ICA-27243: A novel, selective KCNQ2/Q3 potassium channel activator

Authors: AD Wickenden, JL Krajewski, B. London, PK Wagoner, WA Wilson, S Clark, R Roeloffs, G. McNaughton-Smith, GC Rigdon.

Current Laboratory Affiliations:

ADW: Johnson & Johnson PRD, 3210 Merryfield Row, San Diego CA 92121
JLK, BL, PKW, RR, GMS, GCG: Icagen Inc, 4222 Emperor Blvd. Durham N.C. 27703
WAW: Neurology Research Laboratory, Veterans Administration Medical Center, Durham, NC, USA.
SC: University of Wyoming School of Pharmacy Laramie, Wyoming 82071
Running Title: **ICA-27243: A novel and selective KCNQ2/Q3 activator**

Corresponding Author:

Jeffrey Krajewski

Icagen, Inc.

4222 Emperor Blvd Durham, NC 27703 USA

(919) 941-5206

Fax (919) 941-0813

jkrajewski@icagen.com

Number of text pages: 32

Tables: 1, Supplementary

Figures: 8

References: 40

Abstract Word Count: 238

Introduction Word Count: 500

Discussion Word Count: 780

Abbreviations: GABA, \( \gamma \)-aminobutyric acid; TEA, tetraethylammonium; I-V, current-voltage; ACSF, artificial cerebrospinal fluid; EGS, electrographic seizure
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 (Singh et al., 1998; Biervert et al., 1998), suggesting that KCNQ2/Q3 channels may be attractive targets for novel anti-epileptic drugs. In the present study we have identified the compound ICA-27243, (N-(6-Chloro-pyridin-3-yl)-3,4-difluoro-benzamide) 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 XE-911 and linopirdine. ICA-27243 enhanced both ⁸⁶Rb⁺ efflux (EC⁵₀ = 0.2 µM) and whole-cell currents in Chinese Hamster Ovary cells stably expressing heteromultimeric KCNQ2/Q3 channels (EC⁵₀ =0.4 µM). Activation of KCNQ2/Q3 channels was associated with a hyperpolarizing shift of the voltage dependence of channel activation (V₁/₂ 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 (1-10µM) was found to reversibly suppress seizure-like activity in an ex vivo hippocampal slice model of epilepsy, and demonstrated in vivo anti-convulsant activity (ED₅₀ of 8.4 mg/kg) in the mouse maximal electroshock (MES) 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.
Introduction

It is estimated that up to 30% of people with epilepsy are inadequately treated with available anti-epileptic drugs (AEDs) (Schmidt and Loscher, 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 anti-epileptic mechanism that has yet to produce an approved AED is the selective opening of K⁺-channels.

Heterotetrameric KCNQ2/Q3 channels are thought to underlie the neuronal M-current, a non-inactivating, slowly deactivating, sub-threshold 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 they may represent particularly attractive targets for novel AEDs (reviewed by Wickenden, et al., 2004). First, mutations in KCNQ2/Q3 channels lead to a rare form of neonatal epilepsy in humans (Singh et al., 1998; Biervert 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 post-synaptically 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 novel AEDs and suggest that agents capable of
selectively enhancing the activation of KCNQ2/Q3 channels may represent novel anti-
convulsant 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 (Rundfeldt and Netzer, 2000; Main et al., 2000; 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 anti-
convulsant 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 out of 35 patients with refractory epilepsy (Porter et al., 2007).
Although it seems likely that KCNQ2/Q3 opening plays a significant role in the anti-
convulsant 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
(Rundfelt and Netzer, 2000), cannot be excluded. Pharmacological validation of
KCNQ2/Q3 channels as anti-epileptic drug targets would be further aided by the
availability of a more selective activator. In the present study we show that ICA-27243,
(N-(6-Chloro-pyridin-3-yl)-3,4-difluoro-benzamide) is a novel, potent and selective
KCNQ2/Q3 activator structurally distinct from other known KCNQ/M-current activators
(Figure 1). ICA-27243 also suppresses seizure-like activity in an ex vivo hippocampal
slice model, and protects against maximal electroshock-induced seizures in mice.
Methods

Materials

ICA-27243 (N-(6-Chloro-pyridin-3-yl)-3,4-difluoro-benzamide; US Patent 6,372,767) and retigabine (N-(2-Amino-4-[fluorobenzylamino]-phenyl) carbamic acid; D-23129) were synthesized in the laboratories of Icagen Inc, Durham NC. Linopirdine and XE-991 were purchased from Tocris. Unless specified otherwise, all other chemicals were purchased from Sigma-Aldrich. The SH-SY5Y cell line (CRL-2266), the WSS-1 cell line (CRL-2029) stably expressing rat α1γ2 GABA A receptors (Wong et al., 1992), and the GH3 rat pituitary cell line (CCL-82.1) were purchased from ATCC.

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 to GenBank Accession number NM_002250. CHO-K1 KCNQ cell lines were maintained in Ham’s F-12 supplemented with 10% heat-inactivated fetal bovine serum and 400µg ml−1 G418 sulphate in an incubator at 37°C with a humidified atmosphere of 5 % CO2. A HEK293 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 EMEM supplemented with 20 µM non-essential amino acids, 2.4 mM sodium pyruvate, 0.05 mg/ml gentamicin, 1 mM glutamax, 250 mL of unsupplemented Ham’s F12 media and FBS to final concentration of 10%. To differentiate cells, 10 mM retinoic acid in DMSO was added to
Molecular Pharmacology Fast Forward. Published on December 18, 2007 as DOI: 10.1124/mol.107.043216
This article has not been copyedited and formatted. The final version may differ from this version.

7

cells to a final concentration of 10 µM and incubated for 5-8 days. Cells were plated at 1.5x10^6 cells/96-well plate for experiments. Membrane potential changes in SH-SY5Y neuroblastoma cell lines were measured using the membrane potential-sensitive dye, DiBAC₄. In brief, cells were loaded with DiBac₄ (5 µM) by incubation in Earls Balanced Salt Solution (EBSS: 132 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 10 mM HEPES and 5 mM glucose) for 1h. Following loading, drug solution containing 5 µM DiBAC₄ was added to each well and changes in fluorescence following compound addition recorded. Concentration-response curves were constructed by averaging data from multiple independent assays and fitting the averaged data with a logistic function as described below:

(Equation 1) \( \text{Response} = \left\{ \frac{A_1-A_2}{(1+x/x_0)^p} \right\} + A_2 \)

Where \( A_1 \) is the initial response, \( A_2 \) is the final response, \( x_0 \) is the mid-point (i.e., EC₅₀) and \( p \) is power (slope factor).

\(^{86}\text{Rb}^+ \text{Efflux.}

CHO cells stably expressing KCNQ2/Q3, KCNQ4, or KCNQ3/Q5 were grown as confluent monolayers in 96 well tissue culture plates (Corning) in MEM alpha with 10% heat inactivated fetal bovine serum and 400 µg/mL G418. Cells were incubated overnight in growth media containing 1 µCi mL⁻¹ \(^{86}\text{Rb} \) (Perkin Elmer) to permit intracellular uptake of the isotope. At the end of the incubation period, the \(^{86}\text{Rb} \) solution was aspirated and the cells washed three times with EBSS. The cells were then pre-incubated for 10 minutes at room temperature in 100 µL/well of EBSS or EBSS containing test compound at concentrations varying from 1 nM to 100µM. At the end of this period, the supernatant from the wells was aspirated and 100 µL of EBSS containing a sub-maximal 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% sodium dodecyl sulfate 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 plate were then counted using a Wallac MicroBeta liquid scintillation counter. Enhancement of efflux was expressed as a percentage of that induced by 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 (Equation 1).

Electrophysiology

For electrophysiological studies, cells were removed from the culture flask by brief trypsinization and replated at low density onto glass cover slips. Cover slips were placed in a bath on the stage of an inverted microscope and superfused (approximately 1 ml min⁻¹) with EBSS. Membrane currents were measured using the whole cell configuration of the patch-clamp technique. Pipettes were filled with intracellular solution of the following composition (mM): KCl (140), MgCl₂ (2), EGTA (10), HEPES (10), K₂ATP (5), pH 7.3-7.4 with KOH, and had a resistance of 1-3 MΩ. Uncompensated series resistance was typically 3-10 MΩ, and >90% series resistance compensation routinely achieved. As a result, voltage errors were negligible. Current records were acquired at 2–10 kHz and filtered at 1-2 kHz. All recordings were made at room temperature (22-24 °C) using an AXOPATCH 200B amplifier and PCLAMP software. Compounds were resuspended in DMSO and tested at a final DMSO concentration of 0.1%, which did not cause changes in KCNQ current kinetics (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 –20mV (for KCNQ4). KCNQ channel activation is sub-maximal 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 (Equation 1) to derive average EC50 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 (Gapp) was calculated according to:

\[
\text{(Equation 2) } G_{\text{app}} = \frac{I}{V-E_K}
\]

Where I = steady-state current, V = step potential and E\textsubscript{K} = 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:

\[
\text{(Equation 3) } Y = \frac{1}{1+\exp\left[\frac{(V_h-V_m)}{k}\right]}
\]

where V\textsubscript{h} is the voltage for half activation, V\textsubscript{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 (Equation 1).
**GABA-activated chloride currents**

For recordings from WSS-1 cells, pipettes were filled with an intracellular solution of the following composition (mM): KCl (140), MgCl₂ (2), EGTA (5), HEPES (10), MgATP (5), pH 7.3-7.4 with KOH, and had a resistance of 2-3 MΩ. Inward chloride currents were evoked with a sub-maximal concentration of γ-amino-butyric acid (GABA, 10 µM) at a holding potential of −60 mV. GABA activated currents were measured before 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 percent inhibition, calculated with the following equation:

\[
\text{(Equation 4) } \% \text{ Inhibition} = ((\text{Control-Drug})/\text{Control}) \times 100
\]

**hNav1.2**

For hNav1.2 recordings, pipettes were filled with an intracellular solution of the following composition (mM): CsCl (40), CsF (100), NaCl (5), MgCl₂ (2), EGTA (5), HEPES (10), glucose (5), pH 7.3-7.4 with KOH, and had a resistance of 1-2 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 following an 8 sec prepulse to the V₁/₂ 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 prior to the test step. Drug effects are presented as percent inhibition, calculated with Equation 4.

Use-dependence of compound block was performed by holding at −120 mV then stepping to 0 mV for 50 ms at 0.1 second intervals (10 Hz). Currents were normalized to the peak...
current at time zero \( (I_0) \) and presented as percent available current. All data are expressed as mean \( \pm \) standard error of the 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 (mM) Cs-Asp (110), CsCl (20), HEPES (10), EGTA (5), MgCl\(_2\) (1), Mg-ATP (5), Na-GTP (0.1), pH 7.3 with CsOH and the external solution was (mM) NaCl (138), KCl (5.4), HEPES (10), BaCl\(_2\) (20), MgCl\(_2\) (1), 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 application of drug, during application of drug and after washout of drug, and effects are calculated using Equation 4.

**Hippocampal slice electrographic seizure (EGS) model of epilepsy**

Juvenile male Sprague-Dawley rats (P14 & P15) were used for these experiments because robust seizure-like activity occurs during this age range (Clark *et al.*, 1993). All protocols were approved by the VA 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 (mM): NaCl 120; KCl 3.3; NaH\(_2\)PO\(_4\) 1.23; MgSO\(_4\) \( \cdot \) 7 H\(_2\)O 0.9; NaHCO\(_3\) 25; CaCl\(_2\) 1.3; dextrose 10. Hippocampi were removed and cut into nominally 650 \( \mu \)m thick transverse slices and allowed to equilibrate in oxygenated ACSF at room temperature (RT) for at least one hour. Slices were then transferred to a Haas-style recording chamber and perfused with oxygenated ACSF \(~ 2 \) ml/min at 30.5°C-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) connected to a Grass S88 Stimulator. Extracellular field potentials in the stratum pyramidale of area CA3 were recorded with a single barrel thin-walled 1-2 MΩ capillary glass recording electrode (A-M Systems) filled with 150 mM NaCl, filtered and amplified (Duo 773 electrometer, World Precision Instrument), 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 VCR).

Single stimulus pulses (0.1 ms duration and 100-600 µA intensity) were applied to define intensity necessary to evoke a maximal field response in area CA3. Then, 60 Hz, 2-second stimulus trains were delivered at two-fold this intensity (see 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 5-6 stimulus trains, until the responses stabilized. This concentration of DMSO has been previously found to have no effect on in vitro EGS activity (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 6 trains at 10 minute intervals. In some experiments a drug-application phase was followed by a wash-out phase in control ACSF. EGS data were analyzed by measuring the seizure duration of the clonic stage at the 1st burst-event to the last burst-event where the trace crossed the pre-burst baseline, while the tonic phase was measured as burst-event-frequency calculated as the highest number of burst-events during any one second 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 (1-100mg/kg) or vehicle (5%DMSO/95% Olive oil) by oral gavage 10 minutes prior to experiment. Following 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 second. If extension did not occur within 6 seconds, the animal was scored as protected and a value of 6 seconds was recorded. The data are presented as mean ±SEM and are the result of n= 7-15 animals. The ED$_{50}$ values for ICA-27243 effects were calculated by fitting the data to a logistic function (Equation 1).

**Receptor Binding Panel**

ICA-27243 was tested by CEREP (Le Bios l’Evêque-B.P.1- 86600 Celle l’Evescault, France) in a broad receptor binding panel at 10 µM (Supplemental Table 1).

**RESULTS**

**ICA-27243 is an M-Current Activator**

In order to identify novel M-current activators we developed a high-throughput fluorescence-based assay utilizing 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 (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 (Figure 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 (Figure 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 since 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 (IC50 60 ± 5 nM; n = 4), linopirdine (IC50 2.8 ± 0.5 µM; n = 4) and tetraethylammonium (IC50 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; Wickenden et al., 2000; Hadley et al., 2000).

ICA-27243 is a KCNQ2/Q3 subtype selective opener

86Rb+-efflux studies

In order 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 86Rb+ efflux assay. ICA-27243 caused a concentration dependent increase in 86Rb+ efflux from CHO cells expressing KCNQ2/Q3 channels (Figure 3). Threshold activity was observed with concentrations of approximately 0.03µM and on average, half maximal channel activation (EC50) occurred at a concentration of 0.20 ± 0.03µM, n=27. Maximal 86Rb efflux was observed at 10 µM,
and was similar in magnitude to the flux induced by depolarization with 70mM KCl. At concentrations of 30 µM and 100 µM, ICA-27243 caused a small reduction in $^{86}$Rb efflux compared to 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 (Figure 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 (Figure 3B, p<0.05, n=8). Based on the threshold for current amplitude increases, it appears that selectivity of ICA-27243 for KCNQ2/Q3 channels over KCNQ3/Q5 channels is 100 - 300 fold.

KCNQ1 (KvLQT1, Kv7.1) is a member of the KCNQ potassium channel family which, when co-expressed 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**

In order 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 Figure 4A,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 (Wickenden *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 (Figure 4A). This limited effect of ICA-27243 was not due to poor expression of KCNQ3/Q5 channels, since the non-selective KCNQ channel opener, retigabine (10µM), produced a robust increase in outward current in the same cells (Figure 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= -10mV for KCNQ4 compared to $V_{1/2}$ activation = -30mV 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 (Figure 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 EC50 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 (Figure 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 −80 mV, stepped to voltages in the range of −100 mV 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, non-inactivating outward current (Figure 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 Figure 5B. Multiple experiments show the KCNQ2/Q3 V1/2 activation value was $-32.2 \pm 1.5$ mV (n = 7) under control conditions and was shifted to $-51.4 \pm 3.6$ mV (n = 4) in the presence of 10 µM ICA-27243. The slopes of the activation curves appeared 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
(ΔV$_{1/2}$) in the mid-point of the activation curve is shown in Figure 5C. The half maximal shift in ΔV$_{1/2}$ for activation with ICA-27243 was observed at 4.8 µM ± 1.6 µM (slope factor was 0.78±0.22).

**ICA-27243 does not interact with other CNS ion channels targeted by anti-epileptic drugs.**

In order to address broader ion channel selectivity, we examined the effects of ICA-27243 on other channels that are molecular targets of known anti-epileptic agents. For example, GABA$_A$-activated chloride channels, voltage-dependent sodium channels and some types of calcium channels are thought to be modulated by anti-epileptic drugs such as benzodiazepines, carbamazepine and lamotrigine (for reviews see Trieman DM, 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.*, 1992). Currents were activated using a sub-maximal concentration of GABA (10 µM) at a holding potential of −60 mV in symmetrical chloride solutions (Figure 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 (HVAC)

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 (\(\omega\)-agatoxin IVA, \(\omega\)-conotoxin MVIIC sensitive) and the remainder undefined (Glassmeier et al., 2001). Barium currents were recorded in the absence and presence of ICA-27243 (Figure 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 \(\mu\)M (1.0 ± 9.6%; \(n = 3\)).

Nav1.2 Voltage-dependent Sodium Channels

One of the more widely expressed voltage-dependent sodium channels in the mammalian central nervous system is Na\(_{\text{v}}\)1.2. The effect of ICA-27243 on human Nav1.2 channel currents recombinantly expressed in HEK-293 cells is shown in Figure 6 C, 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 \(\mu\)M) exhibited no significant state- or use-dependent inhibition human Nav1.2. ICA-27243 (10\(\mu\)M) inhibited Nav1.2 by only 5 ± 2\% (\(n = 4\)) in experiments designed to assess inactivated state block (Figure 6C) and by only 5 ± 3 \% block (compared to 4 ± 1 \% n=4 in control experiments) in a 10Hz use-dependent protocol (Figure 6D). In contrast, the positive control sodium blocker, amitriptyline (1
µM), blocked Nav1.2 by 54 ± 2% (n=4) in the inactivated state protocol (Figure 6C), and by 20 ± 1% (n=3, p<0.001) in the 10Hz protocol (Figure 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 10µM, this compound inhibited radioligand binding to adenosine A1 receptors, A2 receptors, opiate receptors, PCP receptors, norepinephrine and 5-HT transporters by ≤ 33% (Supplemental Table 1).

ICA-27243 suppresses seizure-like activity in vitro

In order 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-minute 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 electrographic seizures (EGSs) have two distinct firing patterns: the tonic phase is the early, high-frequency firing pattern seen within the first 2-6 seconds of the EGS and the 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 4-6 trains (Figure 7) and is typical for slices from P14 & P15 rats (control EGS duration: 36.5 ± 3.18 sec.; tonic phase: 15.0 ± 1.26 events/sec; n=6). Superfusion of the slice for 40 minutes with 1µM ICA-27243 suppressed EGS duration by 48.6 ± 5.25% and the tonic phase by 22.94 ± 10.33% (p < 0.01; n=4). At the highest dose tested (10 µM), EGS activity was almost completely blocked (99.8 ± 0.21%; n=2) and was reversible, with EGS activity returning within approximately 70 minutes 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**

In order to more directly evaluate anti-seizure 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 (Loscher and Schmidt, 1994). When administered orally at 10 mg/kg, ICA-27243 achieved a maximal plasma concentration (C_{max}) of 5.6 ± 0.2 µM and 2.6µM ± 0.1 in the brain (data not shown). A ten minute pretreatment with ICA-27243 (1-100 mg/kg p.o.) produced a dose-dependent increase in the latency to hind limb extension, exhibiting an ED_{50} value of 8.4 mg/kg (95% CI 5.6 to 12.5 mg/kg).

**Discussion**

There is an unmet medical need for broad-spectrum, safe anticonvulsants since at least 30% of patients are refractory to current therapies (Schmidt and Loscher, 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 inhibits EGS activity in hippocampal slices. Furthermore, ICA-27243 is orally bioavailable and exhibits anti-convulsant 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 pre-clinical epilepsy models further validating KCNQ channels as novel anti-epileptic drug targets. The mechanism of action of ICA-27243 involves a hyperpolarizing shift in the voltage-dependence of activation of KCNQ2/Q3 channels (-19mV at 10µM). Other KCNQ channel activators such as retigabine (Rundfeldt and Netzer, 2000; Main et al., 2000; Wickenden et al., 2000), BMS-204352 (Schroder et al., 2001), acrylamide S-1 (Bentzen et al., 2006) and meclofenamate (Peretz et al., 2004) also induce hyperpolarizing shifts in the voltage-dependence of activation and act in a qualitatively similar manner. The magnitude of this shift appears greater for retigabine (~-33mV at 10µM) than ICA-27243. Whether this represents a true difference in efficacy or a difference in potency is not clear since true maximum shifts were not established in the present study and the study of Wickenden et al., 2000. It is possible ICA-27243 acts directly on the channel to modify the voltage-dependence of channel activation or, alternatively, 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; 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, PLC, PIP2, DAG and cAMP (For a recent review see Delmas and Brown, 2005.)

ICA-27243 is a potent KCNQ2/Q3 channel opener but is less potent as a KCNQ4 activator and only very weakly active against KCNQ3/Q5 channels. This profile is quite distinct from that of previously described KCNQ channel activators, which do not discriminate among neuronal KCNQ sub-types (i.e., KCNQ2 – KCNQ5) (Schroder et al., 2001, Wickenden et al., 2001). A conserved tryptophan in the N-terminal part of S5 has been identified as a key residue responsible for the actions of retigabine, BMS-204352 and acrylamide S-1 (Wuttke et al., 2005; Schenzer et al., 2005; Bentzen et al., 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 sub-types. The finding that ICA-27243 can discriminate among KCNQ sub-types 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 sub-type selectivity, we also evaluated the effects of ICA-27243 against validated anti-epileptic 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 non-selective 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 (Rundfelt and Netzer 2000) 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 \textit{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 (Loscher and Schmidt 1994). Our results are the first to demonstrate that selective pharmacological opening of KCNQ2/3 channels is efficacious in pre-clinical 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 anti-epileptic drug target.

**Acknowledgements.**

h-KCNQ2 was kindly provided by Dr TJ Jensch, Zentrum fur Molekulare Neurobiologie (ZMNH), University of Hamburg, Hamburg, Germany.

The authors wish to thank Neil Castle, PhD and Doug Krafte, PhD (Icagen) for their insightful discussion regarding this manuscript.
References


Ca2+ channels in clonal rat anterior pituitary cells (GH3/B6). Pflugers Arch 442(4):577-
587.

Differential tetraethylammonium sensitivity of KCNQ1-4 potassium channels. Br J
Pharmacol 129(3):413-415.

new experimental anticonvulsant drug, on neurotransmitter amino acids in the rat

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(2):253-262.

Passmore GM, Selyanko AA, Mistry M, Al-Qatari M, Marsh SJ, Matthews EA,

Meclofenamic acid and diclofenac, novel templates of KCNQ2/Q3 potassium channel


Rundfeldt C and Netzer R (2000) 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(12):1063-1070.


Figure Legends

Figure 1. ICA-27243 is structurally distinct from other known KCNQ/M-current activators.

Figure 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 DiBAC₄. Grey bar indicates pre-incubation time with EBSS (solid line) or 10 µM XE-991 (dashed line). Black bar indicates compound addition. (A) 10 µM retigabine (solid line), a known M-current activator, induces a hyperpolarization in SH-SY5Y cells that is blocked by pre-incubation of 10 µM XE-991 (dashed line). (B) 10 µM ICA-27243 (solid line) causes a similar XE-991-sensitive hyperpolarization in differentiated SH-SY5Y cells (dashed line). Time bar = 500 seconds.

Figure 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 an ⁸⁶Rb⁺-efflux assay with increasing doses of ICA-27243. The data was normalized to Eₘₐₓ and fit to a logistic function. The calculated EC₅₀ value for KCNQ2/Q3 activity was 0.20 ± 0.03 µM, n=27, and for homomeric KCNQ4 the EC₅₀ was 7.1±0.1 µM, n=11. An Eₘₐₓ could not be achieved with KCNQ3/Q5 cells even with 100 µM ICA-27243, but a significant level of ⁸⁶Rb⁺-efflux was observed at 10 µM ICA-27243 (asterisk p<0.05, n=8) (B).

Figure 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. Dotted line denotes 0 pA. Drug-induced currents from
KCNQ3/Q5 did not reach $E_{\text{max}}$ even with 30µM ICA-27243. 10µM retigabine substantially increased outward currents in ICA-27243-insensitive cells. Vertical bar for KCNQ2/Q3, KCNQ4 and KCNQ3/Q5 is 200pA, 250 pA and 50 pA respectively. Horizontal bar for KCNQ2/Q3, KCNQ4 and KCNQ3/Q5 is 5s, 15s and 10s respectively.

(B) ICA-27243-induced outward currents were normalized to $E_{\text{max}}$ and fit to a logistic function. ICA-27243 exhibited an EC$_{50}$ of $0.4 \pm 0.1$µM (mean ± sem) (slope = $1.0 \pm 0.1$; n = 7) on KCNQ2/Q3, and an EC$_{50}$ of $9.7 \pm 1.2$µM (slope=0.9± 0.1; n=5) on KCNQ4.

**Figure 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 mV to +30 mV for 3 seconds, 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 10µM ICA-27243 and fit with a Boltzmann function. Data from multiple experiments show the KCNQ2/Q3 $V_{1/2}$ activation value was $-32.2 \pm 1.5$; $n_H=9.1 \pm 0.9$ (n = 7) under control conditions and was shifted to $-51.4 \pm 3.6$; $n_H= 9.7 \pm 1.5$ (n = 4) in the presence of 10 µM ICA-27243. (C) The magnitude of the ICA-27243-induced shifts in the mid-point 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 $4.8\mu M \pm 1.6\mu M$; $n_H= 0.78 \pm 0.22$.

**Figure 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 sub-maximal concentration of GABA
(10µM) at a holding potential of –60 mV in symmetrical chloride solutions generated inward currents (black line). Preincubation with 30µM 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 5µM diazepam (gray bar). (B) High voltage-activated calcium channel (HVAC) 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 HVAC blocking activity at 30µM (1.0 ± 9.6%; n = 3). ICA-27243 was tested on hSCNIIA channels at half-inactivation (C) and 10 Hz stimulus protocol (D) (see methods). At 10 µM ICA-27243 had little effect on I_Na, regardless of the voltage protocol. (C). 1µM amitriptyline was used as a positive control in both assays.

**Figure 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 methods). High frequency stimulation produces a clonic-phase firing pattern with a 36.5 ± 3.18 second duration, followed by a tonic phase frequency of 15.0 ± 1.26 events/sec; n=6 (Control). Following a 10-minute incubation with 1 µM ICA-27243, the clonic phase duration was reduced by 48.6 ± 5.25% and tonic phase frequency by 22.94 ± 10.33% (p = 0.006; n=4). A 40 minute incubation with 10 µM ICA-27243 blocked 99.8 ± 0.21% of the EGS seizure duration (n=2). The blocking effect of ICA-27243 was almost completely reversed with a 70 minute wash-out (Wash). Vertical bar: 1 mV, Horizontal bar: 2 seconds.

**Figure 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 maximal electroshock (MES) assay by measuring the latency to seizure activity (see
methods). Increasing doses of ICA-27243 given orally 10 minutes prior to electroshock stimuli protects mice in a dose-dependent manner, with an ED$_{50}$ of 8.4 mg/kg (95% CI 5.6 to 12.5 mg/kg). Shaded region denotes vehicle range (5% DMSO, 95% olive oil), asterisk denotes a p>0.05. Number of animals = 7-15.
Figure 2
Figure 4

A.

KCNQ2/Q3

- 10μM ICA-27243
- 1μM
- 100nM
- 30nM

KCNQ4

- 100μM ICA-27243
- 3μM
- 10μM

KCNQ3/Q5

- 10 μM retigabine
- 30 μM ICA-27243
- 0.3 μM

B.

Normalized to $E_{\text{max}}$

- Log [ICA-27243]
- KCNQ2/Q3
- KCNQ4
A.

B.

C.

Figure 5
Figure 6

A. 30μM ICA-27243

B. 200μM Cd²⁺

C. 10μM ICA-27243

D. Percent Available Current

- 10μM ICA-27243
- Control
- 1μM amitriptyline
Figure 7
Figure 8