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SPECIAL REPORT

Characterization of KCNQ5/Q3 potassium channels expressed in mammalian cells

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Heteromeric KCNQ5/Q3 channels were stably expressed in Chinese Hamster ovary cells and characterized using the whole cell voltage-clamp technique. KCNQ5/Q3 channels were activated by the novel anticonvulsant, retigabine (EC_{50} 1.4 μM) by a mechanism that involved drug-induced, leftward shifts in the voltage-dependence of channel activation (−31.8 mV by 30 μM retigabine). KCNQ5/Q3 channels were inhibited by linopirdine (IC_{50} 7.7 μM) and barium (IC_{50} 0.46 mM), at concentrations similar to those required to inhibit native M-currents. These findings identify KCNQ5/Q3 channels as a molecular target for retigabine and raise the possibility that activation of KCNQ5/Q3 channels may be responsible for some of the anti-convulsant activity of this agent. Furthermore, the sensitivity of KCNQ5/Q3 channels to linopirdine supports the possibility that potassium channels comprised of KCNQ5 and KCNQ3 may make a contribution to native M-currents.

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Introduction The M-current is a slowly activating, slowly deactivating potassium current that plays an important role in controlling neuronal excitability. KCNQ2/KCNQ3 potassium channels share similar biophysical and pharmacological properties and exhibit similar expression patterns to native M-currents, observations that had led to the suggestion that KCNQ2/Q3 potassium channels may represent the molecular correlates of M-currents (Wang *et al*., 1998). Whether KCNQ2/Q3 channels are the sole contributors to the M-current however, is unclear. Recently two additional members of the KCNQ family, KCNQ4 and KCNQ5, have been cloned (Kubisch *et al*., 1999; Lerche *et al*., 2000; Schroeder *et al*., 2000). KCNQ4 shows restricted expression (Kharkovets *et al*., 2000) and, as such, seems unlikely to be a major contributor to M-currents in most brain regions. KCNQ5 however, shows widespread expression in the CNS, with many regions showing overlapping expression of KCNQ2, KCNQ3 and KCNQ5 (Lerche *et al*., 2000; Schroeder *et al*., 2000). These findings, along with the observation that KCNQ5 and KCNQ3 form functional heteromeric channels in *Xenopus* oocytes (Lerche *et al*., 2000; Schroeder *et al*., 2000), raise the possibility that heterotetramers of KCNQ5 and KCNQ3 may make a significant contribution to the M-current in many brain regions (Jentsch, 2000). In the present study, we have characterized heteromeric KCNQ5/Q3 channels expressed in a mammalian cell line. We report that these channels are potently activated by the novel anticonvulsant, retigabine (Rostock *et al*., 1996), raising the possibility that some of the anticonvulsant activity of this agent may be mediated by KCNQ5/Q3 channels. In addition, KCNQ5/Q3 channels are inhibited by linopirdine and barium, at similar concentrations to those required to inhibit M-currents.

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Methods *Plasmid constructs and stable expression in mammalian cells* KCNQ5 was cloned from human brain mRNA using a combination of RT–PCR and RACE PCR techniques. A complete open reading frame for KCNQ5 was assembled using overlap extension PCR and cloned in pCDNA3.1 (Invitrogen). The sequence of the KCNQ5 cDNA used in these experiments is identical to that of splice variant 1 described in Schroeder *et al*. (2000). A h-KCNQ3 expression vector was constructed as previously described (Wickenden *et al*., 2000). In order to make a KCNQ5-KCNQ3 tandem construct, the open reading frame of h-KCNQ3 was linked to the downstream h-KCNQ5 open reading frame by a nucleic acid sequence encoding the polypeptide GSQQQQQQQQQ. A Chinese Hamster ovary cell line stably expressing the h-KCNQ5/Q3 tandem construct (CHO-KCNQ5/Q3) was generated using standard techniques (see Wickenden *et al*., 2000). For electrophysiological studies, cells were removed from the culture flask by brief trypsinization and replated at low density onto glass cover slips 24–72 h prior to study.

Electrophysiological recording Cover slips containing CHO-KCNQ5/Q3 cells were placed in a bath on the stage of an inverted microscope and perfused with extracellular solution of the following composition (mM): NaCl 138, $CaCl_2$ 2, KCl 5.4, $MgCl_2$ 1, glucose 10, HEPES 10, pH 7.4 with NaOH. Pipettes were filled with an intracellular solution of the following composition (mM): KCl 140, $MgCl_2$ 2, EGTA 10, HEPES 10, K_2ATP 5, pH 7.3–7.4 with KOH, and had a resistance of 1–3 mΩ. All recordings were made at room temperature (22–24°C) using an AXOPATCH 200B amplifier and PCLAMP 6.1 software. KCNQ5/Q3 currents were measured using the whole cell configuration of the patch-clamp technique. Uncompensated series resistance was typically 2–5 MΩ and >90% series resistance compensation was routinely achieved. Current records were acquired at 2–10 KHz and filtered at 1–2 KHz. Steady state current amplitude was measured at the end of a series of 3 s

depolarizing steps (−100 to +30 mV in 10-mV increments from a holding potential of −80 mV). Whole-cell conductance (G) was calculated according to the equation $G = I/(V - E_k)$, where I is the steady-state current, V is the step potential and E_k is the reversal potential for potassium, which was calculated to be −82.9 mV. Activation curves were generated by plotting conductance against the step potential and were fitted with a Boltzmann distribution. In order to describe the time course of current deactivation, KCNQ5/Q3 tail currents, elicited by repolarizing to −100 mV following a 3 s depolarization to 0 mV, were fit with a double exponential function. Retigabine-induced currents were measured as increases in outward current at a holding potential of −40 mV. For experiments involving potassium channel blockers, h-KCNQ5/Q3 currents were elicited by depolarization to +20 mV from a holding potential of −80 mV, every 10 s. Cells were superfused with blocker until steady-state inhibition was achieved. Semi-log plots of drug concentration against effect were fit with a logistic function. The equations used for curve fitting have been described previously (Wickenden *et al*., 2000).

Drugs Retigabine was synthesized in the laboratories of ICAgen Inc. A 10-mM stock solution of retigabine in distilled water was prepared on the day of the experiment. Linopirdine (Sigma Research Biochemicals International, Natick, MA, U.S.A.) was stored at −20°C as a 10 mM stock solution in DMSO. $BaCl_2$ was prepared as a 1 M stock in distilled water. All drugs were diluted to the desired concentrations in extracellular solution (composition as described above) on the day of the experiment.

Statistics All data are expressed as mean ± standard error of the mean (s.e.mean) for $n \geq 3$ observations. Statistical significance was determined using a 2-tailed unpaired *t*-test. A $P < 0.05$ was considered significant.

Results In order to characterize KCNQ5/KCNQ3 hetero-multimeric channels, we generated an h-KCNQ5/Q3 tandem construct. In *Xenopus* oocytes, the tandem construct gave rise to potassium currents that exhibited little inward rectification at positive membrane potentials (whole cell conductance at +30 mV was 99 ± 3% of the whole cell conductance at 0 mV, $n = 6$). In this regard, currents induced by expression of the tandem construct were similar to those induced by co-expression of KCNQ5 and KCNQ3 (whole cell conductance at +30 mV was 93 ± 4% of that at 0 mV, $n = 4$) but differed from those induced by KCNQ5 alone (where whole cell conductance at +30 mV was only 78.0 ± 6% of that at 0 mV, $n = 6$; see also Lerche *et al*., 2000). In CHO-KCNQ5/Q3 cells, voltage steps positive to −60 mV elicited slowly activating, non-inactivating outward currents (Figure 1A). Similar currents were never recorded from untransfected CHO-K1 cells (see Wickenden *et al*., 2000). Activation curves were generated for a total of six control cells and the average voltage at which half-maximal channel activation occurred ($V_{\frac{1}{2}}$) was −34.8 ± 1.6 mV, with an average slope factor of 11.5 ± 1.0.

Retigabine is a novel anti-convulsant, which has previously been shown to enhance activation of KCNQ2/KCNQ3 heteromeric channels (Rundfeldt & Netzer, 2000; Main *et al*., 2000; Wickenden *et al*., 2000). In order to determine whether this compound could exert similar effects on

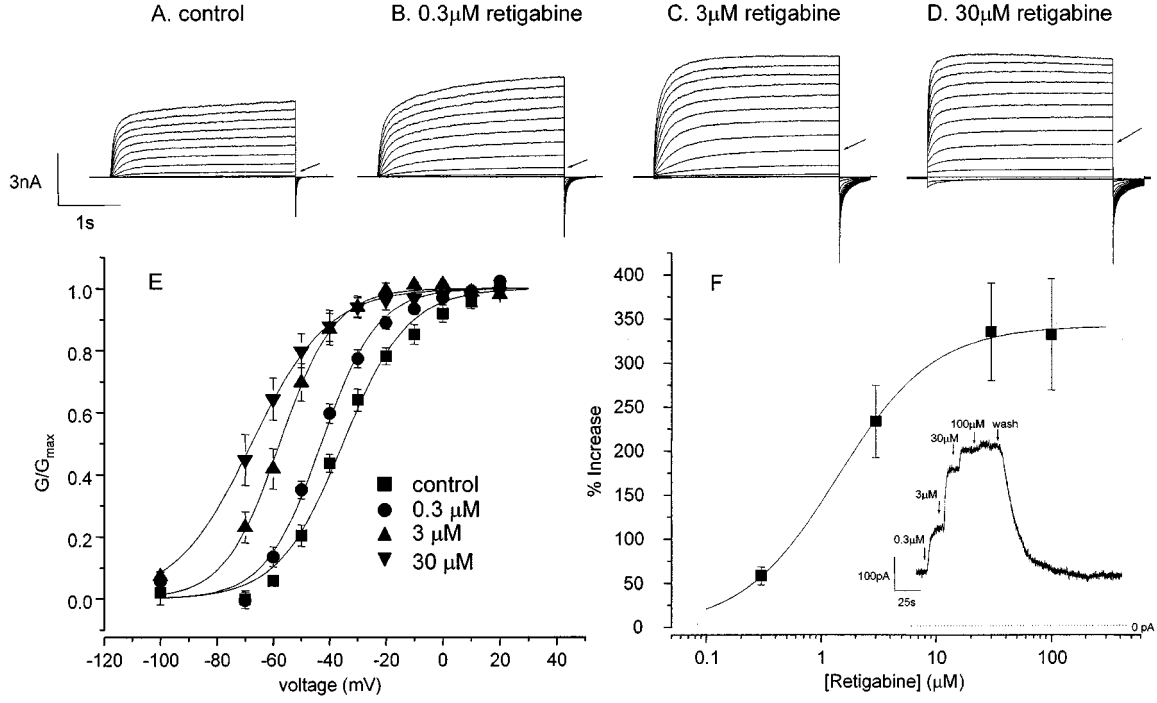

Figure 1 Representative currents from a CHO-KCNQ5Q3 cells (elicited by a series of 3-s depolarizing steps, in the range −100 to +30 mV, in 10-mV increments from a holding potential of −80 mV) in the absence (A) and presence of 0.3 μM (B), 3 μM (C) and 30 μM (D) retigabine (arrows represent −50 mV). Retigabine induced concentration dependent leftward shifts in the KCNQ5/Q3 channel activation curve (E) and increased outward current at −40 mV (F, inset). Mean percentage increases in outward current was plotted against retigabine concentration (F) and an EC_{50} value was calculated to be 1.4 ± 0.17 μM.

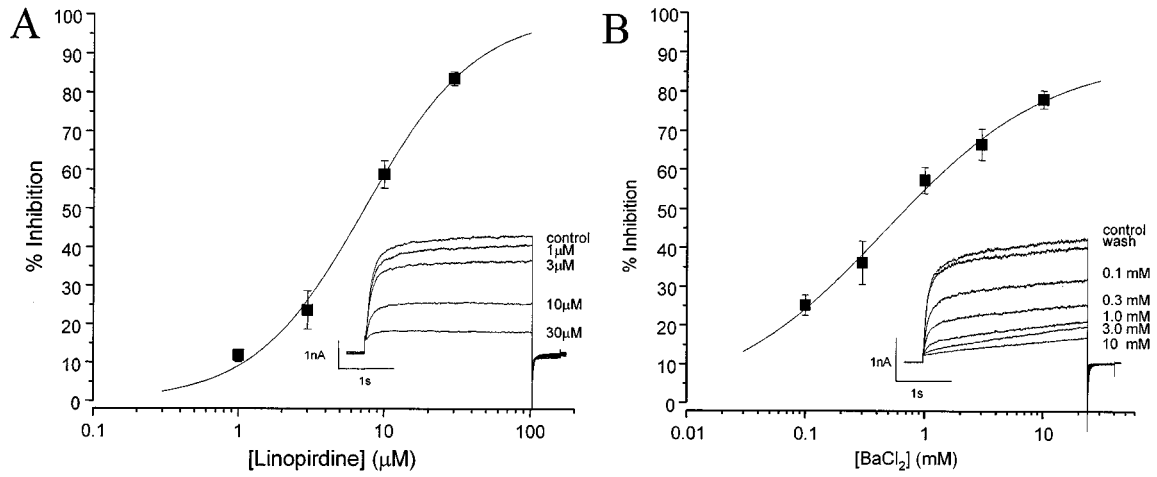

Figure 2 Representative currents from CHO-KCNQ5/Q3 (elicited by depolarization to +20 mV from a holding potential of −80 mV), in the absence or presence of linopirdine (1–30 μM) or Ba^{2+} (0.1–10 mM) are shown in the insets to (A) and (B), respectively. Mean percentage inhibition of KCNQ5/Q3 current was calculated and plotted against the linopirdine (A) or Ba^{2+} (B) concentration. The linopirdine IC_{50} value was 7.7±1.1 μM, and the Ba^{2+} IC_{50} value was 0.46±0.16 μM. The symbols represent the mean±s.e.mean of 3–5 cells.

KCNQ5/Q3, we generated families of KCNQ5/Q3 currents in the absence and presence of retigabine (0.3–30 μM). Voltage clamp records from a representative experiment are shown in Figure 1A–D. In the absence of retigabine (A), voltage steps positive to −60 mV activated an outward current, as described above. Superfusion of the same cell with retigabine (0.3–30 μM) shifted the threshold for channel activation to more negative potentials (arrows in Figure 1 represent −50 mV) and increased the amplitude of the outward current at all voltages. Retigabine induced statistically significant, concentration-dependent leftward shifts in the KCNQ5/Q3 channel activation curve as shown in Figure 1E. Half-maximal channel activation occurred at −34.8±1.6 mV ($n=6$) in control cells, and −42.9±1.3 mV ($n=4$), −56.8±2.4 mV ($n=4$) and −66.6±4.2 mV ($n=6$) mV in the presence of 0.3, 3 and 30 μM retigabine, respectively. Slope values were not significantly altered by retigabine. In addition to shifting the $V_{\frac{1}{2}}$, analysis of the conductance–voltage relationship also revealed that retigabine increased maximal whole cell conductance and slowed KCNQ5/Q3 channel deactivation. Retigabine increased maximal whole cell conductance by 21.6±4.8, 48.1±11.4 and 60.1±12.3% at 0.3, 3 and 30 μM, respectively. In the absence of retigabine, deactivation was dominated by a fast component ($\tau_{fast}=22\pm2$ ms accounting for 68±3%, $n=4$). Retigabine induced a concentration-dependent increase in the contribution of slow deactivation, from 32±3% in the absence of retigabine, to 42±5, 61±8 and 63±7% in the presence of 0.3, 3 and 30 μM retigabine, respectively.

In order to determine the retigabine EC_{50} value, CHO-KCNQ5/Q3 cells were voltage clamped at −40 mV and superfused with increasing concentrations of drug. As shown in the inset to Figure 1F, retigabine (0.3–100 μM) induced a concentration-dependent increase in outward current that was completely reversible upon removal of the drug. Retigabine-induced increases in current were never observed in wild-type CHO-K1 cells (see Wickenden *et al*., 2000). A retigabine concentration-response curve was generated by plotting the mean percentage increase in outward current from 4–6 independent experiments against retigabine concentration. From these data, the retigabine EC_{50} value was calculated to be 1.4±0.17 μM and the average slope factor was 1.04±0.11.

Linopirdine is a blocker of KCNQ2/Q3 currents (Wang *et al*., 1998). In order to determine whether linopirdine could also block KCNQ5/Q3 currents, CHO- KCNQ5/Q3 cells were repeatedly depolarized and superfused with increasing concentrations of linopirdine. As illustrated in the representative voltage clamp records shown in the inset to Figure 2A, linopirdine blocked KCNQ5/Q3 currents in a concentration-dependent manner. The effects of linopirdine were not reversible over the timecourse of the assay (5–20 min). On average the linopirdine IC_{50} value was 7.7±1.1 μM and the slope value was 1.2±0.05 ($n=3-5$ cells per concentration, Figure 2A). KCNQ2 currents are also blocked by barium ions (Jow & Wang, 2000). Therefore, we determined whether barium could inhibit KCNQ5/Q3 currents. As shown in the inset to Figure 2B, barium blocked KCNQ5/Q3 currents in a concentration-dependent and fully reversible manner. Barium-induced block was associated with an apparent slowing of KCNQ5/Q3 activation, which was particularly marked at concentrations greater than 1 mM. The IC_{50} value for barium was 0.46±0.16 mM (slope=0.64±0.11, $n=4$ cells per concentration, Figure 2B).

Discussion In the present study we show, for the first time, that KCNQ5/Q3 channels are potently activated by the novel anticonvulsant, retigabine. Retigabine-induced activation of KCNQ5/Q3 channels involved large hyperpolarizing shifts in the voltage dependence of channel activation, along with an increase in the maximal whole-cell conductance and enhanced slow channel deactivation. Retigabine has recently been shown to exert similar effects on heteromeric KCNQ2/Q3 channels and homomeric KCNQ2 channels, at concentrations similar to those shown to affect KCNQ5/Q3 channels in the present study (Rundfeldt & Netzer, 2000; Main *et al*., 2000; Wickenden *et al*., 2000). Retigabine also increases homomeric

KCNQ4 and KCNQ5 currents in *Xenopus* oocytes (Wickenden & Jegla, unpublished observations), suggesting that this compound is a non-selective activator of the KCNQ family of potassium channels. *In vivo*, retigabine exerts anticonvulsant activity in a broad range of seizure models. At doses several fold higher than those required for anticonvulsant activity, retigabine induces muscle relaxation and motor incoordination (Rostock *et al*., 1996). The exact contribution of KCNQ5/Q3 activation to any, or all, of these effects is unclear at present.

Linopirdine blocks native M-currents with an IC_{50} value in the range 1.2–36 μM (Wang *et al*., 1998; Selyanko *et al*., 1999; Aiken *et al*., 1995; Lamas *et al*., 1997; Noda *et al*., 1998). The variability in the linopirdine IC_{50} value may be indicative of heterogeneity in the molecular composition of M-currents. Linopirdine blocks KCNQ2 and KCNQ2/3 channels at similar concentrations to those required to block some M-currents, providing pharmacological evidence for a role of these channels in the generation of these native currents (Wang *et al*., 1998). In the present study we show that linopirdine also blocks KCNQ5/Q3 channels at concentrations similar to those required to block M-currents, supporting the possibility that potassium channels comprised of KCNQ5 and KCNQ3 may also contribute to native M-currents. In a previous report, the IC_{50} for linopirdine block of KCNQ3/Q5 was reported to be 15 μM (Lerche *et al*., 2000). This value is slightly higher than the IC_{50} value determined in the present study, possibly as a result of the different expression system (*Xenopus* oocytes) used in the study of Lerche *et al*. Barium blocks native M-currents with an IC_{50} value of 470 μM (Robbins *et al*., 1992), a value very similar to the IC_{50} value for block of KCNQ5/Q3 channels determined in the present study. The slope of the barium concentration–response curve was rather shallow, which may suggest that barium interacts with KCNQ5/Q3 channels at more than one site. A two binding site model has previously been proposed to explain the complex interaction between barium and M-currents in NG108-15 cells (Robbins *et al*., 1992). Overall, the similarity between barium block of KCNQ5/Q3 currents and M-currents is consistent with the suggestion that KCNQ5/Q3 channels may contribute to native M-currents.

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