. *Proc Natl Acad Sci U S A.* **110:** 2129-2134.

Cain SM, Meadows HJ, Dunlop J, and Bushell TJ (2008) mGlu4 potentiation of K(2P)2.1 is dependant on C-terminal dephosphorylation. *Mol Cell Neurosci* **37(1):** 32-39.

Cao L, Veale EL, Mathie A, and Stevens E (2010). Differential modulation of TREK-1, TASK-3 and TRESK K2P ion channels by BL-1249. Program No. 174.6/KK13. 2010 Neuroscience Meeting Planner. San Diego, CA: *Society for Neuroscience Online*.

Cohen A, Ben-Abu Y, and Zilberberg N (2009) Gating the pore of potassium leak channels. *Eur Biophys J* **39:** 61-73.

Duprat F, Lesage F, Patel AJ, Fink M, Romey G, and Lazdunski M (2000) The neuroprotective agent riluzole activates the two P domain K(+) channels TREK1 and TRAAK. *Mol Pharmacol* **57(5):** 906-912.

Eckert M, Egenberger B, Döring F, and Wischmeyer E (2011) TREK1 isoforms generated by alternative translation initiation display different susceptibility to the antidepressant fluoxetine. *Neuropharmacology* **61(5-6):** 918-923.

Enyedi P and Czirjak G (2010) Molecular background of leak K^+ currents: Two-pore domain potassium channels. *Physiol Rev* **90:** 559-605.

Farrugia G, Rae JL, and Szurszewski JH (1993) Characterization of an outward potassium current in canine jejunal circular smooth muscle and its activation by fenamates. *J Physiol* **468:** 297-310.

Fernandez D, Sargent J, Sachse FB, and Sanguinetti MC (2008) Structural basis for ether-a-go-go-related gene K+ channel subtype-dependent activation by niflumic acid. *Mol Pharmacol* **73(4):** 1159-1167.

Fink M, Duprat F, Lesage F, Reyes R, Romey G, Heurteaux C, and Lazdunski M (1996) Cloning, functional expression and brain localization of a novel unconventional outward rectifier K+ channel. *EMBO J* 15(24): 6854-6862.

Garg P and Sanguinetti MC (2012) Structure-activity relationship of fenamates as Slo2.1 channel activators. *Mol Pharmacol* **82(5):** 795-802.

Garg V, Stary-Weinzinger A, Sachse F, and Sanguinetti MC (2011) Molecular determinants for activation of human ether-à-go-go-related gene 1 potassium channels by 3-nitro-n-(4-phenoxyphenyl) benzamide. *Mol Pharmacol* **80(4):** 630-637.

Gruss M, Bushell TJ, Bright DP, Lieb WR, Mathie A, and Franks NP (2004) Two-pore-domain K+ channels are a novel target for the anesthetic gases xenon, nitrous oxide, and cyclopropane. *Mol Pharmacol* **65(2):** 443-452.

Heurteaux C, Guy N, Laigle C, Blondeau N, Duprat F, Mazzuca M, Lang-Lazdunski L, Widmann C, Zanzouri M, Romey G, and Lazdunski M (2004) TREK1, a K+ channel involved in neuroprotection and general anesthesia. *EMBO J* **23(13):** 2684-2695.

Heurteaux C, Lucas G, Guy N, El Yacoubi M, Thümmler S, Peng XD, Noble F, Blondeau N, Widmann C, Borsotto M, Gobbi G, Vaugeois JM, Debonnel G, and Lazdunski M (2006) Deletion of the background potassium channel TREK1 results in a depression-resistant phenotype. *Nat Neurosci* **9(9):** 1134-1141.

Higgins DG, Thompson JD, and Gibson TJ (1996) Using CLUSTAL for multiple sequence alignments. *Methods Enzymol* **266:** 383–402.

Honoré E (2007) The neuronal background K2P channels: focus on TREK1. *Nat Rev Neurosci* **8(4):** 251-261.

Honoré E, Maingret F, Lazdunski M, and Patel AJ (2002) An intracellular proton sensor commands lipid- and mechano-gating of the K(+) channel TREK1. *EMBO J* **21(12):** 2968-2976.

Hosaka Y, Iwata M, Kamiya N, Yamada M, Kinoshita K, Fukunishi Y, Tsujimae K, Hibino H, Aizawa Y, Inanobe A, Nakamura H, and Kurachi Y (2007) Mutational analysis of block and facilitation of HERG current by a class III anti-arrhythmic agent, nifekalant. *Channels* **1(3):** 198-208.

Kennard LE, Chumbley JR, Ranatunga KM, Armstrong SJ, Veale EL, and Mathie A (2005) Inhibition of the human two-pore domain potassium channel, TREK1, by fluoxetine and its metabolite norfluoxetine. *Br J Pharmacol* **144(6):** 821-829.

Ma XY, Yu JM, Zhang SZ, Liu XY, Wu BH, Wei XL, Yan JQ, Sun HL, Yan HT, and Zheng JQ (2011) External Ba^{2+} block of the two-pore domain potassium channel TREK1 defines conformational transition in its selectivity filter. *J Biol Chem* **286(46):** 39813-39822.

Maingret F, Lauritzen I, Patel AJ, Heurteaux C, Reyes R, Lesage F, Lazdunski M, and Honoré E (2000a) TREK1 is a heat-activated background K(+) channel. *EMBO J* **19(11):** 2483-2491.

Maingret F, Patel AJ, Lesage F, Lazdunski M, and Honoré E (2000b) Lysophospholipids open the two-pore domain mechano-gated K(+) channels TREK1 and TRAAK. *J Biol Chem* **275(14):** 10128-10133.

Marsh B, Acosta C, Djouhri L, and Lawson SN (2012) Leak K^+ channel mRNAs in dorsal root ganglia: relation to inflammation and spontaneous pain behaviour. *Mol Cell Neurosci* **49(3):** 375-386.

Mathie A (2010) Ion channels as novel therapeutic targets in the treatment of pain. *J Pharm Pharmacol* **62(9):** 1089-1095.

Mathie A, Al-Moubarak E, and Veale EL (2010) Gating of two pore domain potassium channels. *J Physiol* **588:** 3149-3156.

Noël J, Sandoz G, and Lesage F (2011) Molecular regulations governing TREK and TRAAK channel functions. *Channels* **5(5):** 402-409.

Noël J, Zimmermann K, Busserolles J, Deval E, Alloui A, Diochot S, Guy N, Borsotto M, Reeh P, Eschalier A, and Lazdunski M (2009) The mechano-activated K+ channels TRAAK and TREK1 control both warm and cold perception. *EMBO J* **28(9):**1308-1318.

Ozaita A and Vega-Saenz de Miera E (2002) Cloning of two transcripts, HKT4.1a and HKT4.1b, from the human two-pore K+ channel gene KCNK4. Chromosomal localization, tissue distribution and functional expression. *Brain Res Mol Brain Res* **102:** 18-27.

Patel AJ, Honoré E, Maingret F, Lesage F, Fink M, Duprat F, and Lazdunski M (1998) A mammalian two pore domain mechano-gated S-like K+ channel. *EMBO J* **17(15):** 4283-4290.

Patel AJ, Honoré E, Lesage F, Fink M, Romey G, and Lazdunski M (1999) Inhalational anesthetics activate two-pore-domain background K+ channels. *Nat Neurosci* **2(5):** 422-426.

Peretz A, Degani N, Nachman R, Uziyel Y, Gibor G, Shabat D, and Attali B (2005) Meclofenamic acid and diclofenac, novel templates of KCNQ2/Q3 potassium channel openers, depress cortical neuron activity and exhibit anticonvulsant properties. *Mol Pharmacol* **67(4):** 1053-1066.

Peretz A, Degani-Katzav N, Talmon M, Danieli E, Gopin A, Malka E, Nachman R, Raz A, Shabat D, and Attali B (2007) A tale of switched functions: from cyclooxygenase inhibition to M-channel modulation in new diphenylamine derivatives. *PLoS One* **2(12):** e1332.

Perry M, Sanguinetti M, and Mitcheson J (2010) Revealing the structural basis of action of hERG potassium channel activators and blockers. *J Physiol* **588(Pt 17):** 3157-3167.

Piechotta PL, Rapedius M, Stansfeld PJ, Bollepalli MK, Erhlich G, Andres-Enguix I, Fritzenshaft H, Decher N, Sansom MS, Tucker SJ, and Baukrowitz T (2011) The pore structure and gating mechanism of K2P channels. *EMBO J* 30: 3607-3619.

Rapedius M, Schmidt MR, Sharma C, Stansfeld PJ, Sansom MS, Baukrowitz T, and Tucker SJ (2012) State-independent intracellular access of quaternary ammonium blockers to the pore of TREK-1. *Channels* **6:** 473-478.

Rinné S, Renigunta V, Schlichthörl G, Zuzarte M, Bittner S, Meuth SG, Decher N, Daut J, and Preisig-Müller R (2013) A splice variant of the two-pore domain potassium channel TREK-1 with only one pore domain reduces the surface expression of full-length TREK-1 channels. *Pflugers Arch* in press.

Sali A and Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* **234:** 779-815.

Sandoz G, Thümmler S, Duprat F, Feliciangeli S, Vinh J, Escoubas P, Guy N, Lazdunski M, and Lesage F (2006) AKAP150, a switch to convert mechano-, pH- and arachidonic acid-sensitive TREK K(+) channels into open leak channels. *EMBO J* **25(24):** 5864-5872.

Sandoz G, Levitz J, Kramer RH, and Isacoff EY (2012) Optical control of endogenous proteins with a photoswitchable conditional subunit reveals a role for TREK1 in GABA(B) signaling. *Neuron* **74(6):** 1005-1014.

Simkin D, Cavanaugh EJ, and Kim D (2008) Control of the single channel conductance of K2P10.1 (TREK-2) by the amino-terminus: role of alternative translation initiation. *J Physiol* **586(Pt 23):** 5651-5663.

Staudacher K, Baldea I, Kisselbach J, Staudacher I, Rahm AK, Schweizer PA, Becker R, Katus HA, and Thomas D (2011) Alternative splicing determines mRNA translation initiation and function of human K(2P)10.1 K+ channels. *J Physiol* **589(Pt 15):** 3709-3720.

Takahira M, Sakurai M, Sakurada N, and Sugiyama K (2005) Fenamates and diltiazem modulate lipid-sensitive mechano-gated 2P domain K(+) channels. *Pflugers Arch* **451(3):** 474-478.

Talley EM, Solorzano G, Lei Q, Kim D and Bayliss DA (2001) Cns distribution of members of the two-pore-domain (KCNK) potassium channel family. *J Neurosci* 21: 7491-7505.

Tertyshnikova S, Knox RJ, Plym MJ, Thalody G, Griffin C, Neelands T, Harden DG, Signor L, Weaver D, Myers RA, and Lodge NJ (2005) BL-1249 [(5,6,7,8-tetrahydro-naphthalen-1-yl)-[2-(1H-tetrazol-5-yl)-phenyl]-amine]: a putative potassium channel opener with bladder-relaxant properties. *J Pharmacol Exp Ther* **313(1):** 250-259.

Thomas D, Plant LD, Wilkens CM, McCrossan ZA, and Goldstein SA (2008) Alternative translation initiation in rat brain yields K2P2.1 potassium channels permeable to sodium. *Neuron* **58(6):** 859-870.

Veale EL, Hassan M, Walsh Y, Al-Moubarak E, and Mathie A (2014) Recovery of current through mutated TASK3 potassium channels underlying Birk Barel syndrome. *Mol Pharmacol* **85(3):** 397-407.

Veale EL, Rees KA, Mathie A, and Trapp S (2010) Dominant negative effects of a non-conducting TREK1 splice variant expressed in brain. *J Biol Chem* **285(38):** 29295-29304.

Woolf CJ and Ma Q (2007) Nociceptors-noxious stimulus detectors. *Neuron* **55(3):** 353-364.

Footnotes

AM is a Royal Society Industry Fellow [Grant IF080012/AM].

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) [Grant BB/J000930/1, AM], [Grant BB/J00037X/1, SJT] and Pfizer [AM & SJT].

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. Activation of TREK1 currents by fenamates. A, Histogram of percentage enhancement of current by FFA, MFA, NFA, diclofenac (100 µM) and BL-1249 (1 µM) for WT TREK1. B left, Representative time course for enhancement by FFA (100 µM) of current through WT TREK1 channels. Inset, Currents evoked by the step-ramp voltage protocol recorded through WT TREK1 channels in the absence and presence of FFA (100 µM). Right, Current-voltage relationships for WT TREK1 channels in the absence (black) and presence (gray) of FFA (100 µM). C, as B for diclofenac (100 µM) D, as B for BL-1249 (1 µM).

Figure 2. Activation of TREK1 currents by fenamates is not via activation of COX. A, Histogram of percentage enhancement of current by FFA, ibuprofen and indomethacin (100 µM) for WT TREK1. B left, Representative time course for effect of ibuprofen (100 µM) on WT TREK1 channels. Inset, Currents evoked by the step-ramp voltage protocol recorded through WT TREK1 channels in the absence and presence of ibuprofen (100 µM). Right, Current-voltage relationships for WT TREK1 channels in the absence (black) and presence (gray) of ibuprofen (100 µM). C, as B for indomethacin (100 µM).

Figure 3. Reduced activation of mutated, gain of function TREK1 currents by fenamates. A, B, Histogram of percentage enhancement of current by FFA (A), and BL-1249 (1 µM) (B) for WT TREK1 and TREK1_W275S and TREK1_E306A. C left, Representative time course for enhancement by BL-1249 (1 µM) of current through TREK1_W275S channels. Inset, Currents evoked by the step-ramp voltage protocol recorded through TREK1_W275S channels in the absence and presence of BL-1249 (1 µM). Right, Current-voltage relationships for TREK1_W275S channels in the absence (black) and presence (gray) of BL-1249 (1 µM). D, as C for TREK1_E306A channels.

Figure 4. Reduced activation of M1, M2 and M4 mutated, TREK1 currents by FFA. A, Histogram of percentage enhancement of current by FFA for WT TREK1 and various mutated TREK1 channels. Those with a significantly reduced enhancement compared to WT TREK1 are indicated by gray bars. B, Model of hTREK1 dimer based on hTRAAK crystal structure. Mutated amino acids are illustrated in colour. W275 (gain of function mutation to S) in green, W44, L174, Y284 (mutated to A reduces FFA effectiveness) in red. Note that E306 in the C terminus is beyond the solved structure of TRAAK. C, Representative time

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.

course for enhancement by FFA (100 μM) of current through TREK1_W44A channels. D, as C for TREK1_L174A channels, E as C for TREK1_Y284A channels.

Figure 5. Activation of TRAAK currents by FFA. A, Histogram of percentage enhancement of current by FFA (100 μM) for WT TRAAK and TRAAK_Y271A currents. B, Representative time course for enhancement by FFA (100 μM) of current through WT TRAAK channels. C, as B for TRAAK_Y271A channels.

Figure 6. Activation of TREK1_M42I currents by fenamates. A, Histogram of percentage enhancement of current by FFA, MFA, NFA, diclofenac (100 μM) and BL-1249 (1 μM) for WT TREK1 and TREK1_M42I channels. B left, Representative time course for enhancement by FFA (100 μM) of current through TREK1_M42I channels. Right, Current-voltage relationships for TREK1_M42I channels in the absence (black) and presence (gray) of FFA (100 μM). C, as B for BL-1249 (1 μM), D, as B for diclofenac (100 μM).

Figure 7. Activation of TREK1ΔN currents by fenamates. A, Histogram of percentage enhancement of current by FFA, MFA, NFA, diclofenac (100 μM) and BL-1249 (1 μM) for WT TREK1 and TREK1ΔN channels. B left, Representative time course for enhancement by FFA (100 μM) of current through TREK1ΔN channels. Right, Current-voltage relationships for TREK1ΔN channels in the absence (black) and presence (gray) of FFA (100 μM). C, as B for BL-1249 (1 μM). D, as B for diclofenac (100 μM).

Figure 8. Activation of mutated TREK1ΔN currents by FFA. A, Histogram of zero current potential (left) and current density (right) for WT TREK1 and various mutant channels in the absence and presence of FFA (100 μM). B left, Representative time course for enhancement by FFA (100 μM) of current through TREK1ΔN_Y284A channels. Right, Current-voltage relationships for TREK1ΔN_Y284A channels in the absence (black) and presence (gray) of FFA (100 μM). C, as B for TREK1ΔN _E306A channels.

Figure 1

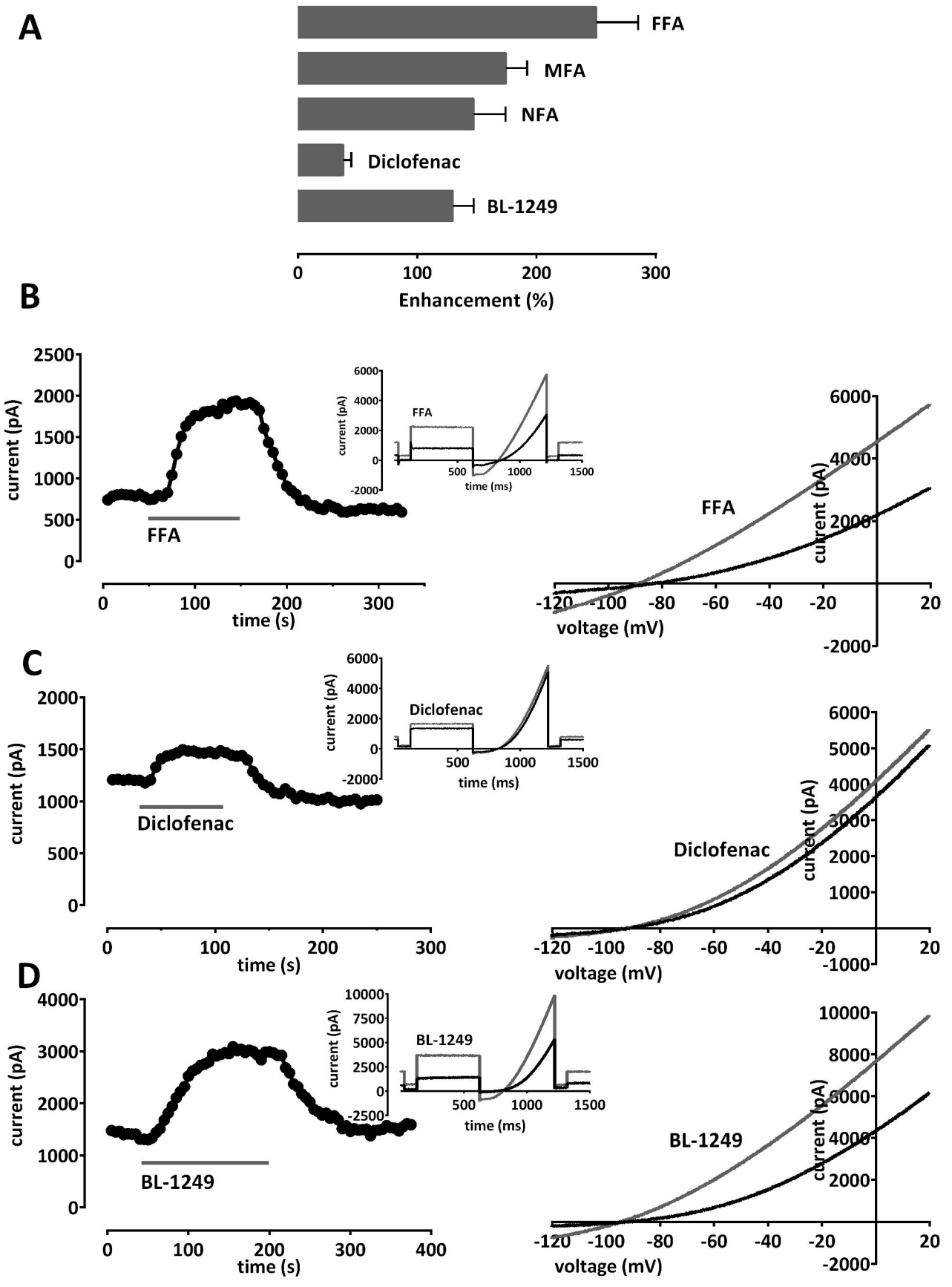

A
FFA
MFA
NFA
Diclofenac
BL-1249
0
100
200
300
Enhancement (%)
B
current (pA)
time (s)
FFA
time (ms)
voltage (mV)
C
Diclofenac
D
BL-1249

Figure 2

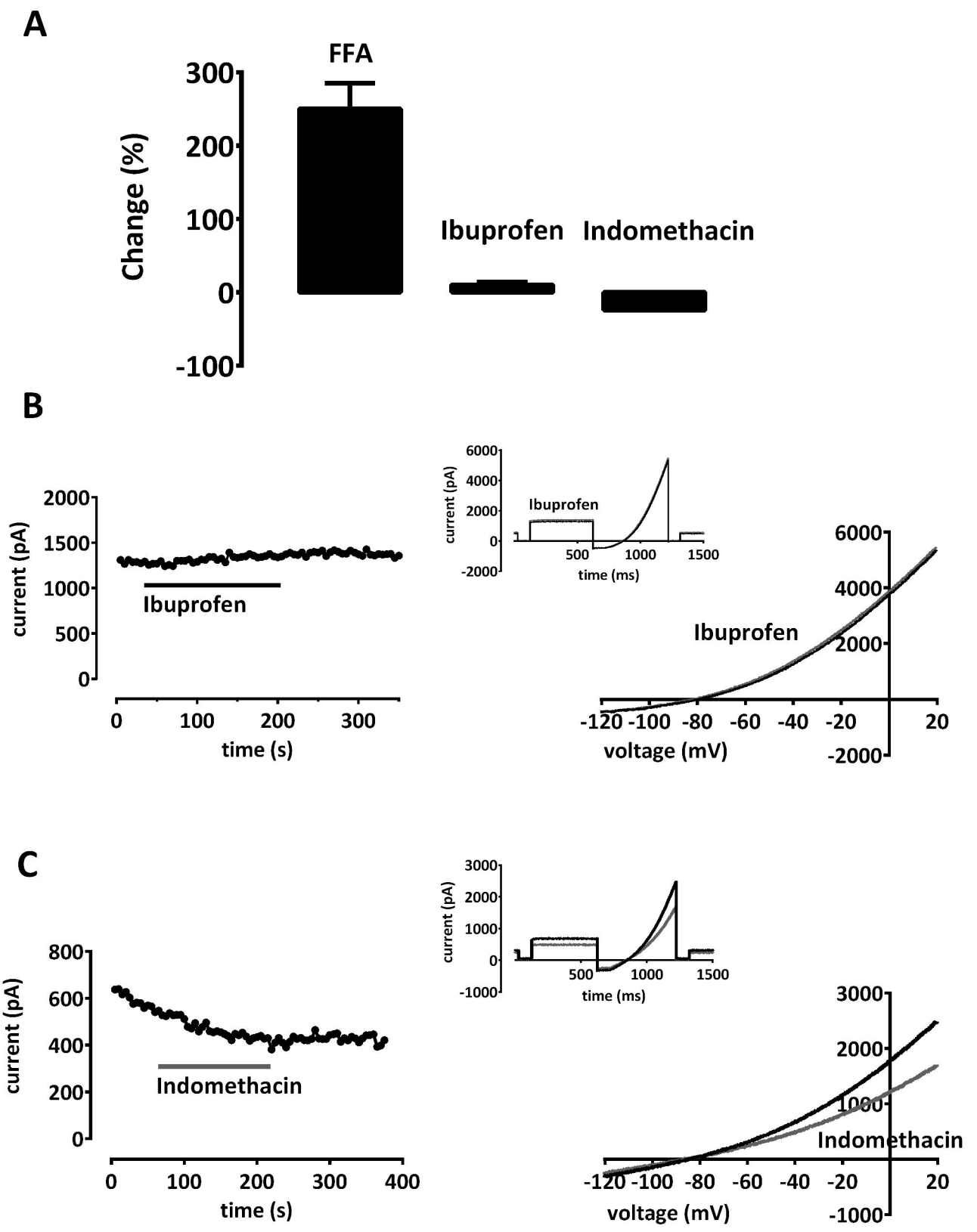
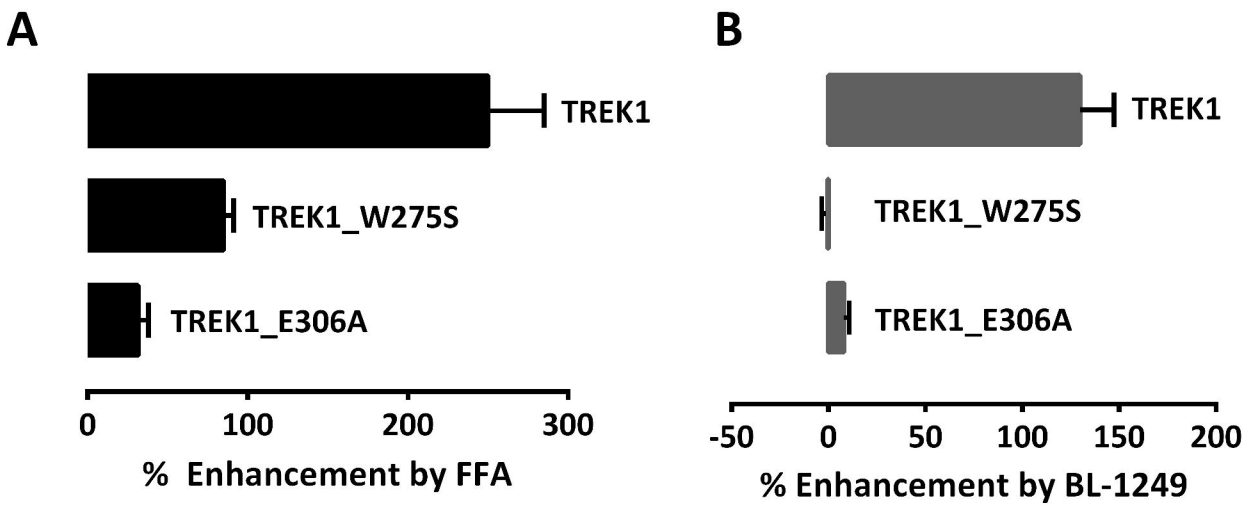
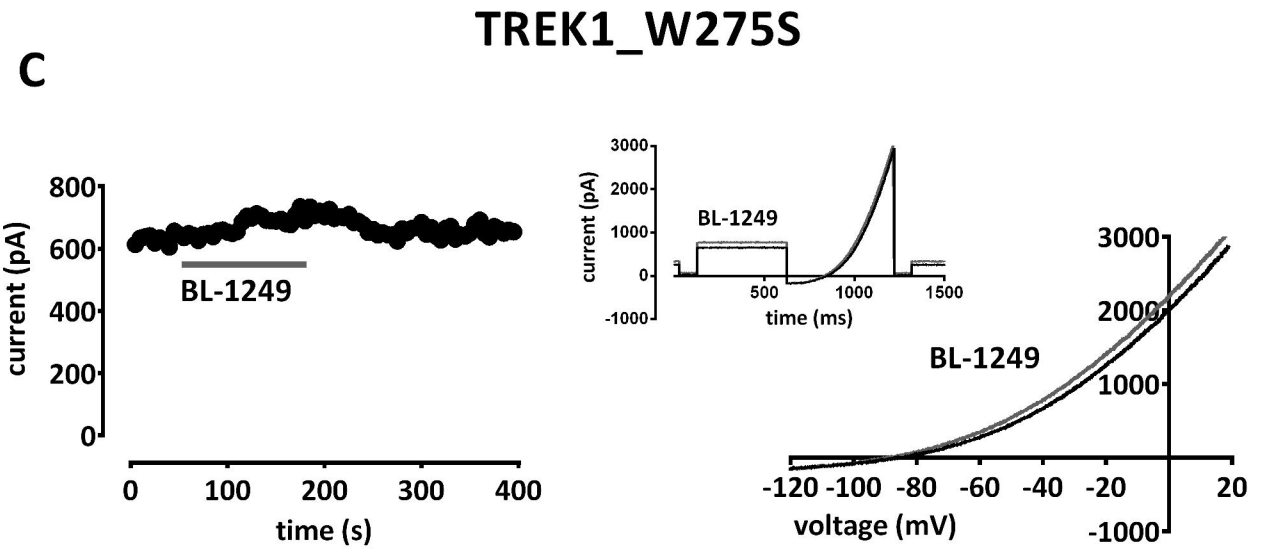

A
FFA
Change (%)
300
200
100
0
-100
Ibuprofen
Indomethacin
B
current (pA)
2000
1500
1000
500
0
Ibuprofen
0
100
200
300
time (s)
6000
4000
2000
0
-2000
current (pA)
Ibuprofen
500
1000
1500
time (ms)
6000
4000
2000
Ibuprofen
-120 -100 -80 -60 -40 -20
20
-2000
voltage (mV)
C
current (pA)
800
600
400
200
0
Indomethacin
0
100
200
300
400
time (s)
3000
2000
1000
0
-1000
current (pA)
500
1000
1500
time (ms)
3000
2000
1000
Indomethacin
-120 -100 -80 -60 -40 -20
20
-1000
voltage (mV)

Figure 3


A
TREK1
TREK1_W275S
TREK1_E306A
0
100
200
300
% Enhancement by FFA
B
TREK1
TREK1_W275S
TREK1_E306A
-50
0
50
100
150
200
% Enhancement by BL-1249


TREK1_W275S
C
current (pA)
800
600
400
200
0
BL-1249
0
100
200
300
400
time (s)
3000
2000
1000
0
-1000
current (pA)
BL-1249
500
1000
1500
time (ms)
BL-1249
3000
2000
1000
-120
-100
-80
-60
-40
-20
20
-1000
voltage (mV)

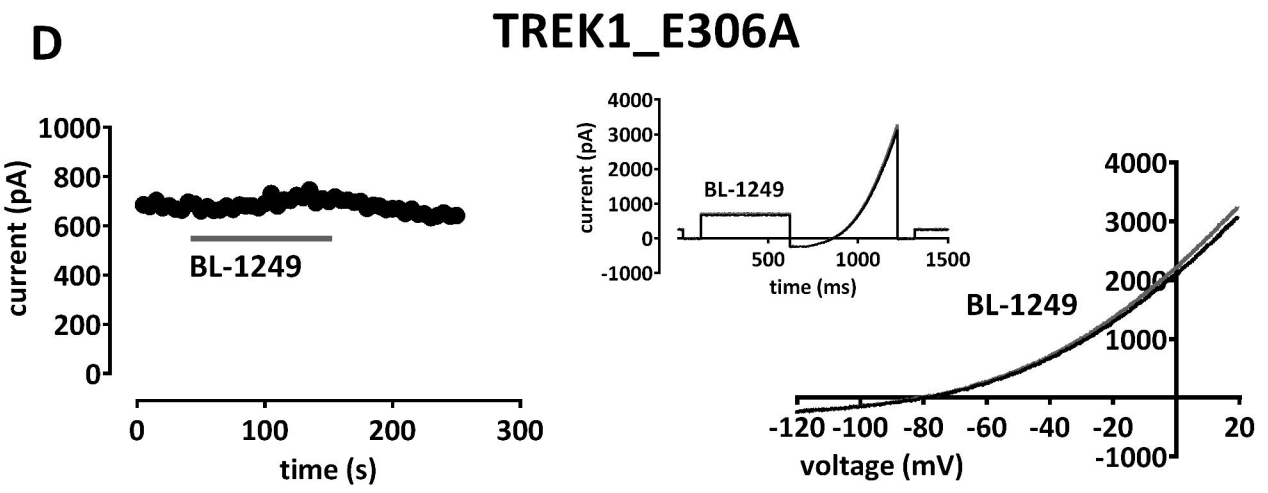
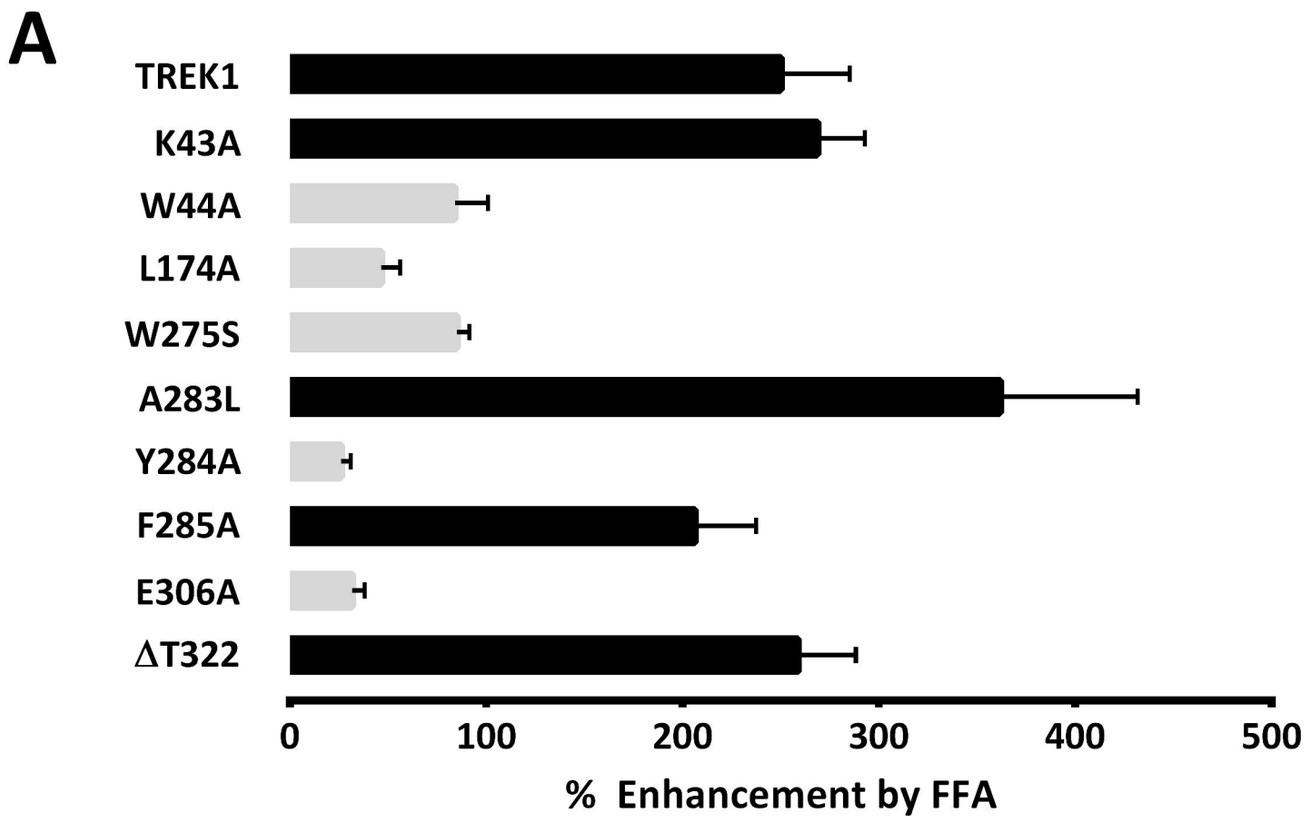
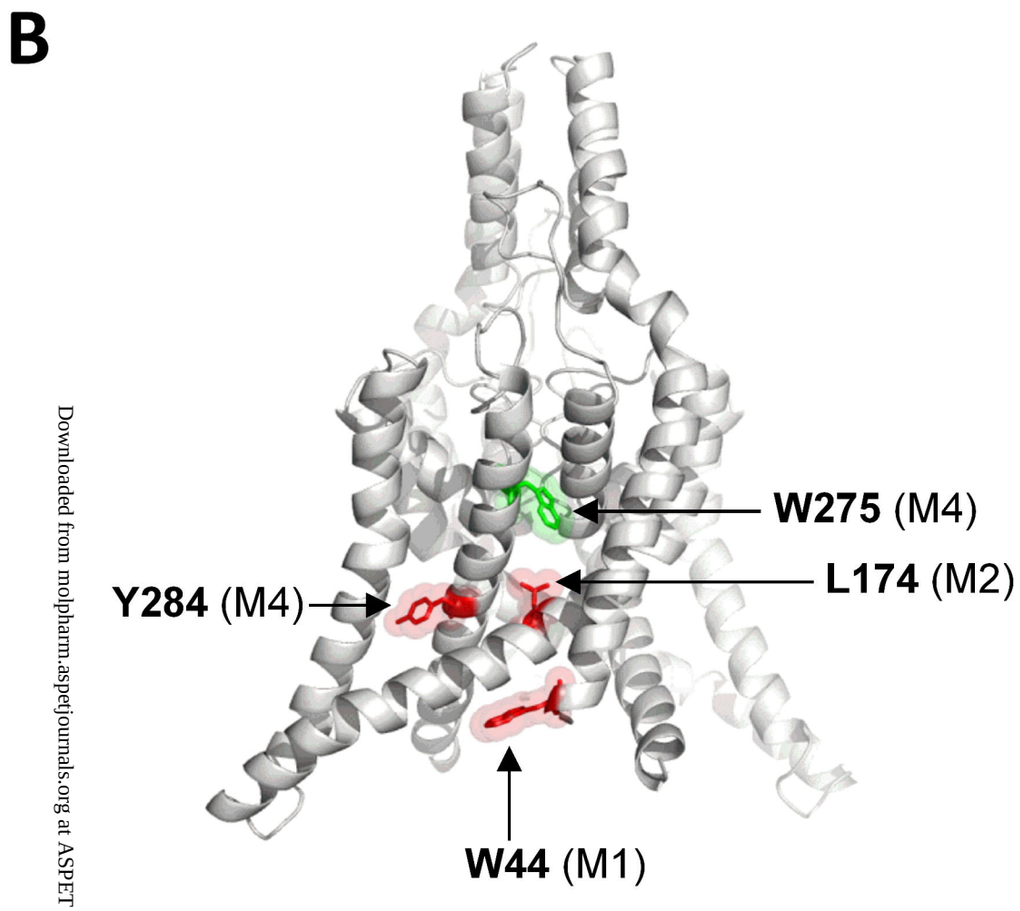
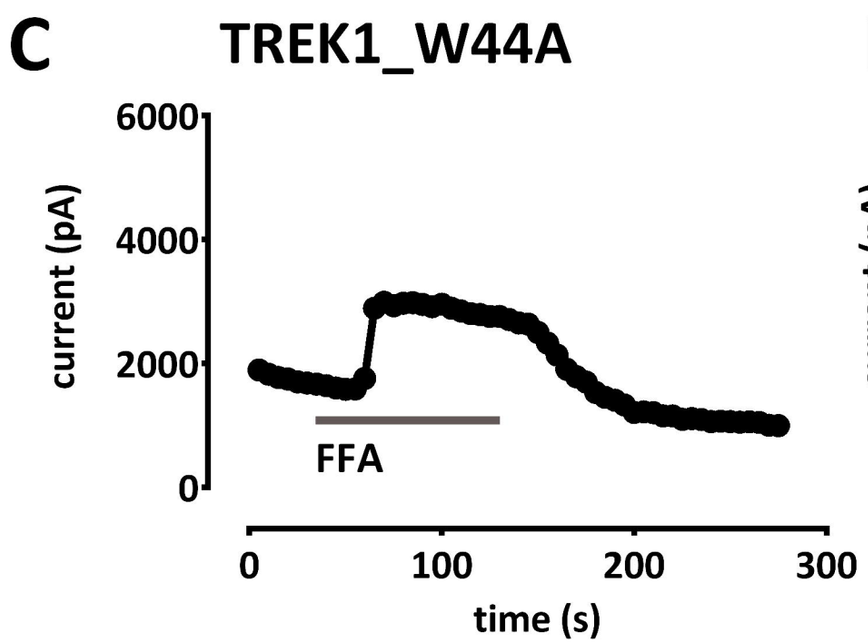

TREK1_E306A
D
current (pA)
1000
800
600
400
200
0
BL-1249
0
100
200
300
time (s)
4000
3000
2000
1000
0
-1000
current (pA)
BL-1249
500
1000
1500
time (ms)
BL-1249
4000
3000
2000
1000
-120
-100
-80
-60
-40
-20
20
-1000
voltage (mV)

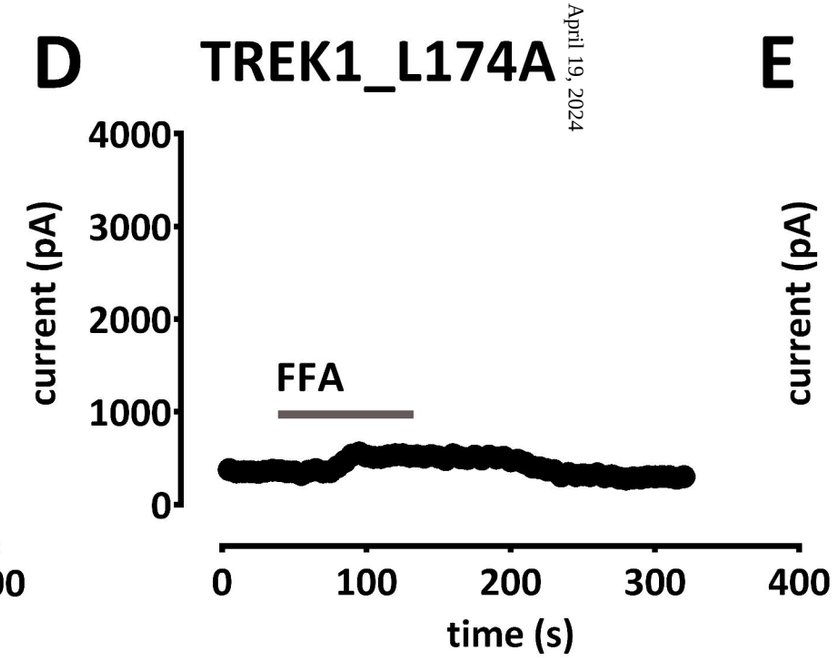
Figure 4

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.









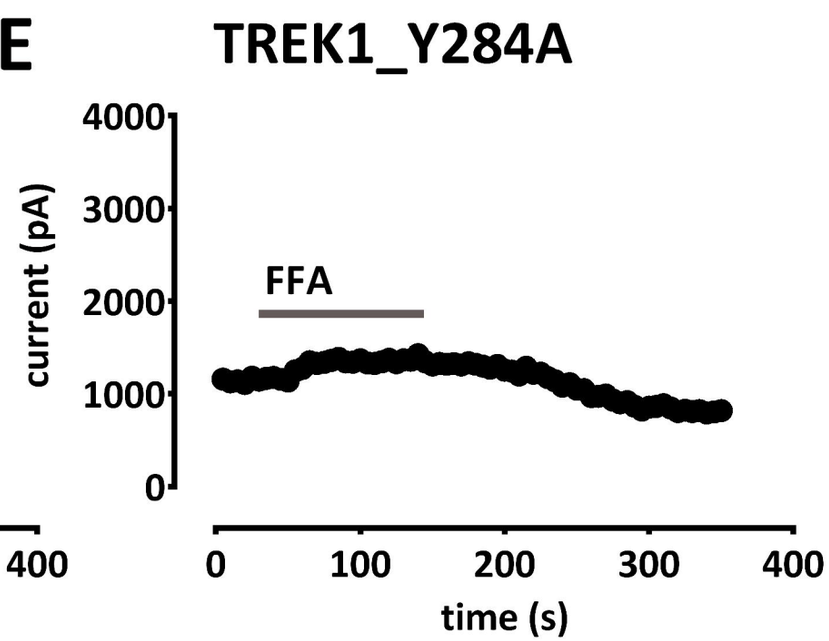
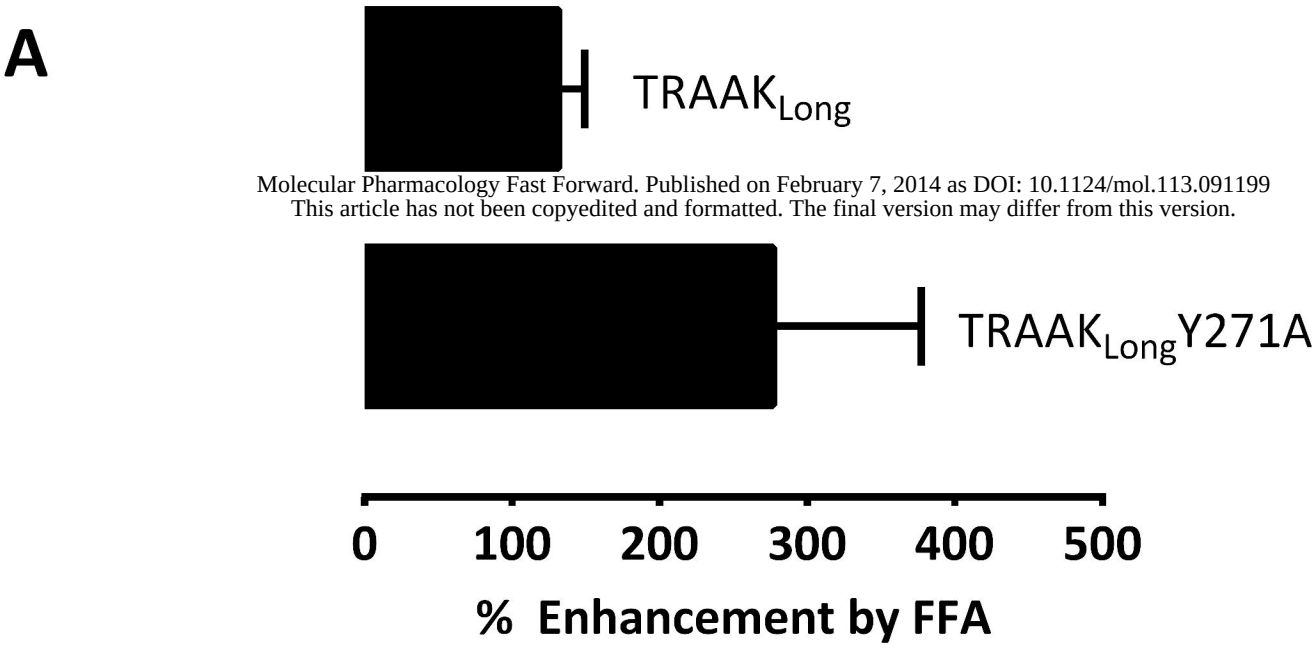
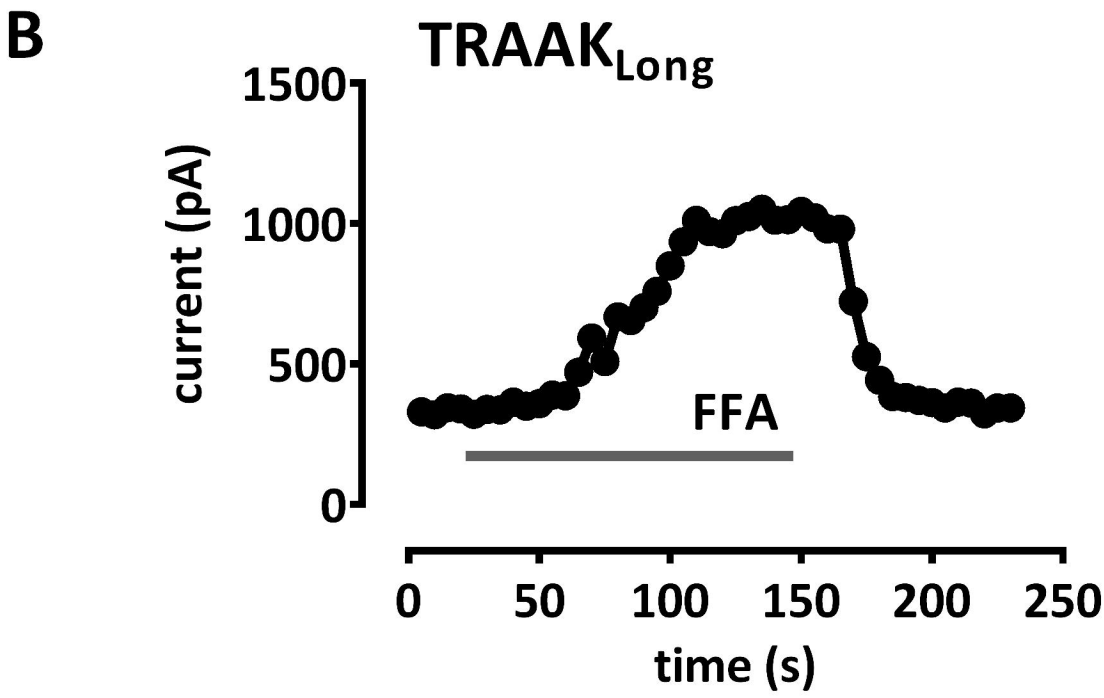


Figure 5

Molecular Pharmacology Fast Forward. Published on February 7, 2014 as DOI: 10.1124/mol.113.091199
This article has not been copyedited and formatted. The final version may differ from this version.





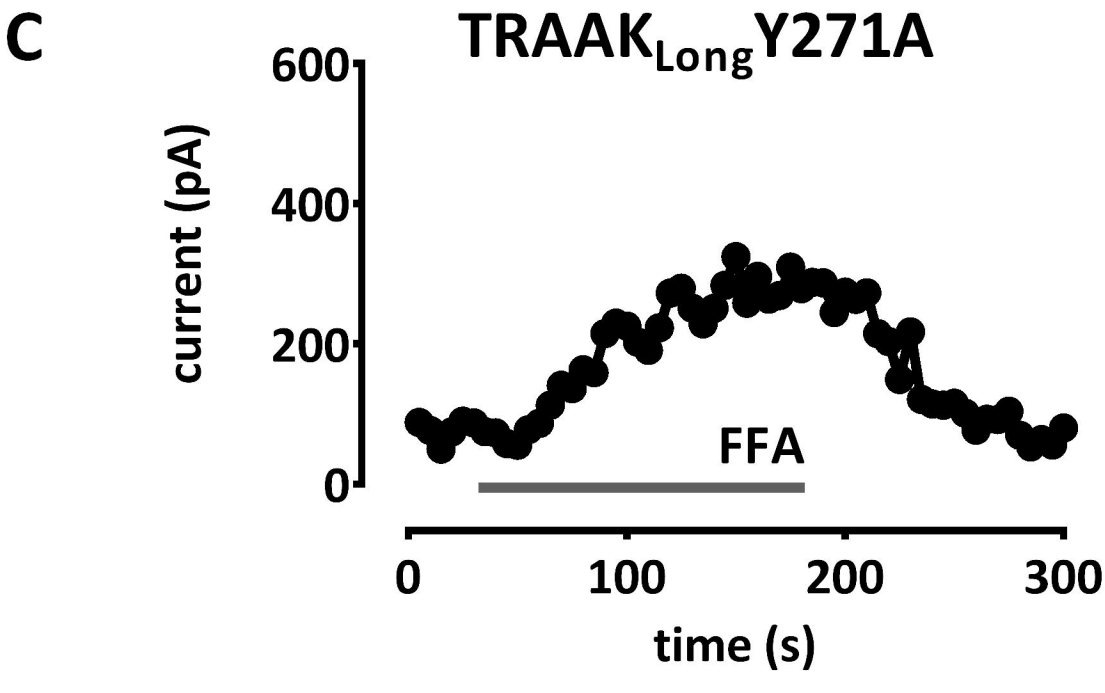


Figure 6

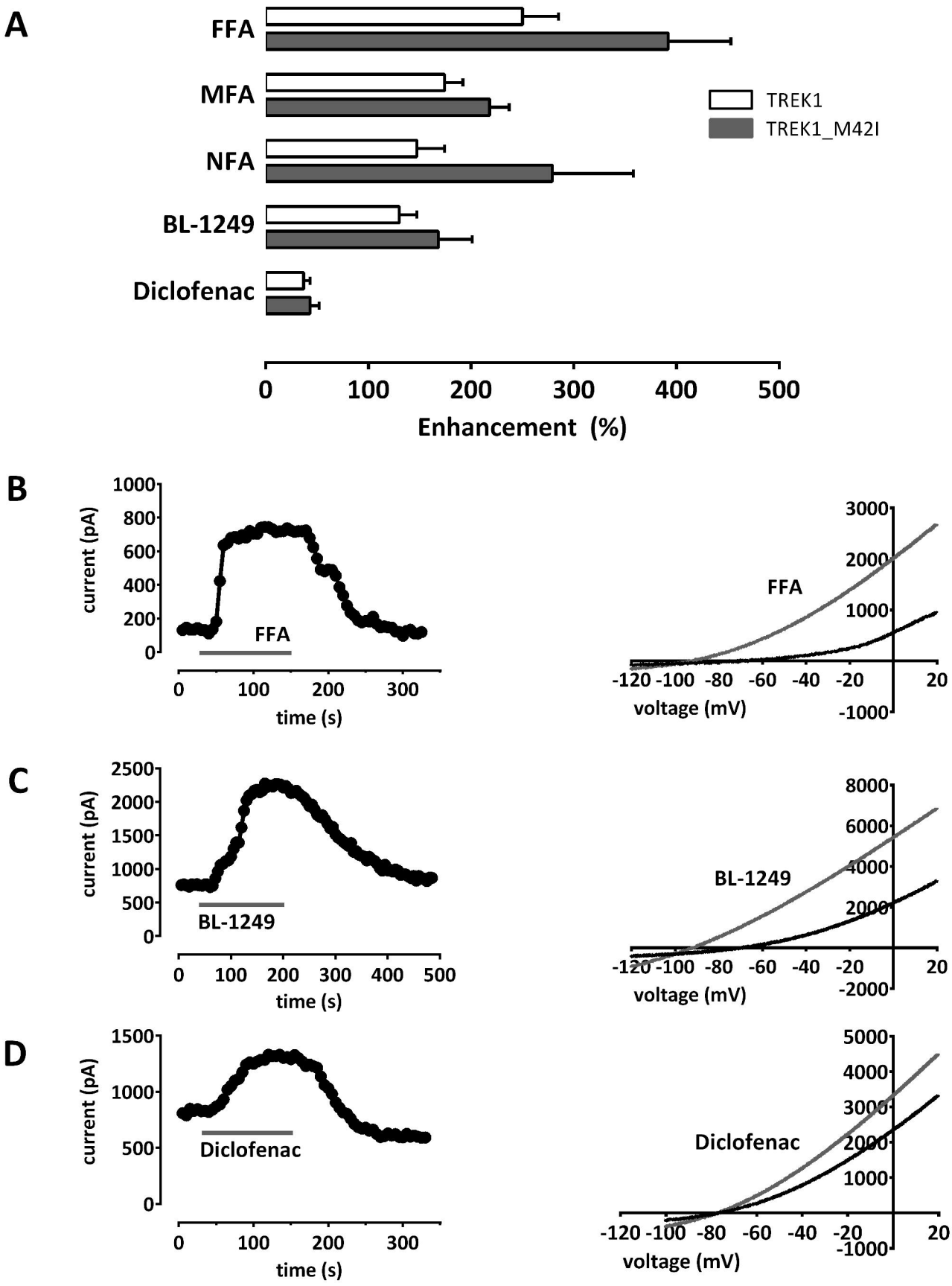

A
FFA
MFA
NFA
BL-1249
Diclofenac
TREK1
TREK1_M42I
0
100
200
300
400
500
Enhancement (%)
B
current (pA)
1000
800
600
400
200
0
FFA
0
100
200
300
time (s)
3000
2000
1000
FFA
-120 -100 -80 -60 -40 -20
20
-1000
voltage (mV)
C
current (pA)
2500
2000
1500
1000
500
0
BL-1249
0
100
200
300
400
500
time (s)
8000
6000
4000
2000
BL-1249
-120 -100 -80 -60 -40 -20
20
-2000
voltage (mV)
D
current (pA)
1500
1000
500
0
Diclofenac
0
100
200
300
time (s)
5000
4000
3000
2000
1000
Diclofenac
-120 -100 -80 -60 -40 -20
20
-1000
voltage (mV)

Figure 7

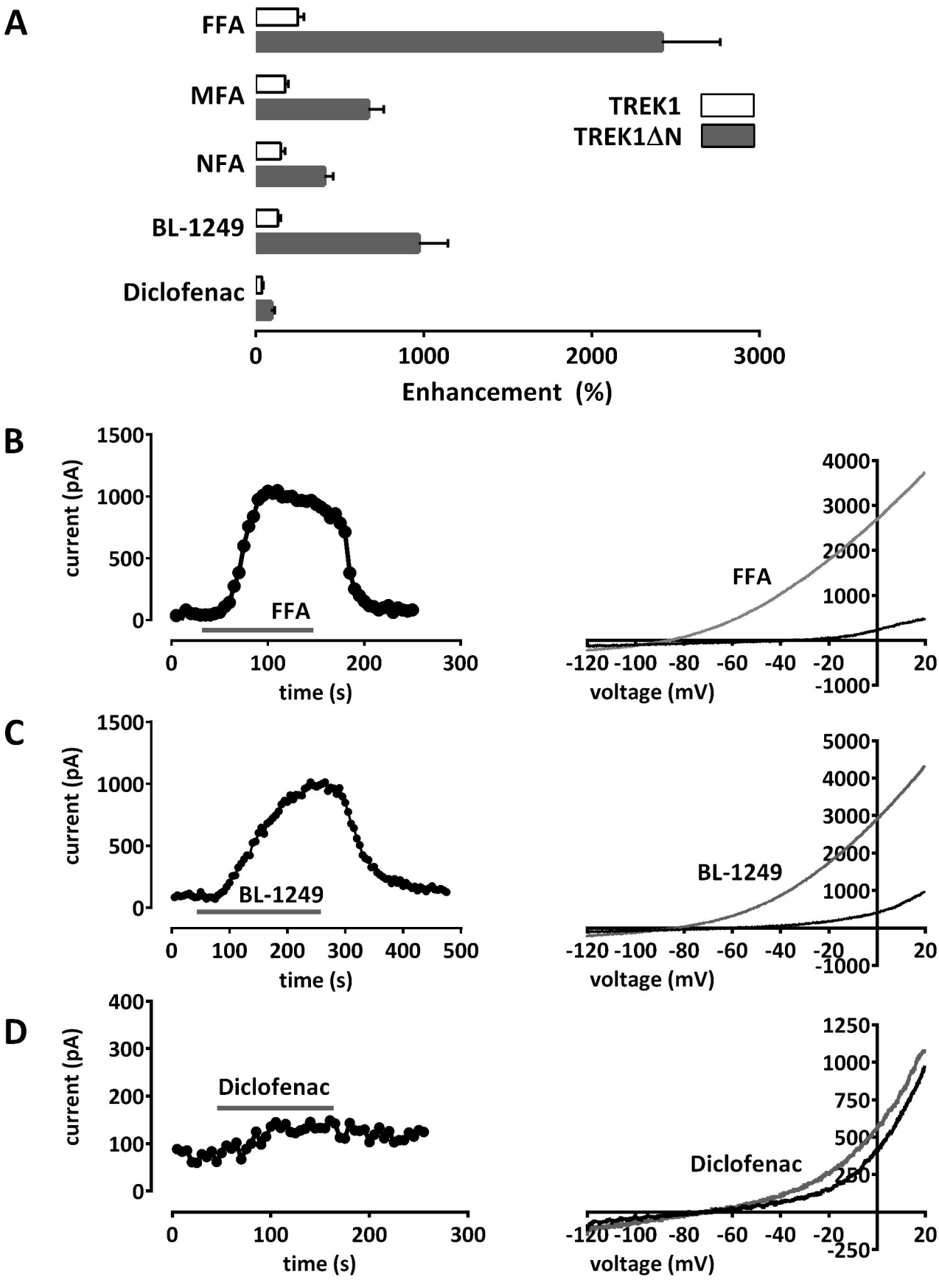

A
FFA
MFA
NFA
BL-1249
Diclofenac
TREK1
TREK1ΔN
0
1000
2000
3000
Enhancement (%)
B
current (pA)
1500
1000
500
0
FFA
0
100
200
300
time (s)
4000
3000
2000
1000
FFA
-120 -100 -80 -60 -40 -20 20
-1000
voltage (mV)
C
current (pA)
1500
1000
500
0
BL-1249
0
100
200
300
400
500
time (s)
5000
4000
3000
2000
1000
BL-1249
-120 -100 -80 -60 -40 -20 20
-1000
voltage (mV)
D
current (pA)
400
300
200
100
0
Diclofenac
0
100
200
300
time (s)
1250
1000
750
500
250
Diclofenac
-120 -100 -80 -60 -40 -20 20
-250
voltage (mV)

Figure 8