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TITLE PAGE

Meclofenamic acid and diclofenac, novel templates of KCNQ2/Q3 potassium channel openers, depress cortical neuron activity and exhibit anticonvulsant properties

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RUNNING TITLE PAGE

Running title: activation of KCNQ2/Q3 channels by novel openers

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Abbreviations used: Kv, voltage-dependent K^+ channels; CHO, Chinese hamster ovary; BFNC, benign familial neonatal convulsions; Meclofen, meclofenamic acid; TEA, tetraethylammonium; 4-AP, 4-aminopyridine; MES, maximal electroshock seizure model; NSAID, non-steroidal anti-inflammatory drugs; COX, cyclooxygenease.

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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 (BFNC), a neonatal form of epilepsy. Enhancement of KCNQ2/Q3 potassium currents may provide an important target for anti-epileptic drug development. Here we show that meclofenamic acid (meclofen) and diclofenac, two related molecules previously used as anti-inflammatory drugs, act as novel KCNQ2/Q3 channel openers. Extracellular application of meclofen (EC_{50} = 25 μM) and diclofenac (EC_{50} = 2.6 μM) resulted in the activation of KCNQ2/Q3 K^+ currents, heterologously expressed in Chinese hamster ovary (CHO) cells. Both openers activated KCNQ2/Q3 channels by causing a hyperpolarizing shift of the voltage activation curve (-23 mV 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, meclofen and diclofenac enhanced the M-current, reduced evoked and spontaneous action potentials, while *in vivo* diclofenac exhibited an anti-convulsant 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.

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INTRODUCTION

Voltage-dependent K^+ (Kv) 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 sub-threshold, 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, non-inactivating, 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; Cooper and Jan, 2003; Marrion, 1997). Modulation of the M-current has profound effects on brain excitability as this non-inactivating 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; Cooper and Jan, 2003; Jentsch, 2000; Marrion, 1997; Rogawski, 2000).

Recently, the KCNQ2/Q3 channel complex belonging to the KCNQ family of voltage-dependent K^+ channels was 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 produce currents that are similar to the M-current (Jentsch, 2000; Rogawski, 2000; Wang et al., 1998). In addition, KCNQ4 and KCNQ5 α subunits can co-assemble with KCNQ3 to produce K^+ currents whose properties are very similar to those of the M-current (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

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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; Castaldo et al., 2002; Charlier et al., 1998; Dedek et al., 2001; Singh et al., 1998). Interestingly, 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; Dost et al., 2004; Passmore et al., 2003; 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. Recently, the anti-convulsant drug Retigabine (D-23129; N-(2-amino-4-(4-fluorobenzylamino)phenyl) carbanic acid ethylester)) and BMS-204352 undergoing clinical trials, were found to be KCNQ2/Q3 and KCNQ4 channels openers, respectively, and therefore were suggested to act as potential anti-epileptic, anti-nociceptive and/or neuroprotective drugs (Blackburn-Munro and Jensen, 2003; Dost et al., 2004; Main et al., 2000; Passmore et al., 2003; Rivera-Arconada et al., 2004; Schroder et al., 2001; Tatulian et al., 2001; Wickenden et al., 2000).

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 (Figure 1). Meclofenamic acid and diclofenac are well known and widely used non-steroidal anti-inflammatory drugs (NSAID) acting as non selective inhibitor of COX-1 and COX-2 cyclooxygenases (Furst and Munster, 2001). Here we show that meclofenamic acid (meclofen)

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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. Meclofen and diclofenac reduce evoked and spontaneous neuronal action potentials and enhance M-current in rat cortical neurons. Diclofenac also exhibit *in vivo* an anti-convulsant activity. These compounds may serve as lead molecules for the treatment of neuronal hyperexcitability including migraine, epilepsy and neuropathic pain.

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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. Briefly, 40,000 cells seeded on poly-D-lysine-coated glass coverslips (13 mm diameter) in a 24-multiwell plate were transfected with pIRES-CD8 (0.5µg) as a marker for transfection and with KCNQ2 (0.5 µg) and /or KCNQ3 (0.5µg). For electrophysiology, transfected cells were visualized approximately 40 hours following transfection, using the anti-CD8 antibody-coated beads method (Jurman et al., 1994). Transfection was performed using 3.5 µl of lipofectamine (Gibco-BRL) according to the manufacturer's protocol.

Neuronal cortical culture

Sprague Dawley rat embryos (E18) were removed by caesarian section and their cortices were dissected out. The tissue was digested with papain (100 U; Sigma, 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 µg/ml poly-L-lysine in 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 µg/ml streptomycin, and 2 mM glutamine. D-Glucose was supplemented to a final concentration of 6 g/l. Cytosine-1-D-arabinofuranoside (5 µM) 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

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The anti-convulsant effect of diclofenac and meclofen was measured by the maximal electroshock seizure model (MES) 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 U.S. 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 D.C). Minimal electroshock were induced in adult mice by means of two transcorneal electrodes delivering an alternative current of 50 mA at 60Hz for 0.2 sec using rodent shocker (Hugo Sachs Electronik, type 221). 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 hours before the electroshock was performed. Animals failing to show tonic hindlimb extension were scored as protected and were expressed in percentage.

Electrophysiology

For current measurements in CHO cells, recordings were performed 40 h following 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), sampled at 2 kHz and filtered at 800 Hz via a 4-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. Corp, USA) with a resistance of 2-5 MΩ and were filled with (in mM): 130 KCl, 1 $MgCl_2$, 5 K_2ATP, 5 EGTA, 10 HEPES, adjusted with KOH at pH 7.4 (290 mOsm). The external solution contained (in mM): 140 NaCl, 4 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 11

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glucose, 5.5 HEPES, adjusted with NaOH at pH 7.4 (310 mOsm). Series resistances (3-13 MΩ) were compensated (75-90%) and periodically monitored. For current-clamp measurements of rat cortical neurons, recordings were performed 10-14 days after plating. The patch pipettes were filled with (in mM): 135 KCl, 1 K_2ATP, 1 MgATP, 2 EGTA, 1.1 $CaCl_2$, 5 glucose, 10 HEPES, adjusted with KOH at pH 7.4 (315 mOsm). The external solution contained (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 glucose, 10 HEPES, adjusted with NaOH at pH 7.4 (325 mOsm). For evoking the neuronal action potentials, 50-300 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 4-pole Bessel low pass filter. For voltage-clamp measurements of rat cortical neurons, the patch pipettes were filled with (in mM): 90 K-acetate, 40 KCl, 3 $MgCl_2$, 2 K_2ATP, 20 HEPES, adjusted with KOH at pH 7.4 (310-315 mOsm). The external solution contained (in mM): 120 NaCl, 23 $NaHCO_3$, 3 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 11 glucose, 0.0005 tetrodotoxin (TTX), 5 HEPES, adjusted with NaOH at 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) in order 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 (Baranauskas et al., 2003; Du et al., 1996). TEA, 4-AP, meclofenamic acid and diclofenac were purchased from Sigma Chemicals (St. Louis, MO). Retigabine was kindly provided by Dr. H. Lerche (Ulm University).

Current measurements in *Xenopus* oocytes were performed as previously described (Peretz et al., 2002). Briefly, two-electrode voltage-clamp measurements were performed 3-5 days following cRNA microinjection into oocytes as previously described. Oocytes were bathed in a modified ND96 solution containing (in mM: 96 NaCl, 2 KCl, 1 MgCl2, 0.1 CaCl2 and 5 HEPES titrated to

pH = 7.4 with NaOH). Whole-cell currents were recorded at room temperature (20°C-22°C) using a GeneClamp 500 amplifier (Axon Instruments). Glass microelectrodes (A-M systems, Inc) were filled with 3M KCl and had tip resistances of 0.5-1.5 MΩ. 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), Axograph 4.6 (Axon Instruments) and Prism 4.0 (GraphPad). Leak subtraction was performed off-line, using the Clampfit program of the pClamp 8.1software. 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:

$G = I/(V- Vrev)$

where I corresponds to the current amplitude measured at the end of the pulse and Vrev, the calculated reversal potential assumed to be -90 mV in CHO cells and –98 mV in *Xenopus* oocytes. G was estimated at various test voltages V and then, normalized to a maximal conductance value, Gmax. Activation curves were fitted by one Boltzmann distribution:

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$G/Gmax = 1/\{1+\exp[(V_{50}-V)/s]\}$

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 ± SEM. Statistically significant differences were assessed by Student's t-test.

Immunocytochemistry

Cortical neurons were grown in culture for 10-14 days on 13mm diameter coated glass coverslips in 24-well plates. Cells were carefully rinsed for 10 minutes in phosphate buffered saline (PBS) and the neurons subsequently fixed for 20 minutes in 4% paraformaldehyde in PBS. Following 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 minutes 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) was combined with a goat polyclonal antibody to KCNQ3 (N19: 1:50; Santa Cruz Biotechnology); 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). 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) and RRX-conjugated anti-goat IgG (1:100; Jackson Immunoresearch). Neurons were viewed and digital images taken using a Zeiss LSM 410 confocal microscope.

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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 co-expressed in CHO cells by co-transfecting their respective cDNAs at an equimolar ratio. Figure 2A (left panel) 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). Due to its opener properties, mefenamic acid can rescue the dominant-negative suppression of I_{KS} produced by LQT-related KCNE1 mutations (Abitbol et al., 1999). Consequently, 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 (meclofen) and diclofenac were potent and specific openers of KCNQ2/Q3 channels (see below and Table 1). Meclofen and diclofenac are well known NSAID drugs (Figure 1) that inhibit non selectively COX-1 and COX-2 cyclooxygenases (Furst and Munster, 2001). The external application of meclofen activated KCNQ2/Q3 K^+ currents at more hyperpolarized potentials (Figures 2A right panel, 2B and 3C). In untreated CHO cells, KCNQ2/Q3 channels were activated above a threshold of –50 mV, while they were activated above –70 mV in the presence of meclofen (Figure 3C).

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Meclofen 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 μM meclofen induced an increase of the current amplitude by up to 72 %, from 844 ± 130 pA to 1451 ± 164 pA (n = 15, $p < 0.01$), for control and meclofen-treated CHO cells, respectively (Figure 3A and 3B). To measure the potency of meclofen, we determined the leftward shift of the activation curves produced by increasing concentrations of the drug as previously described (Tatulian et al., 2001). The relation between the concentration of meclofen and the left-shift in the half-activation potential (ΔV_{50}) was deduced and normalized to the maximal shift produced by 200 μM meclofen (Figure 2C). The values were fitted to a Hill equation with a slope of 1.7 and an EC_{50} = 25 ± 2 μM (n = 8). The onset of the drug action was fast since within less than one minute of external application of 25 μM meclofen, there was a large increase in KCNQ2/3 current amplitude across a range of test potentials between -50 to 0 mV (Figures 2B and 3C). The effect of meclofen 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 meclofen on KCNQ2/3 current amplitude became non-existent (Figure 2A and 2B). Meclofen produced a marked leftward shift of -22.7 mV in the voltage-dependence of KCNQ2/Q3 current activation, from V_{50} = -19.6 ± 1.9 mV (n = 24) to V_{50} = -42.3 ± 2.1 mV (n = 14) in control and meclofen-treated cells, respectively (Figure 2B, $p < 0.01$). The slope parameters of the Boltzmann fitting curve did not change significantly with s = -9.5 ± 0.4 mV/e fold and s = -10.50 ± 0.93 mV/e fold, for control and meclofen-treated cells, respectively. Clearly, the most pronounced action of meclofen is exerted at physiologically relevant negative potentials. At -50 mV, -40 mV and -30 mV, 25 μM meclofen increased KCNQ2/3 current amplitude by more than 10-fold, 5-fold and 2.5-fold, respectively (Figure 3C). While meclofen slightly accelerated the KCNQ2/Q3

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activation kinetics, it markedly slowed down the deactivation process (Figures 2A, 3A and 3D). 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 μM meclofen. Meclofenamic acid reduced by about 2-fold the speed of KCNQ2/Q3 channel closure with the time constant of deactivation increasing from τ_{deact} = 79.6 ± 4.5 msec to τ_{deact} = 167.5 ± 11.6 msec (n = 10, p <0.01) for control and meclofen-treated CHO cells, respectively (Figure 3D and 3E). Deactivation kinetics were also measured at command voltages of equivalent G/Gmax values since meclofen produced a left-shift of the activation curve (~ -20 mV). Under these conditions, the deactivation time constant still increased from τ_{deact} = 85.5 ± 6.6 msec (at -20 mV prepulse command) to τ_{deact} = 151.2 ± 8.7 msec (at -40 mV prepulse command) in the presence of meclofen (n = 15, p < 0.01).

To make sure that the effects of meclofen on KCNQ2/Q3 K^+ currents were not dependent on a particular cell type, we also checked its action on the *Xenopus* oocyte expression system. As in CHO cells, external application of 25 μM meclofen produced a 53 ± 8 % (n =7, p < 0.01) increase in KCNQ2/Q3 current amplitude when the oocyte membrane was stepped from –80 mV to –40 mV (Figure 4A). Similarly, meclofen (25 μM) produced a leftward shift of –15.9 mV in the voltage-dependence of KCNQ2/Q3 current activation, from V_{50} = -28.6 ± 2.9 mV (n = 10) to V_{50} = -44.5 ± 3.1 mV (n = 10) in control and meclofen-treated cells, respectively (Figure 4B). As a result of this leftward shift of the KCNQ2/Q3 activation curve, incubation of the oocytes with increasing concentrations of meclofen led to a progressive hyperpolarization of the oocyte resting membrane potential from –56 ± 2 mV to –72 ± 3 mV, with an EC_{50} = 11.7 ± 5.2 μM (n = 6) (Figure 4C).

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Similar results were obtained with diclofenac, a structurally related compound, having a benzeneacetic acid moiety instead of a benzoic acid group for meclofenamic acid that also bears a methyl group in its dichlorophenyl ring (Figure 1). Adding 50 μM diclofenac externally (Figure 5D) produced a significant leftward shift of –14.5 mV in the voltage-dependence of KCNQ2/3 activation, from V_{50} = -30.9 ± 4.1 mV to V_{50} = -45.4 ± 2.7 mV (n = 7, p <0.01). As with meclofen, treatment of CHO cells with diclofenac slowed down the deactivation kinetics of KCNQ2/Q3 channels (Figure 5A). The deactivation time constant increased from τ_{deact} = 85.0 ± 5.0 msec (at -20 mV prepulse command) to τ_{deact} = 172.0 ± 18.4 msec (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 mV to -50 mV the application of diclofenac increased the current amplitude by up to 262 ±26 % (n = 6) (Figure 5A and 5B). The effect of diclofenac was fully reversible and dose-dependent. The potency of diclofenac was measured by the normalized left-shift in the half-activation potential (ΔV_{50}) as a function of the drug concentration (Figure 5C). The curve fitting to a Hill equation yielded an EC_{50} = 2.6 ± 1.3 μM and a Hill slope of 1.3 (n = 8). Like meclofen, diclofenac produced a hyperpolarization (-10.4 mV) of the resting membrane potential in CHO cells from -44.6 ± 2.3 mV to -55.0 ± 1.8 mV (n = 7, p<0.001) in control and diclofenac-treated cells, respectively.

Selectivity of meclofen and diclofenac action and synergy with retigabine

The opener properties of meclofen 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 μM meclofen on homomeric KCNQ2 and homomeric KCNQ3 channels expressed separately in CHO cells (Figure 6). In general, meclofen

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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 ± 2.2 mV to V_{50} = -50.5 ± 1.4 mV (n = 8) in control and meclofen-treated cells, respectively (Figure 6C left panel, $p < 0.01$). The leftward shift produced by meclofen on the activation curve of KCNQ3 channels was weaker (-15 mV) from V_{50} = -39.0 ± 3.5 mV to V_{50} = -54.0 ± 2.0 mV (n = 11) in control and meclofen-treated cells, respectively (Figure 6C right panel, $p < 0.01$). Meclofen significantly reduced the speed of KCNQ2 channel closure with the time constant of deactivation increasing from τ_{deact} = 92.6 ± 3.9 msec to τ_{deact} = 152.7 ± 4.9 msec (Figure 6A and 6D; n = 8, $p <0.001$). In contrast, it did not affect the deactivation kinetics of KCNQ3 channels (τ_{deact} = 319.6 ± 40.8 msec and τ_{deact} = 317.2 ± 30.2 msec for control and meclofen-treated cells, respectively, n = 8). Reflecting the stronger effect of the opener on KCNQ2 versus KCNQ3 channels, the external application of 50 μM meclofen produced an increase of 240 ± 26 % and 120 ± 4 % of the KCNQ2 and KCNQ3 current amplitudes, respectively, when cells were stepped from –85 mV to -40 mV (Figure 6A and 6B; n = 8, $p<0.001$). Similar results were obtained with diclofenac (not shown). To further address the selectivity of the drugs, the effects of meclofen and diclofenac towards other Kv channels were tested in *Xenopus* oocytes by measuring at non-saturating depolarizing potentials, the current amplitude of various Kv channels including Kv1.2, Kv1.5, Kv2.1, KCNQ1 and KCNQ2/Q3 (Table 1). The results indicate that while meclofen (25 μM) and diclofenac (25 μM) increased KCNQ2/Q3 currents (at -40 mV) by 1.75 fold and 2.25 fold, respectively, they did not affect the current amplitude of other delayed-rectifier channels like, Kv1.2, Kv1.5 and Kv2.1 (Table 1). Interestingly, homomeric KCNQ1 and heteromeric KCNQ1/KCNE1 currents were not enhanced by diclofenac and meclofen across a range of test potentials between -50 to 0 mV (Table 1 and data not shown).

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The comparable opener properties exhibited by meclofen or diclofenac and those displayed by the anti-convulsant 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 the half-activation potential (ΔV_{50}) as a function of the drug concentration in the absence or presence of a fixed EC_{50} concentration (25 μM) of meclofen (Figure 7). The results show that co-application of the two compounds produced additive effects. While dose-dependent applications of retigabine alone and meclofen alone elicited maximal left-shifts of the half-activation potential (ΔV_{50}) of -24 ± 3 mV and -20 ± 3 mV, respectively, co-exposure of 25 μM meclofen with increasing concentrations of retigabine produced a maximum ΔV_{50} of -32 ± 4 mV ($n = 8$; $p < 0.05$; Figure 7). Clearly, the effects of both drugs are not mutually occlusive but additive, which suggests that retigabine and meclofen act independently on KCNQ2/Q3 channels.

Meclofen enhances M-currents in rat cortical neurons

Considering the action of meclofen 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 (Shah et al., 2002; Tatulian et al., 2001; Wang et al., 1998). Here we examined the effects of meclofen on native M-currents of cultured rat cortical neurons. We first checked whether KCNQ2 and KCNQ3 α subunits were co-expressed in rat primary cortical neurons grown in culture for 10-14 days by means of double staining immunofluorescence (Figure 8A). The polyclonal antibodies we used were specific and recognized selectively KCNQ2 and KCNQ3 channel proteins when expressed in CHO cells (Figure 8A, right panel). Confocal

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immunofluorescence microscopy showed co-localization of KCNQ2 and KCNQ3 immunoreactive proteins in cortical neurons with various morphologies, including pyramidal-like cells (Figure 8A, left panel). The staining for both KCNQ2 and KCNQ3 was most prominent in the somata but was also present along the neuronal processes.

Then, we attempted to record the native M-current from pyramidal-like neurons though it was very tiny and subject to run-down. For this purpose, we used an external solution containing 0.5 μM TTX to block voltage-gated Na^+ channels and 1 mM 4-AP plus 0.2 mM TEA in order 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 (Baranauskas et al., 2003; Du et al., 1996). 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 (Figure 8B). Two different 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 (Figure 8B). 2/ or by holding the cell at -80 mV, stepping to -40 mV and then back to -60 mV to activate and deactivate the M-currents (Figure 8C). In the latter protocol, the subtracted traces (with and without 10 μM linopirdine) show that 10 μM meclofen produced a potent increase of the outward current generated by the non saturating step depolarization to –40 mV (Figure 8C). Meclofen (10 μM) enhanced the outward current by 465 ± 134 % of control at –40 mV. These enhanced currents were blocked by 10 μM linopirdine (Figure 8D; $n = 5$, $p < 0.01$).

Inhibition of evoked and spontaneous neuronal activity by meclofen and diclofenac

As 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

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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 meclofen 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 μM meclofen hyperpolarized the cortical neurons by –8 ± 3 mV (n = 8). Figure 9 A shows representative experiments of how 10 μM meclofen drastically reduced the number of evoked action potentials in cortical neurons that exhibited regular spiking patterns with no significant spike adaptation. Within less than one minute, external exposure of 10 μM meclofen produced a widening of inter-spike intervals in the action potentials fired by the cortical neurons (Figure 9A, 2nd row). Following 2 minutes of opener exposure, only one spike could be evoked by the same depolarizing current (Figure 9A, 3rd row). Following 100 pA depolarizing current injection for 800 msec, 10 μM meclofen 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 meclofen (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 minute, neurons recovered their initial spiking activity (25 Hz, Figure 9A, 5th row). Using higher density cultures of rat cortical neurons, we could record spontaneous spiking activity (Figure 9B and 9C). While 10 μM linopirdine, a blocker of M-currents, significantly enhanced the frequency of spontaneous spiking (Figure 9B), the perfusion of 10 μM meclofen produced within less than 2 minutes a profound depression of spontaneous action potentials (Figure 9C). The depressing action of meclofen could be quickly reversed by washout of the compound (Figure 9C).

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 sec superfusion with 25 μM diclofenac, there was a marked reduction in the number of evoked action potentials and a delay in first spike generation (Figure 10A, 2nd row). Following 1 min exposure to diclofenac, no spike could be evoked upon identical current injection (Figure 10, 3rd row). The depressing action of diclofenac could be quickly reversed by washout of the compound (Figure 10A, 4th-6th row). Similar to meclofen, the perfusion of 25 μM diclofenac quickly and reversibly depressed the spontaneous spiking activity (Figure 10B).

Anti-convulsant effect of diclofenac and meclofen in the maximal electroshock seizure test

Considering the opener properties of meclofen and diclofenac on recombinant KCNQ2/Q3 channels and their dampening action on neuronal spiking discharges, we checked for a possible anti-convulsant effect of meclofen 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 hours before the electroshock dose-dependently (25-200 mg/kg) suppressed the tonic extension induced by MES, with an ED_{50} of 43 mg/kg (Figure 10C). For comparison, intraperitoneal injection of phenytoin and sodium valproate 30 min before the electroshock fully prevented hind limb extension at doses of 20 mg/kg and 500 mg/kg, respectively (n = 10 each). Meclofen had a weaker anti-convulsant action than diclofenac in the MES test (Table 2). For example, intraperitoneal injection of meclofen at 50 mg/kg 30 min before the electroshock produced only 17% protection from hind limb extension, while diclofenac elicited 64% protection. In addition, at high doses (200 mg/kg) meclofen induced proconvulsive toxic effects and hyperactivity.

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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, meclofen and diclofenac. These fenamate molecules were previously used as anti-inflammatory drugs. Here we showed that meclofen 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. Importantly, diclofenac and to a lower extent meclofen displayed anti-convulsant activity in mice as measured by the MES test.

Meclofen 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, meclofen leads to a progressive hyperpolarization of the resting membrane potential as measured in *Xenopus* 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. Following 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 meclofen or diclofenac and those displayed by the anti-convulsant 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; Tatulian et al., 2001; Wickenden et al., 2000). 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 meclofen and diclofenac towards KCNQ2/Q3 channels with crossover voltage point at about 0 mV. Third, from a structural perspective and though substantial chemical differences exist, the pseudosymmetrical moiety of the diphenylamine group of the fenamate compounds exhibits some similarities with the two phenyl rings of the retigabine molecule (Figure 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 meclofen and diclofenac (Figure 1). However, there is an interesting difference between retigabine and meclofen or diclofenac with respect to their selectivity towards the KCNQ2 and KCNQ3 α subunits. While retigabine was found to exert its strongest opener action on KCNQ3 homomeric channels (Tatulian et al., 2001), we showed here that meclofen is more potent on KCNQ2 homomeric channels. There are also some similarities between the properties exhibited by meclofen 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; Wu et al., 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 co-application of retigabine with meclofen indicate that the opener effects of the two compounds are additive and not mutually occlusive. These data suggest that retigabine and meclofen 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 meclofen and diclofenac towards other Kv channels, it is interesting to note that they did not affect the current amplitude of other delayed-rectifier channels like, Kv1.2, Kv1.5 and Kv2.1. Notably both compounds did not affect the current properties of KCNQ1 and KCNQ1/KCNE1. These results place meclofen and diclofenac as potential templates for designing novel molecules specifically targeted to neuronal KCNQ2/Q3 channels (manuscript in preparation).

Meclofen and diclofenac are derivatives of *N*-phenylanthranilic acid and the well known mechanism mediating their anti-inflammatory effects is inhibition of the cyclooxygenases 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, though at high concentrations, ASIC channels on sensory nociceptor neurons (Voilley et al., 2001). At high concentrations, meclofen 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 anti-convulsant retigabine, which acts as an opener of M-channels, also exhibits a pleiotropic action on other ionic conductances, for

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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 meclofen and diclofenac) further adds to the functional relevance of these compounds. Thus, meclofen and diclofenac exposure will lead to membrane hyperpolarization. In addition, since the M-current (KCNQ2/3) is non-inactivating, its marked activation by the openers will contribute to a significant steady-state potassium conductance at sub-threshold and threshold potentials, acting as a brake for neuronal firing. Indeed, meclofen and diclofenac depress the evoked and spontaneous cortical neuron spiking activity. The voltage range through which meclofen and diclofenac operate, makes them also well-suited drug templates for the treatment of neuropathic pain. Recently, it was demonstrated that KCNQ/M-channels are also present in nociceptive sensory neurons (Passmore et al., 2003). Furthermore, it was 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 towards 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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FOOTNOTES

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LEGENDS FOR FIGURES

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

Figure 2. Meclofen shifts the voltage dependence of KCNQ2/Q3 activation in CHO cells.

A. Representative traces recorded from the same cell before (left panel, control) and after (right panel, meclofen) external application of 100 μM meclofenamic acid. Cells were held at -85 mV and the membrane potential was stepped from -70 mV to +40 mV for 1.5 sec pulse duration, in 10 mV increments, followed by a 0.75 sec step to -60 mV. **B.** The normalized conductance was plotted as a function of the test voltages, for control (open squares) and meclofen-treated cells (closed squares). The activation curves were fitted using one Boltzmann function. **C.** Dose-response curve of meclofen opener activity for KCNQ2/Q3 channels. The potency of meclofen 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 μM meclofen. The data were fitted to a Hill equation, yielding EC_{50} = 25 ± 2 μM and η_H = 1.8 ± 0.4 (n =8).

Figure 3. Meclofen increases current amplitude and slows down deactivation of KCNQ2/Q3 channels in CHO cells. A. Current traces were recorded in the absence (control) and presence of 25 μM meclofen. In this train protocol, the cells were stepped every 30 sec to -30 mV for 1.5 sec pulse duration. **B.** The percentage of the current is shown in the presence (+) or absence (-) of 25 μM meclofenamic acid, the latter being the control of 100%. **C.** The current amplitude (nA) was plotted against the step voltage (-70 mV to -20 mV) to illustrate the negative shift of the threshold for channel activation, following meclofen application. **D.** Zoom of the tail

current of a CHO cell before (control) and following application of 25 μM meclofen. The step pulse was -20 mV while the tail potential was -60 mV. **E.** The deactivation time constants resulting from the mono-exponential fit of the tail current decay (τ_{deact}), are shown in the absence (-) and the presence of meclofen (+).

Figure 4. Meclofen activates KCNQ2/Q3 channels and hyperpolarizes the membrane potential in *Xenopus* oocytes. A. Current traces were recorded in *Xenopus* oocytes in the absence (control) and presence of 25 μM meclofen. Cells were stepped every 30 sec to -40 mV for 2 sec pulse duration. **B.** The normalized conductance was plotted as a function of the test voltages, for control (open squares) and meclofen-treated *Xenopus* oocytes (closed squares). The activation curves were fitted using one Boltzmann function. **C.** The resting membrane potential (RMP) was recorded in *Xenopus* oocytes and plotted as a function of meclofen concentration. The curve was fitted to a sigmoidal binding function, yielding an EC_{50} = 11.7 ± 5.2 μM (n = 6).

Figure 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 sec to -50 mV for 1.5 sec pulse duration. **B.** The 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 figure 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 EC_{50} = 2.6 ± 1.3 μM and η_H = 1.3 ± 0.3 (n =8).

D. The normalized conductance was plotted as a function of the test voltages, for control (open squares) and diclofenac-treated CHO cells (closed squares). The activation curves were fitted using one Boltzmann function.

Figure 6. Selectivity of meclofen towards 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 meclofen in CHO cells expressing either KCNQ2 (left panel) or KCNQ3 (right panel) homomeric channels. **B.** The percentage of current increase is shown in the absence (-) or presence (+) of 50 μM meclofenamic acid, in cells expressing either KCNQ2 (left panel) or KCNQ3 (right panel) homomeric channels. **C.** The normalized conductance was plotted as a function of the test voltages, for control (open squares) and meclofen-treated CHO cells (closed squares) for cells expressing either KCNQ2 (left panel) or KCNQ3 (right panel) homomeric channels. The activation curves were fitted using one Boltzmann function. **D.** The deactivation time constants resulting from the mono-exponential fit of the tail current decay (τ_{deact}), are shown in the absence (-) and the presence of meclofen (+) for cells expressing either KCNQ2 (left panel) or KCNQ3 (right panel) homomeric channels.

Figure 7. Dose-response curve of meclofen and retigabine opener activity for KCNQ2/Q3 channels and their additive effects upon co-application. The individual potency of retigabine (upward triangles) and meclofen (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 ± 1.3 μM and η_H = 1.20 ± 0.30 (n =8) for retigabine and EC_{50} = 25.7 ± 1.1 μM and η_H = 2.1 ± 0.5 (n =8) for meclofen. For co-application experiments, we checked the potency of retigabine as above in the presence of a fixed concentration of 25 μM meclofen (EC_{50}). Results show that co-application of the two compounds produced additive effects. Co-exposure of 25 μM meclofen with increasing concentrations of retigabine produced a maximum ΔV_{50} of -32 ± 4 mV (n = 8; $p < 0.05$), while dose-dependent applications of retigabine

alone and meclofen alone elicited maximal left-shifts of ΔV_{50} of -24 ± 3 mV and -20 ± 3 mV, respectively.

Figure 8. Meclofen enhances the M-current in rat cortical neurons. A. Immunocytochemical identification of KCNQ2 and KCNQ3 α subunits in rat cortical neurons. Specific immuno-detection of KCNQ2 and KCNQ3 α subunits in CHO cells co-transfected with KCNQ2 and KCNQ3 cDNAs (right panel). Co-localization of KCNQ2 and KCNQ3 α subunits in a pyramidal-like cortical neuron as detected by double immunocytochemistry and illustrated by the merge image (left panel). **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 μM linopirdine (middle). The subtracted traces are shown in the right panel. **C.** Representative trace showing the increase of the linopirdine-sensitive current by 10 μM meclofen. In this protocol, the membrane potential was stepped from –80 mV to -40 mV and the recording was done in 1 mM 4-AP and 0.2 mM TEA with and without 10 μM meclofen, in the absence or presence of 10 μM 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 μM meclofenamic acid, where the control (-) is 100% ($n = 5$, $p < 0.01$).

Figure 9. Meclofen inhibits the spontaneous and evoked neuronal activity in cultured rat cortical neurons. A. Action potentials were evoked by injecting for 800 msec, 100 pA depolarizing currents and were recorded using the current-clamp mode of the patch-clamp technique in the absence (control), following superfusion with 10 μM meclofen and after drug washout. **B.** 10 μM linopirdine enhances spontaneous spiking activity and this action is reversible following washout of the drug. **C.** 10 μM meclofen markedly depresses spontaneous

spiking activity recorded from rat cortical neurons. The depressing effect is quickly reversed upon washout of the drug.

Figure 10. Diclofenac inhibits the spontaneous and evoked neuronal activity in cultured rat cortical neurons and displays anti-convulsant action in mice. A. Action potentials were evoked by injecting for 800 msec, 100 pA depolarizing currents and were recorded using the current-clamp mode of the patch-clamp technique in the absence (control), following superfusion with 25 μM diclofenac and after drug washout. **B.** Diclofenac (25 μM) 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 the MES test. 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} of 43 mg/kg.

TABLES

TABLE 1

Specificity of meclofenamic acid and diclofenac effects

The specificity of meclofen and diclofenac towards Kv channels was tested in *Xenopus* 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), KCNQ1 and KCNQ2/Q3 (quantified at -40 mV). The effects of meclofen and diclofenac were expressed as percentage of the control amplitude, measured at the same potential in the absence of the drug. Data are expressed as mean ± SEM of 5-8 separate experiments.

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

* significant change compared to control at $p<0.01$ paired Student's *t* test

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TABLE 2

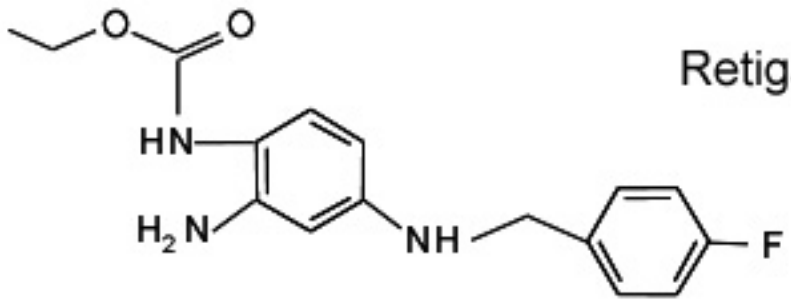
Anti-convulsant effect of diclofenac and meclofenamic acid (meclofen) measured by the maximal electroshock seizure model (MES) in mice.

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 hours before the electroshock was performed. Animals failing to show tonic hindlimb extension were scored as protected and were expressed in percentage. Numbers in parentheses correspond to the number of animals tested. ND, not determined.

Dose	0 (mg/kg)	25 (mg/kg)	50 (mg/kg)	100 (mg/kg)	200 (mg/kg)
			% protection		
Compound					
diclofenac, 30 min	0 (10)	10 (10)	64 (11)	92 (12)	100 (15)
2 hrs	0 (9)	ND	50 (4)	83 (6)	100 (6)
meclofen, 30 min	0 (10)	ND	17 (6)	75 (5)	toxic
2 hrs	0 (10)	ND	ND	25 (4)	toxic

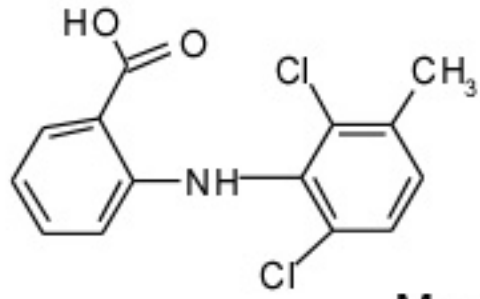
Fig. 1

A



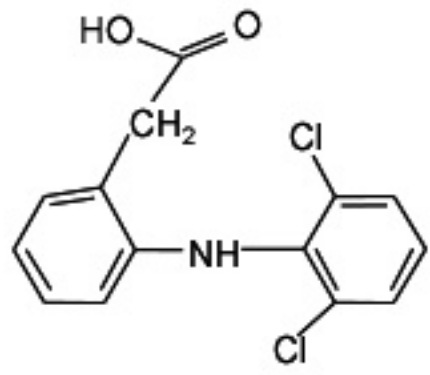
Retigabine

B



Meclofenamic acid

C



Diclofenac

Fig. 2

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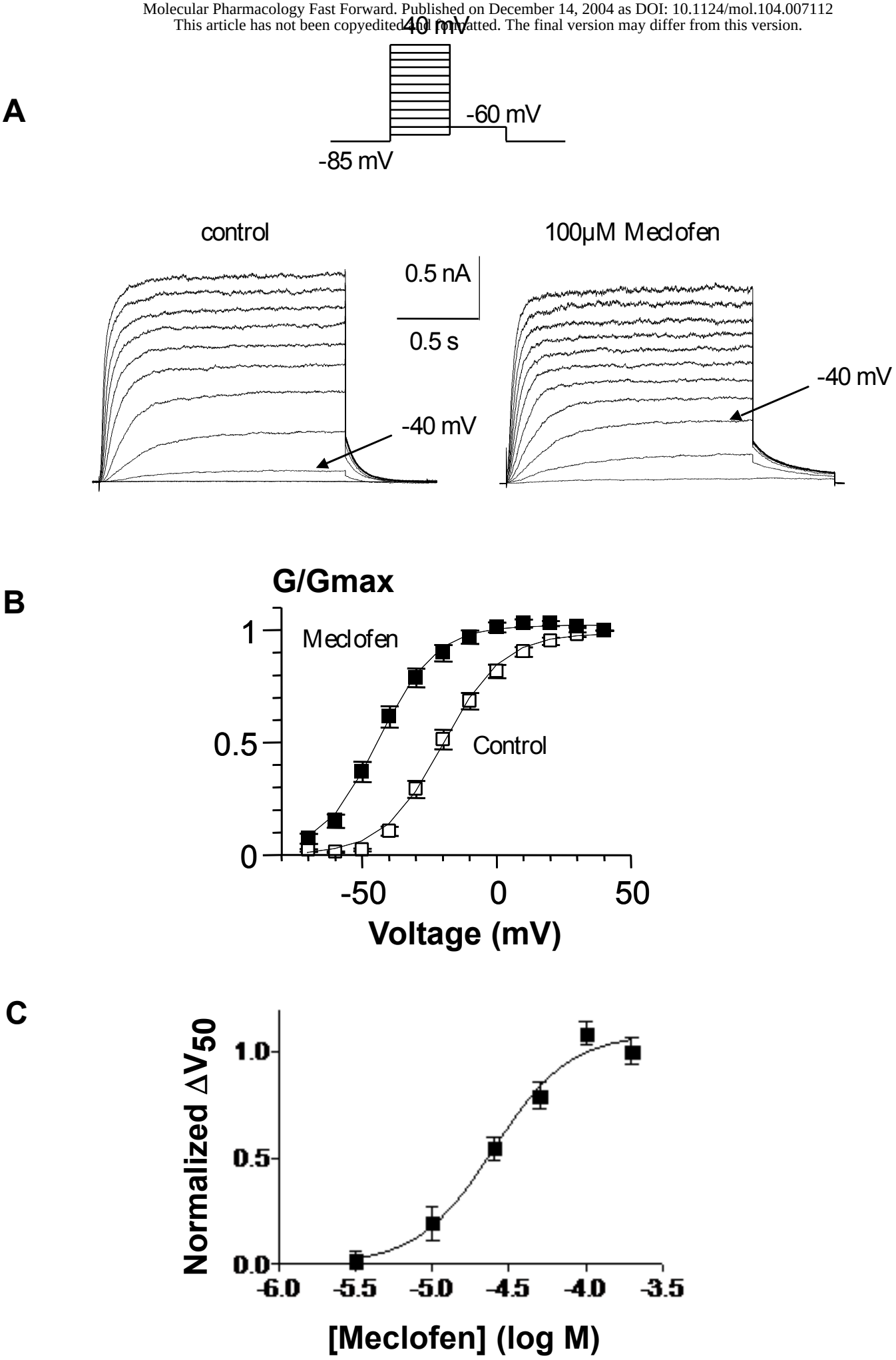

Fig. 3

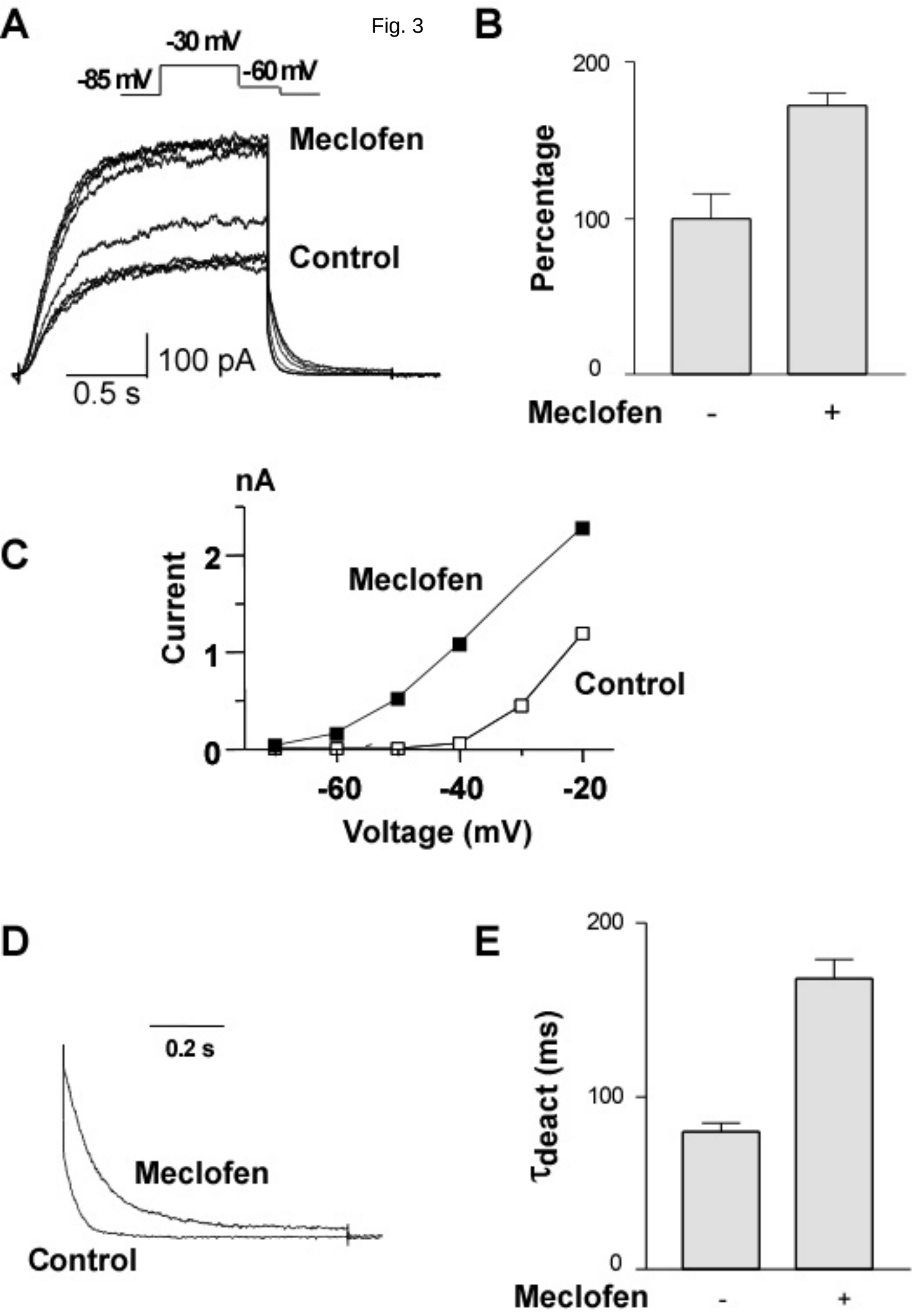
A
-30 mV
-85 mV
-60 mV
Meclofen
Control
100 pA
0.5 s
B
200
100
0
Percentage
Meclofen
-
+
C
nA
2
1
0
Current
Meclofen
Control
-60
-40
-20
Voltage (mV)
D
0.2 s
Meclofen
Control
E
200
100
0
τdeact (ms)
Meclofen
-
+

Fig. 4

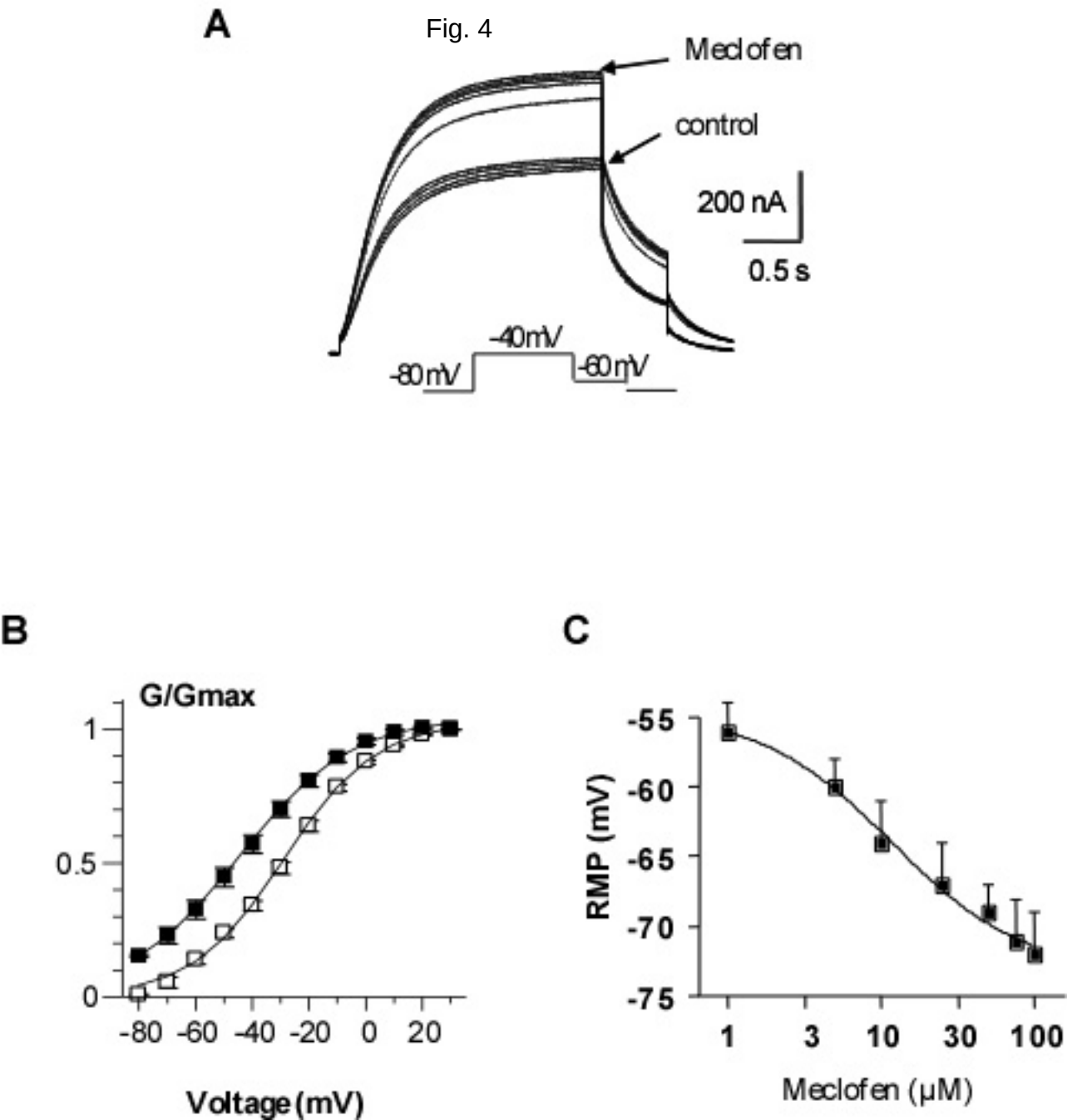
A
Meclofen
control
200 nA
0.5 s
-40mV
-80mV
-60mV
B
G/Gmax
1
0.5
0
-80 -60 -40 -20 0 20
Voltage (mV)
C
RMP (mV)
-55
-60
-65
-70
-75
1 3 10 30 100
Meclofen (μM)

Fig. 5

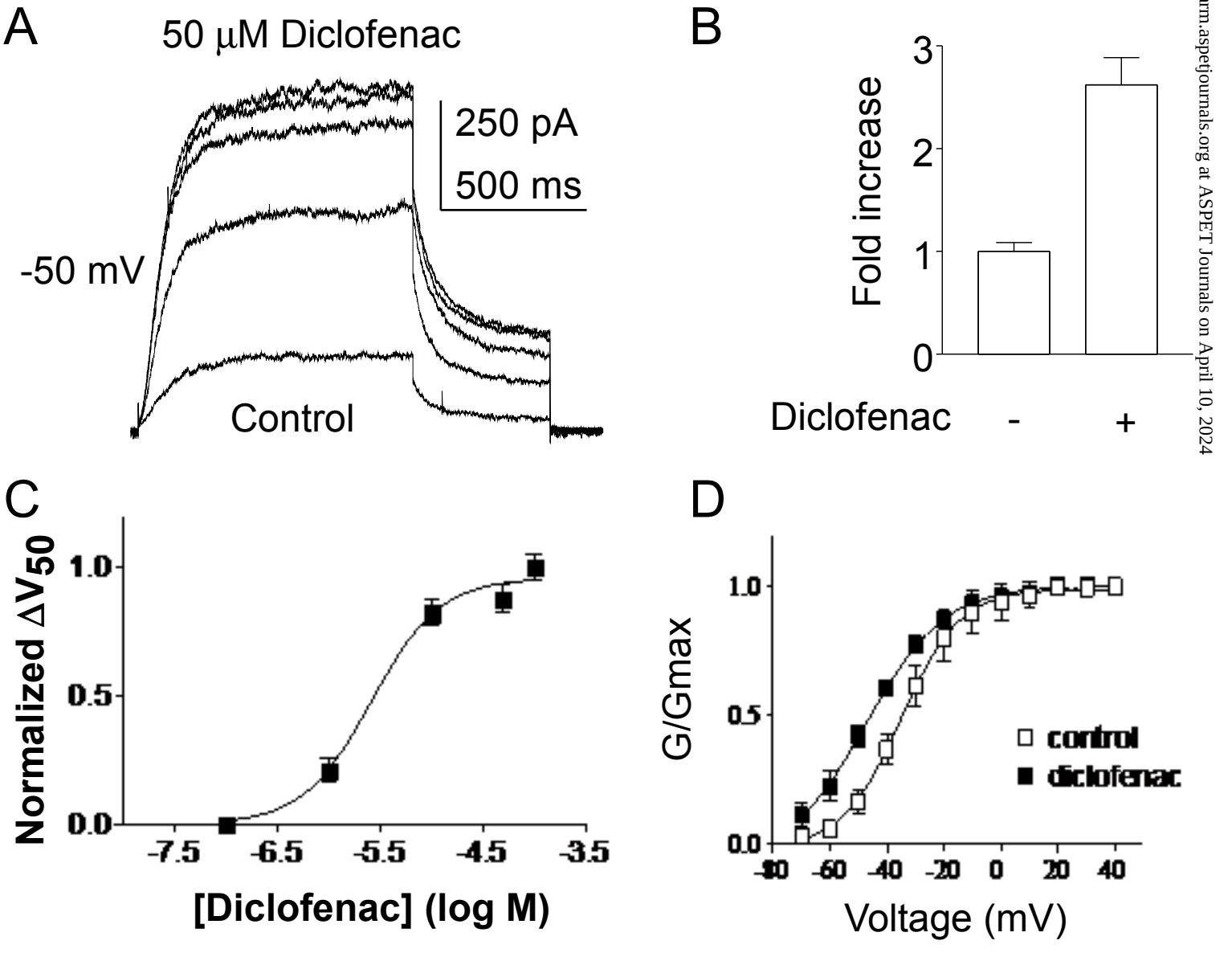

Fig. 6

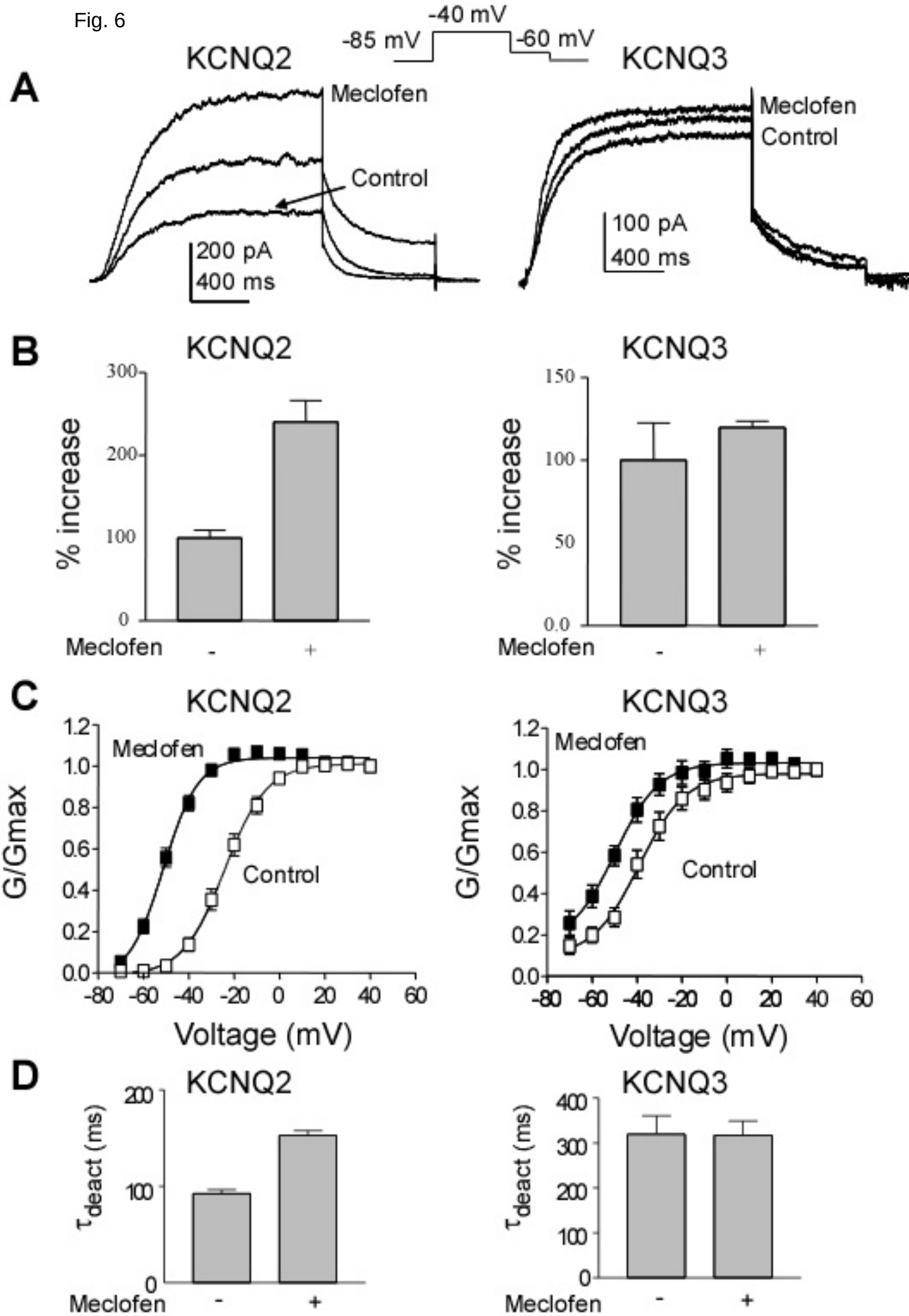
A
-40 mV
-85 mV
-60 mV
KCNQ2
Meclofen
Control
200 pA
400 ms
KCNQ3
Meclofen
Control
100 pA
400 ms
B
KCNQ2
% increase
300
200
100
0
Meclofen
-
+
KCNQ3
% increase
150
100
50
0.0
Meclofen
-
+
C
KCNQ2
Meclofen
Control
G/Gmax
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-80 -60 -40 -20 0 20 40 60
Voltage (mV)
KCNQ3
Meclofen
Control
G/Gmax
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-80 -60 -40 -20 0 20 40 60
Voltage (mV)
D
KCNQ2
τdeact (ms)
200
100
0
Meclofen
-
+
KCNQ3
τdeact (ms)
400
300
200
100
0
Meclofen
-
+

Fig. 7

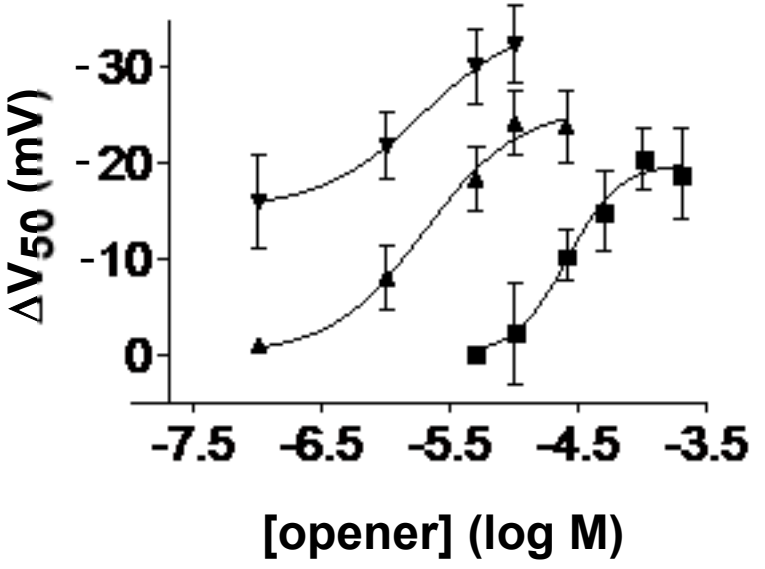

Fig. 8

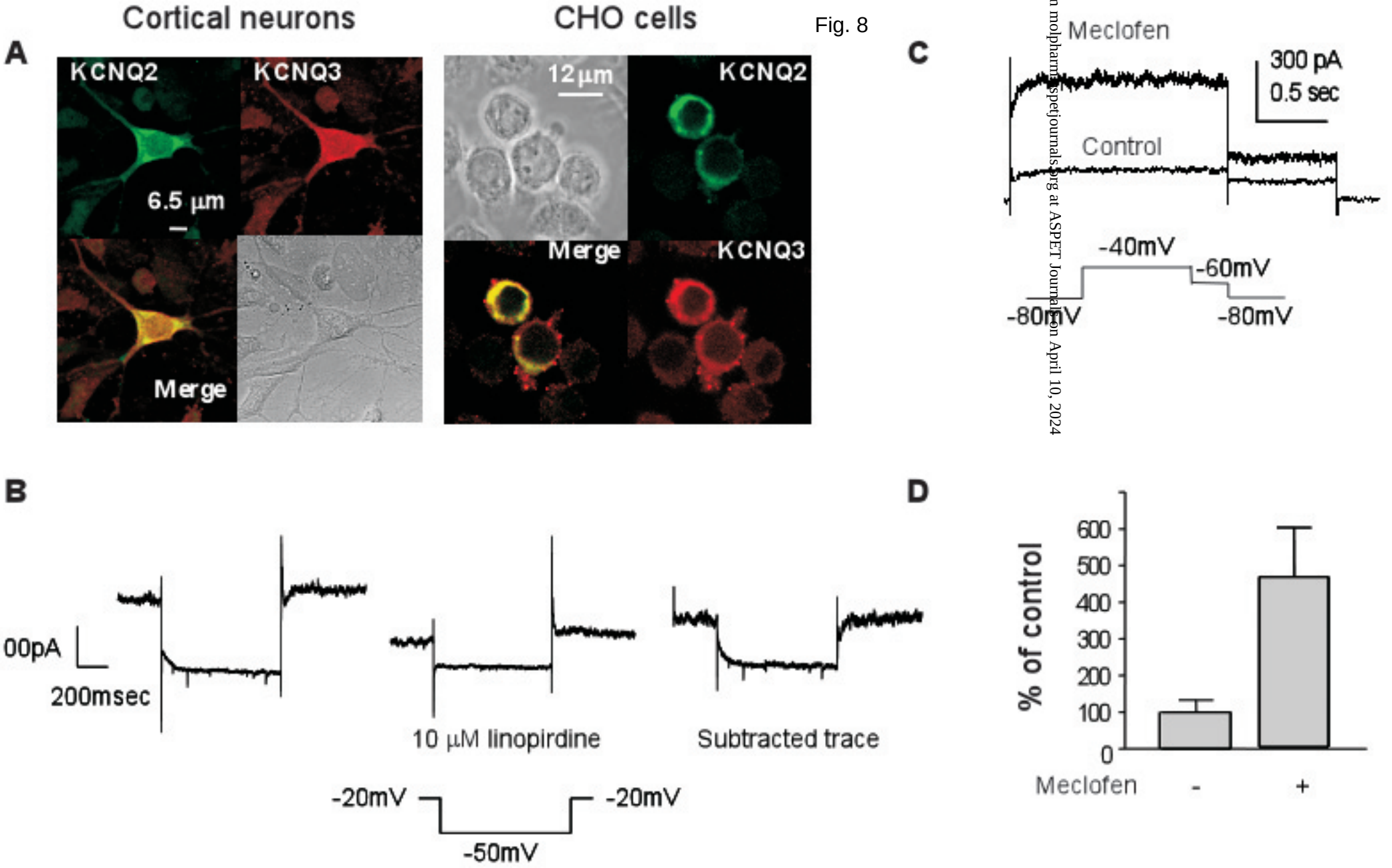

Fig. 9

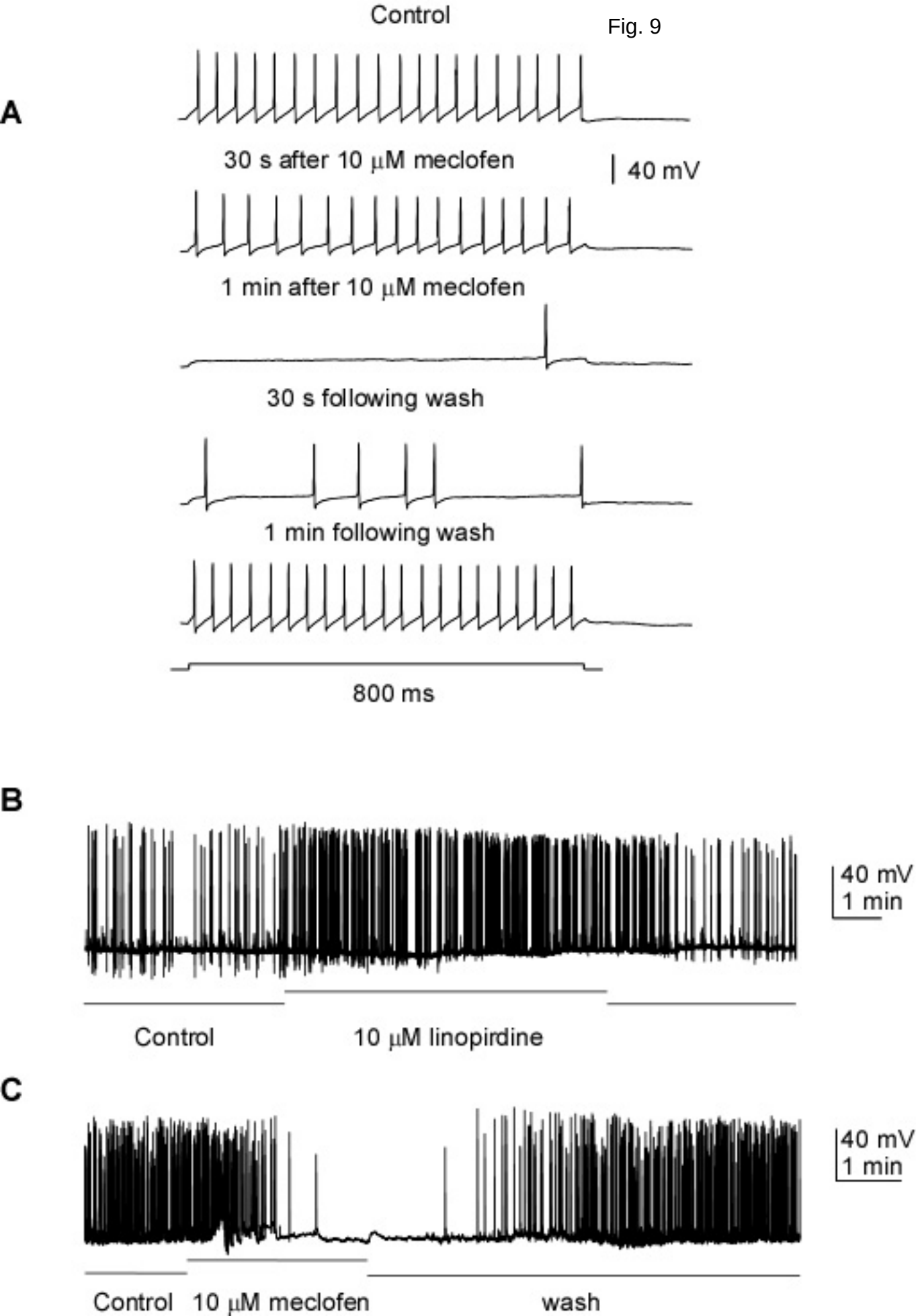
A
Control
30 s after 10 μM meclofen
40 mV
1 min after 10 μM meclofen
30 s following wash
1 min following wash
800 ms
B
40 mV
1 min
Control
10 μM linopirdine
C
40 mV
1 min
Control
10 μM meclofen
wash

Fig.10