Modulation of KCNQ2/3 Potassium Channels by the Novel Anticonvulsant Retigabine

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ABSTRACT

Retigabine is a novel anticonvulsant with an unknown mechanism of action. It has recently been reported that retigabine modulates a potassium channel current in nerve growth factor-differentiated PC12 cells (Rundfeldt, 1999), however, to date the molecular correlate of this current has not been identified. In the present study we have examined the effects of retigabine on recombinant human KCNQ2 and KCNQ3 potassium channels, expressed either alone or in combination in Xenopus oocytes. Application of 10 μM retigabine to oocytes expressing the KCNQ2/3 heteromeric channel shifted both the activation threshold and voltage for half-activation by approximately 20 mV in the hyperpolarizing direction, leading to an increase in current amplitude at test potentials between −80 mV and +20 mV. Retigabine also had a marked effect on KCNQ current kinetics, increasing the rate of channel activation but slowing deactivation at a given test potential. Similar effects of retigabine were observed in oocytes expressing KCNQ2 alone, suggesting that KCNQ2 may be the molecular target of retigabine. Membrane potential recordings in oocytes expressing the KCNQ2/3 heteromeric channel showed that application of retigabine leads to a concentration-dependent hyperpolarization of the oocyte, from a resting potential of −63 mV under control conditions to −85 mV in the presence of 100 μM retigabine (IC50 = 5.2 μM). In control experiments retigabine had no effect on either resting membrane potential or endogenous oocyte membrane currents. In conclusion, we have shown that retigabine acts as a KCNQ potassium channel opener. Because the heteromeric KCNQ2/3 channel has recently been reported to underlie the M-current, it is likely that M-current modulation can explain the anticonvulsant actions of retigabine in animal models of epilepsy.

Retigabine (D-23129; N-(2-amino-4-(4-fluorobenzylamino)-phenyl) carbamic acid ethyl ester) (see Fig. 1D) is a novel anticonvulsant compound that is currently undergoing clinical trials for the treatment of epilepsy (Bialer et al., 1999). Experiments in vivo have shown that retigabine is effective in a broad range of epilepsy and seizure models (Tober et al., 1994b; Rostock et al., 1996) as well as in genetic models of epilepsy (Tober et al., 1994a, 1996; Dailey et al., 1995). Retigabine has been reported to act through several mechanisms, which may underlie its anticonvulsant activity: first, retigabine has been shown to be effective in a broad range of epilepsy and seizure models (Tober et al., 1994b; Rostock et al., 1996) as well as in genetic models of epilepsy (Tober et al., 1994a, 1996; Dailey et al., 1995). Retigabine has been reported to act through several mechanisms, which may underlie its anticonvulsant activity: first, retigabine has been shown to augment GABA-activated currents in cultured neuronal cells (Rundfeldt et al., 1995), possibly through a stimulatory effect on GABA synthesis (Kapetanovic et al., 1995). Second, retigabine blocks sodium channels at high concentrations (Rundfeldt et al., 1995). Finally, retigabine has been shown to activate a potassium current in nerve growth factor-treated PC12 cells and in rat cortical neurons (Rundfeldt, 1999). Preliminary pharmacological characterization showed that the retigabine-activated potassium current is blocked by Ba2+, but is unaffected by high concentrations of 4-aminopyridine (10 mM), a blocker of Kv1, Kv2 and Kv3 channels; and is only weakly inhibited by tetraethylammonium (TEA) (62% at 10 mM). Retigabine-activated currents were recorded at a holding potential of −40 mV, suggesting that retigabine acts on a channel that is activated, but not inactivated, at this membrane potential. On review of these data, we hypothesized that retigabine may be an activator of the M-current, which is a noninactivating potassium current characterized by a relatively negative activation threshold, and which is blocked by Ba2+ and TEA (approximate IC50 = 10 mM), but is insensitive to 4-aminopyridine (Selyanko et al., 1999).

ABBREVIATIONS: TEA, tetraethylammonium; CHO, Chinese hamster ovary; GABA, γ-aminobutyric acid.
The M-current is a low threshold, slowly activating potassium conductance that was first recorded in rat sympathetic ganglia (Brown and Adams, 1980) and has subsequently been identified in a variety of neuronal and nonneuronal cells. It was reported in a recent publication that a combination of two cloned potassium channels—KCNQ2 and KCNQ3—showed virtually identical biophysical and pharmacological properties to M-current following coexpression in *Xenopus* oocytes (Wang et al., 1998). Similarities between the KCNQ2/3 heteromeric channel and M-current include biophysical properties, inhibition of KCNQ2/3 current following muscarinic receptor activation, sensitivity to TEA and Ba$^{2+}$, and pharmacological block by the selective compound linopirdine (Wang et al., 1998). A novel member of the KCNQ family, KCNQ4, has recently been cloned, which shares several biophysical and pharmacological properties of other family members, including heteromerization with KCNQ3 (Kubisch et al., 1999) and inhibition via M1 muscarinic acetylcholine receptors (Selyanko et al., 2000). It is therefore likely that KCNQ3/4 is responsible for M-current in at least some neuronal tissues.

In the present study, we have examined the effects of retigabine on KCNQ2 and KCNQ3 potassium channels recombinantly expressed in *Xenopus* oocytes and Chinese hamster ovary (CHO) cells. Our data demonstrate that retigabine is a potent KCNQ channel opener. These results provide a molecular mechanism that can explain the anticonvulsant actions of retigabine in in vivo animal models.

**Fig. 1.** Expression of KCNQ2 and KCNQ3 potassium channels in oocytes. A, typical whole-cell currents recorded in an oocyte expressing KCNQ2 (left panel), KCNQ3 (middle panel), and KCNQ2 plus KCNQ3 (right panel). Currents recorded from an oocyte not injected with RNA resembled those for KCNQ3 alone. B, mean peak outward current amplitude recorded at a test potential of +20 mV. K2 denotes KCNQ2. C, representative current-voltage curves plotted for an oocyte expressing KCNQ2 (●) and KCNQ2/3 (○). D, structure of retigabine (D-23129; N-(2-amino-4-(4-flurobenzylamino)phenyl) carbamic acid ethyl ester.)
Materials and Methods

Chemistry. Retigabine [D-23129; N-(2-amino-4-(4-fluorobenzylamino)phenyl) carbamic acid ethyl ester or 2-amino-4-(4-fluorobenzylamino)-1-ethoxycarboxyaminobenzene] as its dihydrochloride salt was prepared in a four-stage process from 2-nitro-4-aminooaniline and 4-fluorobenzaldehyde according to the method outlined in US patent 5,384,330 (Deiter et al., 1995).

Molecular Biology and Stable Cell Line Construction. Oligonucleotide primers were designed to the published KCNQ2 (accession number AF033348) and KCNQ3 (accession numbers AF033347 and AF071478) sequences to amplify the complete coding sequence of each gene. A Kozak sequence (GCGGCCACC) was included immediately 5' to the initiating ATG codon to maximize expression levels (Kozak, 1989). Human total brain RNA was reverse transcribed using the Superscript protocol (Life Technologies Inc., Paisley, UK). KCNQ2 and KCNQ3 open reading frames were then amplified using the Advantage GC melt kit (CLONTECH, Basingstoke, UK). Amplified fragments were cloned using the TOPO kit (Invitrogen, Groningen, The Netherlands), and sequence errors were corrected using the Quickchange protocol (Stratagene, Amsterdam, The Netherlands). The KCNQ2 and KCNQ3 expression cassettes were then subcloned into the oocyte expression vector pSP64t (Kreig and Melton, 1984). The KCNQ2 expression cassette was also cloned into the mammalian expression vector pCIN3 (Rees et al., 1996), encoding neomycin resistance, whereas the KCNQ3 expression cassette was subcloned into the mammalian expression vector pCIH5 (Rees et al., 1996), encoding hygromycin resistance.

To generate CHO cell lines stably coexpressing KCNQ2 and KCNQ3, 2 μg of pCIN3/KCNQ2 and 2 μg of pCIH5/KCNQ3 were mixed and electroporated into 10^7 CHO cells. Cells were incubated for 48 h in nonselective media [Dulbecco's modified Eagle's medium plus 10% fetal bovine serum; 2 mM L-glutamine and 100 μg/ml gentamycin] was then added, and the cells cultured for 3 to 4 weeks. Resistant clones were ring cloned and expanded in selective media. Clonal cell lines were screened for functional expression using patch clamp. Cells were then propagated in media containing reduced concentrations of selection compounds (0.4 mg/ml neomycin and 0.2 mg/ml hygromycin).

Oocyte Expression and Electrophysiology. Adult female X. laevis (Blades Biologicals, Ebenbridge, UK) were anesthetized using 0.2% Tricaine (3-aminobenzoic acid ethyl ester), sacrificed, and the oocytes rapidly removed. Oocytes were defolliculated by collagenase (Sigma type I, 1.5 mg ml^-1) in divalent cation-free OR2 solution (in mM): 82.5 NaCl, 2.5 KCl, 1.2 NaH2PO4, 5 HEPES, pH 7.5, at 25°C. Single stage V and VI oocytes were then transferred to medium plus 10% fetal bovine serum; 2 mM L-glutamine and 100 μg/ml gentamycin and 2 μM hygromycin. When filled and the pipette-filling solution contained (in mM): 130 KCl, 3 NaCl, 1 MgCl2, 1 CaCl2, 11 glucose, 5 HEPES (pH 7.4). Patch electrodes had resistances of 2 to 6 MΩ when filled and the pipette-filling solution contained (in mM): 130 KCl, 3 NaCl, 1 MgCl2, 5 potassium + EGTA, 10 HEPES, 5 glucose, 3 Mg-ATP, pH 7.3. Currents were recorded at room temperature using an Axopatch 200B amplifier (Axon Instruments Inc.). Retigabine was applied by addition to the superfusate.

Data Analysis. All data are quoted as mean ± S.E.M. All data comparisons between control and retigabine data were analyzed using a paired Student's t test, and differences were significant at the P < .05 level. Curve fitting was carried out using pClamp (Axon Instruments Inc.) and Origin (MicroCal Inc., Northampton, MA) software.

Threshold for KCNQ current activation was calculated as follows. For each individual current trace, a cursor was placed visually through the data at the holding potential of ~100 mV. Measurements of current were then made at the end of the test pulse, and KCNQ current amplitude was calculated as (current at end of test pulse minus baseline value at ~100 mV). In an attempt to generate an accurate value for the threshold of KCNQ2/3 channel activation, a series of pulses were applied to test potentials between -90 and -42.5 mV at 2.5 mV increments (see Fig. 2B). Measurements of KCNQ current amplitude were taken at each test potential (as described above) and “threshold voltage” was taken as the test potential in which KCNQ current was first recorded.

In a number of experiments the “delay” in KCNQ2/3 current activation was quantified as follows. Exponential curves were fitted to the current data between the end of the test pulse (defined as 100%) and the point at which current amplitude reached 20% of this value. The exponential curve was then extrapolated to the point at which it intersected with the current = zero value. Delay in KCNQ activation was then measured as the difference in time between the start of the voltage-clamp pulse and the point at which the extrapolated curve intersects with the zero current value.

Results

Voltage-clamp recordings were made from oocytes injected with RNA encoding the KCNQ2 and KCNQ3 potassium channels, both alone and in combination. Figure 1A shows representative current traces recorded from an oocyte expressing KCNQ2 alone (left panel), KCNQ3 alone (middle panel), and a combination of KCNQ2 and KCNQ3 (KCNQ2/3, right panel) during a voltage-clamp pulse from the holding potential of ~100 mV to test potentials between ~90 mV and +30 mV. As reported previously by other groups (e.g., Wang et al., 1998), large slowly activating outward currents were recorded in oocytes expressing KCNQ2 alone or KCNQ2/3. These currents did not show any inactivation during the 700-ms voltage-clamp pulse. Oocytes expressing the KCNQ3 channel in isolation showed a current-voltage relationship indistinguishable from that seen in uninjected oocytes. Thus, peak current at +20 mV was 125 ± 43 nA in uninjected oocytes (n = 3) and 123 ± 13 nA in oocytes expressing KCNQ3 (n = 4; Fig. 1B). A similar lack of functional expression of KCNQ3 has been reported previously (Wang et al., 1998), although other groups have shown that KCNQ3 can form a functional channel following expression in oocytes.
Effects of retigabine on the KCNQ2/3 current-voltage relationship. A, current-voltage (I-V) curve for a typical oocyte expressing KCNQ2/3 in control conditions (●) and in the presence of 10 μM retigabine (○). Note the augmentation of KCNQ2/3 current at all test potentials. B, I-V curve from a data shown is mean ± S.E.M for five oocytes. In each oocyte, whole-cell conductance was calculated at each test potential and a sigmoidal (Boltzmann) curve was fitted to this data (y max constrained to zero). Conductance data were then normalized to y max for each individual cell, and mean normalized data are shown. Mean values for peak conductance taken from the individual Boltzmann fits were 93.5 ± 17.8 μS in control, and 91.2 ± 19.9 μS in the presence of 10 μM retigabine.

Fig. 2. Effects of retigabine on the KCNQ2/3 current-voltage relationship. A, current-voltage (I-V) curve for a typical oocyte expressing KCNQ2/3 in control conditions (●) and in the presence of 10 μM retigabine (○). Note the augmentation of KCNQ2/3 current at all test potentials. B, I-V curve from a different oocyte demonstrating the marked shift in threshold for KCNQ2/3 activation following addition of 10 μM retigabine (○). C, conductance curve for KCNQ2/3 in control (●) and in the presence of 10 μM retigabine (○). The data shown is mean ± S.E.M for five oocytes. In each oocyte, whole-cell conductance was calculated at each test potential and a sigmoidal (Boltzmann) curve was fitted to this data (y max constrained to zero). Conductance data were then normalized to y max for each individual cell, and mean normalized data are shown. Mean values for peak conductance taken from the individual Boltzmann fits were 93.5 ± 17.8 μS in control, and 91.2 ± 19.9 μS in the presence of 10 μM retigabine.
ing a 600-ms test pulse from −100 to −55 mV under control conditions, and in the presence of 100 nM, 1 μM, and 10 μM retigabine (construction of a full concentration-response curve in this oocyte gave an EC_{50} of 3.6 μM). It is notable that in addition to a marked concentration-dependent increase in current amplitude (10 μM retigabine increased KCNQ current amplitude 2.9-fold, from 1583 nA to 4655 nA), addition of retigabine also altered the kinetics of current activation.

Under control conditions KCNQ2/3 activation appeared to follow a somewhat sigmoidal time course, with a notable delay in current activation following depolarization to −55 mV. In contrast, in the presence of retigabine KCNQ2/3 current activation was rapid, with little or no delay following depolarization. These changes in current kinetics are shown more clearly in panel B (data taken from a different oocyte at a test potential of −40 mV). Figure 3B (left panel) shows sample data and Fig. 3B (right panel) shows the same data-set normalized to the peak current amplitude in the presence of retigabine. It was not possible to fully describe KCNQ2/3 current activation kinetics using either a standard exponential curve (1, 2, or 3 terms) or an exponential power function. We have therefore quantified KCNQ2/3 activation in two ways. First, we have measured the delay in KCNQ2/3 current activation following depolarization to the test potential. Details of these calculations are provided under Materials and Methods. Second, we have measured time-to-half-maximal current activation, where “maximal activation” is taken as KCNQ2/3 current amplitude at the end of the voltage-clamp pulse. The results of these analyses are as follows. At a test potential of −40 mV, the delay in KCNQ2/3 current activation was 97.5 ± 15.6 ms in control conditions and 60.5 ± 11.8 ms in the presence of 10 μM retigabine (n = 4). At the same test potential, time-to-half-maximal activation was 316.6 ± 27.9 ms in controls and 173.6 ± 32.4 ms in the presence of retigabine (n = 5). Thus, using either of these measures of current activation, there is a marked increase in the rate of KCNQ2/3 current activation following application of retigabine.

Calculations of the delay in KCNQ2/3 current activation were made at a range of test potentials and the results of these analyses are shown in Fig. 3C, where delay in current activation is plotted against test potential. In both control conditions and in the presence of retigabine the delay in current activation showed significant voltage dependence, with the duration of the delay decreasing at more positive potentials. Addition of retigabine shifted the relationship between delay and voltage to the left, such that at a given test potential the delay in current activation is reduced compared with that under control conditions (Fig. 3C). Interestingly, the amplitude of this shift in the relationship between delay and voltage was approximately 20 to 25 mV, which is very similar to the shift in voltage dependence of KCNQ2/3 activation, which we have calculated in our experiments (shown in Fig. 2C).

A series of experiments were carried out to examine whether retigabine also modulates KCNQ2/3 channel deactivation, which is the transition from open to closed state. Figure 4A shows sample data from an oocyte expressing KCNQ2/3. In these experiments the voltage-clamp protocol comprised a 1-s prepulse from −100 mV to +40 mV to fully activate the KCNQ2/3 channel (as demonstrated in Fig. 2C), followed by a 6-s pulse to various test potentials between −30 and −110 mV. As shown in Fig. 4A (left panel), under control conditions a large KCNQ current was recorded during the prepulse, with a series of tail currents recorded following repolarization to the test potential. At each test potential a clear deactivation of the tail current (seen as a decrease in current amplitude with time) was recorded during the 6-s test pulse (Fig. 4A, left panel). Application of 10 μM retigabine led to a marked alteration in the properties of the
KCNQ2/3 tail currents. Thus, at the most positive test potentials (−30 and −40 mV) a steady-state KCNQ2/3 tail current was recorded during the test pulse, suggesting that channel deactivation does not occur at these potentials (Fig. 4A, right panel). With subsequent pulses to less positive potentials (−50 to −110 mV) channel deactivation was seen, however, the rate of deactivation appeared to be significantly slowed in comparison to control current data. These results suggest that in the presence of retigabine, the voltage dependence of channel deactivation is shifted to more hyperpolarized membrane potentials. Physiologically, these effects of retigabine are highly significant, because a hyperpolarizing shift in the voltage dependence of both KCNQ2/3 channel activation (Fig. 2B) and channel deactivation provides a mechanism whereby sustained KCNQ2/3 currents will contribute to cellular excitability over a relatively negative range of membrane potentials.

Analysis of the extremely slow deactivation of KCNQ2/3 at more positive test potentials in the presence of retigabine posed a number of practical experimental problems. Therefore, in an attempt to quantify the changes in KCNQ2/3 deactivation recorded in the presence of retigabine, further experiments were carried out recording tail currents at more hyperpolarized membrane potentials and during shorter test pulses (Fig. 4B). In these experiments, the voltage-clamp protocol comprised a 200-ms prepulse from −100 mV to +50 mV, followed by a 150-ms test pulse to various potentials between −60 and −130 mV. As with the longer voltage-clamp pulses shown in Fig. 4A, a clear slowing in the rate of decline of the KCNQ2/3 tail current was recorded over a range of potentials. This effect is shown more clearly in Fig. 4C where data at a test potential of −130 mV have been normalized to the peak tail current amplitude. To quantify these changes in KCNQ2/3 deactivation, exponential curves were fitted to the KCNQ2/3 tail currents in control conditions and following application of 10 μM retigabine. As shown in Fig. 4C, retigabine decreased the rate of channel deactivation at all test potentials between −110 and −130 mV. Thus, at −130 mV the mean rate of deactivation (τ deactivation) was 34.9 ± 3.3 ms under control conditions and 90.1 ± 10.6 ms in the presence of retigabine (n = 5).

To examine whether KCNQ2 or KCNQ3 is the molecular target for retigabine, a number of experiments were carried out in oocytes expressing KCNQ2 alone. As shown in Fig. 5, retigabine had a qualitatively similar action on KCNQ2 as that seen with the KCNQ2/3 heteromeric channel. Thus 10 μM retigabine shifted the threshold for current activation (from approximately −60 to −80 mV, Fig. 5C) increased KCNQ current amplitude over a range of test potentials (control = 145 ± 34 nA; retigabine = 501 ± 116 nA at −50

**Fig. 4.** Effects of retigabine on channel deactivation. A, currents recorded during a voltage-clamp protocol comprising a 1-s prepulse to +40 mV, followed by a 6-s test pulse to potentials between −30 and −110 mV. A family of KCNQ2/3 tail currents was recorded during the test pulse. Application of 10 μM retigabine (right panel) led to a shift in the voltage dependence of channel deactivation and to a slowing in the rate of decline of the KCNQ2/3 tail current. Note that in the presence of retigabine, currents show no deactivation during a test pulse to either −30 or −40 mV. B, KCNQ2/3 tail currents recorded during a 150-ms test pulse to potentials between −60 and −130 mV. Each test pulse was preceded by a voltage step to +50 mV to fully activate the KCNQ2/3 channels. Note the slowed deactivation in the presence of retigabine. C, normalized tail currents at a test potential of −130 mV; D, mean data showing the effects of retigabine on the rate of channel deactivation.

○, control; ○, 10 μM retigabine. Tau (τ) deactivation was calculated by fitting a single exponential curve to KCNQ2/3 tail currents generated using similar protocols to that described in panel B.
mV, n = 7, Fig. 5, A and C) and increased the rate of current activation (Fig. 5, A and B; mean delay at −40 mV: control = 132 ± 16 ms; retigabine = 75 ± 7 ms). Interestingly, a reduction in current amplitude was consistently recorded at positive test potentials in the presence of retigabine (Fig. 5C). For example, at +20 mV mean KCNQ2 current was 2955 ± 418 nA under control conditions and 2303 ± 322 nA in the presence of 10 μM retigabine (n = 7; conductance values were 26.9 ± 3.8 μS and 20.9 ± 2.9 μS, respectively). This inhibitory effect of retigabine was not seen in oocytes co-expressing the combination of KCNQ2 and KCNQ3 (see Fig. 2), and it is unclear whether this observation reflects a difference in mechanism of retigabine action between KCNQ family members. Although it was not possible to study the effects of retigabine on the KCNQ3 channel in isolation (the KCNQ3 channel gave no functional expression in the absence of KCNQ2, see Fig. 1), our data suggest that at least a part of retigabine’s actions on the KCNQ2/3 heteromer occurs through an interaction with the KCNQ2 channel.

It was observed that in oocytes expressing KCNQ2/3 the resting membrane potential was significantly hyperpolarized compared with that in control (uninjected) oocytes (mean resting $E_{m}$: control = −30 to −40 mV; KCNQ2/3 = −62 ± 2 mV). A number of experiments were therefore carried out to look at the effects of retigabine, which we have shown to be a KCNQ2/3 channel opener, on membrane potential. As shown in Fig. 6A application of retigabine led to a concentration-dependent hyperpolarization of the oocyte from a control value of −63 mV, to −85 mV in the presence of 100 μM retigabine. It is interesting to note that the measured values of resting membrane potential lie close to the activation threshold for KCNQ2/3, both under control conditions and in the presence of 10 μM retigabine (−81.5 mV in this cell, see Fig. 2B for activation thresholds plus or minus retigabine). These data support the hypothesis that M-current (KCNQ2/3) plays a key role in setting the resting potential of a cell. Figure 6B shows mean concentration-response data from four oocytes. A mean IC$_{50}$ of 5.3 μM (95% confidence interval 5.0–5.6 μM; n = 4 cells) was observed.

Fig. 5. Effects of retigabine on the KCNQ2 potassium channel. A, sample current traces are shown from an oocyte expressing KCNQ2 in isolation. The test potential is −50 mV, and currents are shown under control conditions and following application of 10 μM retigabine. B, normalized data showing the increase in rate of KCNQ2 activation in the presence of retigabine; C, mean current-voltage curve for KCNQ2 in control conditions (●) and in the presence of retigabine (○). Data are normalized to peak KCNQ2 current under control conditions at a test potential of +20 mV for each cell. Mean data are shown and the error bars lie within the data point. Note the shift in threshold for channel activation, and the augmented KCNQ current at test potentials between −80 and −20 mV.

Fig. 6. Effects of retigabine on resting membrane potential. Membrane potential recordings were made in an oocyte expressing KCNQ2/3. Application of retigabine led to concentration-dependent hyperpolarization of the oocyte. A, sample trace; B, mean concentration-response curve. Retigabine concentration is plotted on a logarithmic axis. Mean IC$_{50}$ was 5.2 ± 1.3 μM and mean slope was 1.1 ± 0.1.
intervals = 4.0 to 6.6 μM) was calculated, which is close to the value of between 1 and 5 μM reported for retigabine activation of a native potassium current in PC12 cells (Rundfeldt, 1999).

All the experiments described above were carried out in *Xenopus* oocytes expressing KCNQ2 or KCNQ2/3. However, oocytes have been shown to express endogenous KCNQ channels (Barhanin et al., 1996), which may interact functionally with our cloned channels. We have therefore examined the effects of retigabine in an alternative expression system to rule out any oocyte-specific actions of retigabine. Figure 7 shows the effects of retigabine on KCNQ2/3 current in a KCNQ2/3 CHO stable cell line generated in our laboratory. It has previously been reported that CHO cells provide a null background for voltage-gated potassium channels (Yu and Kerchner, 1998), and in our experiments we have seen no evidence of delayed rectifier type currents in native CHO cells. Whole-cell patch clamp recordings revealed that the KCNQ2/3 CHO stable cell line expresses potassium channel currents with qualitatively similar biophysics to those seen in oocytes (Fig. 7, A and C). The effects of retigabine on KCNQ2/3 in this cell line were also similar to those recorded in oocytes. Thus, application of 10 μM retigabine shifted the threshold for channel activation from approximately −50 to −70 mV and led to a marked increase in current amplitude over a range of test potentials (Fig. 7C). However, a significant difference in the effects of retigabine at positive test potentials was seen between oocytes and the CHO KCNQ2/3 stable cell line. Thus, whereas in oocytes retigabine had little effect on KCNQ current/conductance at +20 mV (Fig. 2C), a significant increase in KCNQ2/3 current was recorded in CHO cells (control current = 2287 ± 869 pA; retigabine = 3070 ± 1366 pA, n = 5; Fig. 7C). The mechanism underlying these differences is unclear, although there are many examples in the literature of ion channels behaving differently in oocytes and mammalian expression systems. Finally, as observed in oocytes, application of 10 μM retigabine to CHO KCNQ2/3 cells led to approximately a 20-mV hyperpolarization of the cell’s resting membrane potential (data not shown).

**Discussion**

In this study we have demonstrated that the anticonvulsant compound retigabine is an opener of KCNQ potassium channels. The effects of retigabine on the KCNQ2/3 channel are 3-fold: retigabine shifts the voltage dependence of channel activation to more hyperpolarized membrane potentials, increases the rate of channel activation, and slows channel deactivation. Although further experiments are clearly required to fully elucidate the mechanism of action of retigabine, our results suggest that retigabine shifts the equilibrium between the open and closed states of the KCNQ channel such that an increased number of channels lie in the open state at any given membrane potential. Retigabine could act to stabilize the KCNQ2/3 channel in the open conformational state, or alternatively it could modify the channel-gating mechanism such that the voltage dependence of S4 voltage-sensor movement is shifted in the hyperpolarizing direction. With reference to the latter point, it was noticeable that the voltage dependence of both channel activation and deactivation appeared to be left-shifted by approximately 20 mV, as was the voltage dependence of the delay in current activation. A third possibility is that there could be a charge screening effect whereby the voltage-field sensed by the KCNQ channel during a voltage-clamp test pulse is altered in the presence of retigabine.

A number of striking similarities can be drawn between the effects of retigabine on the KCNQ2/3 heteromeric channel, and the effects of the benzodiazepine compound L-364,373 on the KCNQ1 channel (Salata et al., 1998). These authors studied the effects of L-364,373 on native $I_{Ks}$ current in isolated guinea pig ventricular myocytes, as well as on the molecular correlate of $I_{Ks}$, KCNQ1, in *Xenopus* oocytes. L-364,373 shifted the voltage dependence of channel activation to the left by 25 mV, increased the rate of channel activation, and markedly slowed the rate of channel deactivation. It therefore appears that there may be a common mechanism of action for KCNQ channel openers from diverse chemical series. Similarities can also be seen between the effects of retigabine on the KCNQ2/3 channel, and those of other channel openers on voltage-gated calcium and sodium channels. Thus, Bay K 8644 has been shown to shift the threshold of L-type calcium channel activation to more hyperpolarized potentials, to increase the rate of channel activation, and to slow deactivation (McDonald et al., 1994). Similarly, in *Xenopus* oocytes expressing the rat skeletal muscle sodium channel, β-scorpion venom has been shown to augment sodium channel current through a hyperpolarizing shift in the voltage dependence of activation (Tsushima et al., 1999).

In addition to characterizing the effects of retigabine on KCNQ channel function under voltage-clamp, we also mea-
ured membrane potential in oocytes expressing KCNQ2/3 and demonstrated that application of retigabine leads to a hyperpolarization. This result highlights the key role which KCNQ2/3 channels (and therefore M-current) play in setting the resting membrane potential. KCNQ channels have a relatively negative threshold for channel activation (~60 to −65 mV in our experiments), therefore small depolarizations from the resting potential will lead to KCNQ activation and a reduction in cellular excitability. Furthermore, because KCNQ channels are nonactivating, they contribute a significant, steady-state potassium conductance at all membrane potentials positive to the threshold for activation and can therefore act as a brake on action potential firing. Two recent papers highlight the importance of M-current in controlling cellular excitability. In a series of intracellular recordings from rat sympathetic neurones, Wang and McKinnon (1995) found that neuronal firing pattern shows a marked dependence on M-current expression. Thus, the M-current was present in all phasic neurones (i.e., neurones that respond to a stimulus with a single action potential) but was weak or absent in tonic neurones (which respond with a train of action potentials). More recently, Wang and coworkers (Wang et al., 1998) demonstrated that application of XE991, which is a selective blocker of the M-current and KCNQ2/3, can convert the firing pattern of a rat sympathetic neurone from phasic to tonic. Taken together, these results suggest that pharmacological modulation of the KCNQ2/3 potassium channel is likely to have a profound effect on cellular excitability in vivo.

It has been reported that retigabine has a diverse set of actions against a number of systems. Thus, retigabine has been reported to have effects on GABAergic pathways, where it increases de novo GABA synthesis in hippocampal slices (Kapetanovic et al., 1995) and potentiates GABA-induced currents in cultured neuronal cells (Rundfeldt et al., 1995). At higher concentrations, retigabine has been reported to block voltage-gated sodium and calcium channels (Rundfeldt et al., 1995). In the present study, we have shown that retigabine activates the KCNQ2/3 channel over the concentration range 0.3 to 100 μM. This concentration range is comparable to that reported previously for retigabine activation of an undefined potassium channel in PC12 cells (1 to 5 μM; Rundfeldt, 1999), and is also similar to the values quoted for retigabine-induced GABA release in hippocampal slices (5 to 40 μM; Kapetanovic et al., 1995). The estimated effective plasma concentration of retigabine in animal models of epilepsy is between 0.1 and 3 μM (Jainta et al., 1995; Rostock et al., 1996; Tober et al., 1996), therefore it is possible that the anticonvulsant activity of retigabine could occur through an activation of M-current (KCNQ2/3), an augmentation of GABA release, or a combination of the two mechanisms.

It is now established that the heteromeric KCNQ2/3 potassium channel forms a molecular basis for the M-current (Wang et al., 1998; Selyanko et al., 1999), although more recently the Erγ1 potassium channel subunit has also been linked to M-current recorded in NG108-15 cells (Selyanko et al., 1999). Openers of the KCNQ2/3 channel may therefore provide a good target for the treatment of disorders of hyperexcitability such as epilepsy. There is also genetic evidence linking KCNQ channels to epilepsy. Mutations in both KCNQ2 and KCNQ3 have been linked to a rare form of epilepsy known as benign neonatal familial convulsions (Biervert et al., 1998; Charlier et al., 1998; Singh et al., 1998). These findings suggest that KCNQ channels may also play a role in more common forms of the disease. In the present study, by demonstrating that retigabine is an opener of KCNQ2/3 channels, we have made another link between KCNQ potassium channels and epilepsy. This provides validation for KCNQ2/3 channel openers as a new mechanism for the rational development of antiepileptic drugs.

References


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