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Inhibition of hKv2.1, a major human neuronal voltage-gated K^+ channel, by meclofenamic acid

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Abstract

Using the standard patch clamp whole cell recording method, we assessed the pharmacological activity of four fenamate nonsteroidal anti-inflammatory drugs, meclofenamic acid, flufenamic acid, mefenamic acid and niflumic acid, on hKv2.1, a major human neuronal voltage-gated potassium channel stably expressed heterologously in Chinese hamster ovary cells. Meclofenamic acid inhibited hKv2.1 in a concentration-dependent manner whereas the other three fenamates had weaker or no effect on these channels at a concentration of 100 μM. The estimated IC_{50} of meclofenamic acid was 56.0 μM for hKv2.1 compared an IC_{50} of 155.9 μM for another human neuronal K channel (hKv1.1). Meclofenamic acid reached its maximum inhibition within 5 min of bath application and its effect was readily reversed upon wash. Kinetic analysis revealed that this drug did not alter the channel activation or deactivation time courses. Moreover, the effect of meclofenamic acid on hKv2.1 channels was not voltage-dependent. Indomethacin, another inhibitor of the cyclooxygenase that catalyses the synthesis of prostaglandin from arachidonic acid, had no effect on either hKv2.1 or hKv1.1. These results indicate that meclofenamic acid inhibits hKv2.1 more potently than hKv1.1 and it is likely that this compound acts directly on the channel proteins.

Keywords: K^+ channel, voltage-gated; hKv2.1; Meclofenamic acid; Nonsteroidal anti-inflammatory drug; Ovary cell, Chinese hamster

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as fenamate, aspirin, acetaminophen and ibuprofen, are widely used substances. The fenamate NSAIDs such as meclofenamic, flufenamic, mefenamic and niflumic acids are all derivatives of *N*-phenylanthranilic acid (Fig. 1). The potential effects of NSAIDs on the central nervous system, such as neuro-protection in Alzheimer's disease (Rich et al., 1995; Stewart et al., 1997), have spurred several basic and clinical studies. It is well known that the major mechanism mediating anti-inflammatory effects of fenamate and other NSAIDs is inhibition of the cyclooxygenase that catalyzes the biosynthesis of prostaglandins (e.g., prostaglandin E_2) from arachidonic acid in both peripheral and central tissues (Wu, 1998). However, accumulating evidence indicates that fenamates also modulate a diversity of ion channels through a pathway which may be independent of the cyclooxygenase-prostaglandin mechanism. For example, it has been reported that meclofenamic acid (5–10 μM) is a potent blocker of an ATP-sensitive K^+ channel (Grover et al., 1994). The effect of niflumic acid on ion channels seems more complex as it blocks both anionic and cationic channels in a number of species at various concentrations from 10–100 μM (White and Aylwin, 1990; Gögelein et al., 1990; Hogg et al., 1994; Wang et al., 1997a,b). In addition, niflumic acid and other fenamates potentiate a large conductance Ca^{2+}-activated K^+ channel current, possibly by acting directly on the channel proteins at concentrations from 75 μM to 1 mM (Farrugia et al., 1993; Ottolia and Toro, 1994; Greenwood and Large, 1995). Effects of fenamates on ion channels in the central nervous system have also been reported. For example, fenamates modulate the $GABA_A$ receptor-gated Cl^- channels in a concentration-dependent fashion. When Cl^- currents were induced by low concentrations of GABA, fenamates potentiated this ligand-gated current with EC_{50}s of 5–10 μM. In contrast, the maximum Cl^- currents

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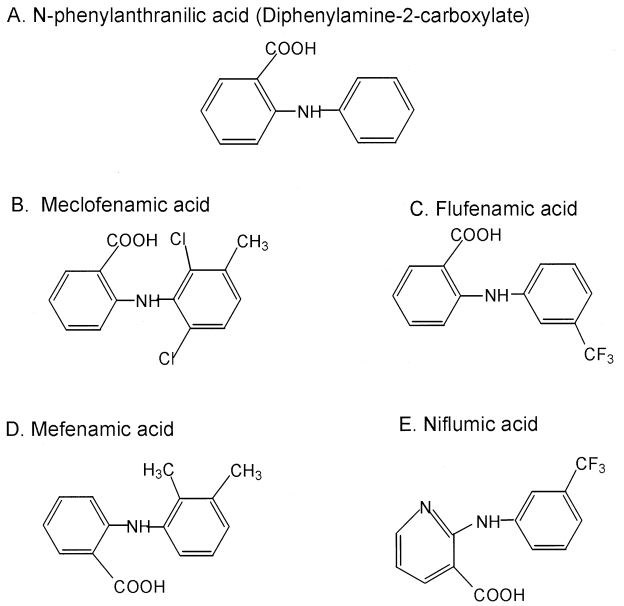

Fig. 1. Structures of *N*-phenylanthranilic acid (A) and the NSAIDs tested on hKv1.1 and hKv2.1 K^+ channels in the present study: meclofenamic acid (B), flufenamic acid (C), mefenamic acid (D) and niflumic acid (E).

stimulated by a high GABA concentration (3 mM) were inhibited by fenamates with IC_{50}s of 7–50 μM (Woodward et al., 1994).

Despite the above-mentioned reports, little is known about the effects of fenamates on neuronal voltage-gated K^+ channels (Kv). It is well established that Kv channels are crucial in shaping action potentials (Hille, 1992; Pongs, 1992). Ultimately, Kv channels are involved in the regulation of intracellular calcium level and thereby neurotransmitter release (Boireau et al., 1991; Schechter, 1997). To date, at least five subfamilies of functional Kv channel α subunits have been cloned (Kv1-5) and their expression pattern in mammalian central nervous system has been extensively investigated (Pongs, 1992; Jan and Jan, 1997). Recent data from Wang et al. (1997a) have shown that some fenamates at high concentration (1 mM) may act as blockers of the Kv4 subfamily which encode ''A-type'' transient K currents. However, Kv4 subfamily exists not only in central nervous system, but also in the heart. The majority Kv channels in the soma are Kv2.1 and virtually every neuron in the brain expresses Kv2.1 whereas Kv1 subfamily is expressed in the axonal terminals (Awan and Dolly, 1991; Pongs, 1992; Trimmer, 1993; Rhodes et al., 1997; Rasband et al., 1998; Murakoshi and Trimmer, 1999). Physiological significance of these Kv channels is evident as behavioral changes in mice were observed when a Kv channel was ''knocked-down'' using an intracerebroventricular injection of antisense oligonucleotide (Galeotti et al., 1997) or knocked out genetically (Galeotti et al., 1997; Smart et al., 1998). Therefore, the primary goal of this investigation was to find out if fenamates were blockers of Kv2.1 K^+ channels. For comparison, we also performed some experiments with another delayed rectifier K channel, the hKv1.1. Both channels were stably expressed in the Chinese hamster ovary (CHO) cells. The results demonstrate that meclofenamic acid preferentially inhibits hKv2.1 in a concentration-dependent manner. This inhibition seems independent of the cyclooxygenase-prostaglandin pathway.

2. Methods

2.1. Construction of CHOK1-hKv1.1 and CHOK1-hKv2.1 cell line

Methods for the construction of cell lines were similar to what had been reported previously (Wang et al., 1998). Human Kv1.1 cDNA (kind gift of Dr. Bruce Tempel) was excised from pGexHG2 with *Bgl*II/*Eco*RI and inserted into the *Bam*HI/*Eco*R1 sites of mammalian expression vector pWE1 to yield pWE1/Kv1.1. The construct was characterized by restriction enzyme mapping and DNA sequencing (dideoxynucleotide chain termination method). CHOK1 cells were transfected with linearized pWE1/Kv1.1 by electroporation using a single pulse at 250 V, 1180 μF in a Gibco BRL Cell-Porator. Stably transfected cells were selected in DMEM supplemented with 10% dialyzed FCS (Summit Biotechnologies), 1% NEAA, 1% HT (Gibco BRL), 50 μg/ml mycophenolic acid and 250 μg/ml xanthine (Sigma, St. Louis, MO, USA). Selected clones were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) for Kv1.1 mRNA expression and by Western Blot analysis for protein expression. Clones with the highest levels of protein expression were analyzed for delayed rectifier type channel activity by standard whole cell patch clamp recording. Clone 2–13 exhibited the highest frequency and amplitude of current and was further sub-cloned by limiting dilution to ensure stability of line. Subclones 2–13 and 1–19 were used for further experiments.

Similarly, CHOK1-hKv2.1 (kind gift of Dr. P. Sokol) was sub-cloned by limiting dilution to increase stability of the cell line. The resulting subclones were analyzed for hKv2.1 mRNA by RT-PCR. Clones exhibiting the highest expression levels were further analyzed for delayed rectifier type channel activity by standard whole cell patch clamp recording. Subclone 2–3 was selected for further experiments.

Flasks of cells were maintained at 37°C in an incubator shaker (Innova 4330, New Brunswick Scientific, NJ, USA) at a shaking speed of 140 rpm. Subsequently, cells were plated onto 5-mm glass cover slips at least 30 min prior to electrophysiological recordings. The cover slips were then kept at 37°C in a humidified atmosphere and used within 72 h after plating.

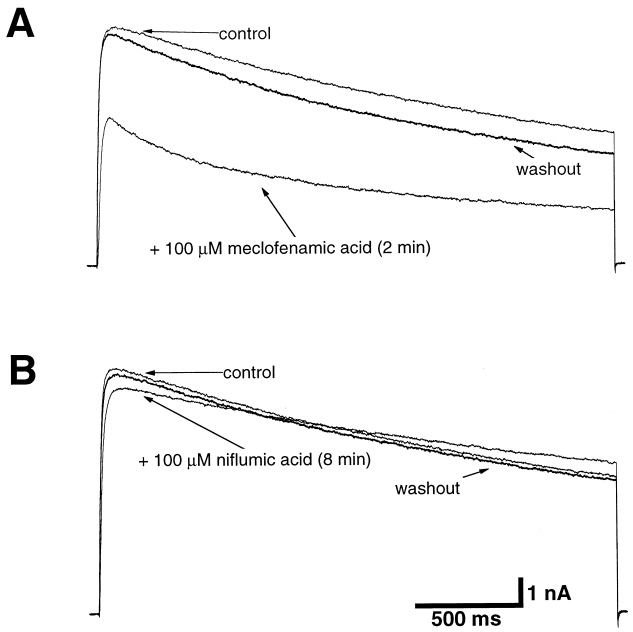

Fig. 2. The effect of meclofenamic acid (A) and niflumic acid (B) on hKv2.1 current recorded from the same CHO cell. The current amplitude was readily recovered to control level upon washout of the drug. Cell was voltage-clamped at −60 mV and stepped to +50 mV for 2.5 s.

2.2. Electrophysiology

The electrophysiological experiments were performed at room temperature (22–23°C) as described previously (Wang et al., 1998). Using the standard patch clamp whole-cell recording method, currents were recorded from CHO cells that stably expressed hKv2.1 or hKv1.1. Patch pipettes were pulled from thin-wall borosilicate glass capillaries (TW-150-4, World Precision Instruments, Sarassota, FL, USA) on a horizontal Flaming Brown Micropipette Puller (P.80/PC, Sutter Instruments, San Rafael, CA, USA). When filled with pipette solutions, the resistance of the patch pipettes was in a range of 3–5 MΩ. The membrane currents were elicited by membrane depolarization from −60 to +50 mV for 2.5 s, sampled at a rate of 1 ms per point and filtered by a low-pass Bessel filter set at 1.0 kHz. Data was acquired and stored on a Power Macintosh 7500/100 computer using an EPC-9 amplifier and the HEKA Pulse 8.0 software (HEKA Elektronik, Lambrecht, Germany). Membrane currents were leak-subtracted and capacity-compensated on line with a p/4 subtraction protocol. Series resistance compensation was set at 50%/10 μs (HEKA Pulse 8.0). Hard copies were printed out on a LaserJet 5Si Mx printer using Igor Pro software (Version 3.0, Wavemetrics, Lake Oswego, OR, USA).

2.3. Solutions and chemicals

The pipette solution used in this study contained (in mM): K Aspartate, 125; KCl, 20; KH_2PO_4, 1; NaCl, 5; $MgCl_2$, 6; *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (HEPES), 5; ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10; ADP 5. The pH was adjusted to 7.4 with KOH. The bath solution was made from modified Hanks' balanced salt solution (14060, 10× liquid, Life Technologies, Gaitherburg, MD, USA) and consisted of (in mM): KCl, 5.4; NaCl, 136.9; $NaHPO4_4 \cdot 7H_2O$, 0.34; $MgCl_2 \cdot 6H_2O$, 0.5; $MgSO_2$, 0.41; $CaCl_2$, 1.26; HEPES, 10; glucose, 11, and 0.01 g/l of Phenol Red. NaOH was used to adjust the pH to 7.4. When tail currents were recorded, 20 mM external KCl was used to yield a larger current for precise analysis. Fenamates and indomethacin were purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO) as stock solutions. The final vehicle concentration was below 0.3% at which DMSO did not have any effect on the membrane currents. Drugs were applied to the cells via a gravity flow system at a speed of 1 ml/min and washed with a peristaltic pump (Dynamax Model RP-1, Rainin Instrument, Woburn, MA, USA). The change from control solution to the final drug-concentration in the bath was completed within 1 min.

2.4. Data analysis

The steady level of ion currents was measured on line utilizing the HEKA Pulsefit 8.10 software. A mono-exponential fit in the HEKA PulseFit software or Igor Pro (version 3, WaveMetrics) was applied on line to obtain channel activation (from the outward currents) and deactivation (from the tail currents) time constant values. In the concentration–effect curve, each point represented the mean ± S.E.M. of several experiments and mean concentration–effect curves were obtained by fitting the data using an equation as below:

$$y = E_{\min} + E_{\max}/\left(1 + \left[D/\mathrm{IC}_{50}\right]\right)^{\mathrm{Hill}}$$

where y is the percent response to the control obtained in the absence of the drug; $E_{\min}$ is the minimum response in

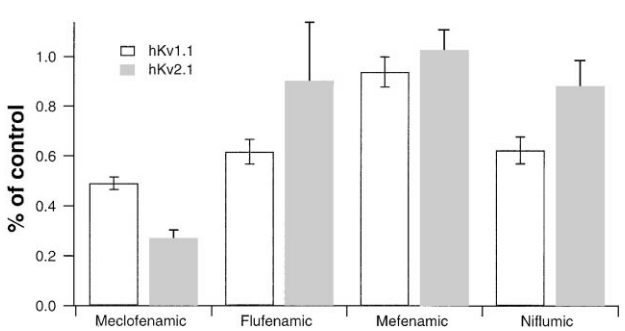

Fig. 3. Effects of meclofenamic acid, mefenamic acid, flufenamic acid and niflumic acid on hKv2.1 and hKv1.1 current amplitudes. The concentration for each drug was 100 μM. The unfilled bars represent hKv1.1 current and the grey bars represent hKv2.1 current. Error bars are ±S.E.M. The mean values were obtained from 3–4 cells for each drug tested.

the presence of the highest concentration of the drug and E_{max} is the response in the presence of lowest concentration of the drug used in this study; D is the drug concentration; IC_{50} is the concentration of the drug that produced 50% inhibition of the control current; and Hill is the Hill coefficient. The data were expressed as mean ± S.E.M. and either paired or unpaired *t*-tests were performed. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Inhibition of Kv channel currents by fenamates

In order to produce a preliminary pharmacological profile of the four fenamates on Kv channels, we used a single dose (100 μM) of fenamates in our first set of experiments. Fig. 2A shows that meclofenamic acid (100 μM)

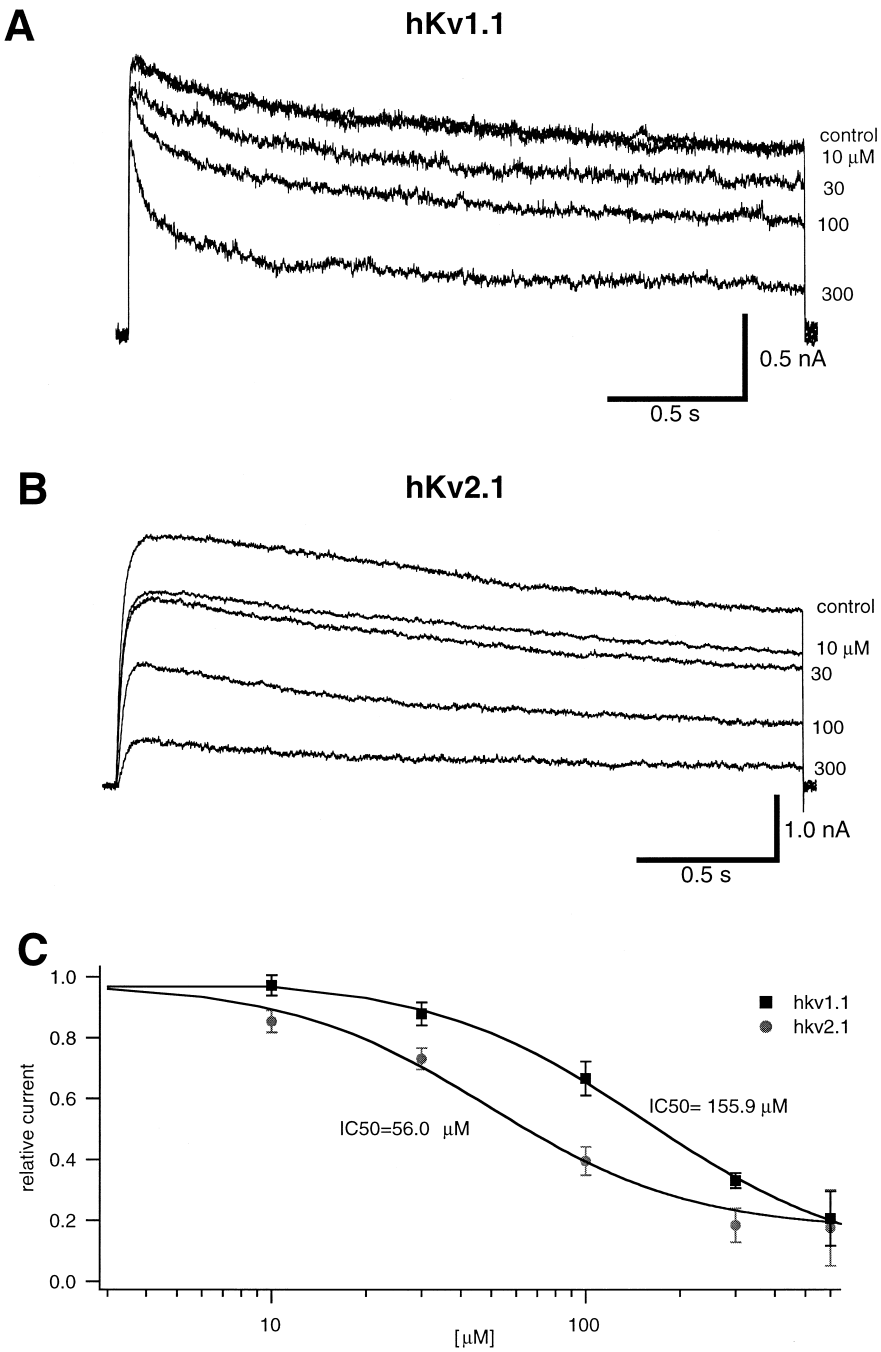

Fig. 4. Concentration-dependent inhibition of Kv channel currents by meclofenamic acid. Representative traces show the effects of 10, 30, 100 and 300 μM meclofenamic acid on hKv1.1 (A) and hKv2.1 (B) currents. Current was induced by a voltage step to +50 mV for 2.5 s. C: Concentration–effect curves of meclofenamic acid on hKv1.1 (squares) and hKv2.1 (circles) channel current. Smooth lines represent logistic curve fit of the data points (see the equation in text). Error bars represent ± S.E.M.

inhibited hKv2.1 current by over 50%. Another fenamate NSAID, niflumic acid (100 μM), was almost without effect on hKv2.1 current (Fig. 2B). The effects of meclofenamic acid on Kv channels were readily reversible upon wash. In three cells at a test potential of +50 mV 100 μM meclofenamic acid reduced hKv2.1 and hKv1.1 currents to about 30% and 50% of the control current amplitudes, respectively. Three other fenamates were also tested on hKv2.1 and hKv1.1 currents and the results are summarized in Fig. 3. The rank order of potencies for inhibiting hKv2.1 was meclofenamic acid ≫ niflumic acid > flufenamic acid. Mefenamic acid at 100 μM did not show any inhibition on hKv2.1 current. For inhibition of hKv1.1 currents, the rank order of potencies was meclofenamic acid > niflumic acid ≈ flufenamic acid ≫ mefenamic acid. Pilot experiments using primary cultured rat hippocampal neurons also showed that 100 μM meclofenamic acid reversibly inhibited voltage-gated K^+ currents (mainly Kv2.1) by approximately 50% (data not shown). The single-dose experiments demonstrated that meclofenamic acid seemed to be the most potent fenamate to inhibit Kv channels, especially hKv2.1. Therefore, a more extensive study of the properties of this drug was carried out.

3.2. Concentration-dependent inhibition of Kv current amplitude by meclofenamic acid

Meclofenamic acid inhibited both hKv2.1 and hKv1.1 in a concentration-dependent fashion. As shown in Fig. 4A and B, representative current traces of hKv2.1 and hKv1.1 were recorded at a test potential of +50 mV in the absence and presence of varying concentrations of the fenamate. The concentration–response curves in Fig. 4C show that the estimated IC_{50}s were 56.0 and 155.9 μM, respectively for blocking hKv2.1 and hKv1.1 currents. Also obtained from the concentration–response curves were the Hill coefficients: 1.35 for hKv2.1 and 1.26 for hKv1.1.

To determine if meclofenamic acid inhibits the Kv channel by inhibiting the cyclooxygenase or by directly acting on the channel proteins, we tested indomethacin, another inhibitor of cyclooxygenase. We found that indomethacin (up to 100 μM) was without any effect on either Kv2.1 channel currents ($n = 3$) or on the delayed rectifier K^+ currents in cultured rat hippocampal neuronal cells ($n = 3$).

3.3. Voltage-independent inhibition of Kv channels by meclofenamic acid

We had found that meclofenamic acid reached its maximum inhibition of hKv2.1 within 5 min of drug application. The inhibition was partially reversed immediately after washout of the drug. To test if stimulus frequency may affect either the time course of drug effect or the maximum inhibition of the current amplitude or both, we used two frequencies of test pulse. In one group of experiments, a test pulse from −60 to +50 mV was used every 2 min whereas in another group, 5-min intervals separated the test pulses. Neither the maximum inhibition of current amplitude nor the time course of drug effect was dependent on pulse frequency. The impact of clamp voltage on the drug potency was also analyzed (Fig. 5). The I/V plot indicates that the threshold potential for activation of hKv2.1 was approximately +20 mV. We used normalized current–voltage curves in Fig. 5 because it is more obvious to detect if there is any voltage-dependent inhibition of Kv channels by this fenamate. In Fig. 5, the I/V curves were normalized to the maximum current obtained in the absence of the drug (+40 mV). Clearly, meclofenamic acid inhibited Kv currents at various test potentials. We also used a ''non-crossing'' normalization method and found that the two curves overlapped, i.e., meclofenamic acid inhibited the Kv2.1 current in a voltage-independent manner (data not shown). Fig. 5 also shows that neither the activation threshold potential nor the I/V relationship was altered by meclofenamic acid.

3.4. Influence of meclofenamic acid on the Kv channel deactivation kinetics

The Kv channel deactivation time constant (τ_d), which reflects the channel closing, was studied using a typical tail current analysis. The membrane potential was depolarized to +40 mV for 80 ms and stepped to varying test potentials for 150 ms. In Fig. 6A, the tail currents were scaled to the same size for a better comparison. As can be seen from the original current traces (Fig. 6A), meclofenamic acid did not have any influence on the tail current kinetics of hKv2.1 at −120 mV. The plots of τ_d against membrane potentials in Fig. 6B illustrate that τ_d of Kv channel current increased with membrane depolarization and meclofenamic acid did not significantly alter this voltage-dependent pattern of τ_d from −120 to −60 mV. In addition, we also measured the activation time constant

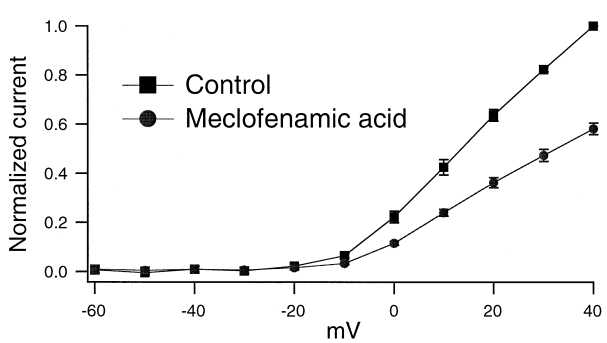

Fig. 5. Meclofenamic acid inhibits K^+ channels in a voltage-independent manner. Current–voltage relationship of hKv2.1 was obtained respectively in the absence (squares) and presence (circles) of 100 μM meclofenamic acid. The data are responses normalized to the control response generated at +40 mV. The membrane potential was held at −60 mV and depolarized to +40 mV for 200 ms in 10-mV increments. Error bars represent the ±S.E.M.

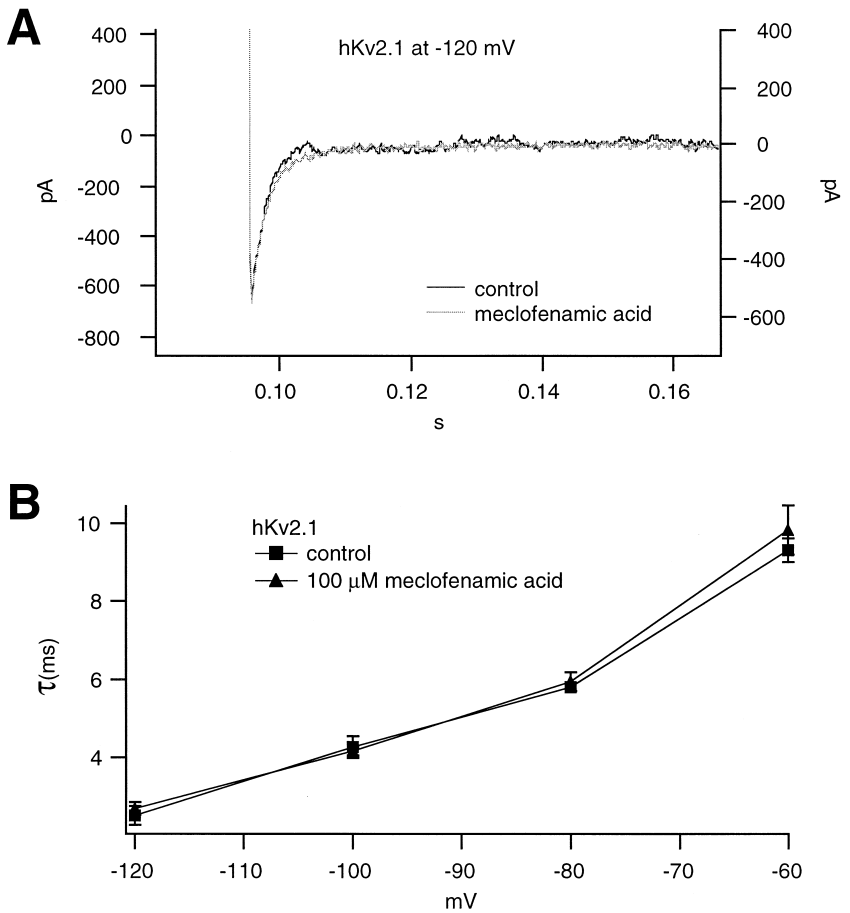

Fig. 6. Meclofenamic acid has no effect on the closing rate of hKv2.1 channels. Tail currents were generated by stepping voltage from −60 to +40 mV for 80 ms (pre-pulse) and then returning to membrane potentials at a range of −120 to −60 mV in 20-mV increments so that all of the tail currents measured in this range were inward. (A) Tail current of hKv2.1 in the absence (black trace) and presence (gray trace) of 100 μM meclofenamic acid. The left and right axes represent the inward tail current amplitude (pA) in the absence and presence of meclofenamic acid, respectively. (B) Graph of the deactivation time constant (τ_d) values of the hKv2.1 tail current in the presence (triangles) and absence (squares) of meclofenamic acid. Error bars represent the ±S.E.M.

(τ_a) of Kv channel current and no significant difference was found in τ_a after application of meclofenamic acid ($n = 3$, data not shown).

4. Discussion

The current study clearly showed that the NSAID meclofenamic acid inhibits hKv2.1 (a delayed rectifier K^+ channel) current amplitude in a concentration-dependent manner with little effect on the channel activation and deactivation kinetics. The data also suggested that this inhibition is likely due to a direct interaction of the drug with the channel proteins.

NSAIDs including fenamates are known to block the cyclooxygenase-prostaglandin pathway to achieve their anti-inflammatory effects in both peripheral and central tissues (Wu, 1998). In this study, we have also tested indomethacin, another cyclooxygenase-prostaglandin pathway inhibitor with markedly different structure, on the same channel currents and found that indomethacin at similar concentrations did not inhibit either of the expressed Kv channels. This result agreed well with a previous report (Grover et al., 1994) which showed that meclofenamic acid seemed to inhibit an ATP-sensitive K^+ channel in cardiac cells without involvement of the cyclooxygenase-prostaglandin pathway. Moreover, although the mechanisms of anti-inflammatory effects for the four fenamates tested in the current study are similar, minor alteration of the base structure (Fig. 1) drastically changed their potency and selectivity in inhibiting the two expressed Kv channels. Thus, meclofenamic acid was found to be a more potent inhibitor of hKv2.1 than hKv1.1 and niflumic acid seemed to be more selective as a hKv1.1 inhibitor (Fig. 3). According to the structure of meclofenamic acid (Fig. 1), it is reasonable to presume that the presence of chloro- (Cl^-) at the 2, 6 positions is important for this NSAID to possess the inhibitory effect on the Kv channels. On the other hand, drugs lacking of the chloro- at these positions (such as mefenamic acid) seem to have weaker or even no

effects on these channels. In addition, drugs with a trifluoromethyl group (CF_3–) at the position 3 (e.g., flufenamic acid and niflumic acid) are not as potent as meclofenamic acid in inhibiting the Kv2.1 channels. This structure–activity profile also supports a direct interaction between the drug and the channel proteins.

At this stage, we did not intend to perform extensive biophysical analysis of the mechanism of meclofenamic acid-induced inhibition of hKv2.1. Nevertheless, meclofenamic acid did not change the channel activation threshold potential (≈ -20 mV for hKv2.1) and therefore did not shift the current–voltage (I/V) relationship. The normalized I/V curves in the absence and presence of the blocker overlapped, reflecting a ''proportional'' inhibition of the current at each test potential. In other words, the meclofenamic acid-induced inhibition of hKv2.1 channels was not voltage-dependent. In this study, we also analyzed the time constants of activation (τ_a) and deactivation (τ_d) and found that the mono-exponential rise or decay of the Kv currents was not reshaped (e.g., to a double exponential) even in the presence of the drug. This analysis suggests that the binding site for meclofenamic acid on the channel proteins is unlikely to be inside the pore, i.e., meclofenamic acid is unlikely to be a pore blocker. In fact, evidence from the original recording traces in Fig. 4B supports this speculation. By examining the traces in Fig. 4B, one can see that even in the presence of high concentration (300 μM) of the drug, the current was reduced ''evenly'' from the beginning to the end of the voltage pulse (2.5 s). To compare with its effect on hKv1.1 (Fig. 4A), one can see that there is an obvious difference regarding the mechanisms of channel inhibition. This is more apparent when a high concentration of meclofenamic acid (300 μM) was used, i.e., it inhibited more hKv1.1 current at the end of the voltage pulse (Fig. 4A) than the peak current. By comparing the effect of meclofenamic acid on these two K^+ channels, one may argue that this agent might be a pore blocker for hKv1.1 but is very unlikely to be a pore blocker for hKv2.1. This is because a pore blocker usually exhibits ''use-dependent'' blockade of a channel, i.e., it blocks the channel more readily when the channels are open (e.g., by prolonged depolarization). This was the reason that we used longer depolarization pulses (2.5 s) in our study, aiming to see the difference (if there is any) of the drug effects on the peak and the steady level of the currents measured at respectively the beginning and the end of the depolarization pulse.

The importance of Kv channels in the maintenance of neuronal membrane potential and neurotransmitter release is widely appreciated. It is conceivable that modulation of Kv channels may provide another avenue for developing novel drugs and understanding the mechanism of action of existing drugs. Meclofenamic acid serves as a good example. Although some side effects of NSAIDs (e.g., on the gastrointestinal system) are unwanted, some may be beneficial to the patients as indicated in recent studies which showed that NSAIDs somehow reduced the occurrence of Alzheimer's disease (Rich et al., 1995; Stewart et al., 1997). While the mechanism remains unknown, the drug-induced block of Kv channels and subsequent membrane depolarization and enhancement of transmitter release could certainly influence neural plasticity. Indeed, a recent study has shown that some ''classic'' antipsychotic drugs such as pimozide and fluspirilene also potently blocked these Kv channels (Wible et al., 1997 and the author's unpublished data). In addition, it has also been reported that meclofenamic acid was effective in preventing glutamate-induced neurotoxicity in cultured neuronal cells (Hewett et al., 1996). This effect did not seem to involve the glutamate receptors or voltage-gated Ca channels (Hewett et al., 1996). Whether there is a link between these effects of NSAIDs and their interactions with other ion channels (such as K channels) needs to be fully evaluated.

Finally, it should be mentioned that the concentration of fenamates required to inhibit the cyclooxygenase is usually lower that what is needed to block ion channels. For instance, the IC_{50} of meclofenamic acid is about 1 μM in inhibiting cyclooxygenase (Boctor et al., 1986) and about 56 μM in blocking the voltage-gated K^+ channel (hKv2.1, see our current study). This may partially explain the fact that ion channel modulation by fenamate may account only part of their advantageous or unwanted side effects. Nevertheless, the current study has provided evidence that this old drug may have new perspectives which are worthy of exploration.

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