MOL PHARM

N-(6-Chloro-pyridin-3-yl)-3,4-difluoro-benzamide (ICA-27243): A Novel, Selective KCNQ2/Q3 Potassium Channel Activator[S]

A. D. Wickenden, J. L. Krajewski, B. London, P. K. Wagoner, W. A. Wilson, S. Clark, R. Roeloffs, G. McNaughton-Smith, and G. C. Rigdon

Johnson & Johnson Pharmaceutical Research and Development, San Diego, California (A.D.W.); Icagen Inc., Durham, North Carolina (J.L.K., B.L., P.K.W., R.R., G.M.S., G.C.G.); Neurology Research Laboratory, Veterans Administration Medical Center, Durham, North Carolina (W.A.W.); and University of Wyoming School of Pharmacy, Laramie, Wyoming (S.C.)

MOLECULAR PHARMACOLOGY

ABSTRACT

KCNQ2 (Kv7.2) and KCNQ3 (Kv7.3) are voltage-gated K^+ channel subunits that underlie the neuronal M current. In humans, mutations in these genes lead to a rare form of neonatal epilepsy (Biervert et al., 1998; Singh et al., 1998), suggesting that KCNQ2/Q3 channels may be attractive targets for novel antiepileptic drugs. In the present study, we have identified the compound N-(6-chloro-pyridin-3-yl)-3,4-difluoro-benzamide (ICA-27243) as a selective activator of the neuronal M current and KCNQ2/Q3 channels. In SH-SY5Y human neuroblastoma cells, ICA-27243 produced membrane potential hyperpolarization that could be prevented by coadministration with the M-current inhibitors 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone dihydrochloride (XE-991) and linopirdine. ICA-27243 enhanced both $^{86}Rb^+$ efflux (EC_{50} = 0.2 μM) and whole-cell currents in Chinese hamster ovary cells stably expressing heteromultimeric KCNQ2/Q3 channels (EC_{50} = 0.4 μM). Activation of KCNQ2/Q3 channels was associated with a hyperpolarizing shift of the voltage dependence of channel activation ($V_{1/2}$ shift of -19 mV at 10 μM). In contrast, ICA-27243 was less effective at activating KCNQ4 and KCNQ3/Q5 and was selective over a wide range of neurotransmitter receptors and ion channels such as voltage-dependent sodium channels and GABA-gated chloride channels. ICA-27243 (1-10 μM) was found to reversibly suppress seizure-like activity in an ex vivo hippocampal slice model of epilepsy and demonstrated in vivo anticonvulsant activity (ED_{50} = 8.4 mg/kg) in the mouse maximal electroshock epilepsy model. In conclusion, ICA-27243 represents the first member of a novel chemical class of selective KCNQ2/Q3 activators with anticonvulsant-like activity in experimental models of epilepsy.

It is estimated that up to 30% of people with epilepsy are inadequately treated with available antiepileptic drugs (AEDs) (Schmidt and Löscher, 2005). Thus, there is a clear medical need for new treatments with novel mechanisms of action to serve as alternate or adjunct therapy for the treatment of drug-resistant or refractory epilepsy. One potential antiepileptic mechanism that has yet to produce an approved AED is the selective opening of K^+ channels.

Heterotetrameric KCNQ2/Q3 channels are believed to underlie the neuronal M current, a noninactivating, slowly deactivating, subthreshold current (Wang et al., 1998). Considerable genetic, molecular, physiological, and pharmacological evidence now exists to support a role for these channels in the control of neuronal excitability, suggesting that they may represent particularly attractive targets for novel AEDs (Wickenden et al., 2004). First, mutations in KCNQ2/Q3 channels lead to a rare form of neonatal epilepsy in humans (Biervert et al., 1998; Singh et al., 1998). Second, targeted deletion or dominant-negative suppression of KCNQ2 in mice results in spontaneous seizures and hypersensitivity to the convulsant pentylenetetrazole (Watanabe et al., 2000; Peters et al., 2005). Third, KCNQ2/Q3 can be found both pre- and postsynaptically in brain regions that are known to be important for the control of neuronal network oscillations and synchronization (Cooper et al., 2001). Finally, inhibition of the M current by muscarinic agonists or linopirdine, a relatively selective M-current and KCNQ2/Q3 inhibitor, initiates seizure activity in vitro and in vivo (Turski et al., 1984; Aiken et al., 1995). Collectively, these data identify KCNQ2/Q3 channels as potentially attractive targets for mechanistically

aspet

ABBREVIATIONS: AED, antiepileptic drug; ACSF, artificial cerebrospinal fluid; EGS, electrographic seizure; CNS, central nervous system; MES, maximal electroshock; CHO-K1, Chinese hamster ovary K1; DMSO, dimethyl sulfoxide; EBSS, Earle's balanced salt solution; HERG, human *ether-a-go-go*-related gene; XE-991, 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone dihydrochloride.

novel AEDs and suggest that agents capable of selectively enhancing the activation of KCNQ2/Q3 channels may represent novel anticonvulsant drugs.

The first reported KCNQ2/Q3 opener was retigabine [*N*-(2-amino-4-[fluorobenzylamino]-phenyl) carbamic acid; D-23129]. This agent potently enhances KCNQ2/Q3 currents by inducing a leftward shift in the voltage-dependence of channel activation (Main et al., 2000; Rundfeldt and Netzer, 2000b; Wickenden et al., 2000). Consistent with the widespread CNS distribution of KCNQ2/Q3 channels and the apparent role played by these channels in neuronal activity, retigabine exerts anticonvulsant activity in a broad range of seizure models (Rostock et al., 1996; Tober et al., 1996). Furthermore, in phase II clinical trials, retigabine has been reported to reduce seizure frequency in 12 of 35 patients with refractory epilepsy (Porter et al., 2007). Although it seems likely that KCNQ2/Q3 opening plays a significant role in the anticonvulsant actions of retigabine, potential contributions from other mechanisms, including retigabine-mediated enhancement of GABAergic transmission in the central nervous system (Kapetanovic et al., 1995) and interactions with other ion channels (Rundfeldt and Netzer, 2000a), cannot be excluded. Pharmacological validation of KCNQ2/Q3 channels as antiepileptic drug targets would be further aided by the availability of a more selective activator. In the present study we show that ICA-27243 is a novel, potent, and selective KCNQ2/Q3 activator structurally distinct from other known KCNQ/M-current activators (Fig. 1). ICA-27243 also suppresses seizure-like activity in an ex vivo hippocampal slice model, and protects against maximal electroshock-induced seizures in mice.

Materials and Methods

Materials. ICA-27243 (McNaughton-Smith et al., 2002) and retigabine were synthesized in the laboratories of Icagen Inc. (Durham, NC). Linopirdine and XE-991 were purchased from Tocris (Ellisville, MO). Unless specified otherwise, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The SH-SY5Y cell line (CRL-2266), the WSS-1 cell line (CRL-2029) stably expressing rat -α1,γ2 GABA A receptors (Wong et al., 1994), and the GH3 rat pituitary cell line (CCL-82.1) were purchased from American Type Culture Collection (Manassas, VA).

Plasmid Constructs and Generation of Stable Cell Lines. The generation of KCNQ2, KCNQ3, and KCNQ5 plasmid constructs and Chinese hamster ovary-K1 (CHO-K1) cell lines stably expressing KCNQ2/Q3 and KCNQ3/Q5 tandem constructs has been described in detail elsewhere (Wickenden et al., 2000, 2001). KCNQ4 was cloned from human brain cDNA using standard techniques. The resulting sequence was identical with GenBank accession number NM_002250. CHO-K1 KCNQ cell lines were maintained in Ham's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum and 400 μg/ml G418 sulfate in an incubator at 37°C with a

humidified atmosphere of 5% CO_2. An human embryonic kidney 293 cell line stably expressing human Nav1.2 was generated at Icagen.

Membrane Potential Recordings in SH-SY5Y Cells. SH-SY5Y cells were maintained in a modified media made from 250 ml of Eagle's minimal essential medium supplemented with 20 μM nonessential amino acids, 2.4 mM sodium pyruvate, 0.05 mg/ml gentamicin, 1 mM GlutaMAX, 250 ml of unsupplemented Ham's F-12 media, and fetal bovine serum to final concentration of 10%. To differentiate cells, 10 mM retinoic acid in DMSO was added to cells to a final concentration of 10 μM and incubated for 5 to 8 days. Cells were plated at 1.5×10^6 cells/96-well plate for experiments. Membrane potential changes in SH-SY5Y neuroblastoma cell lines were measured using the membrane potential-sensitive dye DiBAC$_4$. In brief, cells were loaded with DiBac$_4$ (5 μM) by incubation in Earle's balanced salt solution (EBSS; 132 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl$_2$, 0.8 mM MgCl$_2$, 10 mM HEPES, and 5 mM glucose) for 1 h. After loading, drug solution containing 5 μM DiBAC$_4$ was added to each well, and changes in fluorescence after compound addition were recorded. Concentration-response curves were constructed by averaging data from multiple independent assays and fitting the averaged data with a logistic function as described by

$$\text{Response} = \{A_1 - A_2/[(1 + x/x_0)_p]\} + A_2 \qquad (1)$$

where A_1 is the initial response, A_2 is the final response, x_0 is the midpoint (i.e., EC$_{50}$), and p is power (slope factor).

^{86}Rb$^+$ Efflux. CHO cells stably expressing KCNQ2/Q3, KCNQ4, or KCNQ3/Q5 were grown as confluent monolayers in 96-well tissue culture plates (Corning Glassworks, Corning, NY) in minimal essential medium-α with 10% heat-inactivated fetal bovine serum and 400 μg/ml G418. Cells were incubated overnight in growth media containing 1 μCi/ml ^{86}Rb (PerkinElmer Life and Analytical Sciences, Waltham, MA) to permit intracellular uptake of the isotope. At the end of the incubation period, the ^{86}Rb solution was aspirated, and the cells were washed three times with EBSS. The cells were then preincubated for 10 min at room temperature in 100 μl/well EBSS or EBSS containing test compound at concentrations varying from 1 nM to 100 μM. At the end of this period, the supernatant from the wells was aspirated, and 100 μl of EBSS containing a submaximal concentration of KCl (typically 20–40 mM) and the test compound was added to each well. After incubation in the KCl buffer, the solution was removed and placed into the appropriate well of a 96-well counting plate for analysis. Finally, 100 μl of 0.1% SDS was added to each well to lyse the cells. The lysate was also placed in a 96-well counting plate for analysis. Both the efflux and lysate plates were then counted using a Wallac MicroBeta liquid scintillation counter (PerkinElmer Wallac, Gaithersburg, MD). Enhancement of efflux was expressed as a percentage of that induced by the addition of 70 mM KCl. Concentration-response curves were constructed by averaging data from multiple independent assays and fitting the averaged data with a logistic function (eq. 1).

Electrophysiology. For electrophysiological studies, cells were removed from the culture flask by brief trypsinization and replated at low density onto glass coverslips. Coverslips were placed in a bath on the stage of an inverted microscope and superfused (approximately 1 ml/min) with EBSS. Membrane currents were measured
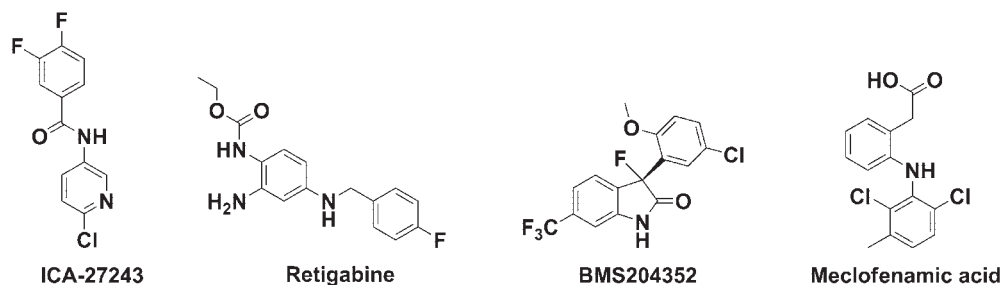
Fig. 1. ICA-27243 is structurally distinct from other known KCNQ/M-current activators.

ICA-27243   Retigabine   BMS204352   Meclofenamic acid

using the whole-cell configuration of the patch-clamp technique. Pipettes were filled with intracellular solution of the following composition: 140 mM KCl, 2 mM MgCl$_2$, 10 mM EGTA, 10 mM HEPES, and 5 mM K$_2$ATP, pH 7.3 to 7.4 with KOH, and had a resistance of 1 to 3 MΩ. Uncompensated series resistance was typically 3 to 10 MΩ, and >90% series resistance compensation was routinely achieved. As a result, voltage errors were negligible. Current records were acquired at 2 to 10 kHz and filtered at 1 to 2 kHz. All recordings were made at room temperature (22–24°C) using an Axopatch 200B amplifier and pCLAMP software (Molecular Devices, Sunnyvale, CA). Compounds were resuspended in DMSO and tested at a final DMSO concentration of 0.1%, which did not cause changes in KCNQ current kinetics (A. D. Wickenden, unpublished observations). ICA-27243-induced currents were measured as increases in outward current at a holding potential of −40 mV (for KCNQ2/Q3 and KCNQ3/Q5) or −20 mV (for KCNQ4). KCNQ channel activation is submaximal at these potentials, thereby providing a window for drug-induced current increases. Multiple concentrations of ICA-27243 were tested per cell. Drug-induced current amplitude (current amplitude in the presence of drug minus baseline current) was expressed as a percentage of the maximum drug-induced current in each cell. Normalized drug-induced currents from multiple cells were averaged and plotted against drug concentration, and these data were fit using a logistic function (eq. 1) to derive average EC$_{50}$ and slope values.

To construct KCNQ2/Q3 activation curves, steady-state current amplitude was measured at the end of a series of 3-s depolarizing steps (−100 mV to +30 mV in 10-mV increments from a holding potential of −80 mV). Apparent whole-cell conductance (G_{app}) was calculated according to the following equation:

$$G_{app} = I/(V − E_K) \tag{2}$$

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 −83 mV. Activation curves were generated by plotting normalized conductance against the step potential and were fitted with a Boltzmann distribution according to the following equation:

$$Y = 1/\{1 + \exp[(V_h − V_m)/k]\} \tag{3}$$

where V_h is the voltage for half-activation, V_m is the test potential and k is the slope factor. Semi-log plots of drug concentration against effect were fit with a logistic function as described above (eq. 1).

GABA-Activated Chloride Currents. For recordings from WSS-1 cells, pipettes were filled with an intracellular solution of the following composition: 140 mM KCl, 2 mM MgCl$_2$, 5 mM EGTA, 10 mM HEPES, and 5 mM MgATP, pH 7.3 to 7.4 with KOH, and had a resistance of 2 to 3 MΩ. Inward chloride currents were evoked with a submaximal concentration of GABA (10 μM) at a holding potential of −60 mV. GABA-activated currents were measured before the application of drug, during application of drug, and after washout of drug. For each cell, the control current amplitude was calculated as the average of the baseline and washout current amplitudes. Drug effects are presented as the percentage of inhibition, calculated with the following equation:

$$\% \text{ Inhibition} = ((\text{Control} − \text{Drug})/\text{Control}) \times 100 \tag{4}$$

hNav1.2. For hNav1.2 recordings, pipettes were filled with an intracellular solution of the following composition: 40 mM CsCl, 100 mM CsF, 5 mM NaCl, 2 mM MgCl$_2$, 5 mM EGTA, 10 mM HEPES, and 5 mM glucose, pH 7.3 to 7.4 with KOH, and had a resistance of 1 to 2 MΩ. To determine the ability of ICA-27243 to block hNav1.2 channels in the open or inactivated state, sodium currents were elicited with 20-ms test steps to 0 mV after an 8-s prepulse to the $V_{1/2}$ of activation in the absence or presence of drug. The prepulse was followed by a brief (2 ms) repolarization to −120 mV, to relieve

inactivation before the test step. Drug effects are presented as the percentage of inhibition, calculated with eq. 4.

Use-dependence of compound block was performed by holding at −120 mV then stepping to 0 mV for 50 ms at 0.1-s intervals (10 Hz). Currents were normalized to the peak current at time 0 (I_0) and presented as the percentage of available current. All data are expressed as mean ± S.E.M. for $n = 3$ observations.

GH3 Cell Line. For measuring barium currents through native calcium channels in GH3 cells, the internal solution was 110 mM Cs-Asp, 20 mM CsCl, 10 mM HEPES, 5 mM EGTA, 1 mM MgCl$_2$, 5 mM Mg-ATP, and 0.1 mM Na-GTP, pH 7.3 with CsOH, and the external solution was 138 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 20 mM BaCl$_2$, and 1 mM MgCl$_2$, pH 7.4 with NaOH. GH3 cells were held at −40 mV and depolarized to +10 mV for 200 ms. Barium currents were measured before the application of drug, during application of drug, and after washout of drug, and effects were calculated using eq. 4.

Hippocampal Slice Electrographic Seizure Model of Epilepsy. Juvenile male Sprague-Dawley rats (P14 and P15) were used for these experiments because robust seizure-like activity occurs during this age range (Clark and Wilson, 1992). All protocols were approved by the Veterans' Administration animal facility. Hippocampal slices were prepared as follows. Rats were anesthetized with halothane. The brain was removed rapidly, rinsed, and placed briefly in ice-cold, oxygenated (95% O$_2$/5% CO$_2$) artificial cerebrospinal fluid (ACSF) containing 120 mM NaCl, 3.3 mM KCl, 1.23 mM NaH$_2$PO$_4$, 0.9 mM MgSO$_4$ · 7 H$_2$O, 25 mM NaHCO$_3$, 1.3 mM CaCl$_2$, and 10 mM dextrose. Hippocampi were removed and cut into nominally 650 μm thick transverse slices and allowed to equilibrate in oxygenated ACSF at room temperature for at least 1 h. Slices were then transferred to a Haas-style recording chamber and perfused with oxygenated ACSF at ~2 ml/min at 30.5 to 31.5°C.

Stimulus trains were delivered to the fimbria in stratum radiatum of area CA3 at the level of the Schaffer collateral pathway via a tungsten monopolar stimulating electrode (A-M Systems, Sequim, WA) connected to a Grass S88 Stimulator (Grass Technologies, West Warwick, RI). Extracellular field potentials in the stratum pyramidale of area CA3 were recorded with a single-barrel thin-walled 1 to 2 MΩ capillary glass recording electrode (A-M Systems) filled with 150 mM NaCl, filtered and amplified (Duo 773 electrometer; World Precision Instruments, Sarasota, FL), and then displayed on an oscilloscope (Nicolet 430) and a Gould Brush chart recorder. Responses were also recorded on videotape for later playback and analysis (Unitrade-modified digital Toshiba video cassette recorder).

Single stimulus pulses (0.1-ms duration and 100–600 μA intensity) were applied to define the intensity necessary to evoke a maximal field response in area CA3. Then, 60-Hz, 2-s stimulus trains were delivered at 2-fold this intensity (Stasheff et al., 1989).

For experiments testing compound effects, hippocampal slices were initially exposed to ACSF containing 0.1% DMSO (control ACSF) during the first five to six stimulus trains until the responses stabilized. This concentration of DMSO has been found previously to have no effect on in vitro electrographic seizure (EGS) activity (S. Clark, unpublished finding). The last stable train was considered the control response. ACSF solution containing ICA-27243 (0.3, 1.0, or 10 μM) was bath-applied for at least six trains at 10-min intervals. In some experiments, a drug-application phase was followed by a washout phase in control ACSF. EGS data were analyzed by measuring the seizure duration of the clonic stage at the first burst event to the last burst event where the trace crossed the preburst baseline, whereas the tonic phase was measured as burst-event frequency calculated as the highest number of burst events during any 1-s period of the seizure. Parameters were compared using paired two sample t-tests.

Mouse Maximal Electroshock Seizure Model. All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee at Icagen, Inc. and according to the National Institutes of Health guidelines. Male CD-1 mice were administered ICA-27243 (1–100 mg/kg) or vehicle (5% DMSO/95%

MOLECULAR PHARMACOLOGY

olive oil) by oral gavage 10 min before experiment. After a drop of 1% proparacaine solution in each eye, a 50 mA, 200-ms shock was delivered through eye electrodes. Latency to hind limb extension was measured to the nearest 0.1 s. If extension did not occur within 6 s, the animal was scored as protected, and a value of 6 s was recorded. The data are presented as mean ± S.E.M. and are the result of n = 7 to 15 animals. The ED_{50} values for ICA-27243 effects were calculated by fitting the data to a logistic function (eq. 1).

Receptor Binding Panel. ICA-27243 was tested by CEREP (Celle l'Evescault, France) in a broad receptor binding panel at 10 μM (Supplemental Table S1).

Results

ICA-27243 Is an M-Current Activator

To identify novel M-current activators, we developed a high-throughput fluorescence-based assay using the human neuroblastoma cell line SH-SY5Y. A semiquantitative PCR-mRNA analysis of neuronal cell lines indicated that differentiated SH-SY5Y cells robustly express KCNQ2, KCNQ3, and KCNQ5 mRNA (B. Londen, unpublished observations). Furthermore, using the membrane potential-sensitive fluorescent probe DiBac4, we were able to show that SH-SY5Y cells exhibit a hyperpolarizing response to the known KCNQ channel/M-current activator retigabine, which could be inhibited by the selective M-current inhibitor XE-991 (Fig. 2A). Using the same fluorescence-based membrane potential assay, we characterized ICA-27243, which also produced a robust hyperpolarization in human SH-SY5Y cells that was fully reversed with XE-991 (Fig. 2B). SH-SY5Y cells have been reported to express the HERG potassium channel (Finlayson et al., 2001). However, the ICA-27243-induced hyperpolarization observed in this study is not due to HERG activation because 10 μM ICA-27243 actually exhibits weak inhibition of HERG current in transiently transfected COS-7 cells (12.0 ± 2.9%, n = 4). In separate experiments, membrane hyperpolarizations induced by 10 μM ICA-27243 could be prevented in a concentration-dependent manner by coadministration with known M-current inhibitors like XE-991 (IC_{50} = 60 ± 5 nM; n = 4), linopirdine (IC_{50} = 2.8 ± 0.5 μM; n = 4), and tetraethylammonium (IC_{50} = 1.8 ± 0.3 mM; n = 3). These values are consistent with known potencies for inhibiting M currents in other studies (Aiken et al., 1995; Wang et al., 1998; Hadley et al., 2000; Wickenden et al., 2000).

ICA-27243 Is a KCNQ2/Q3 Subtype-Selective Opener

$^{86}Rb^+$-Efflux Studies. To further understand the molecular targets of ICA-27243, we assessed its ability to modulate recombinant homomeric and heteromultimeric human KCNQ channels stably expressed in CHO cells using a $^{86}Rb^+$ efflux assay. ICA-27243 caused a concentration-dependent increase in

$^{86}Rb^+$ efflux from CHO cells expressing KCNQ2/Q3 channels (Fig. 3). Threshold activity was observed with concentrations of approximately 0.03 μM and on average, half-maximal channel activation (EC_{50}) occurred at a concentration of 0.20 ± 0.03 μM, n = 27. Maximal ^{86}Rb efflux was observed at 10 μM and was similar in magnitude to the flux induced by depolarization with 70 mM KCl. At concentrations of 30 and 100 μM, ICA-27243 caused a small reduction in ^{86}Rb efflux compared with the effect observed at 10 μM, suggesting that ICA-27243 may possess KCNQ2/Q3 inhibitory activity at high concentrations. ICA-27243 also enhanced ^{86}Rb efflux through homomeric human KCNQ4 channels and, to a much lesser extent, through heteromeric human KCNQ3/Q5 channels (Fig. 3). For KCNQ4, threshold activity was observed at concentrations between 1 and 3 μM with half-maximal activation (EC_{50}) at 7.1 ± 0.1 μM (n = 11). Because maximal activation of KCNQ3/Q5 could not be achieved with ICA-27243 concentrations up to 100 μM, an EC_{50} value could not be accurately determined. However a significant increase in baseline current amplitude was observed at 10 μM (Fig. 3B, p < 0.05, n = 8). Based on the threshold for current amplitude increases, it seems that the selectivity of ICA-27243 for KCNQ2/Q3 channels over KCNQ3/Q5 channels is 100- to 300-fold.

KCNQ1 (KvLQT1, Kv7.1) is a member of the KCNQ potassium channel family, which, when coexpressed with the auxiliary subunit KCNE1 (minK), forms the important cardiac repolarizing potassium current, IKs. The effect of ICA-27243 on KCNQ1/KCNE1 channels stably expressed in CHO cells was also assessed using a ^{86}Rb efflux assay. ICA-27243 exhibited no activation of KCNQ1/KCNE1 concentrations up to 100 μM. However, at concentrations greater than 10 μM, inhibition of ^{86}Rb efflux through KCNQ1/KCNE1 was observed (IC_{50} = 40 ± 3 μM, n = 8).

Whole-Cell Electrophysiology. To further characterize the activation of KCNQ channels by ICA-27243, we examined its electrophysiological properties in CHO cells expressing KCNQ2/Q3. Previous studies by Wickenden et al. (2000) have shown that the threshold for activation of KCNQ2/Q3 channels occurs between −50 and −40 mV. Therefore, when these cells were voltage-clamped at −40 mV, a small outward current was observed, consistent with activation of the KCNQ2/Q3 channel. Application of ICA-27243 induced a reversible concentration-dependent increase in KCNQ2/Q3 current amplitude beginning at a concentration of 30 nM and a maximal effect at 10 μM. The EC_{50} value was 0.4 ± 0.1 μM (slope = 1.0 ± 0.1; n = 7; Fig. 4, A and B). No increase in outward potassium currents was observed in wild-type CHO-K1 cells exposed to ICA-27243 (data not shown). KCNQ3/Q5 currents activate over a voltage range similar to that for KCNQ2/Q3 currents (Wick-
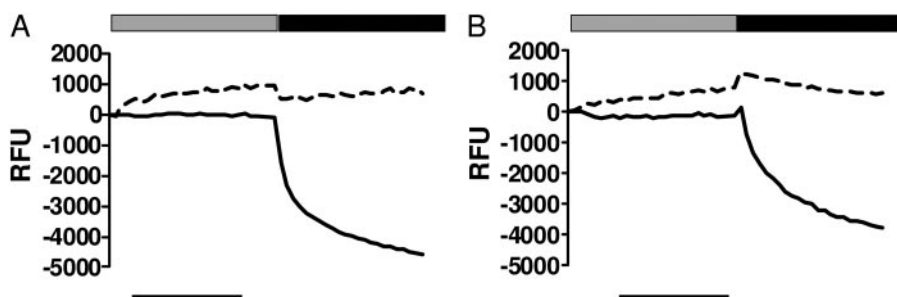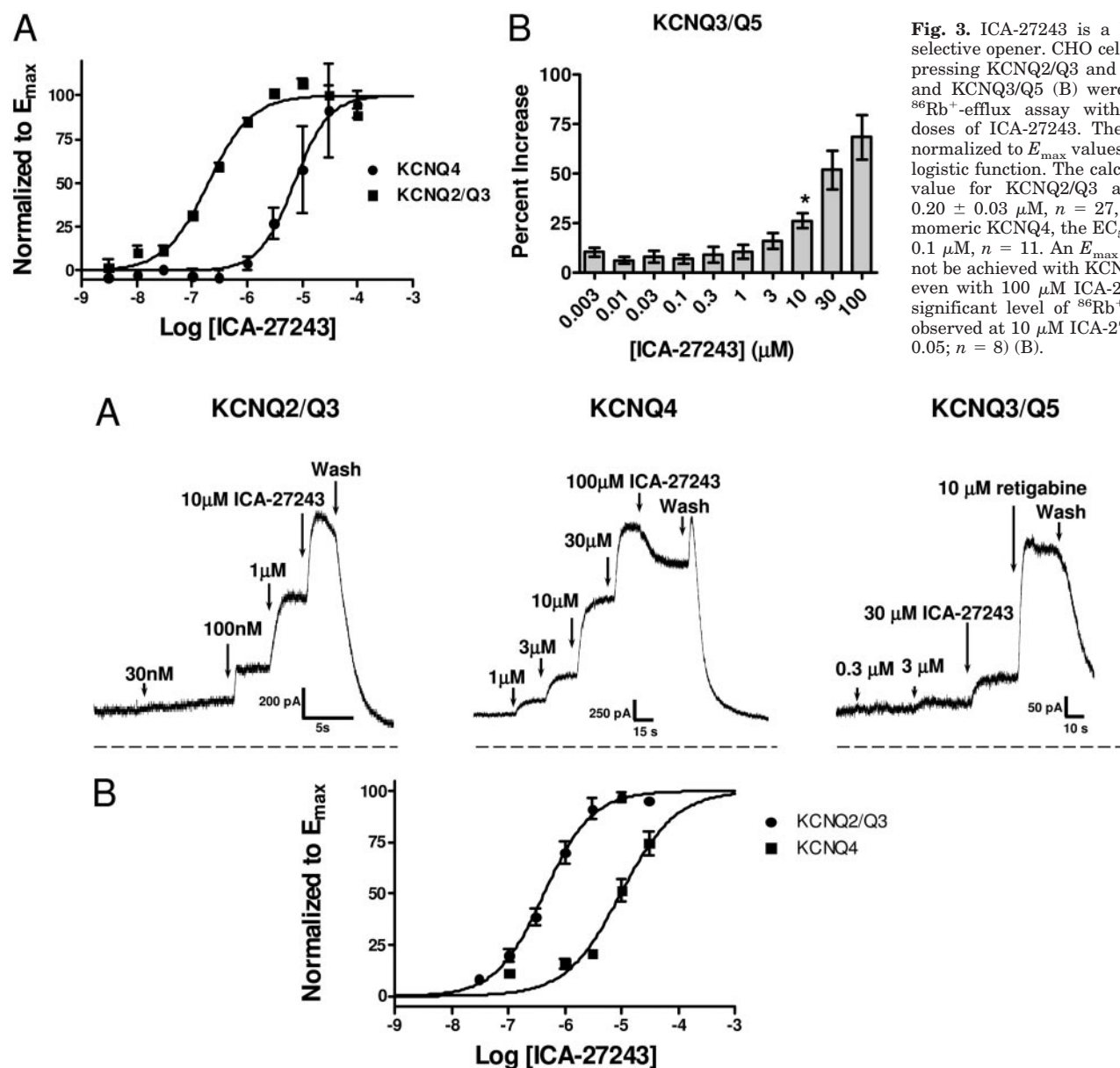

Fig. 2. ICA-27243 is an M-current activator in SH-SY5Y neuroblastoma cells. Differentiated human SH-SY5Y neuroblastoma cells were loaded with the voltage sensor DiBAC$_4$. Gray bar indicates preincubation time with EBSS (solid line) or 10 μM XE-991 (broken line). ■, compound addition. A, 10 μM retigabine (solid line), a known M-current activator, induces a hyperpolarization in SH-SY5Y cells that is blocked by preincubation of 10 μM XE-991 (broken line). B, 10 μM ICA-27243 (solid line) causes a similar XE-991-sensitive hyperpolarization in differentiated SH-SY5Y cells (broken line). Time bar, 500 s.

enden et al., 2001). However, in contrast to its effect on KCNQ2/Q3, ICA-27243 induced only minor increases in outward current in KCNQ3/Q5-expressing cells, even at concentrations as high as 30 μM (Fig. 4A). This limited effect of ICA-27243 was not due to poor expression of KCNQ3/Q5 channels, because the nonselective KCNQ channel opener, retigabine (10 μM), produced a robust increase in outward current in the same cells (Fig. 4A).

The threshold for activation of KCNQ4 potassium channels occurs at more positive membrane potentials than for either KCNQ2/Q3 or KCNQ3/Q5 ($V_{1/2}$ activation = −10 mV for KCNQ4 compared with $V_{1/2}$ activation = −30 mV for KCNQ2/Q3). Therefore, the effect of ICA-27243 on KCNQ4 currents was examined at a holding membrane

potential of −20 mV. At this holding potential, ICA-27243 produced a concentration-dependent increase in outward current (Fig. 4A). At a concentration of 100 μM, the increase in current amplitude was less than that seen with 30 μM, suggesting that ICA-27243 may possess additional weak inhibitory activity at high concentrations. The EC_{50} value for activation of KCNQ4 by ICA-27243 was 9.7 ± 1.2 μM (slope = 0.9 ± 0.1; n = 5), which is more than 20-fold less potent than observed for KCNQ2/Q3 currents (Fig. 4B).

ICA-27243 Shifts the Voltage-Dependence of KCNQ2/Q3 Activation to Hyperpolarized Potentials. The mechanism of KCNQ channel activation by ICA-27243 was assessed by examining the effect of the compound on the voltage-dependence of channel activation. CHO-KCNQ2/Q3 cells were held at

Fig. 3. ICA-27243 is a KCNQ2/Q3-selective opener. CHO cells stably expressing KCNQ2/Q3 and KCNQ4 (A) and KCNQ3/Q5 (B) were used in a $^{86}Rb^+$-efflux assay with increasing doses of ICA-27243. The data were normalized to E_{max} values and fit to a logistic function. The calculated EC_{50} value for KCNQ2/Q3 activity was 0.20 ± 0.03 μM, n = 27, and for homomeric KCNQ4, the EC_{50} was 7.1 ± 0.1 μM, n = 11. An E_{max} value could not be achieved with KCNQ3/Q5 cells even with 100 μM ICA-27243, but a significant level of $^{86}Rb^+$-efflux was observed at 10 μM ICA-27243 (∗, $p <$ 0.05; n = 8) (B).

Fig. 4. ICA-27243 enhances outward currents in cells stably transfected with KCNQ subtypes. CHO cells stably transfected with KCNQ2/Q3, KCNQ4, and KCNQ3/Q5 were studied using whole-cell electrophysiology in voltage-clamp mode (A). KCNQ2/Q3 and KCNQ3/Q5 were held at −40 mV, and KCNQ4 was held at −20 mV to generate tonic outward current. Broken line denotes 0 pA. Drug-induced currents from KCNQ3/Q5 did not reach E_{max} even with 30 μM ICA-27243. Retigabine (10 μM) substantially increased outward currents in ICA-27243-insensitive cells. Vertical bar for KCNQ2/Q3, KCNQ4, and KCNQ3/Q5 is 200, 250, and 50 pA, respectively. Horizontal bar for KCNQ2/Q3, KCNQ4, and KCNQ3/Q5 is 5, 15, and 10 s, respectively. B, ICA-27243-induced outward currents were normalized to the E_{max} value and fit to a logistic function. ICA-27243 exhibited an EC_{50} value of 0.4 ± 0.1 μM (mean ± S.E.M.) (slope = 1.0 ± 0.1; n = 7) on KCNQ2/Q3 and an EC_{50} value of 9.7 ± 1.2 μM (slope = 0.9 ± 0.1; n = 5) on KCNQ4.

−80 mV, stepped to voltages in the range of −100 to +30 mV for 3 s and then repolarized to −100 mV for 0.5 s. In the absence of ICA-27243, voltage steps positive to −50 mV induced a slowly activating, noninactivating outward current (Fig. 5A). Superfusion with 0.1, 1, and 10 μM ICA-27243 increasingly shifted the threshold for channel activation to more hyperpolarized potentials. This resulted in increased current amplitude with drug, most notably around the threshold membrane potential for activation. A representative plot of the ICA-27243-induced hyperpolarizing shift in the voltage-dependence of activation is illustrated in Fig. 5B. Multiple experiments show the KCNQ2/Q3 $V_{1/2}$ activation value was −32.2 ± 1.5 mV ($n = 7$) under control conditions and was shifted to −51.4 ± 3.6 mV ($n = 4$) in the presence of 10 μM ICA-27243. The slopes of the activation curves seemed relatively unaffected by ICA-27243; 9.1 ± 0.9 ($n = 7$) and 9.7 ± 1.5 ($n = 4$) in the absence and presence of 10 μM ICA-27243, respectively. The concentration-dependence of the ICA-27243-induced shift ($\Delta V_{1/2}$) in the midpoint of the activation curve is shown in Fig. 5C. The half-maximal shift in $\Delta V_{1/2}$ for activation with ICA-27243 was observed at 4.8 ± 1.6 μM (slope factor was 0.78 ± 0.22).

ICA-27243 Does Not Interact with Other CNS Ion Channels Targeted by Antiepileptic Drugs. To address broader ion channel selectivity, we examined the effects of ICA-27243 on other channels that are molecular targets of known antiepileptic agents. For example, GABA$_A$-activated

chloride channels, voltage-dependent sodium channels, and some types of calcium channels are believed to be modulated by antiepileptic drugs such as benzodiazepines, carbamazepine, and lamotrigine (Treiman, 2001; Armijo JA et al., 2005; Biton V, 2006). Clarifying the effects of ICA-27243 on these channels would help in the mechanistic interpretation of effects in in vitro and in vivo models of epilepsy.

GABA$_A$-Induced Chloride Currents in WSS-1 Cells. Inward GABA$_A$-induced chloride currents were measured in WSS-1 cells stably expressing rat -α1,γ2 GABA$_A$ receptors (Wong et al., 1994). Currents were activated using a submaximal concentration of GABA (10 μM) at a holding potential of −60 mV in symmetrical chloride solutions (Fig. 6A). In contrast to the benzodiazepine diazepam (5 μM), which increased the amplitude of GABA-activated currents by 99 ± 7% ($n = 14$), 30 μM ICA-27243 had no effect on GABA-mediated currents (4 ± 5%; $n = 5$).

High Voltage-Activated Calcium Channels. The mammalian CNS expresses several classes of voltage-gated calcium channel. We examined the effect of ICA-27243 on endogenous calcium currents in the GH3 pituitary cell line, which is known to express a mixture of different high voltage-activated calcium channels [approximately 50% L-type (dihydropyridine-sensitive), 40% P/Q type (ω-agatoxin IVA, ω-conotoxin MVIIC-sensitive), and the remainder undefined (Glassmeier et al., 2001)]. Barium currents were recorded
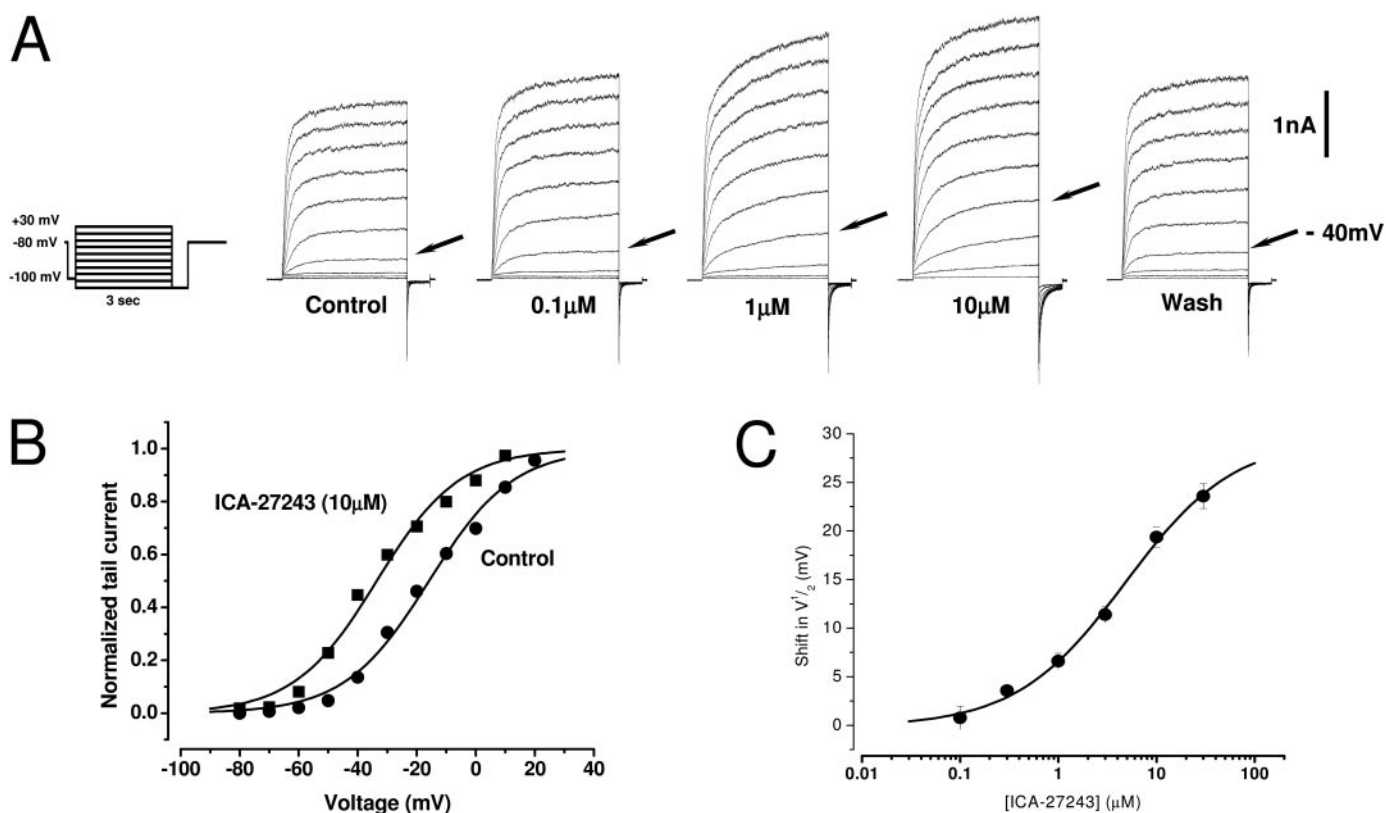
Fig. 5. ICA-27243 shifts the voltage dependence of KCNQ2/Q3 activation. A, series of outward currents elicited by depolarizing voltage steps (hold at −80 mV, steps from −100 to +30 mV for 3 s; see inset) with increasing concentrations of ICA-27243. Comparisons of the current traces show that ICA-27243 increases the outward current at all voltages, most notably at voltages that are threshold for channel opening in control (see arrow). B, a representative plot of the activation curves were constructed for KCNQ2/Q3 in the absence and presence of 10 μM ICA-27243 and fit with a Boltzmann function. Data from multiple experiments show the KCNQ2/Q3 $V_{1/2}$ activation value was −32.2 ± 1.5, $n_H = 9.1 ± 0.9$ ($n = 7$), under control conditions, and was shifted to −51.4 ± 3.6, $n_H = 9.7 ± 1.5$ ($n = 4$), in the presence of 10 μM ICA-27243. C, the magnitude of the ICA-27243-induced shifts in the midpoint of the activation curve ($\Delta V_{1/2}$) were calculated, plotted against concentration, and fit to a logistic function, giving an EC$_{50}$ value of 4.8 ± 1.6 μM; $n_H = 0.78 ± 0.22$.

in the absence and presence of ICA-27243 (Fig. 6B) using 200-ms depolarizing steps to +10 mV from a holding potential of −40 mV. ICA-27243 had no inhibitory effect on whole-cell barium channel currents at 30 μM (1.0 ± 9.6%; $n = 3$).

Na$_v$1.2 Voltage-Dependent Sodium Channels. One of the more widely expressed voltage-dependent sodium channels in the mammalian central nervous system is Na$_v$1.2. The effect of ICA-27243 on human Nav1.2 channel currents recombinantly expressed in human embryonic kidney 293 cells is shown in Fig. 6, C and D, using protocols that assessed use-dependent and inactivated state channel inhibition. All known sodium channel-blocking agents that are used in the treatment of epilepsy exhibit both use-dependent block and a preferential interaction with inactivated state of the channel. However, ICA-27243 (10 μM) exhibited no significant state- or use-dependent inhibition human Nav1.2. ICA-27243 (10 μM) inhibited Nav1.2 by only 5 ± 2% ($n = 4$) in experiments designed to assess inactivated state block (Fig. 6C) and by only 5 ± 3% block (compared with 4 ± 1% $n = 4$ in control experiments) in a 10-Hz use-dependent protocol (Fig. 6D). In contrast, the positive control sodium blocker, amitriptyline (1 μM), blocked Nav1.2 by 54 ± 2% ($n = 4$) in the inactivated state protocol (Fig. 6C) and by 20 ± 1% ($n = 3$, $p < 0.001$) in the 10-Hz protocol (Fig. 6D).

The studies described above indicate that ICA-27243 is selective for KCNQ2/Q3 channels over several other CNS ion channels that are targeted by currently available AEDs. In addition to the electrophysiological assays described above, ICA-27243 was tested for pharmacological activity against a variety of neurotransmitter receptors and transporters using radioligand-binding studies. ICA-27243 (10 μM) did not significantly (i.e., greater than 10%) displace specific radioligand binding to the majority of receptors tested. At 10 μM, this compound inhibited radioligand binding to adenosine A1 receptors, A2 receptors, opiate receptors, phencyclidine receptors, norepinephrine, and 5-hydroxytryptamine transporters by ≤33% (Supplemental Table S1).

ICA-27243 Suppresses Seizure-Like Activity in Vitro. To assess the potential anticonvulsant properties of ICA-27243, we examined the effects of the compound in an in vitro model of seizure-like activity in rat hippocampal slices. Stimulus trains were delivered at 10-min intervals to area CA3 of each hippocampal slice to evoke electrical responses that resemble activity recorded during tonic-clonic seizures in humans or in certain in vivo animal models of epilepsy. These in vitro seizure-like events or EGSs have two distinct firing patterns: the tonic phase is the early, high-frequency firing pattern seen within the first 2 to 6 s of the EGS, and the
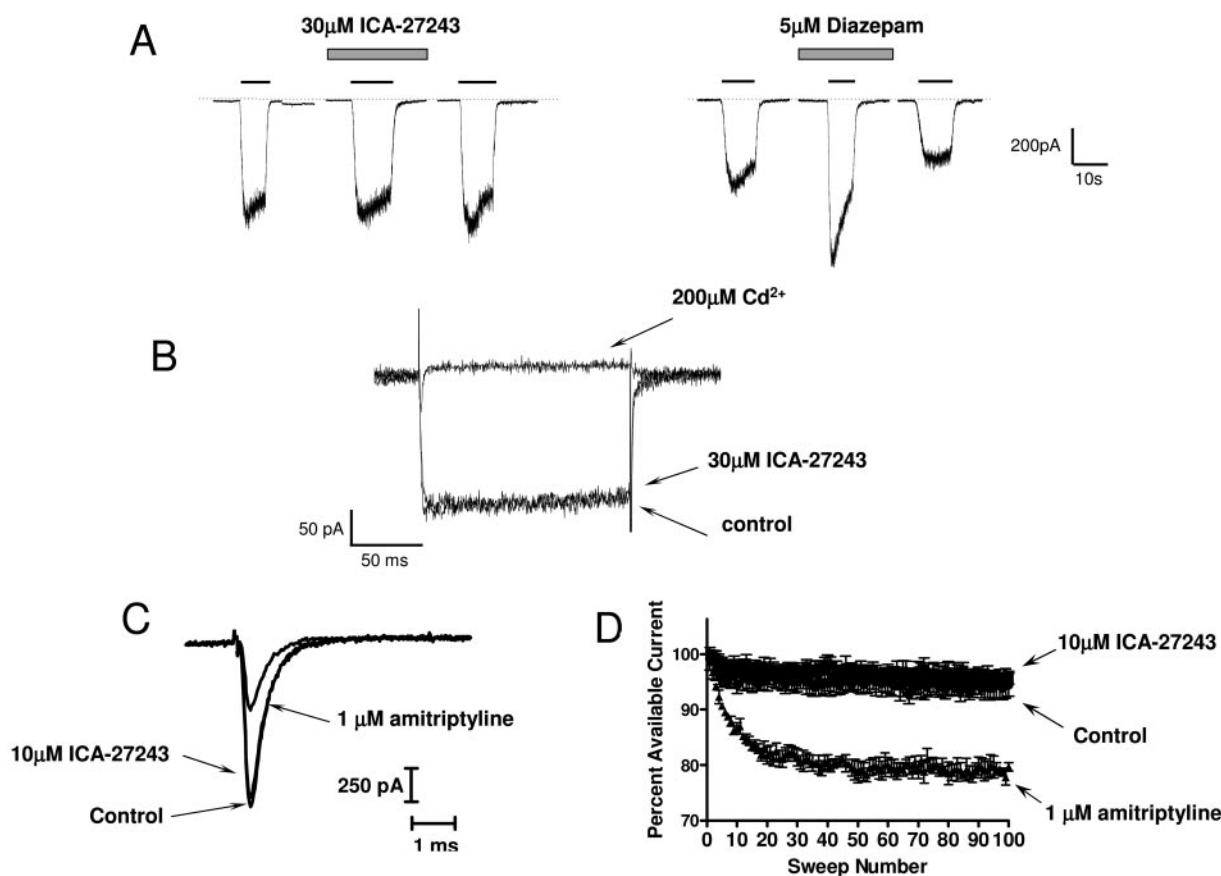
Fig. 6. ICA-27243 is selective over ion channels known to modulate epileptic activity. A, inward GABA-induced chloride currents were measured in WSS-1 cells stably expressing rat -α1,γ2 GABA$_A$ receptors. A submaximal concentration of GABA (10 μM) at a holding potential of −60 mV in symmetrical chloride solutions generated inward currents (black line). Preincubation with 30 μM ICA-27243 had little effect on GABA-activated currents (gray bar). As a control, peak GABA-activated currents were enhanced strongly in the presence of 5 μM diazepam (gray bar). B, high voltage-activated calcium channel currents were elicited with 200-ms depolarizing steps to +10 mV from a holding potential of −40 mV in GH3 cells. ICA-27243 exhibited no high voltage-activated calcium channel blocking activity at 30 μM (1.0 ± 9.6%; $n = 3$). ICA-27243 was tested on hSCNIIA channels at half-inactivation (C) and at 10-Hz stimulus protocol (D) (see *Materials and Methods*). At 10 μM, ICA-27243 had little effect on I_{Na}, regardless of the voltage protocol. C, 1 μM amitriptyline was used as a positive control in both assays.

clonic phase occurs after the tonic phase; the firing pattern is slower, but the phase usually lasts longer (tens of seconds). Consistent with previous reports (Stasheff et al., 1989), robust tonic-clonic EGS activity was evoked within the first four to six trains (Fig. 7) and is typical for slices from P14 and P15 rats (control EGS duration, 36.5 ± 3.18 s; tonic phase, 15.0 ± 1.26 events/s; $n = 6$). Superfusion of the slice for 40 min with 1 μM ICA-27243 suppressed EGS duration by $48.6 \pm 5.25\%$ and the tonic phase by $22.94 \pm 10.33\%$ ($p < 0.01$; $n = 4$). At the highest dose tested (10 μM), EGS activity was almost completely blocked ($99.8 \pm 0.21\%$; $n = 2$) and was reversible, with EGS activity returning within approximately 70 min of washout. At all concentrations tested, no spontaneous epileptiform bursts occurred, suggesting that ICA-27243 does not have inherent proconvulsant properties.

Oral Administration of ICA-27243 Protects Mice in an in Vivo Model of Epilepsy. To more directly evaluate antiseizure effects, ICA-27243 was investigated for its ability to protect against hind limb extension in the in vivo mouse maximal electroshock assay (MES), which is predictive of anticonvulsant drug efficacy against generalized tonic-clonic seizures (Löscher and Schmidt, 1994). When administered orally at 10 mg/kg, ICA-27243 achieved a maximal plasma concentration (C_{max}) of 5.6 ± 0.2 and 2.6 ± 0.1 μM in the brain (data not shown). A 10-min pretreatment with ICA-27243 (1–100 mg/kg p.o.) produced a dose-dependent increase in the latency to hind limb extension (Fig. 8), exhibiting an ED_{50} value of 8.4 mg/kg (95% confidence interval, 5.6–12.5 mg/kg).

Discussion

There is an unmet medical need for broad-spectrum, safe anticonvulsants because at least 30% of patients are refractory to current therapies (Schmidt and Löscher, 2005). This study describes the biophysical properties of ICA-27243, a structurally novel and selective M-current and KCNQ2/Q3 opener. We show that ICA-27243 rapidly and reversibly in-

hibits EGS activity in hippocampal slices. Furthermore, ICA-27243 is orally bioavailable and exhibits anticonvulsant efficacy in the mouse MES-seizure model. As such, ICA-27243 is the first selective KCNQ channel opener reported to date and shows efficacy in preclinical epilepsy models, further validating KCNQ channels as novel antiepileptic drug targets. The mechanism of action of ICA-27243 involves a hyperpolarizing shift in the voltage-dependence of activation of KCNQ2/Q3 channels (-19 mV at 10 μM). Other KCNQ channel activators such as retigabine (Main et al., 2000; Rundfeldt and Netzer, 2000; Wickenden et al., 2000), BMS-204352 (Schröder et al., 2001), acrylamide S-1 (Bentzen et al., 2006), and meclofenamate (Peretz et al., 2005) also induce hyperpolarizing shifts in the voltage-dependence of activation and act in a qualitatively similar manner. The magnitude of this shift seems greater for retigabine (~ -33 mV at 10 μM) than ICA-27243. Whether this represents a true difference in efficacy or a difference in potency is not clear because true maximum shifts were not established in the present study and the study of Wickenden et al. (2000). It is possible that ICA-27243 acts directly on the channel to modify the voltage-dependence of channel activation or may act indirectly through an undefined second-messenger pathway. Although the former explanation is supported by 1) the rapid onset and reversibility of ICA-27243; 2) the effects of ICA-27243 are readily apparent in dialyzed cells, in low intracellular calcium levels, and at room temperature; and 3) the selectivity of the compound for KCNQ2/Q3 over other closely related channels. Collectively, these observations argue against an indirect effect mediated through many of the second-messenger cascades known to regulate KCNQ channels, such as calcium, phospholipase C, phosphatidylinositol bisphosphate, diacylglycerol, and cAMP (Delmas and Brown, 2005).

ICA-27243 is a potent KCNQ2/Q3 channel opener but is less potent as a KCNQ4 activator and is only very weakly active against KCNQ3/Q5 channels. This profile is quite distinct from that of KCNQ channel activators described previously, which do not discriminate among neuronal KCNQ subtypes (i.e., KCNQ2-KCNQ5) (Schröder et al., 2001, Wickenden et al., 2001). A conserved tryptophan in the N-terminal part of Ser5 has been identified as a key residue responsible for the actions of retigabine, BMS-204352, and acrylamide S-1 (Schenzer et al., 2005; Wuttke et al., 2005; Bentzen et al.,
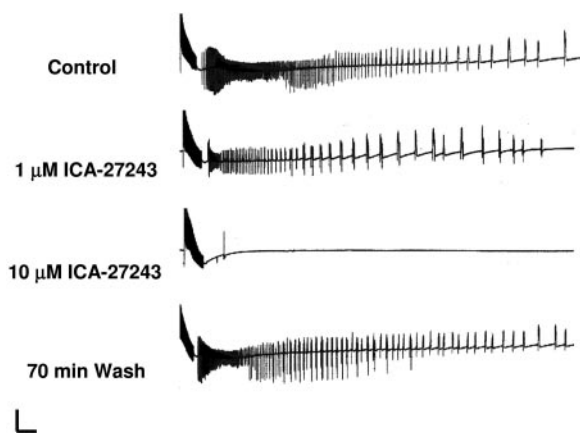
Fig. 7. ICA-27243 reversibly blocks EGS seizure activity in hippocampal slices. EGS activity was elicited in hippocampal slices superfused with ACF by electrical stimulation (see *Materials and Methods*). High-frequency stimulation produces a clonic-phase firing pattern with a 36.5 ± 3.18-s duration followed by a tonic phase frequency of 15.0 ± 1.26 events/s; $n = 6$ (control). After a 10-min incubation with 1 μM ICA-27243, the clonic phase duration was reduced by $48.6 \pm 5.25\%$ and tonic phase frequency by $22.94 \pm 10.33\%$ ($p = 0.006$; $n = 4$). A 40-min incubation with 10 μM ICA-27243 blocked $99.8 \pm 0.21\%$ of the EGS seizure duration ($n = 2$). The blocking effect of ICA-27243 was almost completely reversed with a 70-min washout (Wash). Vertical bar, 1 mV; horizontal bar, 2 s.
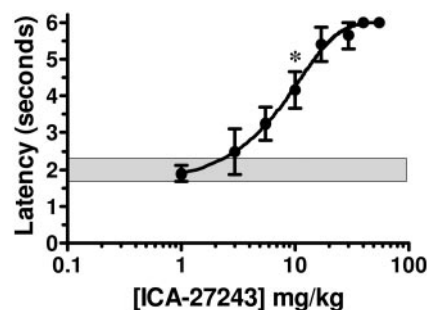
Fig. 8. Orally administered ICA-27243 protects mice in the MES seizure model. ICA-27243 was tested for the ability to protect against hind limb extension in the MES assay by measuring the latency to seizure activity (see *Materials and Methods*). Increasing doses of ICA-27243 given orally 10 min before electroshock stimuli protects mice in a dose-dependent manner, with an ED_{50} value of 8.4 mg/kg (95% confidence interval, 5.6–12.5 mg/kg). Shaded region denotes vehicle range (5% DMSO, 95% olive oil); *, $p > 0.05$. Number of animals, 7 to 15.

2006). An interaction with a conserved residue close to the pore may explain the observation that compounds such as retigabine shift the voltage dependence of activation of all neuronal KCNQ channel subtypes. The finding that ICA-27243 can discriminate among KCNQ subtypes suggests that this compound may interact at a novel binding site on KCNQ2 and/or KCNQ3 channels that is not present in other KCNQ channels. The location of the ICA-27243 binding site and how drug binding to this putative site influences channel gating remains to be investigated.

In addition to KCNQ subtype selectivity, we also evaluated the effects of ICA-27243 against validated antiepileptic targets (e.g., sodium channels, calcium channels, and GABA-activated chloride channels). In electrophysiological studies, ICA-27243 was without effect on voltage-dependent type II sodium channels, calcium currents in GH3 cells, or GABA-activated chloride channels. Furthermore, ICA-27243 had minimal effects on a variety of CNS receptors and transporters. These findings indicate that ICA-27243 is a highly selective KCNQ2/Q3 activator and further differentiate ICA-27243 from retigabine, which, in addition to being a nonselective KCNQ channel activator, also increases levels of newly synthesized GABA in hippocampal slices (Kapetanovic et al., 1995), inhibits GABA transaminase in mouse brain (Sills et al., 2000), potentiates GABA-induced currents in rat cortical neurons (Rundfeldt and Netzer, 2000a), and allosterically enhances GABA binding to rat brain membranes (van Rijn and Willems-van Bree, 2003). Collectively, these data suggest that ICA-27243 represents a valuable pharmacological tool that could be used to further understand the roles of KCNQ2/Q3 channels in epilepsy and other conditions. In that respect, we found that ICA-27243 was effective in an in vitro EGS hippocampal seizure model and when given orally in the mouse MES hind limb extension model, which is predictive of anticonvulsant drug efficacy against generalized tonic-clonic seizures (Löscher and Schmidt 1994). Our results are the first to demonstrate that selective pharmacological opening of KCNQ2/3 channels is efficacious in preclinical models of epilepsy.

In conclusion, ICA-27243 represents a novel KCNQ2/Q3 selective opener. In addition, because of its selectivity over other ion channels, including other KCNQ channel subtypes, our data provide further pharmacological validation of KCNQ2/Q3 as an antiepileptic drug target.

Acknowledgments

References

Aiken SP, Lampe BJ, Murphy PA, and Brown BS (1995) Reduction of spike frequency adaptation and blockade of M-current in rat CA1 pyramidal neurones by linopirdine (DuP 996), a neurotransmitter release enhancer. *Br J Pharmacol* **115:**1163–1168.

Armijo JA, Shushtarian M, Valdizan EM, Cuadrado A, de las Cuevas I, and Adín J (2005) Ion channels and epilepsy. *Curr Pharm Des* **11:**1975–2003.

Bentzen BH, Schmitt N, Calloe K, Dalby Brown W, Grunnet M, and Olesen SP (2006) The acrylamide (S)-1 differentially affects Kv7 (KCNQ) potassium channels. *Neuropharmacology* **51:**1068–1077.

Biervert C, Schroeder BC, Kubisch C, Berkovic SF, Propping P, Jentsch TJ, and Steinlein OK (1998) A potassium channel mutation in neonatal human epilepsy. *Science* **279:**403–406.

Biton V (2006) Pharmacokinetics, toxicology and safety of lamotrigine in epilepsy. *Expert Opin Drug Metab Toxicol* **2:**1009–1018.

Clark S and Wilson WA (1992) Brain slice model of epilepsy: Neuronal networks and actions of antiepileptic drugs, in *Drugs for Control of Epilepsy: Actions of Neuronal Networks Involved in Seizure Disorders* (CL Faingold and GH Fromm eds) pp 89–123, CRC Press, Boca Raton, FL.

Cooper EC, Harrington E, Jan YN, and Jan LY (2001) M channel KCNQ2 subunits are localized to key sites for control of neuronal network oscillations and synchronization in mouse brain. *J Neurosci* **21:**9529–9540.

Delmas P and Brown DA (2005) Pathways modulating neural KCNQ/M (Kv7) potassium channels. *Nat Rev Neurosci* **6:**850–862.

Finlayson K, Pennington AJ, and Kelly JS (2001) [³H]dofetilide binding in SHSY5Y and HEK293 cells expressing a HERG-like K+ channel? *Eur J Pharmacol* **412:**203–212.

Glassmeier G, Hauber M, Wulfsen I, Weinsberg F, Bauer CK, and Schwarz JR (2001) Ca²⁺ channels in clonal rat anterior pituitary cells (GH3/B6). *Pflugers Arch* **442:**577–587.

Hadley JK, Noda M, Selyanko AA, Wood IC, Abogadie FC, and Brown DA (2000) Differential tetraethylammonium sensitivity of KCNQ1–4 potassium channels. *Br J Pharmacol* **129:**413–415.

Kapetanovic IM, Yonekawa WD, and Kupferberg HJ (1995) The effects of D-23129, a new experimental anticonvulsant drug, on neurotransmitter amino acids in the rat hippocampus in vitro. *Epilepsy Res* **22:**167–173.

Löscher W and Schmidt D (1994) Strategies in antiepileptic drug development: is rational drug design superior to random screening and structural variation? *Epilepsy Res* **17:**95–134.

Main MJ, Cryan JE, Dupere JR, Cox B, Clare JJ, and Burbidge SA (2000) Modulation of KCNQ2/3 potassium channels by the novel anticonvulsant retigabine. *Mol Pharmacol* **58:**253–262.

McNaughton-Smith GA, Gross MF, Wickenden AD (2000), inventors; Icagen, Inc., assignee. Benzanilides as potassium channel openers. U. S. Patent No. 6,372,767. 2002 April 16.

Peretz A, Degani N, Nachman R, Uziyel Y, Gibor G, Shabat D, and Attali B (2005) Meclofenamic acid and diclofenac, novel templates of KCNQ2/Q3 potassium channel openers, depress cortical neuron activity and exhibit anticonvulsant properties. *Mol Pharmacol* **67:**1053–1066.

Peters HC, Hu H, Pongs O, Storm JF, and Isbrandt D (2005) Conditional transgenic suppression of M channels in mouse brain reveals functions in neuronal excitability, resonance and behavior. *Nat Neurosci* **8:**51–60.

Porter RJ, Patriot A, Sachdeo R, Nohria V, and Alves WM (2007) Randomized, multicenter, dose-ranging trial of retigabine for partial-onset seizures. *Neurology* **68:**1197–1204.

Rostock A, Tober C, Rundfeldt C, Bartsch R, Engel J, Polymeropoulos EE, Kutscher B, Löscher W, Honack D, White HS, et al. (1996) D-23129: a new anticonvulsant with a broad spectrum activity in animal models of epileptic seizures. *Epilepsy Res* **23:**211–223.

Rundfeldt C and Netzer R (2000a) Investigations into the mechanism of action of the new anticonvulsant retigabine. Interaction with GABAergic and glutamatergic neurotransmission and with voltage gated ion channels. *Arzneimittelforschung* **50:**1063–1070.

Rundfeldt C and Netzer R (2000b) The novel anticonvulsant retigabine activates M-currents in Chinese hamster ovary-cells transfected with human KCNQ2/3 subunits. *Neurosci Lett* **282:**73–76.

Schenzer A, Friedrich T, Pusch M, Saftig P, Jentsch TJ, Grotzinger J, and Schwake M (2005) Molecular determinants of KCNQ (Kv7) K+ channel sensitivity to the anticonvulsant retigabine. *J Neurosci* **25:**5051–5060.

Schmidt D and Löscher W (2005) Drug resistance in epilepsy: putative neurobiologic and clinical mechanisms. *Epilepsia* **46:**858–877.

Schröder RL, Jespersen T, Christophersen P, Strobaek D, Jensen BS, and Olesen SP (2001) KCNQ4 channel activation by BMS-204352 and retigabine. *Neuropharmacology* **40:**888–898.

Sills GJ, Rundfeldt C, Butler E, Forrest G, Thompson GG, and Brodie MJ (2000) A neurochemical study of the novel antiepileptic drug retigabine in mouse brain. *Pharmacol Res* **42:**553–557.

Singh NA, Charlier C, Stauffer D, DuPont BR, Leach RJ, Melis R, Ronen GM, Bjerre I, Quattlebaum T, Murphy JV, et al. (1998) A novel potassium channel gene, KCNQ2, is mutated in an inherited epilepsy of newborns. *Nat Genet* **18:**25–29.

Stasheff SF, Anderson WW, Clark S, and Wilson WA (1989) NMDA antagonists differentiate epileptogenesis from seizure expression in an in vitro model. *Science* **245:**648–651.

Tober C, Rostock A, Rundfeldt C, and Bartsch R (1996) D-23129: a potent anticonvulsant in the amygdala kindling model of complex partial seizures. *Eur J Pharmacol* **303:**163–169.

Treiman DM (2001) GABAergic mechanisms in epilepsy. *Epilepsia* **42 (Suppl 3):**8–12.

Turski WA, Cavalheiro EA, Bortolotto ZA, Mello LM, Schwarz M, and Turski L (1984) Seizures produced by pilocarpine in mice: a behavioral, electroencephalographic and morphological analysis. *Brain Res* **3 21:**237–253.

van Rijn CM and Willems-van Bree E (2003) Synergy between retigabine and GABA in modulating the convulsant site of the GABAA receptor complex. *Eur J Pharmacol* **464:**95–100.

Wang HS, Pan Z, Shi W, Brown BS, Wymore RS, Cohen IS, Dixon JE, and McKinnon D (1998) KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. *Science* **282:**1890–1893.

Watanabe H, Nagata E, Kosakai A, Nakamura M, Yokoyama M, Tanaka K, and Sasai H (2000) Disruption of the epilepsy KCNQ2 gene results in neural hyperexcitability. *J Neurochem* **75:**28–33.

Wickenden AD, Roeloffs R, McNaughton-Smith G, and Rigdon GC (2004) KCNQ potassium channels: drug targets for the treatment of epilepsy and pain. *Expert Opin Ther Pat* **14:**457–469.

Wickenden AD, Yu W, Zou A, Jegla T, and Wagoner PK (2000) Retigabine, a novel anti-convulsant, enhances activation of KCNQ2/Q3 potassium channels. *Mol Pharmacol* **58:**591–600.

Wickenden AD, Zou A, Wagoner PK, and Jegla T (2001) Characterization of KCNQ5/Q3 potassium channels expressed in mammalian cells. *Br J Pharmacol* **132:**381–384.

Wong G, Lyon T, and Skolnick P (1994) Chronic exposure to benzodiazepine receptor ligands uncouples the gamma-aminobutyric acid type A receptor in WSS-1 cells. *Mol Pharmacol* **46:**1056–1062.

Wuttke TV, Seebohm G, Bail S, Maljevic S, and Lerche H (2005) The new anticonvulsant retigabine favors voltage-dependent opening of the Kv7.2 (KCNQ2) channel by binding to its activation gate. *Mol Pharmacol* **67:**1009–1017.

Address correspondence to: Dr. Jeffrey Krajewski, Icagen, Inc., 4222 Emperor Blvd., Durham, NC 27703. E-mail: jkrajewski@icagen.com