

Meclofenamic Acid and Diclofenac, Novel Templates of KCNQ2/Q3 Potassium Channel Openers, Depress Cortical Neuron Activity and Exhibit Anticonvulsant Properties

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ABSTRACT

The voltage-dependent M-type potassium current (M-current) plays a major role in controlling brain excitability by stabilizing the membrane potential and acting as a brake for neuronal firing. The KCNQ2/Q3 heteromeric channel complex was identified as the molecular correlate of the M-current. Furthermore, the KCNQ2 and KCNQ3 channel α subunits are mutated in families with benign familial neonatal convulsions, a neonatal form of epilepsy. Enhancement of KCNQ2/Q3 potassium currents may provide an important target for antiepileptic drug development. Here, we show that meclofenamic acid (meclofenamate) and diclofenac, two related molecules previously used as anti-inflammatory drugs, act as novel KCNQ2/Q3 channel openers. Extracellular application of meclofenamate ($EC_{50} = 25 \mu\text{M}$) and diclofenac ($EC_{50} = 2.6 \mu\text{M}$) resulted in the activation of KCNQ2/Q3 K^+ currents, heterologously expressed in Chinese

hamster ovary cells. Both openers activated KCNQ2/Q3 channels by causing a hyperpolarizing shift of the voltage activation curve (-23 and -15 mV, respectively) and by markedly slowing the deactivation kinetics. The effects of the drugs were stronger on KCNQ2 than on KCNQ3 channel α subunits. In contrast, they did not enhance KCNQ1 K^+ currents. Both openers increased KCNQ2/Q3 current amplitude at physiologically relevant potentials and led to hyperpolarization of the resting membrane potential. In cultured cortical neurons, meclofenamate and diclofenac enhanced the M-current and reduced evoked and spontaneous action potentials, whereas in vivo diclofenac exhibited an anticonvulsant activity ($ED_{50} = 43$ mg/kg). These compounds potentially constitute novel drug templates for the treatment of neuronal hyperexcitability including epilepsy, migraine, or neuropathic pain.

Voltage-dependent K^+ (K_v) channels play a major role in brain excitability through the regulation of action potential generation and propagation, the tuning of neuronal firing patterns, or the modulation of neurotransmitter release. The M-type K^+ channel generates a subthreshold, voltage-gated K^+ current (M-current) that plays an important role in controlling neuronal excitability. Brown and Adams (1980) first identified the M-current in frog sympathetic neurons as a slowly activating, noninactivating, voltage-sensitive K^+ current, which was inhibited by muscarinic acetylcholine receptor stimulation (Brown and Adams, 1980). M-currents were also characterized in hippocampal and cortical neurons

(Brown, 1988; Marrion, 1997; Cooper and Jan, 2003). Modulation of the M-current has profound effects on brain excitability because this noninactivating K^+ channel exhibits significant conductance in the voltage range of action potential initiation. The low-threshold gating and the slow activation and deactivation of the M-current act as a brake for repetitive firing and neuronal excitability (Brown, 1988; Marrion, 1997; Jentsch, 2000; Rogawski, 2000; Cooper and Jan, 2003).

The KCNQ2/Q3 channel complex belonging to the KCNQ family of voltage-dependent K^+ channels has been identified as the molecular correlate of the M-current (Wang et al., 1998). In heterologous expression systems, the complex formed by KCNQ2/Q3 α subunits produces currents that are similar to the M-current (Wang et al., 1998; Jentsch, 2000; Rogawski, 2000). In addition, KCNQ4 and KCNQ5 α subunits can coassemble with KCNQ3 to produce K^+ currents whose properties are very similar to those of the M-current

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ABBREVIATIONS: K_v , voltage-dependent K^+ channel; NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; CHO, Chinese hamster ovary; MES, maximal electroshock seizure; 4-AP, 4-aminopyridine; TEA, tetraethylammonium; PBS, phosphate-buffered saline; NGS, normal goat serum; BMS-204352, (3S)-(+)-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2H-indole-2-one.

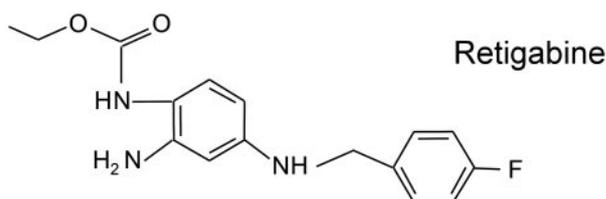
(Lerche et al., 2000; Schroeder et al., 2000; Wickenden et al., 2001). Recent studies showed that calmodulin binds constitutively to the KCNQ2 and KCNQ3 C termini and may function as an auxiliary channel subunit (Wen and Levitan, 2002; Yus-Najera et al., 2002). Consistent with their physiological importance, mutations of the KCNQ2 and KCNQ3 genes have been identified as causes of myokymia and of benign familial neonatal convulsions, a neonatal form of epilepsy (Biervert et al., 1998; Charlier et al., 1998; Singh et al., 1998; Dedek et al., 2001; Castaldo et al., 2002). It is interesting that M-channels were recently found to be expressed in regions of the nervous system involved in migraine and neuropathic pain such as dorsal and ventral horn of the spinal cord as well as sensory dorsal root and trigeminal ganglion neurons (Blackburn-Munro and Jensen, 2003; Cooper and Jan, 2003; Passmore et al., 2003; Dost et al., 2004; Rivera-Arconada et al., 2004).

In view of the crucial role of KCNQ2 and KCNQ3 channel α subunits in neonatal epilepsy and neuropathic pain, enhancement of KCNQ2/Q3 potassium currents may provide a promising target for the treatment of neuronal hyperexcitability. The anticonvulsant drug retigabine [D-23129; *N*-(2-amino-4-(4-fluorobenzylamino)phenyl) carbanic acid ethylester] and BMS-204352, undergoing clinical trials, have

been found to be KCNQ2/Q3 and KCNQ4 channels openers, respectively, and therefore were suggested to act as potential antiepileptic, antinociceptive, and/or neuroprotective drugs (Main et al., 2000; Wickenden et al., 2000; Schroeder et al., 2001; Tatulian et al., 2001; Blackburn-Munro and Jensen, 2003; Passmore et al., 2003; Dost et al., 2004; Rivera-Arconada et al., 2004).

In this study, we found that two related compounds, meclofenamic acid (2-[(2,6-dichloro-3-methylphenyl) amino] benzoic acid) and diclofenac (benzeneacetic acid, 2-[(2,6-dichlorophenyl)amino]-monosodium salt), act as KCNQ2/Q3 potassium channel openers (Fig. 1). Meclofenamic acid and diclofenac are well known and widely used nonsteroidal anti-inflammatory drugs (NSAIDs) acting as nonselective inhibitors of COX-1 and COX-2 (Furst and Munster, 2001). Here, we show that meclofenamic acid (meclofenamate) and diclofenac are potent openers of the recombinant KCNQ2/Q3 channels expressed in CHO cells. Both compounds activate KCNQ2/Q3 channels, by shifting leftward the voltage activation curve and slowing the deactivation kinetics. This leads to increased KCNQ2/3 current amplitude at physiologically relevant potentials and to a hyperpolarization of the cell resting membrane potential. Meclofenamate and diclofenac reduce evoked and spontaneous neuronal action potentials

A



B

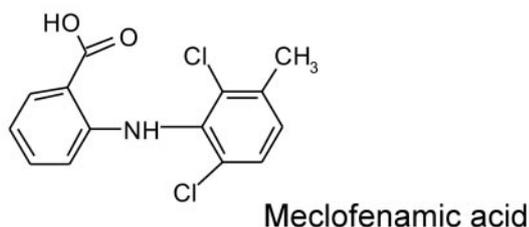
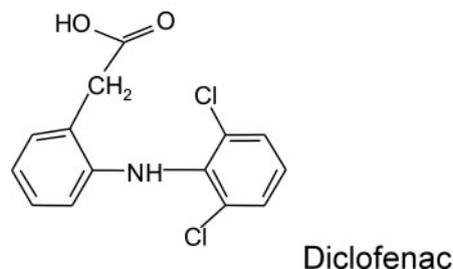


Fig. 1. Chemical structure of retigabine (A), meclofenamic acid (B), and diclofenac (C).

C



and enhance M-current in rat cortical neurons. Diclofenac also exhibits in vivo an anticonvulsant activity. These compounds may serve as lead molecules for the treatment of neuronal hyperexcitability, including migraine, epilepsy, and neuropathic pain.

Materials and Methods

CHO Cell Culture and Transfection. CHO cells were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% fetal calf serum, and antibiotics. In brief, 40,000 cells seeded on poly-D-lysine-coated glass coverslips (13 mm in diameter) in a 24-multiwell plate were transfected with pIRES-CD8 (0.5 μ g) as a marker for transfection and with KCNQ2 (0.5 μ g) and/or KCNQ3 (0.5 μ g). For electrophysiology, transfected cells were visualized approximately 40 h after transfection, using the anti-CD8 antibody-coated beads method (Jurman et al., 1994). Transfection was performed using 3.5 μ l of LipofectAMINE (Invitrogen) according to the manufacturer's protocol.

Neuronal Cortical Culture. Sprague-Dawley rat embryos (embryonic day 18) were removed by caesarian section and their cortices were dissected out. The tissue was digested with papain (100 U; Sigma-Aldrich, St. Louis, MO) for 20 min, triturated to a single-cell suspension, and plated at a density of 40,000 cells per milliliter on a substrate of bovine collagen type IV and 100 μ g/ml poly-L-lysine in a 13-mm-diameter glass coverslip of a 24-multiwell plate. The culture medium consisted of modified Eagle's medium containing 5% horse serum (Biological Industries, Beit HaEmek, Israel), B-27 neuronal supplement (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine. D-Glucose was supplemented to a final concentration of 6 g/l. Cytosine-1-D-arabinofuranoside (5 μ M) was added after 5 days to arrest glial cell proliferation. All cultures were maintained at 37°C in humidified air containing 5% CO₂.

Maximal Electroshock Seizure Test. The anticonvulsant effect of diclofenac and meclufenamate was measured by the maximal electroshock seizure (MES) model in ICR mice. All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The procedures followed for experimentation and maintenance of the animals were approved by the Animal Research Ethics Committee of Tel Aviv University and in accordance with the Guide for the Care and Use of Laboratory Animals (1996, National Academy of Sciences, Washington, DC). Minimal electroshock was induced in adult mice by means of two transcorneal electrodes delivering an alternative current of 50 mA at 60 Hz for 0.2 s using rodent shocker (type 221; Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany). This was shown to cause tonic convulsions in 100% of the animals tested. The drugs dissolved in 0.9% saline were administered intraperitoneally either 30 min or 2 h before the electroshock was performed. Animals failing to show tonic hind limb extension were scored as protected and were expressed in percentage.

Electrophysiology. For current measurements in CHO cells, recordings were performed 40 h after transfection, using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Signals were amplified using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA), sampled at 2 kHz and filtered at 800 Hz via a four-pole Bessel low pass filter. Data were acquired using pClamp 8.1 software (Axon Instruments) and an Elonex Pentium III computer in conjunction with a DigiData 1322A interface (Axon Instruments). The patch pipettes were pulled from borosilicate glass (Warner Instrument, Hamden, CT) with a resistance of 2 to 5 M Ω and were filled with 130 mM KCl, 1 mM MgCl₂, 5 mM K₂ATP, 5 mM EGTA, and 10 mM HEPES, adjusted with KOH to pH 7.4 (290 mOsM). The external solution contained 140 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 11 mM glucose, and

5.5 mM HEPES, adjusted with NaOH to pH 7.4 (310 mOsM). Series resistances (3–13 M Ω) were compensated (75–90%) and periodically monitored. For current-clamp measurements of rat cortical neurons, recordings were performed 10 to 14 days after plating. The patch pipettes were filled with 135 mM KCl, 1 mM K₂ATP, 1 mM MgATP, 2 mM EGTA, 1.1 mM CaCl₂, 5 mM glucose, and 10 mM HEPES, adjusted with KOH to pH 7.4 (315 mOsM). The external solution contained 140 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 5 mM glucose, and 10 mM HEPES, adjusted with NaOH to pH 7.4 (325 mOsM). For evoking the neuronal action potentials, 50- to 300-pA currents were injected into the cells for 800 ms (square pulse). Recordings were sampled at 5 kHz and filtered at 2 KHz via a four-pole Bessel low pass filter. For voltage-clamp measurements of rat cortical neurons, the patch pipettes were filled with 90 mM potassium acetate, 40 mM KCl, 3 mM MgCl₂, 2 mM K₂ATP, and 20 mM HEPES, adjusted with KOH to pH 7.4 (310–315 mOsM). The external solution contained 120 mM NaCl, 23 mM NaHCO₃, 3 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 11 mM glucose, 0.0005 mM tetrodotoxin, and 5 mM HEPES, adjusted with NaOH to pH 7.4 (325 mOsM). When indicated, the voltage-clamp recording external solution (for cortical neurons) also contained 1 mM 4-aminopyridine (4-AP) and 0.2 mM tetraethylammonium (TEA) to block the fast transient A-type K⁺ currents (I_A) and the TEA/4-AP-sensitive delayed rectifier K⁺ currents such as those of the Kv3 family (Du et al., 1996; Baranauskas et al., 2003). TEA, 4-AP, meclufenamic acid, and diclofenac were purchased from Sigma-Aldrich. Retigabine was kindly provided by Dr. H. Lerche (Ulm University, Ulm, Germany).

Current measurements in *Xenopus laevis* oocytes were performed as described previously (Peretz et al., 2002). In brief, two-electrode voltage-clamp measurements were performed 3 to 5 days after cRNA microinjection into oocytes as described previously. Oocytes were bathed in a modified ND96 solution containing 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, and 5 mM HEPES, titrated to pH 7.4 with NaOH. Whole-cell currents were recorded at room temperature (20–22°C) using a GeneClamp 500 amplifier (Axon Instruments). Glass microelectrodes (A-M Systems, Inc., Carlsborg, WA) were filled with 3 M KCl and had tip resistances of 0.5 to 1.5 M Ω . Stimulation of the preparation, data acquisition, and analyses were performed using the pCLAMP 6.02 software (Axon Instruments) and a 586 personal computer interfaced with a Digidata 1200 interface (Axon Instruments). Current signals were filtered at 0.5 kHz and digitized at 2 kHz.

Data Analyses. Data analysis was performed using the Clampfit program (pClamp 8.1; Axon Instruments), Microsoft Excel (Microsoft, Redmond, WA), Axograph 4.6 (Axon Instruments), and Prism 4.0 (GraphPad Software, Inc., San Diego, CA). Leak subtraction was performed off-line, using the Clampfit program of the pClamp 8.1 software. To analyze the KCNQ2/3 channel deactivation, a single exponential fit was applied to the tail currents. Chord conductance (G) was calculated by using the following equation: $G = I/(V - V_{rev})$, where I corresponds to the current amplitude measured at the end of the pulse, and V_{rev} is the calculated reversal potential assumed to be -90 mV in CHO cells and -98 mV in *X. laevis* oocytes. G was estimated at various test voltages (V) and then normalized to a maximal conductance value, G_{max} . Activation curves were fitted by one Boltzmann distribution: $G/G_{max} = 1/(1 + \exp[(V_{50} - V)/s])$, where V_{50} is the voltage at which the current is half-activated and s is the slope factor. All data were expressed as mean \pm S.E.M. Statistically significant differences were assessed by Student's t test.

Immunocytochemistry. Cortical neurons were grown in culture for 10 to 14 days on 13-mm-diameter coated glass coverslips in 24-well plates. Cells were carefully rinsed for 10 min in phosphate-buffered saline (PBS), and the neurons were subsequently fixed for 20 min in 4% paraformaldehyde in PBS. After extensive washes in PBS, the cells were blocked and permeabilized by incubation with 10% normal goat serum (NGS) in PBS containing 0.2% Triton X-100. Cells were then washed for 10 min in PBS containing 1% NGS.

Neurons were incubated at 4°C overnight with anti-KCNQ2 and anti-KCNQ3 channel antibodies diluted in PBS containing 1% NGS. A rabbit polyclonal antibody to KCNQ2 (1:500; Alomone Labs, Jerusalem, Israel) was combined with a goat polyclonal antibody to KCNQ3 (N19, 1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA); alternatively, a rabbit polyclonal antibody to KCNQ3 (1:100; Alomone Labs) was combined with a goat polyclonal antibody to

KCNQ2 (N19, 1:50; Santa Cruz Biotechnology Inc.). After a wash in PBS, cells were incubated for an hour at room temperature with secondary antibodies, Cy2-conjugated anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and rhodamine red X-conjugated anti-goat IgG (1:100; Jackson ImmunoResearch Laboratories Inc.). Neurons were viewed and digital images taken using a Zeiss LSM 410 confocal microscope.

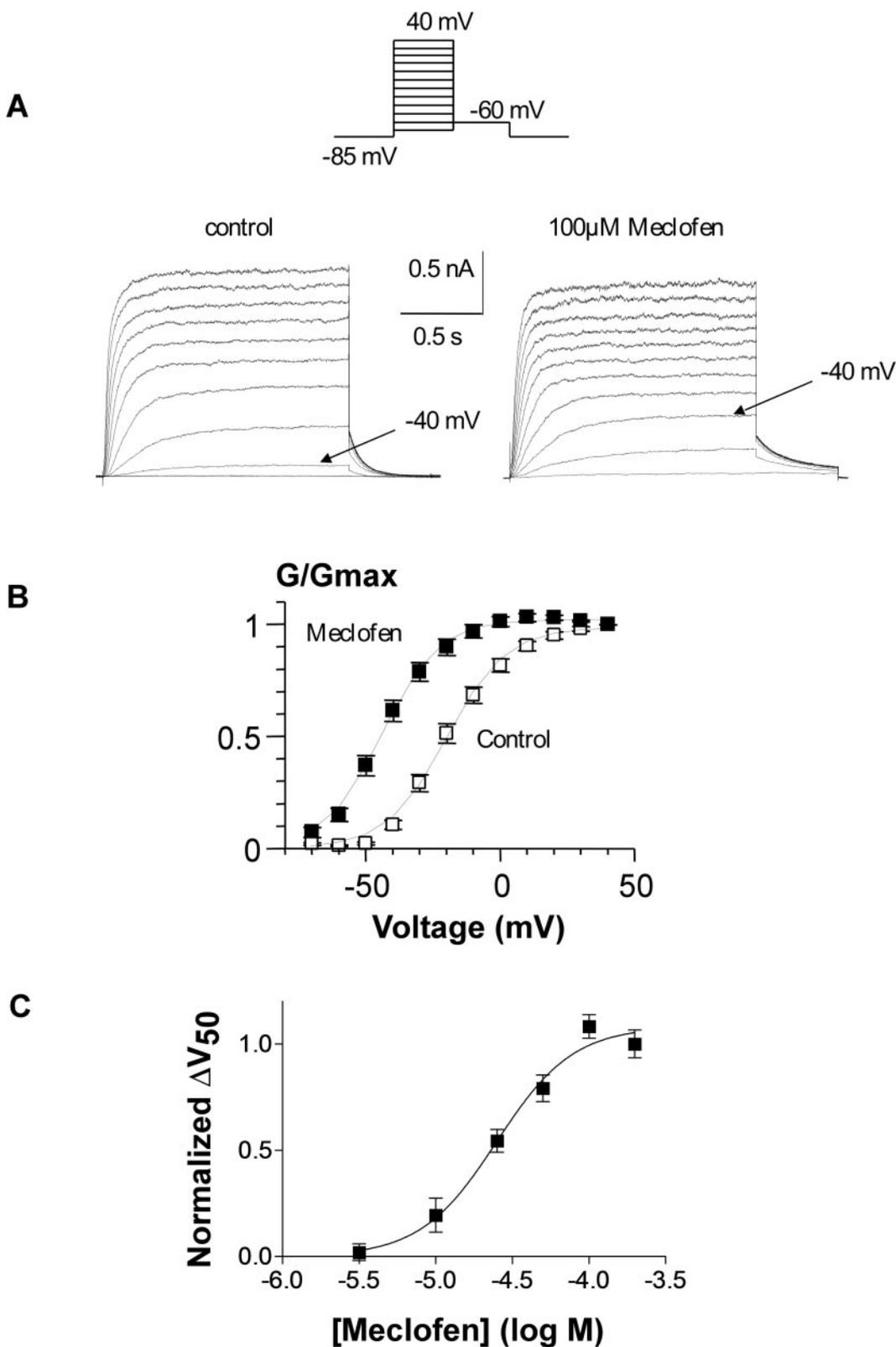


Fig. 2. Meclofenamate shifts the voltage dependence of KCNQ2/Q3 activation in CHO cells. **A**, representative traces recorded from the same cell before (left, control) and after (right, meclofenamate) external application of 100 μ M meclofenamic acid. Cells were held at -85 mV and the membrane potential was stepped from -70 to $+40$ mV for 1.5-s pulse duration, in 10-mV increments, followed by a 0.75-s step to -60 mV. **B**, normalized conductance was plotted as a function of the test voltages for control (\square) and meclofenamate-treated cells (\blacksquare). The activation curves were fitted using one Boltzmann function. **C**, dose-response curve of meclofenamate opener activity for KCNQ2/Q3 channels. The potency of meclofenamate was determined by the leftward shift of the activation curves produced by increasing concentrations of the drug. The magnitude of the leftward shift in the half-activation potential (V_{50}) was calculated with each opener concentration and normalized against the maximum shift produced by 200 μ M meclofenamate. The data were fitted to a Hill equation, yielding $EC_{50} = 25 \pm 2$ μ M and $n_H = 1.8 \pm 0.4$ ($n = 8$).

Results

Meclofenamic Acid and Diclofenac Shift Leftward the Activation Curve and Slow Down the Deactivation of KCNQ2/Q3 Heteromeric K⁺ Currents. The KCNQ2 and KCNQ3 α subunits were coexpressed in CHO cells by cotransfecting their respective cDNAs at an equimolar ratio. Figure 2A (left) shows representative traces of the KCNQ2/Q3 current activated by step depolarization above a voltage threshold of about -50 mV. We previously showed that the fenamate compound mefenamic acid shifts leftward the activation curve of homomeric KCNQ1 channels (about -20 mV) and acts as a strong opener of heteromeric KCNQ1/KCNE1 channels (I_{KS}) (Abitbol et al., 1999). Because of its opener properties, mefenamic acid can rescue the dominant-negative suppression of I_{KS} produced by LQT-related KCNE1 mutations (Abitbol et al., 1999). Therefore, we checked the effects of several fenamate compounds on other members of the KCNQ channel family, mainly, the KCNQ2/Q3 heteromeric channels, known to encode the neuronal M-current (Wang et al., 1998). Among the various fenamates we screened (e.g., mefenamic, flufenamic, tolfenamic, meclofenamic, and niflumic acids and diclofenac), we found that meclofenamic acid (meclofenamate) and diclofenac were potent and specific openers of KCNQ2/Q3 channels (see below; Table 1). Meclofenamate and diclofenac are well known NSAID drugs (Fig. 1) that inhibit nonselectively COX-1 and COX-2 (Furst and Munster, 2001). The external application of meclofenamate activated KCNQ2/Q3 K⁺ currents at more hyperpolarized potentials (Figs. 2A, right, B and 3C). In untreated CHO cells, KCNQ2/Q3 channels were activated above a threshold of -50 mV, whereas they were activated above -70 mV in the presence of meclofenamate (Fig. 3C).

Meclofenamate produced a concentration-dependent increase in KCNQ2/Q3 current amplitude. In a train protocol, when the cells were stepped to -30 mV the application of 25 μ M meclofenamate induced an increase of the current amplitude by up to 72%, from 844 ± 130 to 1451 ± 164 pA ($n = 15$; $p < 0.01$) for control and meclofenamate-treated CHO cells, respectively (Fig. 3, A and B). To measure the potency of meclofenamate, we determined the leftward shift of the activation curves produced by increasing concentrations of the drug as described previously (Tatulian et al., 2001). The relationship between the concentration of meclofenamate and the leftward shift in the half-activation potential (ΔV_{50}) was deduced and normalized to the maximal shift produced by 200 μ M meclofenamate (Fig. 2C). The values were fitted to a Hill equation with a slope of 1.7 and an $EC_{50} = 25 \pm 2$ μ M ($n = 8$). The onset of the drug action was fast because within less than 1 min of external application of 25 μ M meclofen-

amate, there was a large increase in KCNQ2/3 current amplitude across a range of test potentials between -50 and 0 mV (Figs. 2B and 3C). The effect of meclofenamate was fully reversible (see below). As the test potentials were more positive and approached the saturating values of the activation curve (above 0 mV), the effects of meclofenamate on KCNQ2/3 current amplitude became nonexistent (Fig. 2, A and B). Meclofenamate produced a marked leftward shift of -22.7 mV in the voltage-dependence of KCNQ2/Q3 current activation, from $V_{50} = -19.6 \pm 1.9$ mV ($n = 24$) to $V_{50} = -42.3 \pm 2.1$ mV ($n = 14$) in control and meclofenamate-treated cells, respectively (Fig. 2B; $p < 0.01$). The slope parameters of the Boltzmann fitting curve did not change significantly with $s = -9.5 \pm 0.4$ mV/e fold and $s = -10.50 \pm 0.93$ mV/e fold for control and meclofenamate-treated cells, respectively. It is clear that the most pronounced action of meclofenamate is exerted at physiologically relevant negative potentials. At -50 , -40 , and -30 mV, 25 μ M meclofenamate increased KCNQ2/3 current amplitude by more than 10-, 5-, and 2.5-fold, respectively (Fig. 3C). Whereas meclofenamate slightly accelerated the KCNQ2/Q3 activation kinetics, it markedly slowed the deactivation process (Figs. 2A and 3, A and D). Figure 3D shows the normalized tail currents when CHO cells were depolarized to -20 mV and then repolarized to -60 mV, in the absence (control) and presence of 100 μ M meclofenamate. Meclofenamic acid reduced by about 2-fold the speed of KCNQ2/Q3 channel closure with the time constant of deactivation increasing from $\tau_{deact} = 79.6 \pm 4.5$ to $\tau_{deact} = 167.5 \pm 11.6$ ms ($n = 10$; $p < 0.01$) for control and meclofenamate-treated CHO cells, respectively (Fig. 3, D and E). Deactivation kinetics was also measured at command voltages of equivalent G/G_{max} values because meclofenamate produced a leftward shift of the activation curve (approximately -20 mV). Under these conditions, the deactivation time constant still increased from $\tau_{deact} = 85.5 \pm 6.6$ ms (at -20 -mV prepulse command) to $\tau_{deact} = 151.2 \pm 8.7$ ms (at -40 -mV prepulse command) in the presence of meclofenamate ($n = 15$; $p < 0.01$).

To make sure that the effects of meclofenamate on KCNQ2/Q3 K⁺ currents were not dependent on a particular cell type, we also checked its action on the *X. laevis* oocyte expression system. As in CHO cells, external application of 25 μ M meclofenamate produced a $53 \pm 8\%$ ($n = 7$; $p < 0.01$) increase in KCNQ2/Q3 current amplitude when the oocyte membrane was stepped from -80 to -40 mV (Fig. 4A). Likewise, meclofenamate (25 μ M) produced a leftward shift of -15.9 mV in the voltage dependence of KCNQ2/Q3 current activation, from $V_{50} = -28.6 \pm 2.9$ mV ($n = 10$) to $V_{50} = -44.5 \pm 3.1$ mV ($n = 10$) in control and meclofenamate-

TABLE 1
Specificity of meclofenamic acid and diclofenac effects

The specificity of meclofenamate and diclofenac toward Kv channels was tested in *X. laevis* oocytes by measuring from a -80 -mV holding potential, the current amplitude of various Kv channels, including Kv1.2 (quantified at -20 mV), Kv1.5, and Kv2.1 (quantified at 0 mV), and KCNQ1 and KCNQ2/Q3 (quantified at -40 mV). The effects of meclofenamate and diclofenac were expressed as a percentage of the control amplitude, measured at the same potential in the absence of the drug. Data are expressed as mean \pm S.E.M. of five to eight separate experiments.

Compound	Kv1.2 (-20 mV)	Kv1.5 (0 mV)	Kv2.1 (0 mV)	KCNQ1 (-40 mV)	KCNQ2/Q3 (-40 mV)
	% of control current amplitude				
Meclofenamate (25 μ M)	84 ± 6	98 ± 5	105 ± 6	95 ± 6	175 ± 15^a
Diclofenac (25 μ M)	90 ± 7	103 ± 7	100 ± 5	101 ± 6	225 ± 18^a

^a Significant change compared with control at $P < 0.01$ paired Student's *t* test.

treated cells, respectively (Fig. 4B). As a result of this leftward shift of the KCNQ2/Q3 activation curve, incubation of the oocytes with increasing concentrations of meclofenamate led to a progressive hyperpolarization of the oocyte resting membrane potential from -56 ± 2 to -72 ± 3 mV, with an $EC_{50} = 11.7 \pm 5.2 \mu\text{M}$ ($n = 6$) (Fig. 4C).

Similar results were obtained with diclofenac, a structurally related compound, having a benzene acetic acid moiety instead of a benzoic acid group for meclofenamic acid that

also bears a methyl group in its dichlorophenyl ring (Fig. 1). Adding $50 \mu\text{M}$ diclofenac externally (Fig. 5D) produced a significant leftward shift of -14.5 mV in the voltage dependence of KCNQ2/3 activation, from $V_{50} = -30.9 \pm 4.1$ to $V_{50} = -45.4 \pm 2.7$ mV ($n = 7$; $p < 0.01$). As with meclofenamate, treatment of CHO cells with diclofenac slowed down the deactivation kinetics of KCNQ2/Q3 channels (Fig. 5A). The deactivation time constant increased from $\tau_{\text{deact}} = 85.0 \pm 5.0$ ms (at -20 -mV prepulse command) to $\tau_{\text{deact}} = 172.0 \pm 18.4$

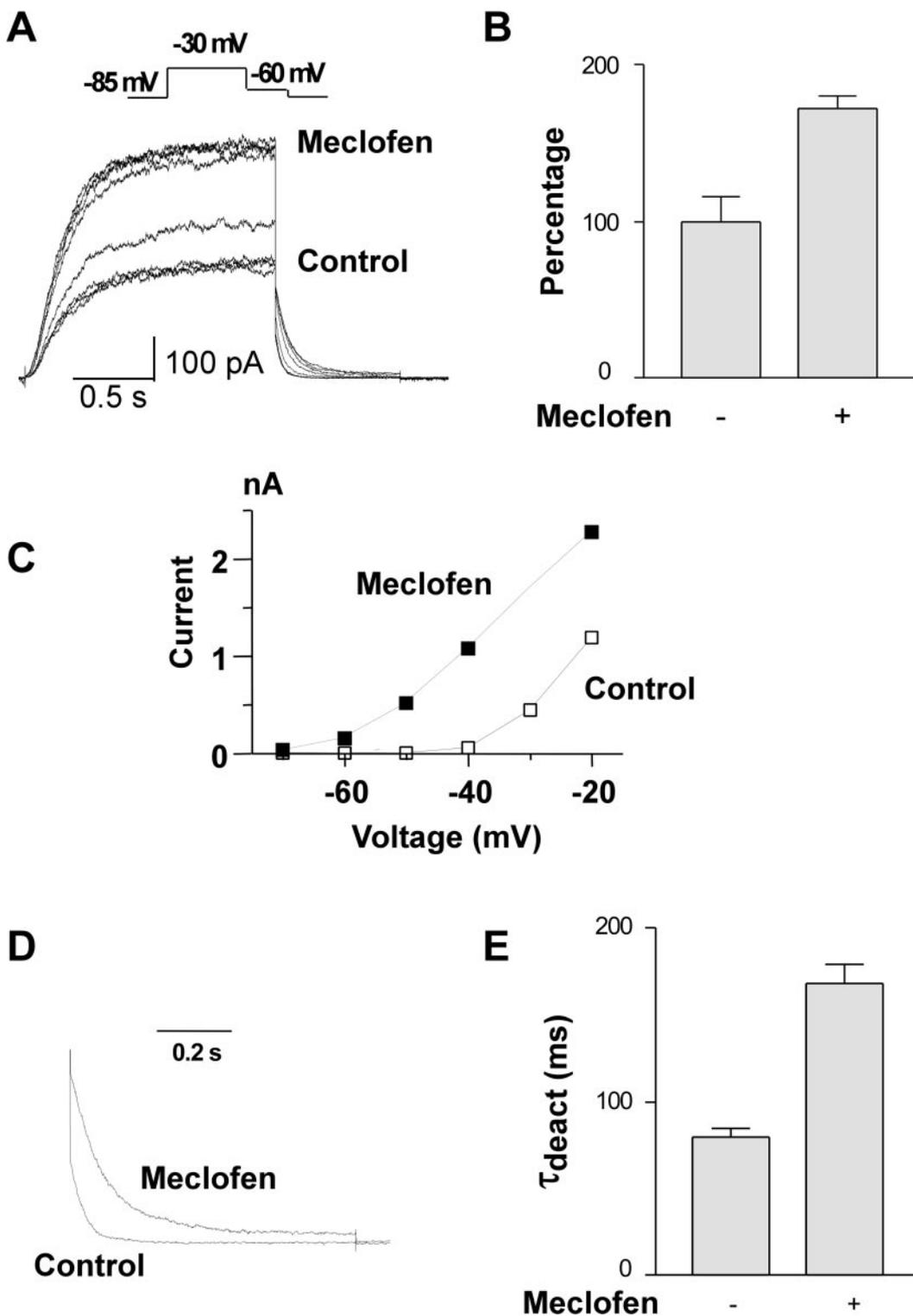


Fig. 3. Meclofenamate increases current amplitude and slows deactivation of KCNQ2/Q3 channels in CHO cells. **A**, current traces were recorded in the absence (control) and presence of $25 \mu\text{M}$ meclofenamate. In this train protocol, the cells were stepped every 30 s to -30 mV for 1.5-s pulse duration. **B**, percentage of the current is shown in the presence (+) or absence (-) of $25 \mu\text{M}$ meclofenamic acid, the latter being the control of 100%. **C**, current amplitude (nanoamperes) was plotted against the step voltage (-70 to -20 mV) to illustrate the negative shift of the threshold for channel activation, after meclofenamate application. **D**, zoom of the tail current of a CHO cell before (control) and after application of $25 \mu\text{M}$ meclofenamate. The step pulse was -20 mV, whereas the tail potential was -60 mV. **E**, deactivation time constants resulting from the monoexponential fit of the tail current decay (τ_{deact}), are shown in the absence (-) and the presence of meclofenamate (+).

ms (at -40 -mV prepulse command) in the presence of diclofenac ($n = 7$; $p < 0.01$).

Likewise, the KCNQ2/3 current amplitude is increased by diclofenac at physiologically relevant potentials. In a train protocol, when CHO cells were stepped from -85 to -50 mV, the application of diclofenac increased the current amplitude by up to $262 \pm 26\%$ ($n = 6$) (Fig. 5, A and B). The effect of diclofenac was fully reversible and dose-dependent. The potency of diclofenac was measured by the normalized leftward shift in the ΔV_{50} as a function of the drug concentration (Fig. 5C). The curve fitting to a Hill equation yielded an $EC_{50} = 2.6 \pm 1.3 \mu\text{M}$ and a Hill slope of 1.3 ($n = 8$). Like meclofenamate, diclofenac produced a hyperpolarization (-10.4 mV) of the resting membrane potential in CHO cells from -44.6 ± 2.3 to -55.0 ± 1.8 mV ($n = 7$; $p < 0.001$) in control and diclofenac-treated cells, respectively.

Selectivity of Meclofenamate and Diclofenac Action and Synergy with Retigabine. The opener properties of meclofenamate and diclofenac on heteromeric KCNQ2/Q3 channels raised the question of whether these compounds act equally well or more selectively on either subunit. To address this issue, we checked the effect of $50 \mu\text{M}$ meclofenamate on homomeric KCNQ2 and homomeric KCNQ3 channels expressed separately in CHO cells (Fig. 6). In general, meclofenamate exerted a stronger action on KCNQ2 than on KCNQ3 channels. It produced a substantial leftward shift of -26.9

mV in the activation curve of KCNQ2 channels, from $V_{50} = -23.6 \pm 2.2$ to $V_{50} = -50.5 \pm 1.4$ mV ($n = 8$) in control and meclofenamate-treated cells, respectively (Fig. 6C, left; $p < 0.01$). The leftward shift produced by meclofenamate on the activation curve of KCNQ3 channels was weaker (-15 mV) from $V_{50} = -39.0 \pm 3.5$ mV to $V_{50} = -54.0 \pm 2.0$ mV ($n = 11$) in control and meclofenamate-treated cells, respectively (Fig. 6C, right; $p < 0.01$). Meclofenamate significantly reduced the speed of KCNQ2 channel closure with the time constant of deactivation increasing from $\tau_{\text{deact}} = 92.6 \pm 3.9$ to $\tau_{\text{deact}} = 152.7 \pm 4.9$ ms (Fig. 6, A and D; $n = 8$, $p < 0.001$). In contrast, it did not affect the deactivation kinetics of KCNQ3 channels ($\tau_{\text{deact}} = 319.6 \pm 40.8$ and $\tau_{\text{deact}} = 317.2 \pm 30.2$ ms for control and meclofenamate-treated cells, respectively; $n = 8$). Reflecting the stronger effect of the opener on KCNQ2 versus KCNQ3 channels, the external application of $50 \mu\text{M}$ meclofenamate produced an increase of 240 ± 26 and $120 \pm 4\%$ of the KCNQ2 and KCNQ3 current amplitudes, respectively, when cells were stepped from -85 to -40 mV (Fig. 6, A and B; $n = 8$; $p < 0.001$). Similar results were obtained with diclofenac (not shown). To further address the selectivity of the drugs, the effects of meclofenamate and diclofenac toward other Kv channels were tested in *X. laevis* oocytes, by measuring at nonsaturating depolarizing potentials, the current amplitude of various Kv channels, including Kv1.2, Kv1.5, Kv2.1, KCNQ1, and KCNQ2/Q3 (Table 1). The results

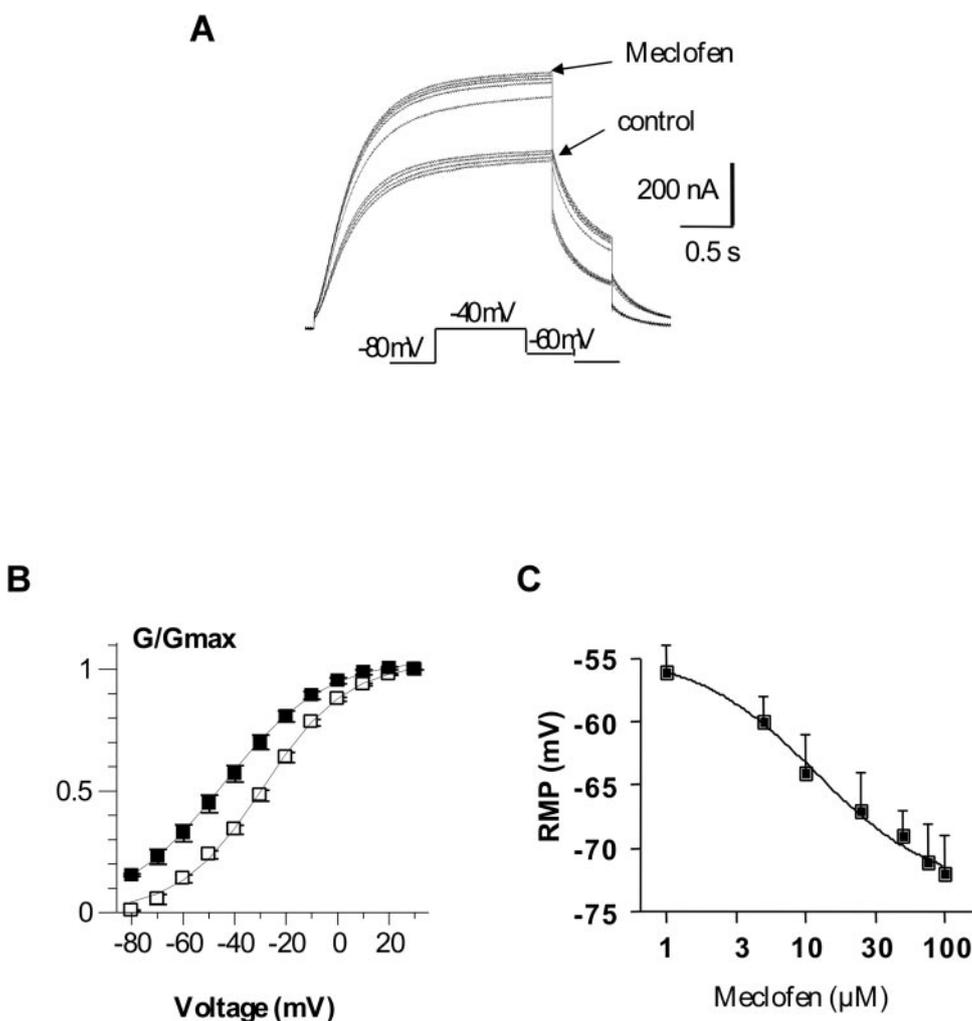


Fig. 4. Meclofenamate activates KCNQ2/Q3 channels and hyperpolarizes the membrane potential in *X. laevis* oocytes. **A**, current traces were recorded in *X. laevis* oocytes in the absence (control) and presence of $25 \mu\text{M}$ meclofenamate. Cells were stepped every 30 s to -40 mV for 2-s pulse duration. **B**, normalized conductance was plotted as a function of the test voltages for control (\square) and meclofenamate-treated *X. laevis* oocytes (\blacksquare). The activation curves were fitted using one Boltzmann function. **C**, resting membrane potential (RMP) was recorded in *X. laevis* oocytes and plotted as a function of meclofenamate concentration. The curve was fitted to a sigmoidal binding function, yielding an $EC_{50} = 11.7 \pm 5.2 \mu\text{M}$ ($n = 6$).

indicate that although meclofenamate (25 μM) and diclofenac (25 μM) increased KCNQ2/Q3 currents (at -40 mV) by 1.75- and 2.25-fold, respectively, they did not affect the current amplitude of other delayed rectifier channels, such as Kv1.2, Kv1.5, and Kv2.1 (Table 1). It is interesting that homomeric KCNQ1 and heteromeric KCNQ1/KCNE1 currents were not enhanced by diclofenac and meclofenamate across a range of test potentials between -50 to 0 mV (Table 1; data not shown).

The comparable opener properties exhibited by meclofenamate or diclofenac and those displayed by the anticonvulsant drug retigabine, including the leftward shift of the activation curve, the slowing of deactivation, and the hyperpolarization of the membrane potential, are suggestive of a common site of action. To test this possibility, we checked the potency of retigabine as measured by the leftward shift of ΔV_{50} as a function of the drug concentration in the absence or presence of a fixed EC_{50} concentration (25 μM) of meclofenamate (Fig. 7). The results show that coapplication of the two compounds produced additive effects. Although dose-dependent applications of retigabine alone and meclofenamate alone elicited maximal leftward shifts of ΔV_{50} of -24 ± 3 and -20 ± 3 mV, respectively, coexposure of 25 μM meclofenamate with increasing concentrations of retigabine produced a maximum ΔV_{50} of -32 ± 4 mV ($n = 8$; $p < 0.05$; Fig. 7). It

is clear that the effects of both drugs are not mutually occlusive but additive, which suggests that retigabine and meclofenamate act independently on KCNQ2/Q3 channels.

Meclofenamate Enhances M-Currents in Rat Cortical Neurons. Considering the action of meclofenamate and diclofenac on recombinant KCNQ2/Q3 channels, we checked their potential impact on native M-currents that were recently found to be encoded in rat sympathetic and hippocampal neurons by the heteromeric assembly of KCNQ2 and KCNQ3 gene products (Wang et al., 1998; Tatulian et al., 2001; Shah et al., 2002). Here, we examined the effects of meclofenamate on native M-currents of cultured rat cortical neurons. We first checked whether KCNQ2 and KCNQ3 α subunits were coexpressed in rat primary cortical neurons grown in culture for 10 to 14 days by means of double staining immunofluorescence (Fig. 8A). The polyclonal antibodies we used were specific and recognized selectively KCNQ2 and KCNQ3 channel proteins when expressed in CHO cells (Fig. 8A, right). Confocal immunofluorescence microscopy showed colocalization of KCNQ2 and KCNQ3 immunoreactive proteins in cortical neurons with various morphologies, including pyramidal-like cells (Fig. 8A, left). The staining for both KCNQ2 and KCNQ3 was most prominent in the somata, but it was also present along the neuronal processes.

Then, we attempted to record the native M-current from

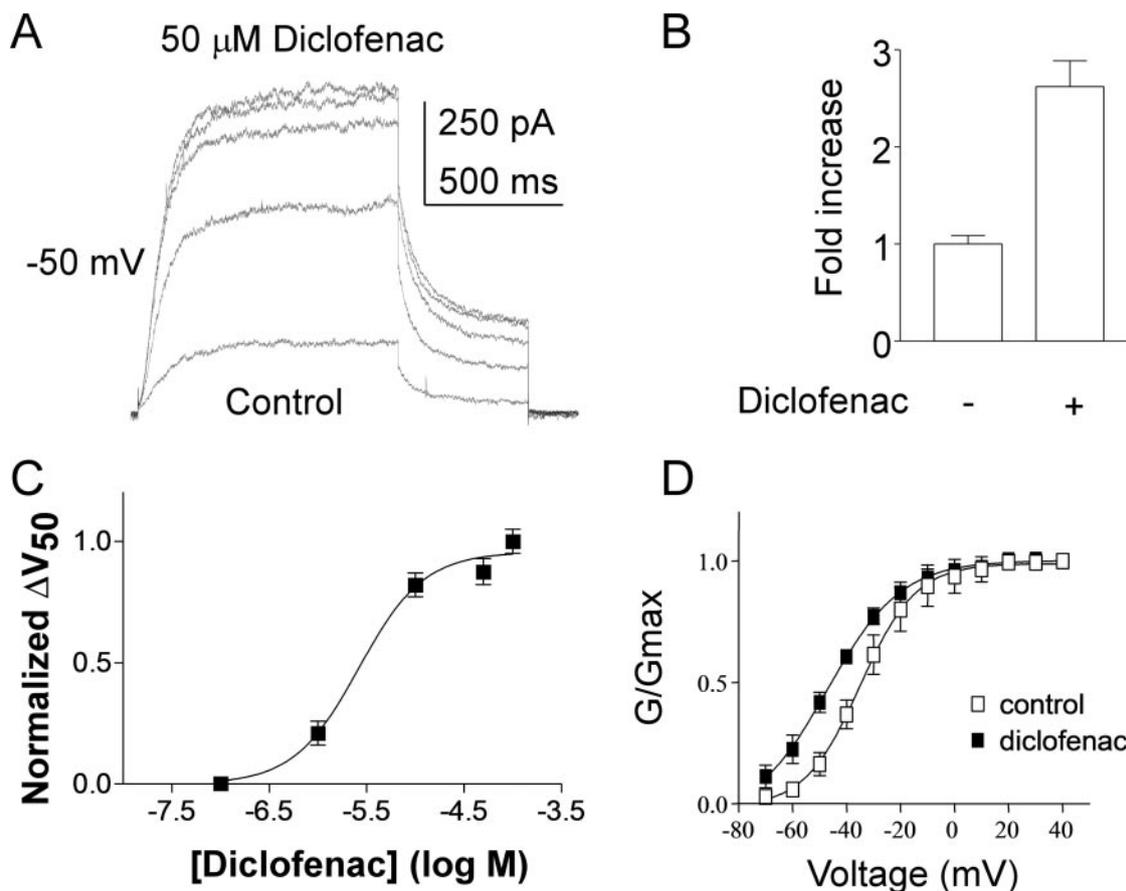


Fig. 5. Diclofenac activates KCNQ2/Q3 channels in CHO cells. A, current traces were recorded in the absence (control) and presence of 50 μM diclofenac. Cells were stepped every 30 s to -50 mV for 1.5-s pulse duration. B, current stimulation (fold increase) is shown in the absence (-) or presence (+) of 50 μM diclofenac. C, dose-response curve of diclofenac for KCNQ2/Q3 channels. The potency of diclofenac was determined as in Fig. 2C. The magnitude of the leftward shift in the half-activation potential (V_{50}) was calculated with each drug concentration and normalized against the maximum shift produced by 200 μM diclofenac. The data were fitted to a Hill equation, yielding $\text{EC}_{50} = 2.6 \pm 1.3 \mu\text{M}$ and $n_H = 1.3 \pm 0.3$ ($n = 8$). D, normalized conductance was plotted as a function of the test voltages for control (\square) and diclofenac-treated CHO cells (\blacksquare). The activation curves were fitted using one Boltzmann function.

pyramidal-like neurons, although it was very tiny and subject to run-down. For this purpose, we used an external solution containing 0.5 μM tetrodotoxin to block voltage-gated Na^+ channels and 1 mM 4-AP plus 0.2 mM TEA to block the fast transient A-type K^+ currents (I_A) and the TEA/4-AP-sensitive delayed rectifier K^+ currents such as those of the $\text{Kv}3$ family (Du et al., 1996; Baranauskas et al., 2003). Then, a reasonable estimate of the contribution of the

M-current was provided by the additional perfusion of 10 μM linopirdine, a blocker of M-channels (Wang et al., 1998), and was revealed by subtracting the current traces (Fig. 8B). Two voltage protocols could be used to reveal the M-current: 1) either by holding the cell at -20 mV and stepping back to -50 mV to activate and deactivate the M-channels, respectively (Fig. 8B), or 2) by holding the cell at -80 mV, stepping to -40 mV, and then back to -60 mV to activate and deac-

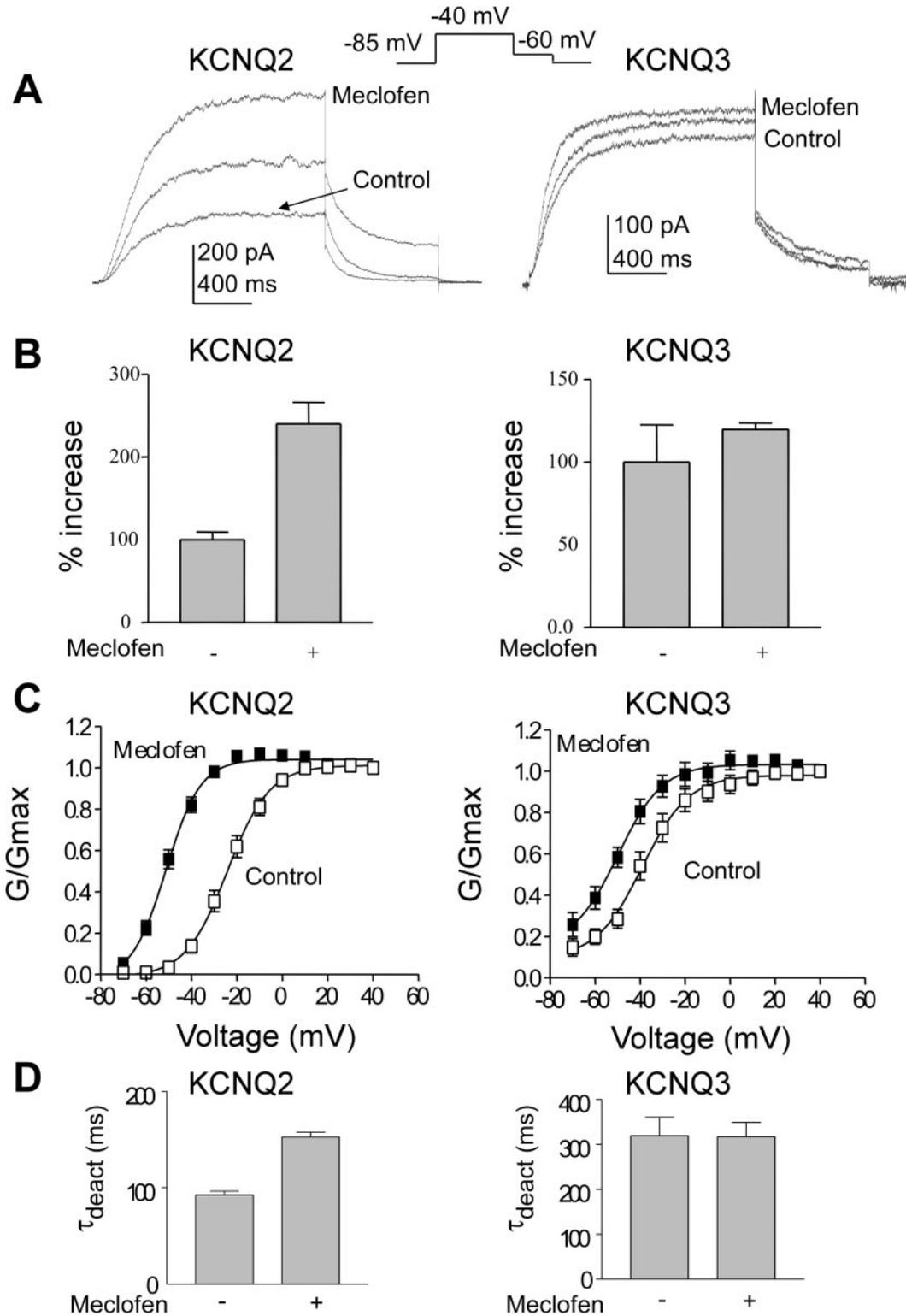


Fig. 6. Selectivity of meclofenamate toward KCNQ2 and KCNQ3 homomeric channels, expressed in CHO cells. **A**, by stepping the membrane potential to -40 mV, current traces were recorded in the absence (control) and presence of 50 μM meclofenamate in CHO cells expressing either KCNQ2 (left) or KCNQ3 (right) homomeric channels. **B**, percentage of current increase is shown in the absence (-) or presence (+) of 50 μM meclofenamic acid in cells expressing either KCNQ2 (left) or KCNQ3 (right) homomeric channels. **C**, normalized conductance was plotted as a function of the test voltages for control (□) and meclofenamate-treated CHO cells (■) for cells expressing either KCNQ2 (left) or KCNQ3 (right) homomeric channels. The activation curves were fitted using one Boltzmann function. **D**, deactivation time constants resulting from the monoexponential fit of the tail current decay (τ_{deact}) are shown in the absence (-) and the presence of meclofenamate (+) for cells expressing either KCNQ2 (left) or KCNQ3 (right) homomeric channels.

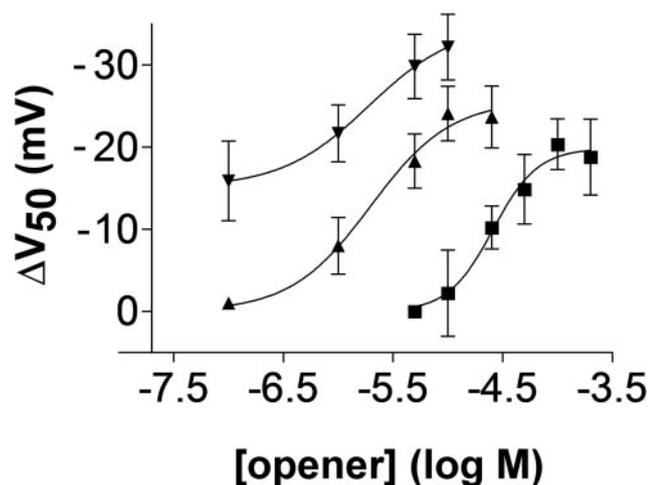


Fig. 7. Dose-response curve of meclofenamate and retigabine opener activity for KCNQ2/Q3 channels and their additive effects upon coapplication. The individual potency of retigabine (upward triangles) and meclofenamate (squares) was measured by the leftward shift of the half-activation potential (ΔV_{50}) as a function of the drug concentration. The data were fitted to a Hill equation, yielding $EC_{50} = 2.0 \pm 1.3 \mu\text{M}$ and $n_H = 1.20 \pm 0.30$ ($n = 8$) for retigabine and $EC_{50} = 25.7 \pm 1.1 \mu\text{M}$ and $n_H = 2.1 \pm 0.5$ ($n = 8$) for meclofenamate. For coapplication experiments, we checked the potency of retigabine as described above in the presence of a fixed concentration of $25 \mu\text{M}$ meclofenamate (EC_{50}). Results show that coapplication of the two compounds produced additive effects. Coexposure of $25 \mu\text{M}$ meclofenamate with increasing concentrations of retigabine produced a maximum ΔV_{50} of $-32 \pm 4 \text{ mV}$ ($n = 8$; $p < 0.05$), whereas dose-dependent applications of retigabine alone and meclofenamate alone elicited maximal left-shifts of ΔV_{50} of -24 ± 3 and $-20 \pm 3 \text{ mV}$, respectively.

tivate the M-currents (Fig. 8C). In the latter protocol, the subtracted traces (with and without $10 \mu\text{M}$ linopirdine) show that $10 \mu\text{M}$ meclofenamate produced a potent increase of the outward current generated by the non saturating step depolarization to -40 mV (Fig. 8C). Meclofenamate ($10 \mu\text{M}$) enhanced the outward current by $465 \pm 134\%$ of control at -40 mV . These enhanced currents were blocked by $10 \mu\text{M}$ linopirdine (Fig. 8D; $n = 5$; $p < 0.01$).

Inhibition of Evoked and Spontaneous Neuronal Activity by Meclofenamate and Diclofenac. Because one of the main functions of the M-current is to dampen the neuronal spiking discharges, we checked whether the new KCNQ2/Q3 channel openers affect the evoked and spontaneous action potential activity of cultured rat cortical neurons. Using the current-clamp configuration of the patch-clamp technique, we first examined the effects of meclofenamate on evoked action potentials. The resting membrane potential was close to -60 mV and, when needed, was maintained at this level by injecting DC current. Superfusion of $10 \mu\text{M}$ meclofenamate hyperpolarized the cortical neurons by $-8 \pm 3 \text{ mV}$ ($n = 8$). Figure 9A shows representative experiments of how $10 \mu\text{M}$ meclofenamate drastically reduced the number of evoked action potentials in cortical neurons that exhibited regular spiking patterns with no significant spike adaptation. Within less than 1 min, external exposure of $10 \mu\text{M}$ meclofenamate produced a widening of interspike intervals in the action potentials fired by the cortical neurons (Fig. 9A, second row). After 2 min of opener exposure, only one spike

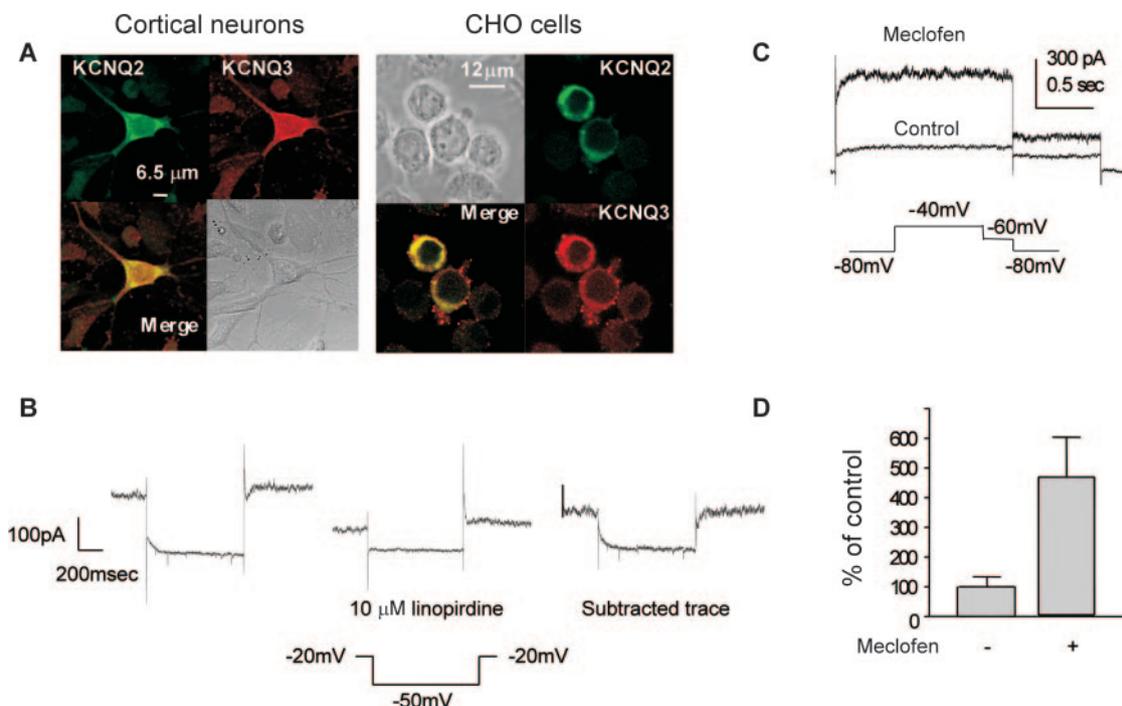


Fig. 8. Meclofenamate enhances the M-current in rat cortical neurons. **A**, immunocytochemical identification of KCNQ2 and KCNQ3 α subunits in rat cortical neurons. Specific immunodetection of KCNQ2 and KCNQ3 α subunits in CHO cells cotransfected with KCNQ2 and KCNQ3 cDNAs (right). Colocalization of KCNQ2 and KCNQ3 α subunits in a pyramidal-like cortical neuron as detected by double immunocytochemistry and illustrated by the merge image (left). **B**, representative recording of M-currents from a pyramidal-like neuron held at -20 mV and where the membrane potential was stepped to -50 mV . Recording was done in 1 mM 4-AP and 0.2 mM TEA in the absence (left) or presence of $10 \mu\text{M}$ linopirdine (middle). The subtracted traces are shown on the right. **C**, representative trace showing the increase of the linopirdine-sensitive current by $10 \mu\text{M}$ meclofenamate. In this protocol, the membrane potential was stepped from -80 to -40 mV , and the recording was done in 1 mM 4-AP and 0.2 mM TEA with and without $10 \mu\text{M}$ meclofenamate, in the absence or presence of $10 \mu\text{M}$ linopirdine. The subtracted traces corresponding to the linopirdine-sensitive current are shown. **D**, percentage of the current potentiated at -40 mV by the presence (+) of $10 \mu\text{M}$ meclofenamic acid, where the control (-) is 100% ($n = 5$; $p < 0.01$).

could be evoked by the same depolarizing current (Fig. 9A, third row). After 100-pA depolarizing current injection for 800 ms, 10 μM meclofenamate reduced the number of action potentials from 20 ± 1 to 1 ± 1 ($n = 10$; $p < 0.001$). Similar results were obtained with even lower concentrations of meclofenamate (5 μM) that consistently reduced the number of evoked action potentials (at 50-pA current injection; not shown). Upon washout of the compound for 1 min, neurons recovered their initial spiking activity (25

Hz; Fig. 9A, fifth row). Using higher density cultures of rat cortical neurons, we could record spontaneous spiking activity (Fig. 9, B and C). Although 10 μM linopirdine, a blocker of M-currents, significantly enhanced the frequency of spontaneous spiking (Fig. 9B), the perfusion of 10 μM meclofenamate produced within less than 2 min a profound depression of spontaneous action potentials (Fig. 9C). The depressing action of meclofenamate could be quickly reversed by washout of the compound (Fig. 9C).

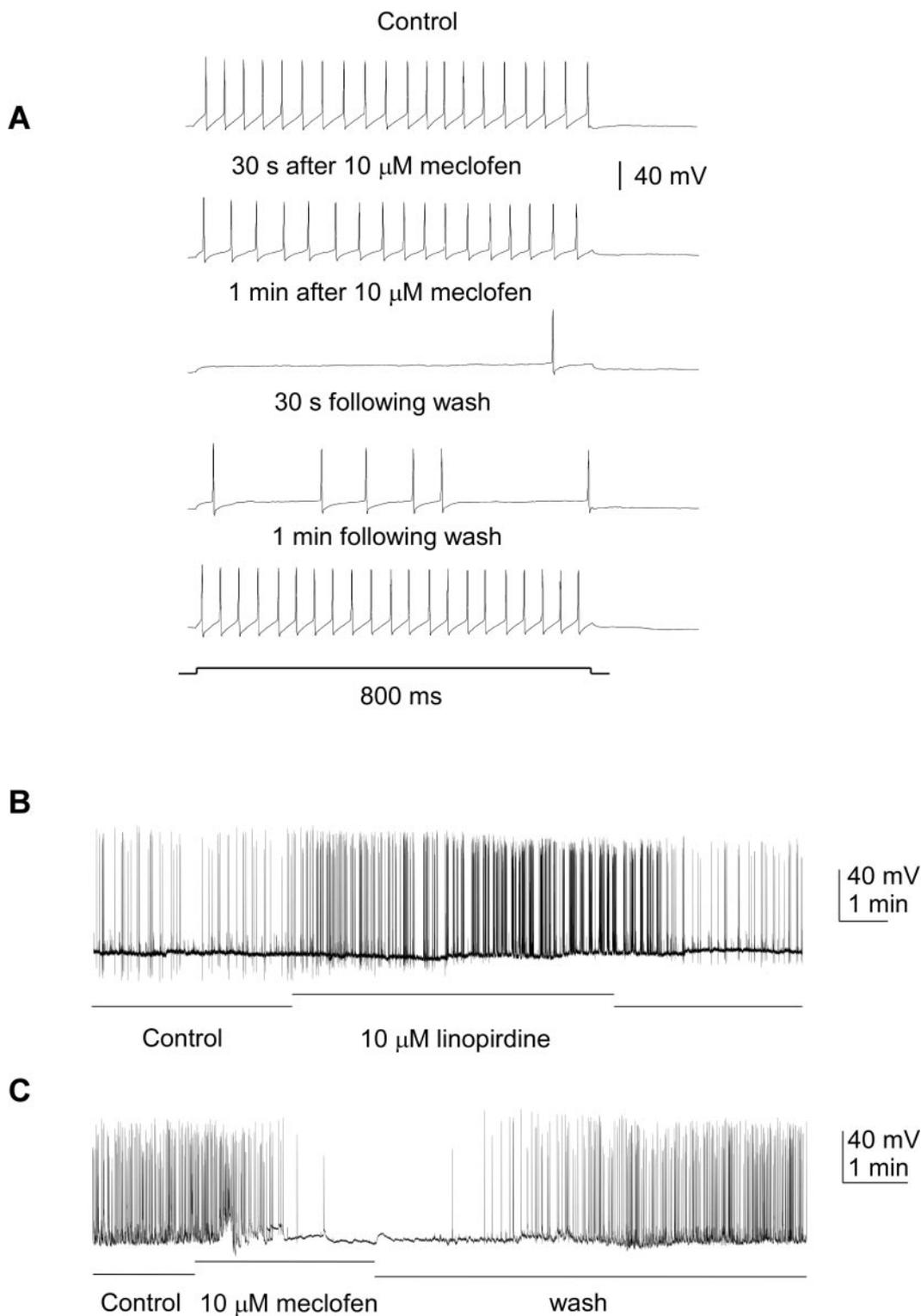


Fig. 9. Meclofenamate inhibits the spontaneous and evoked neuronal activity in cultured rat cortical neurons. **A**, action potentials were evoked by injecting for 800 ms, 100-pA depolarizing currents that were recorded using the current-clamp mode of the patch-clamp technique in the absence (control), after superfusion with 10 μM meclofenamate and after drug washout. **B**, 10 μM linopirdine enhances spontaneous spiking activity and this action is reversible after washout of the drug. **C**, 10 μM meclofenamate markedly depresses spontaneous spiking activity recorded from rat cortical neurons. The depressing effect is quickly reversed upon washout of the drug.

Similar results were obtained with diclofenac. Figure 10A shows a cortical neuron that exhibited a firing pattern with spike adaptation upon current injection (100 pA, 800 ms). Within 30 s of superfusion with 25 μ M diclofenac, there was a marked reduction in the number of evoked action potentials and a delay in first spike generation (Fig. 10A, second row). After 1-min exposure to diclofenac, no spike could be evoked upon identical current injection (Fig. 10, third row). The depressing action of diclofenac could be quickly reversed by washout of the compound (Fig. 10A, fourth to sixth rows). Similar to meclofenamate, the perfusion of 25 μ M diclofenac quickly and reversibly depressed the spontaneous spiking activity (Fig. 10B).

Anticonvulsant Effect of Diclofenac and Meclofenamate in the Maximal Electroshock Seizure Test.

Considering the opener properties of meclofenamate and diclofenac on recombinant KCNQ2/Q3 channels and their dampening action on neuronal spiking discharges, we checked for a possible anticonvulsant effect of meclofenamate and diclofenac. We used the maximal electroshock seizure test in mice, which is generally thought to be a model of generalized tonic-clonic seizure in human (Macdonald and Kelly, 1995). MES produced hind limb extension in all mice that received intraperitoneal injection of vehicle solution (Table 2). Intraperitoneal injection of diclofenac 30 min or even 2 h before the electroshock dose dependently (25–200 mg/kg)

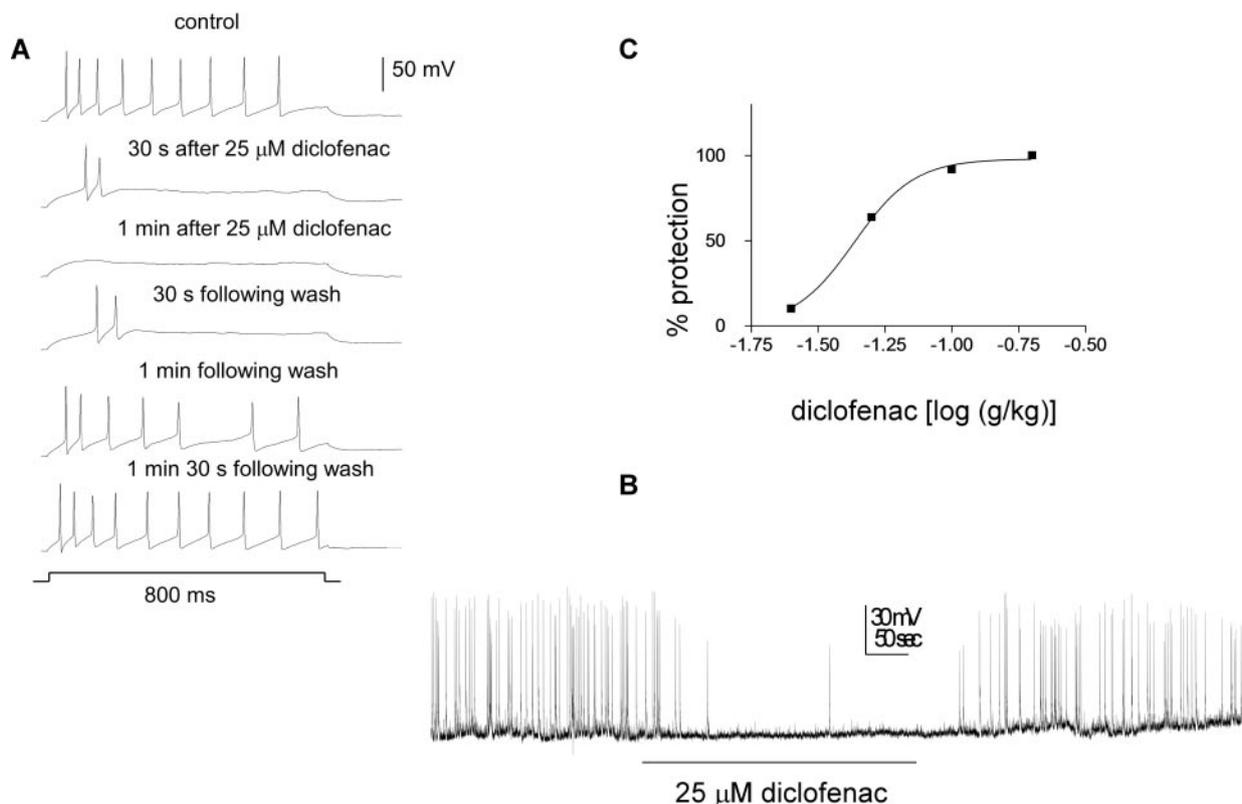


Fig. 10. Diclofenac inhibits the spontaneous and evoked neuronal activity in cultured rat cortical neurons and displays anticonvulsant action in mice. A, action potentials were evoked by injecting for 800 ms, 100-pA depolarizing currents that were recorded using the current-clamp mode of the patch-clamp technique in the absence (control), after superfusion with 25 μ M diclofenac and after drug washout. B, diclofenac (25 μ M) potently depresses spontaneous spiking activity recorded from rat cortical neurons. The depressing effect is quickly reversed upon washout of the drug. C, dose-dependent protecting effect of diclofenac on seizures induced in ICR adult mice by MES. Intraperitoneal injection of diclofenac 30 min before the electroshock, dose dependently (25–200 mg/kg) suppressed the tonic extension induced by MES, with an ED_{50} = 43 mg/kg.

TABLE 2

Anticonvulsant effect of diclofenac and meclofenamic acid (meclofenamate) measured by the MES in mice

MES was shown to cause tonic convulsions in 100% of the tested animals. The drugs dissolved in 0.9% saline were administered intraperitoneally either 30 min or 2 h before the electroshock was performed. Animals failing to show tonic hind limb extension were scored as protected and were expressed in percentage. Numbers in parentheses correspond to the number of animals tested.

Compound	Dose				
	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
	% protection				
Diclofenac					
30 min	0 (10)	10 (10)	64 (11)	92 (12)	100 (15)
2 h	0 (9)	N.D.	50 (4)	83 (6)	100 (6)
Meclofenamate					
30 min	0 (10)	N.D.	17 (6)	75 (5)	Toxic
2 h	0 (10)	N.D.	N.D.	25 (4)	Toxic

N.D., not determined.

suppressed the tonic extension induced by MES, with an $ED_{50} = 43$ mg/kg (Fig. 10C). For comparison, intraperitoneal injection of phenytoin and sodium valproate 30 min before the electroshock fully prevented hind limb extension at doses of 20 and 500 mg/kg, respectively ($n = 10$ each). Meclofenamate had a weaker anticonvulsant action than diclofenac in MES (Table 2). For example, intraperitoneal injection of meclofenamate at 50 mg/kg 30 min before the electroshock produced only 17% protection from hind limb extension, whereas diclofenac elicited 64% protection. In addition, at high doses (200 mg/kg) meclofenamate induced proconvulsive toxic effects and hyperactivity.

Discussion

Increase in potassium channel activity generally reduces neuronal excitability, thus making potassium channel openers potential drug candidates for the treatment of diseases linked to neuronal hyperexcitability. In this study, we have characterized two novel KCNQ2/Q3 channel openers, meclofenamate and diclofenac. These fenamate molecules were previously used as anti-inflammatory drugs. Here, we showed that meclofenamate and diclofenac are openers of the heterologously expressed KCNQ2/3 channels and of neuronal M-currents. They reduce both evoked and spontaneous spiking activity recorded from rat cortical neurons. It is noteworthy that diclofenac and to a lower extent meclofenamate displayed anticonvulsant activity in mice as measured by MES.

Meclofenamate and diclofenac produce two main effects: they shift the voltage dependence of KCNQ2/3 channel activation to more hyperpolarized potentials and slow channel deactivation. As a result of this leftward shift of the KCNQ2/Q3 activation curve, meclofenamate leads to a progressive hyperpolarization of the resting membrane potential as measured in *X. laevis* oocytes, in CHO cells, and as seen in neurons as well. Our data suggest that both openers either destabilize a closed channel conformation or stabilize the KCNQ2/Q3 channel in the open state. After fenamate exposure, the marked slowing of deactivation also contributes to the stabilization of the KCNQ2/3 channel in the open state. There are several interesting similarities between the properties exhibited by meclofenamate or diclofenac and those displayed by the anticonvulsant drug retigabine. First, retigabine, like the fenamate compounds, shifts leftward the voltage dependence of KCNQ2/Q3 channel activation, decelerates deactivation kinetics, and hyperpolarizes the resting membrane potential (Main et al., 2000; Wickenden et al., 2000; Tatulian et al., 2001). Along this line, it is interesting to note that a single-channel study showed that retigabine enhances KCNQ2/Q3 currents by increasing the channel open probability, mainly via the reduction of the contribution of the longest closed state and via an increase of the open times (Tatulian and Brown, 2003). It will be interesting to examine in future experiments, the mechanism by which the fenamate drugs activate the M-current at the single-channel level. Second, retigabine produces at positive potentials (above +20 mV) a secondary inhibitory action on KCNQ channels (Tatulian et al., 2001). We observed the same trend for meclofenamate and diclofenac toward KCNQ2/Q3 channels with crossover voltage point at about 0 mV. Third, from a structural perspective and although substantial chemical differences exist, the pseudosymmetrical moiety of the diphe-

nylamine group of the fenamate compounds exhibits some similarities with the two phenyl rings of the retigabine molecule (Fig. 1). Both structures are constructed from two substituted benzene rings, linked to each other through one or two atom bonds. In addition, one of the bridged atoms in retigabine is a secondary nitrogen, forming an aromatic amine functionality, similar to meclofenamate and diclofenac (Fig. 1). However, there is an interesting difference between retigabine and meclofenamate or diclofenac with respect to their selectivity toward the KCNQ2 and KCNQ3 α subunits. Whereas retigabine was found to exert its strongest opener action on KCNQ3 homomeric channels (Tatulian et al., 2001), we showed here that meclofenamate is more potent on KCNQ2 homomeric channels. There are also some similarities between the properties exhibited by meclofenamate or diclofenac and those displayed by the recently characterized KCNQ2 channel openers, acrylamide derivatives (Wu et al., 2004). Like with retigabine and fenamate compounds, the acrylamide drugs shift leftward the voltage dependence of channel activation, slow down deactivation kinetics, hyperpolarize the resting membrane potential, and produce at depolarized potentials a crossover of the current-voltage relations (Wu et al., 2003, 2004). It is not clear, however, whether all these compounds act by the same mechanisms and bind to the same channel site. Our results of coapplication of retigabine with meclofenamate indicate that the opener effects of the two compounds are additive and not mutually occlusive. These data suggest that retigabine and meclofenamate act on independent sites of KCNQ2/Q3 channels. Yet, the physical mapping of the two drugs on the molecular structure of KCNQ2/Q3 channels will be needed to conclusively solve this issue.

Considering the selectivity of meclofenamate and diclofenac toward other Kv channels, it is interesting to note that they did not affect the current amplitude of other delayed rectifier channels, such as Kv1.2, Kv1.5, and Kv2.1. It is noteworthy that both compounds did not affect the current properties of KCNQ1 and KCNQ1/KCNE1. These results place meclofenamate and diclofenac as potential templates for designing novel molecules specifically targeted to neuronal KCNQ2/Q3 channels (our unpublished data).

Meclofenamate and diclofenac are derivatives of *N*-phenylanthranilic acid, and the well known mechanism mediating their anti-inflammatory effects is inhibition of COX-1 and COX-2 that catalyze the biosynthesis of prostaglandins from arachidonic acid. However, accumulating data suggest that NSAIDs also act on other targets such as ion channels, via mechanisms different from the inhibition of the cyclooxygenase-prostaglandin pathway. For example, it was recently shown that NSAIDs such as diclofenac or flurbiprofen inhibit, although at high concentrations, acid-sensing ion channels on sensory nociceptor neurons (Voilley et al., 2001). At high concentrations, meclofenamate was also found to inhibit ATP-sensitive and Kv2.1 K^+ channels (Grover et al., 1994; Lee and Wang, 1999). Other fenamate compounds were found to potentiate large conductance Ca^{2+} -activated K^+ currents and GABA_A receptor Cl^- channels (Ottolia and Toro, 1994; Woodward et al., 1994). Along this line, the anticonvulsant retigabine, which acts as an opener of M-channels, also exhibits a pleiotropic action on other ionic conductances, for example, by directly potentiating GABA_A receptor Cl^- currents in cultured cortical neurons (Otto et al., 2002).

From a functional point of view, the leftward shift of the activation curve and the slowing of deactivation, produced by the fenamates, will lead to substantial M-current activation at normal resting and subthreshold potentials. The large potentiation of KCNQ2/Q3 currents elicited at potentials around $-60/-50$ mV (more than 10-fold increase in current amplitude produced by meclofenamate and diclofenac) further adds to the functional relevance of these compounds. Thus, meclofenamate and diclofenac exposure will lead to membrane hyperpolarization. In addition, because the M-current (KCNQ2/3) is noninactivating, its marked activation by the openers will contribute to a significant steady-state potassium conductance at subthreshold and threshold potentials, acting as a brake for neuronal firing. Indeed, meclofenamate and diclofenac depress the evoked and spontaneous cortical neuron spiking activity. The voltage range through which meclofenamate and diclofenac operate also makes them well suited drug templates for the treatment of neuropathic pain. KCNQ/M-channels have been demonstrated to also be present in nociceptive sensory neurons (Passmore et al., 2003). Furthermore, it has been shown that retigabine potently reduces the electrophysiological responses of dorsal horn nociceptive neurons and exerts an analgesic action in an animal model of inflammatory pain (Passmore et al., 2003).

In all, the channel opener properties of meclofenamic acid and diclofenac toward the brain KCNQ2/Q3 channels and the voltage range by which they operate provide a novel drug template approach for the treatment of diseases linked to neuronal hyperexcitability such as epilepsy, migraine, and neuropathic pain.

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