A Novel Modulator of Kv3 Potassium Channels Regulates the Firing of Parvalbumin-Positive Cortical Interneurons

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ABSTRACT

Kv3.1 and Kv3.2 high voltage-activated potassium channels, which display fast activation and deactivation kinetics, are known to make a crucial contribution to the fast-spiking phenotype of certain neurons. Pharmacological experiments show that the blockade of native Kv3 currents with low concentrations of tetraethylammonium or 4-aminopyridine impairs the expression of this firing phenotype. In particular, Kv3 channels are highly expressed by fast-spiking, parvalbumin-positive interneurons in corticolimbic brain circuits, which modulate the synchronization of cortical circuits and the generation of brain rhythms. Here, we describe a novel small molecule, (5R)-5-ethyl-3-(6-[[4-methyl-3-(methyloxy)phenyl]oxy]-3-pyridinyl)-2,4-imidazolidinedione (AUT1), which modulates Kv3.1 and Kv3.2 channels in human recombinant and rodent native neurons. AUT1 increased whole currents mediated by human Kv3.1b and Kv3.2a channels, with a concomitant leftward shift in the voltage dependence of activation. A less potent effect was observed on hKv3.3 currents. In mouse somatosensory cortex slices in vitro, AUT1 rescued the fast-spiking phenotype of parvalbumin-positive–fast-spiking interneurons following an impairment of their firing capacity by blocking a proportion of Kv3 channels with a low concentration of tetraethylammonium. Notably, AUT1 had no effect on interneuron firing when applied alone. Together, these data confirm the role played by Kv3 channels in the regulation of the firing phenotype of somato-sensory interneurons and suggest that AUT1 and other Kv3 modulators could represent a new and promising therapeutic approach to the treatment of disorders associated with dysfunction of inhibitory feedback in corticolimbic circuits, such as schizophrenia.

Introduction

The firing pattern of projection neurons (e.g., cortical pyramidal neurons) throughout the central nervous system is critically shaped by synaptic inputs from diverse classes of a local interneuron (Buhl et al., 1994). Specific subclasses of an interneuron, which can fire at high frequencies, are able to synchronize the activity of pyramidal neurons (Cobb et al., 1995; Tamás et al., 2000). High-frequency firing of interneurons requires that both action potentials (APs) and after-hyperpolarizations are brief. This is ensured by the opening of rapidly activating and deactivating voltage-gated potassium channels of the Kv3 family (Du et al., 1996; Erísir et al., 1999). In numerous brain areas, Kv3.1 and Kv3.2 subtypes have been shown to be involved in the fast repolarization of interneuron action potentials and the generation of high-frequency firing in vitro (Erísir et al., 1999; Atzori et al., 2000; Lau et al., 2000; Deuchars et al., 2001; Lien and Jonas, 2003; Joho and Hurlock, 2009; Johnston et al., 2010). Pharmacological experiments show that the blockade of native Kv3 currents with low concentrations of TEA or 4-aminopyridine impairs high-frequency firing (Martina et al., 1998; Wang et al., 1998; Rudy et al., 1999). A similar impairment of firing can be achieved by reducing Kv3-like conductances in in silico neuron models (Lien and Jonas, 2003).

Kv3.1 and Kv3.2 potassium channels are highly expressed by fast-spiking (FS), parvalbumin-positive (PV⁺) interneurons, a subpopulation of the GABAergic neuron that represents a major class of inhibitory interneurons in corticolimbic brain circuits (Chow et al., 1999; Rudy and McBain, 2001). PV⁺ interneurons play a critical role in the synchronization of cortical circuits and in the generation of rhythms (Bartos et al., 2007; Cardin et al., 2009). Thus, activation of Kv3 channels allows FS interneurons to fire accurately at high frequency to orchestrate the activity of cortical networks.
We have identified a series of small molecules, such as the compound (5R)-5-ethyl-3-(6-[(4-methyl-3-methoxyphenyl)oxy]-3-pyridinyl)-2,4-imidazolidinedione (AUT1) (Alvaro et al., 2011), which modulates human Kv3.1 and Kv3.2 channels and which can modify the activity of PV⁺-FS interneurons in the mouse somatosensory cortex in vitro. Selective manipulation of FS interneuron firing by pharmacological manipulation of Kv3 channels has considerable potential for the treatment of a range of neuropsychiatric disorders, such as schizophrenia, which has been associated with functional deficits of these specific neurons (Lewis et al., 2012; Nakazawa et al., 2012). In the present study, we evaluate the pharmacology of AUT1 on recombinant human Kv3.1b and Kv3.2a channels, confirm the localization of these two Kv3 channel subtypes on PV⁺ neurons in the mouse somatosensory cortex, and finally, demonstrate that AUT1 can rescue the FS phenotype of these interneurons in vitro.

Materials and Methods

Recombinant Channel Electrophysiology

Cell Culture.

IonWorks assay. CHO-K1 cells stably expressing hKv3.2a (NM_139136.2) or hKv3.3 (NM_004977.2) and CGE22 frozen cells pretransduced with hKv3.1b (NM_00112741.1) BacMam were used. Cells (5–10 million) were plated in complete Dulbecco’s modified Eagle’s medium and F-12 medium (GibcoLife Technologies, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (PAA), 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA), 1% l-glutamine (Invitrogen), and 1% nonessential amino acids (PAA). The culturing conditions were 37°C under 5% CO₂ for 24 hours and 30°C under 5% CO₂ for 18–24 hours.

Q-Patch assay. BacMam-transduced U2OS cells expressing hKv3.1b or hKv3.2a channels were used. They were transduced by incubation overnight (8 hours) with 1% of a BacMam-hKv3.1b or hKv3.2a reagent (2.3 × 10⁶ pfu/ml). Cells were resuspended in fresh 293 SFM II medium (Gibco) containing 25 mM HEPES (Gibco) and 0.04 mg/ml soy bean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO) and transferred to a Q-Patch recording station.

Voltage-Clamp Recordings.

IonWorks assay. The population patch-clamp mode of an automated voltage clamp recording with IonWorks Quattro (Molecular Devices, Sunnyvale, CA) was used. Briefly, the effects on these channels were tested using 384-well population patch-clamp plates. Devices, Sunnyvale, CA) was used. Briefly, the effects on these channels were tested using 384-well population patch-clamp plates. All experiments were carried out using an intracellular solution containing (in millimolar) NaCl (145), KCl (4), MgCl₂ (1), CaCl₂ (2), glucose (10), and HEPES (10), pH 7.3 adjusted with NaOH, and whole-cell recordings were carried out using an intracellular solution containing (in millimolar) NaCl (10), KGlucosate (100), HEPES (10), EGTA (10), and KF (32), pH 7.2 adjusted with KOH. Cells were transfected with 100–500 mV, and currents were evoked by voltage steps (200-millisecond duration) from –40 to +30 mV (in 10-mV increments). Steady-state inactivation was evaluated using a two-step voltage protocol: a 30-second conditioning prepulse from –100 to +20 mV (20-mV increments) from a holding potential of –60 mV, followed by a test pulse at +40 mV applied for 200 milliseconds. Vehicle (0.1% DMSO) or AUT1 was applied for at least 3 minutes prior to subsequent voltage step protocol application. A P/4 leak subtraction protocol was applied, and serial resistance values were constantly monitored. Any cell where serial resistance exceeded 20 MΩ was eliminated from the subsequent analysis.

Data Analysis. IonWorks assay. Paired comparisons between predrug and postdrug additions of steady-state currents measured at a –15 mV voltage step were used to determine the effect of the compound. For the hKv3.1 and hKv3.2 assays, concentration-response data were normalized to the maximum effect (i.e., 100%) observed with the standard. Normalized data were then plotted against the concentration of the compound and analyzed using GraphPad Prism software 6.03 (GraphPad Software, Inc., La Jolla, CA). The log of concentrations required to increase the current by 50% (pEC50) was determined by fitting data points to the four parameter logistic function (sigmoidal dose response)

\[ Y = \text{Bottom} + \left( \frac{\text{Top} - \text{Bottom}}{1 + 10^{\frac{X-10\log\text{EC}_{50}}{Y}}} \right) \]

where Bottom is the Y value at the bottom plateau, Top is the Y value at the top plateau, and LogEC₅₀ is the X value when the response is halfway between Bottom and Top. \( \tau_{activation} \) values were calculated by fitting the rising phase of the currents evoked at –15 mV to an exponential function (one-phase sloping baseline). Only cells with mean current amplitude at +40 mV greater than 0.5 nA and a seal resistance of less than 20 MΩ were included for data analysis. A similar procedure was used for hKv3.3 channels, although no standard was included in these assays. Thus, data were normalized to the mean value of the currents in the presence of 0.5% DMSO in each assay replication.

Q-Patch assay. Data analysis was performed using Sophion’s QPatch assay software in combination with GraphPad Prism software 6.03 and Microsoft Excel (Redmond, WA). Potentiation of the currents was calculated as percentage potentiation = [current (cpd/current (veh))¹₀₀, where current (cpd) was the amplitude of the steady-state current in the presence of the compound and current (veh) was the amplitude in the presence of vehicle. For voltage dependence of activation and/or inactivation experiments, normalized conductance-voltage plots were obtained by normalizing conductance (G) to maximal conductance (Gₘₐₓ) and fit using the Boltzmann isofrom

\[ G = \frac{G_{\text{max}}}{1 + \exp[(V - V_{1/2})/k]} \]

where \( V_{1/2} \) is the voltage at half-maximal activation and \( k \) is the slope factor. Conductance values were obtained by dividing the current by the K⁺-electrochemical driving force \( [K]/(V - E_{\text{m}}) \), where \( E_{\text{m}} \) is membrane potential. The effect of AUT1 on half-activation voltages was evaluated using a one-way analysis of variance (ANOVA), whereas the effect on \( G_{\text{max}} \) was evaluated using a paired t test. An unpaired t test was performed to compare the inactivation \( V_{1/2} \) during the control period and after AUT1 application. Results are reported as mean ± S.E.M. Differences were considered significant for \( P < 0.05 \).

Cross-Screening Selectivity Functional Assays

These studies were conducted on behalf of Autifony by Scinovo (GlxoxSmithKline, London, UK), and represent a panel of functional assays available within GlaxoSmithKline at the time of the study.
Data were obtained from 11 point concentration-response curves using a four-parameter logistic fitting module in Microsoft Excel within ActivityBase (IDS, London, UK). Potency is reported as pEC_{50}. Experiments were only considered for the analysis when two-thirds of the pharmacological standards had pXC_{50} ≥ 0.5 log units within the respective average assay value of data derived over the previous 3 months. Pharmacological standards were commercially available compounds that were reported as specific modulators of the targets.

**Immunohistochemistry**

**Slice Preparation.** Coronal brain sections (30-μm thickness) from C57BL/6 and glutamate decarboxylase 1 (GAD1)-enhanced green fluorescent protein (EGFP) mice (25–35 days old) were collected at approximately −1.58 mm from the bregma. A heat-induced antigen retrieval step was performed by incubating the slices at 80°C for 20 minutes in preheated 10 mM sodium citrate (pH 9), followed by 10 minutes of cooling at room temperature, and by incubation in a blocking solution (0.1 M PBS + 3% milk + 3% bovine serum albumin + 0.1% cold water fish gelatin + 0.1% Triton X-100). Slices were then incubated with specific antibodies as follows: rabbit anti-Kv3.1b at 1:500 dilution, rabbit anti-Kv3.2 custom-made by Cambridge Research Biochemicals (Billingham, UK) [according to the peptide sequence used by Chow et al. (1999)] and mouse anti-parvalbumin (anti-PV) at 1:2,000 dilution. The tissues were incubated with two antibodies (i.e., anti-Kv3.1b + anti-PV or anti-Kv3.2 + anti-PV) for 48 hours in a blocking solution and then washed with buffer (0.1 M PBS + 0.05% Tween-20) before adding Alexa Fluor fluorescent secondary antibodies (488-goat anti-rabbit and 594-goat anti-mouse).

**Image acquisition.** Cells coexpressing parvalbumin and Kv3.1b or Kv3.2 in the somatosensory cortex of each brain hemisphere were identified, distinguishing among superficial layers (II–III), the intermediate layer (IV), and deep layers (V and VI). Digital images representing PV and Kv3 staining for the same field were merged with a fluorescence lamp with an EGFP filter. Only EGFP-positive targets. In order to compare PV and Kv3 staining for the same field, the contrast video microscopy (Hamamatsu C5985, Hamamatsu City, Japan) was used.

**Somatosensory Cortex Electrophysiology**

**Animals.** GAD1-EGFP transgenic mice (http://jaxmice.jax.org/strain/007673.html) were purchased from the Jackson Laboratory (Bar Harbor, ME). GAD1-EGFP mice selectively express EGFP in PV+ interneurons in the cortex. EGFP expression is not reported in other interneuron classes positive for somatostatin, cholecystokinin, calretinin, and vasoactive intestinal peptide in these mice (Chattopadhyaya et al., 2004).

**Slice Preparation.** Experiments were performed on 250-μm-thick brain slices containing the somatosensory cortex prepared from male or female animals as described (Agmon and Connors, 1991). Briefly, brains were removed from deeply anesthetized (isoflurane) 25- to 35-day-old GAD1-EGFP mice. Slices were cut using a DTK 1000 microslicer (DSK, Kyoto, Japan) in the following solution (in millimolar): KCl (2.5), CaCl2 (0.1), NaH2PO4 (1.2), MgCl2 (5), NaHCO3 (26), sucrose (189), and glucose (10), kept at 2–6°C and gassed with 95% O2–5% CO2. After cutting, the slices were left to equilibrate for at least 1 hour in artificial cerebrospinal fluid containing (in millimolar) NaCl (120), KCl (2.5), CaCl2 (2), NaH2PO4 (2.5), MgCl2 (1.5), NaHCO3 (26), and glucose (10) at room temperature and saturated with 95% O2–5% CO2.

**Current Clamp Recordings.** Somatosensory cortex slices from GAD1-EGFP mice were placed in a submersion chamber mounted on the stage of an upright microscope (Axioskop; Carl Zeiss, Oberkochen, Germany), and cell visualization was accomplished with a 40× objective lens using infrared-differential interference contrast video microscopy (Hamamatsu C5985, Hamamatsu City, Japan). PV+ interneurons were identified by illuminating the slice with a fluorescence lamp with an EGFP filter. Only EGFP-positive neurons were recorded. Whole-cell recordings in current-clamp mode were performed using borosilicate-glass patch pipettes (World Precision Instruments, Sarasota, FL) pulled using a Sutter P-97 electode puller and filled with an internal solution containing (in millimolar) KGlucuronate (144), MgCl2 (3), EGTA (0.2), HEPES (10), Mg-ATP (4), and Na-GTP (0.5), pH 7.3 adjusted with KOH (tip resistance of 4–7 MΩ). Recordings were carried out at room temperature (20–22°C) using a Multiclamp 700B amplifier (Molecular Devices, LLC). Current-command protocols (indicated below) and data acquisition were performed using pClamp 10.0 software and the Digidata 1320A interface (Molecular Devices, LLC). Data were filtered at 3 kHz and sampled at 10 kHz. The firing activity of the cortical interneurons was evaluated by applying long current steps (600 milliseconds) at different intensities, with 50-pA intensity increments. A hyperpolarizing current was injected to maintain the resting potential of the cells (near −80 mV). An online bridge-balance compensation was carried out.

**Data Analysis.** Data were analyzed using Clampfit 10.0 (Molecular Devices, LLC) in combination with GraphPad Prism software 6.03 and Microsoft Excel. The mean action potential frequency for each current step was then calculated. Since the threshold to evoke action potentials differed across the cells, current step intensities were expressed as pA from threshold. The action potential half-width and first derivative (FD) of the action potential waveform were automatically calculated by Clampfit. From the first derivative, the mean amplitude of the first downward peak was calculated as an expression of the rate of repolarization of the action potential.

The effects of the drug treatments on the action potential frequency were evaluated using a two-way ANOVA and, as appropriate, post hoc planned comparisons. Similarly, the effect of the drug on the action potential half-width and amplitude of the negative peak of the first derivative was evaluated using a one-way ANOVA followed by Dunnett’s test or unpaired t test when indicated. All of the statistical analyses were conducted using the statistical tool of GraphPad Prism software 6.03. Results are reported as mean ± S.E.M. Differences were considered significant for P < 0.05.

**Compounds.** The novel small molecule AUT1 (in DMSO; Autifony Therapeutics Ltd.) and tetraethylammonium chloride (in distilled water; Sigma-Aldrich) were dissolved and stored at −20°C to provide stock solutions. The stock solutions were in turn diluted in DMSO or extracellular solution to obtain final compound concentrations (maximal DMSO final concentration of 0.1%). In IonWorks experiments, 0.05% Pluronic P-127 was added to the extracellular solution. Slices were preincubated with the desired final concentration of DMSO, TEA, and/or AUT1 for 1 hour prior to recording to ensure full equilibration of the drugs with the brain slice. No incubation time-related effects were observed on the measured parameters. Compounds were diluted to the test concentrations on the day of the experiment.

**Results**

**Modulation of Recombinant Human Kv3 Channels.** Voltage-clamp experiments performed using the IonWorks automated patch-clamp assay found that AUT1 increased the current mediated by human recombinant Kv3.1b and Kv3.2a channels, measured at −15 mV, in a concentration-dependent manner. pEC_{50} values for AUT1 were 5.35 ± 0.03 (4.7 μM; n = 17) and 5.31 ± 0.03 (4.9 μM; n = 29) for Kv3.1b and Kv3.2a, respectively (Fig. 1). Maximum potentiation in each case was observed at a concentration of 25 μM, whereas a notably lower potentiation was observed with the highest concentration tested (50 μM). AUT1 also enhanced whole-cell currents mediated by human recombinant Kv3.3 channels; however, with an approximately 10-fold lower potency (pEC_{50} = 4.5 ± 0.06; 30 μM; n = 16) measured at the test potential of −15 mV compared with Kv3.1 and Kv3.2 (Supplemental Fig. 1).
Furthermore, maximum potentiation was clearly not reached at the highest concentration of AUT1 tested (50 μM).

Kv3 currents in these assays were fully blocked by TEA in a concentration-dependent manner, with pIC50 values of 4.04 ± 0.03 (91 μM; n = 16) and 3.36 ± 0.03 (436 μM; n = 16) for Kv3.1b and Kv3.2a, respectively (see Supplemental Fig. 1). The effects of TEA on the Kv3.3 cell line were not investigated. No changes in the currents were observed when vehicle alone (0.1% DMSO) was applied (unpublished data).

To evaluate the voltage dependence of AUT1 modulation of Kv3 currents, current–voltage relationship plots were constructed for Kv3.1b (n = 22) and Kv3.2a (n = 17) channels in the absence and presence of AUT1 using the QPatch platform. AUT1 (10 and 30 μM) produced voltage-dependent potentiation of whole-cell currents, with higher levels of potentiation observed at voltages close to the threshold for Kv3 channel activation (i.e., −20 to 0 mV). Little or no potentiation of Kv3 currents was observed at higher test voltages (i.e., > +20 mV) (Fig. 2). For example, at 0 mV, AUT1 (30 μM) increased hKv3.1b and hKv3.2a currents by 1049 ± 198% and 226 ± 39%, respectively, whereas at +30 mV, the potentiation was just 15 ± 7% and 12 ± 11% for Kv3.1b and Kv3.2a, respectively, a profile suggestive of an effect on channel open probability rather than conductance.

Next, the effects of AUT1 on the voltage dependence of activation and inactivation of Kv3.1b and Kv3.2a channels were determined. For this purpose, the half-voltage ($V_{1/2}$) of activation and/or inactivation were calculated from $G/G_{\text{max}}$ − Voltage curves by fitting the data to a Boltzmann equation (Fig. 3). AUT1 produced a significant leftward shift in the voltage dependence of activation of Kv3.1b ($V_{1/2}$ control = 13.3 ± 0.64 mV; $V_{1/2}$ AUT1 10 μM = 8.7 ± 0.62 mV and $V_{1/2}$ AUT1 30 μM = −1 ± 0.42 mV; $F_{2,21} = 164.75, P = 0.0002$, one-way ANOVA) and Kv3.2a curves ($V_{1/2}$ control = 7.1 ± 0.76 mV; $V_{1/2}$ AUT1 10 μM = 2.8 ± 0.81 mV and $V_{1/2}$ AUT1 30 μM = −7.5 ± 0.83 mV; $F_{2,21} = 87.83, P = 0.0005$). Likewise, this modulator was able to shift the voltage dependence of steady-state inactivation toward hyperpolarized potentials. The half-inactivation potential ($V_{1/2}$) values were −32.3 ± 0.72 mV ($V_{1/2}$ control) and −38.2 ± 0.62 mV ($V_{1/2}$ AUT1 10 μM) for Kv3.1b-mediated currents (n = 6; $t_{10} = 3.19; P = 0.0096$, two-tailed paired t test). As for Kv3.2a-mediated currents, these values were −40 ± 0.85 mV ($V_{1/2}$ control) and −55 ± 0.84 mV ($V_{1/2}$ AUT1 10 μM; n = 6; $t_{10} = 7.06; P < 0.0001$, two-tailed paired t test). Additionally, AUT1 produced a small increase in $G_{\text{max}}$ values for Kv3.1b ($G_{\text{CTR}} = 40 ± 6 μS; G_{\text{AUT-10}} = 45 ± 6 μS; t_{13} = −2.21; P = 0.045$, two-tailed paired t test) and Kv3.2a ($G_{\text{CTR}} = 59 ± 7 μS; G_{\text{AUT-10}} = 65 ± 7 μS; t_{8} = 3.18; P = 0.012$, two-tailed paired t test) currents. Finally, given the importance of rapid activation of Kv3 channels to their physiologic role, we investigated the effects of AUT1 on channel activation kinetics using currents measured at −15 mV in the IonWork assay. AUT1 produced a small, although significant, increase in the rate of Kv3 channel activation. Values of $\tau_{\text{activation}}$ for Kv3.1b channels were 6.73 ± 0.64 milliseconds (in DMSO) and 11.8 ± 0.62 milliseconds (in 12.5-μM AUT1), n = 8, $t_{14} = 6.17, P < 0.0001$, two-tailed unpaired t test, whereas for Kv3.2a channels, $\tau_{\text{activation}}$ values were 6.8 ± 0.21 milliseconds (in DMSO) and 8.4 ± 0.39 milliseconds (in 12.5-μM AUT1), n = 8, $t_{14} = 3.33, P = 0.002$, two-tailed unpaired t test. Representative traces and fits for this analysis are shown in Supplemental Fig. 2.

Effects of AUT1 in a Series of Functional Selectivity Assays In Vitro. A series of functional selectivity assays were conducted with AUT1 to investigate its pharmacologic activity across a range of ion channels, receptors, and
The studies were conducted using the enhanced cross-screen profiling panel (GlaxoSmithKline) for selectivity screening. The enhanced cross-screen profiling panel assays are chosen to cover major liability targets, such as cardiac ion channels, including hERG, as well as a range of other transporters and neurotransmitter receptors. Experiments were conducted using functional assays that may detect both positive (agonism) and negative (antagonism) modulation of the targets. Table 1 summarizes the potency values (expressed in pXC50) and the X50 values (in micromolar) obtained in these cross-screening experiments.

In addition to the range of liability targets listed in Table 1, the effects of AUTO1 on recombinant human Kv2.1 channels were investigated using a manual patch clamp, given the likely similarity between this channel class and Kv3 (activation threshold, slow inactivation, and role in high-frequency firing). Current-voltage relationship plots were obtained for hKv3.1b (A) and hKv3.2a-mediated (B) currents in control and treated conditions. This compound, at both tested concentrations of 10 and 30 μM (indicated at the top of the plots), positively modulated current amplitudes at the voltage range where Kv3 channels are operating. Note the voltage-dependent effect of AUTO1 on current amplitude.

Localization of Kv3.1b and Kv3.2 Channels in Parvalbumin-Positive Neurons in the Somatosensory Cortex of C57BL/6 and GAD1-EGFP Mice. Neurons coexpressing Kv3 channels and PV were found in different proportions throughout all of the cell body layers (II–VI) of the mouse somatosensory cortex (Supplemental Fig. 3).
Table 1: Cross-screen evaluation of AUT1 in a series of functional assays

<table>
<thead>
<tr>
<th>Target</th>
<th>Mode</th>
<th>( p_{XC_{50}} )</th>
<th>( XC_{50} )</th>
<th>( \mu M )</th>
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<td>(&lt;4.5 (n = 1))</td>
<td>&gt;31.6</td>
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<tr>
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<td>(&lt;4.4 (n = 1))</td>
<td>&gt;39.8</td>
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<td>Agonist</td>
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<td>&gt;31.6</td>
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<tr>
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<td>Agonist</td>
<td>(&lt;4.5)</td>
<td>&gt;31.6</td>
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<tr>
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<tr>
<td>Serotonin transporter</td>
<td>Antagonist</td>
<td>(&lt;4.3 (n = 1))</td>
<td>&gt;50.1</td>
<td></td>
</tr>
</tbody>
</table>

Double-labeling immunofluorescence experiments demonstrated that in the superficial layers (II–IV), 312 out of 412 cells (76%) that displayed a positive signal for PV also showed Kv3.1b-associated immunofluorescence. In the deep layers (V and VI), the level of coexpression was greater at 81% (363 out of 448 cells). Likewise, Kv3.2 immunofluorescence was observed in PV+ interneurons in 269 out of 358 cells (75%) in the deep layers of the mouse somatosensory cortex; however, in the superficial layers, only 44% of PV fluorescent cells also showed Kv3.2 immunofluorescence (234 out of 528 cells). Although not quantified, similar Kv3.1b and Kv3.2 distributions were observed in the somatosensory cortex of GAD1-EGFP transgenic mice (unpublished data). Immunohistochemistry data are summarized in Table 2.

**Fast-Firing Phenotype of EGFP-Positive Interneurons Is Inhibited by TEA.** Current-clamp recordings from identified EGFP-positive interneurons in the somatosensory cortex of GAD1-EGFP mice confirmed their FS phenotype in response to depolarizing current steps (Fig. 4A). The neurons had an average resting \( V_m \) of \(-67 \pm 0.7 \) mV and an average resting membrane resistance \( (R_m) \) of \( 128 \pm 11 \) M\( \Omega \) (\( n = 27 \) neurons). Prior to the application of the current-step protocol, a hyperpolarizing current was injected to maintain the resting potential of the cells near \(-80 \) mV. The mean maximal firing rate evoked by depolarizing current steps (typically 400 pA) was \( 168 \pm 9 \) Hz, the mean AP half-width was \( 0.46 \pm 0.02 \) milliseconds, and the mean amplitude of the negative peak of the first derivative of the action potential waveform (AP-FD) was \(-182 \pm 9 \) mV/\( \mu \)s (\( n = 6 \) neurons). These values are consistent with values reported previously (see Lau et al., 2000).

Incubation of the slices with 0.5 mM TEA for 3 minutes reduced the mean maximum firing frequency to \( 118 \pm 12 \) Hz (\( t_{10} = 3.34; P = 0.0074 \), two-tailed unpaired \( t \) test), increased the mean action potential half-width to \( 0.63 \pm 0.03 \) milliseconds (\( t_{10} = 4.45; P = 0.0012 \), two-tailed unpaired \( t \) test), and reduced the mean AP-FD to \(-109 \pm 7 \) mV/\( \mu \)s (\( t_{10} = 6.12; P = 0.0001 \), two-tailed unpaired \( t \) test; Fig. 4B), indicating a reduction in the repolarization rate of the action potentials after TEA application. Nevertheless, application of this concentration of TEA did not modify the threshold of the action potential generation.

**AUT1 Rescues the Fast Firing of EGFP-Positive Cortical Interneurons.** In further experiments, brain slices were preincubated for 1 hour with TEA (0.5 mM) and either vehicle (0.1% DMSO) or AUT1 (1 or 10 \( \mu \)M) prior to recordings. In slices incubated with TEA+DMSO, the firing frequency of the recorded neurons was significantly reduced for stimulation currents above 150 pA when compared with control slices (\( F_{1,11} = 12.75; P = 0.0044 \), two-way ANOVA and Sidak’s multiple comparisons test) (Fig. 5A). The value of the maximal firing rate was decreased from \( 168 \pm 9 \) Hz (control; \( n = 6 \)) to 91 Hz \( (P = 0.006) \), two-way ANOVA and Tukey’s multiple comparisons test) (Fig. 5A). The maximal firing frequency of the neurons was also significantly increased in the presence of AUT1 (1 \( \mu \)M: \( 135 \pm 6 \) Hz, \( n = 9 \); 10 \( \mu \)M: \( 138 \pm 7 \) Hz, \( n = 5 \)) compared with the maximum firing frequency of neurons in slices incubated with TEA alone. Incubation with AUT1 alone did not affect the maximum firing frequency of these interneurons when compared with neurons from control slices (166 \pm 6 \) Hz, \( n = 9 \), \( F_{2,18} = 6.997; P = 0.0056 \), two-way ANOVA and Tukey’s multiple comparisons test) (Fig. 5A). The maximal firing frequency of the neurons was also significantly increased in the presence of AUT1 (1 \( \mu \)M: \( 135 \pm 6 \) Hz, \( n = 9 \); 10 \( \mu \)M: \( 138 \pm 7 \) Hz, \( n = 5 \)) compared with the maximum firing frequency of neurons in slices incubated with TEA alone. Incubation with AUT1 alone did not affect the maximum firing frequency of these interneurons when compared with neurons from control slices (166 \pm 6 \) Hz, \( n = 9 \), \( F_{1,13} = 1.408; P = 0.2566 \), two-way ANOVA and Sidak’s multiple comparisons test; unpublished data).

Analysis of action potential parameters showed that preincubation with TEA (0.5 mM) alone significantly increased the mean action potential half-width (control: \( 0.46 \pm 0.02 \) milliseconds, \( n = 6 \); TEA: \( 0.94 \pm 0.1 \) milliseconds, \( n = 7 \) ) (Fig. 4A). Coincubination of slices with TEA+AUT1 significantly reduced the action potential half-width compared with TEA alone (\( \Delta T_{AUT1,p,M} = 0.72 \pm 0.03 \) milliseconds, \( n = 9 \); AUT1 \( \Delta T_{10,p,M} = 0.64 \pm 0.02 \) milliseconds, \( n = 6 \)).

Table 2: Evaluation of neurons coexpressing Kv3.1b/3.2 channels and parvalbumin in mouse somatosensory cortex

<table>
<thead>
<tr>
<th>PV Cells</th>
<th>Kv3.1b Cells</th>
<th>Cells</th>
<th>PV Cells</th>
<th>Kv3.2 Cells</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layers II–IV</td>
<td>412</td>
<td>312</td>
<td>76</td>
<td>528</td>
<td>234</td>
</tr>
<tr>
<td>Layers V and VI</td>
<td>448</td>
<td>363</td>
<td>81</td>
<td>358</td>
<td>269</td>
</tr>
</tbody>
</table>

*The number indicates the percentage of the cells that coexpress parvalbumin and Kv3.1b channels.

*The number indicates the percentage of the cells that coexpress parvalbumin and Kv3.2 channels.
for neurons from slices incubated with AUT1 and TEA when compared with control values (half-width: 0.48 milliseconds; AP-FD: 0.59 and 4.2 mV/ms, n = 8.1 mV/ms, n = 10 mV/ms; control: 2 mV). DMSO significantly reduced the amplitude of the first derivative of the action potential (control: 10 mV, n = 5; 5 mV, n = 5; 2 mV, n = 5; 0 mV, n = 5; unpublished data)

Discussion

AUT1 is a novel, small molecular weight molecule that we have shown can modulate the function of human Kv3.1, Kv3.2, and, to a lesser extent, Kv3.3, voltage-gated ion channels in recombinant cell lines. The compound has been shown to have similar effects on recombinant rat Kv3.1b channels (L. K. Kaczmarek, personal communication), consistent with the highly conserved nature of the channel across mammalian species (Ried et al., 1993). In the present study, we have also shown that under circumstances of reduced Kv3 function through partial blockade by TEA, AUT1 was able to rescue the fast firing and short action potential duration of mouse PV+ interneurons, suggesting that the compound can regulate native Kv3 channels. Furthermore, in vivo studies (M. M. Sidor et al., unpublished data), we have found that AUT1 has effects on mouse behavior and that these effects were not observed in Kv3.1 knockout animals, suggesting the specific interaction with the Kv3.1 channel subtype in vivo. Relevant to the discussion below, active free brain concentrations in the in vivo studies were estimated to be in the region of 0.5–1 μM.

Experiments with recombinant human Kv3.1b and Kv3.2a channels indicate that AUT1 enhances the open probability of the channels, with a concomitant leftward shift in the voltage dependence of activation. Moreover, AUT1 also shifted the voltage dependence of inactivation toward hyperpolarized potentials. The leftward shift in inactivation may account for the apparent reduction in potentiation of Kv3 currents observed at the highest concentration of AUT1 tested in the recombinant channel experiments (e.g., see Fig. 1). However, this effect is unlikely to be relevant at the lower concentrations that were tested on native neurons and which were estimated to be associated with behavioral effects in vivo. Furthermore, since the interneurons were hyperpolarized to around −80 mV prior to application of depolarizing current steps to evoke firing in the present native tissue experiments, significant channel inactivation in the presence of AUT1 is unlikely to have occurred.

Selectivity of AUT1 for Kv3.1 and Kv3.2 channels was determined from a series of cross-screening experiments, in which it did not interact with a wide range of ion channels, receptors, and transporters (Table 1). However, we cannot exclude the possibility that AUT1 might affect other targets that were not included in the cross-screen. A lower potency modulation of human recombinant Kv3.3 channels was observed, but it remains to be determined whether significant interaction with Kv3.3 channels would occur at active concentrations in vivo. Kv3.3 channels are not expressed by PV+ interneurons in the rodent cortex so interaction of AUT1 with this channel subtype is not relevant to the present native tissue experiments.

To evaluate the action of AUT1 on native Kv3 channels, experiments using brain slices obtained from GAD1-EGFP transgenic mice were performed. The immunohistochemical evaluation of the expression of these channels suggests that in the deep layers (V and VI) of the mouse somatosensory cortex, the majority of the cells expressing PV were in good agreement with previously published results (Chow et al., 1999). Consequently, it was probable that all fluorescent neurons identified in the somatosensory cortex would contain either Kv3.1b or Kv3.2a channels or both. This was further confirmed by the observed...
in the presence of AUT1 produced an increment on the frequency of action unpaired sets of experiments in the control (closed circles; \( n = 5 \)) AUT1. AP frequencies were compared with those obtained in treatment, respectively (1 \( \mu M \) of AUT1 producing a lesser effect on AP parameters than 10 \( \mu M \) (see Fig. 5). These experiments suggest that 1 \( \mu M \) of AUT1 is sufficient to produce significant effects on PV\(^*\) interneuron function in vitro, which is consistent with the behavioral effects of the drug observed in vivo (M. M. Sidor et al., unpublished data). Effects of 1 \( \mu M \) of AUT1 on current amplitude in the recombinant channel experiments were modest, producing perhaps only 10–20% current potentiation (see Fig. 1). However, we conclude that this is sufficient to produce relevant effects on native neurons and on behavior in vivo. This may, in part, be due to the density and somatic location of Kv3 channels on PV\(^*\) neurons. Furthermore, these results are consistent with other voltage-gated ion channel modulators, where only small effects on the channels are sufficient to produce significant changes in neural activity and in vivo behavior (Large et al., 2009).

As part of the more detailed assessment of effects of AUT1 on channel function using recombinant channels, we noticed that AUT1 did have a significant slowing effect on the kinetics of activation and deactivation of Kv3 currents, which could potentially reduce the ability of the channels to initiate a rapid repolarization and/or increase their contribution to the afterhyperpolarization, both of which might lead to a slowing of firing. However, since we did not observe a detrimental effect of AUT1 on firing frequency or AP parameters of somatosensory cortex interneurons, we conclude that the numerically small increase in Kv3 activation and deactivation time seen on the recombinant channels was insufficient to alter the role of the channels in native neurons.

In the present study, we have not investigated the site of action of AUT1 on Kv3 channels; however, it is unlikely that the reversal of the TEA-mediated changes in fast firing was due to pharmacological displacement of TEA since the biophysical effects of AUT1 on channel function (i.e., shift in the activation curve) are quite different to TEA, which acts as a pore blocker (Heginbotham and MacKinnon, 1992). Thus, it is likely that AUT1 binds to a separate, most likely intracellular, location of Kv3 channels on PV\(^*\) neurons. Furthermore, these channels display very slow activation and deactivation

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**Fig. 5.** Effect of AUT1 on the firing frequency and action potential features after long incubation with TEA. The effect of AUT1 on the frequency, half-width, and FD of the action potentials after long incubation in the presence of TEA was evaluated. (A) Evoked action potentials by depolarizing current steps (600-millisecond duration and increment of 50 pA) were recorded after long incubation (at least 1 hour) with 0.5 mM TEA + 1 \( \mu M \) (red triangles; \( n = 9 \)) and 10 \( \mu M \) (open triangles; \( n = 5 \)) AUT1. AP frequencies were compared with those obtained in unpaired sets of experiments in the control (closed circles; \( n = 6 \)) and in the presence of 0.5 mM TEA alone (open circles; \( n = 7 \)). Long-time incubation in the presence of AUT1 produced an increment on the frequency of action potentials, with respect to the TEA condition above a stimulation of 150 pA. \(*P < 0.05; \text{Tukey’s multiple comparisons test.} \) (B) Bar chart shows the effect of AUT1 in the presence of TEA on the half-width and AP-FD, with respect to the values obtained in the control condition and in the presence of TEA + DMSO. As it is shown, this Kv3 modulator was able to partially revert the effect of TEA. The mean half-width value was decreased by 24 and 36% after 1- and 10- \( \mu M \) treatment, respectively (1 \( \mu M \): 0.72 ± 0.03 milliseconds, \( n = 9 \); 10 \( \mu M \): 0.64 ± 0.02 milliseconds, \( n = 5 \)), whereas the mean value of AP-FD was increased by 46 and 69% after 1- \( \mu M \) and 10- \( \mu M \) treatment, respectively (1 \( \mu M \): −102.4 ± 9.4 mV/ms, \( n = 9 \); 10 \( \mu M \): −118.8 ± 4.2 mV/ms, \( n = 5 \); \(^*P < 0.05; **P < 0.01; ***P < 0.001, \text{Dunnnett’s multiple comparisons test}. \) Differences were evaluated against TEA effect.
kinetics (time constant of hundreds of milliseconds), which could not be significantly activated to mediate the fast repolarization of these short interneuronal action potentials (Yang et al., 1998). Likewise, BK channels, which are expressed in these cortical interneurons, seem to regulate the release probability at the presynaptic terminals, limiting the calcium influx rather than the firing pattern of these cells (Erisir et al., 1999; Goldberg et al., 2005). Specific blockade of BK channels in neocortical FS interneurons had no significant effect on the evoked firing rate, AP width, and afterhyperpolarization amplitude, which is analogous to the role of these channels observed in deep cerebellar nuclear neurons (Erisir et al., 1999; Pedroarena, 2011). Similarly, it is unlikely that Kv3.3 or Kv3.4 potassium channels were involved since they are not expressed on these cortical interneurons (Wulf et al., 2009). Consequently, we propose that the ability of AUT1 to prevent the effects of 0.5 mM TEA was due to enhanced currents mediated by Kv3.1 and/or Kv3.2 residual channels that were not blocked.

This study, using a novel Kv3 channel modulator, confirms the role of Kv3.1/3.2 channels in the modulation of the action potential duration and firing rate of FS interneurons, as previously demonstrated (Erisir et al., 1999; Atzori et al., 2000; Lau et al., 2000; Lien and Jonas, 2003). Moreover, it shows that small molecule modulators of Kv3 channels, such as AUT1, can rescue the fast firing capability of FS interneurons following a reduction in Kv3 channel availability. We found that TEA inhibited recombiant human Kv3.1 and Kv3.2 channels, with IC50 values of 0.09 and 0.44 mM, respectively, which is in line with previous reports (Hernandez-Pineda et al., 1999). We therefore estimate that the 0.5-mM concentration of TEA used in our native tissue experiments would block approximately 60–80% of Kv3.1/3.2 channels. Kv3.1 channel expression has been shown to reduce with aging (von Huhn et al., 2004; Jung et al., 2005), and recent postmortem studies observed a reduction in Kv3.1 expression in the cortex of patients with schizophrenia (Yanagi et al., 2014). Thus, under conditions of pathologic reduction in Kv3 channel expression or function, which is likely to impair the normal FS phenotype of PV+ interneurons, compounds like AUT1 have the potential to rescue normal function and thus have a therapeutic benefit in disorders like schizophrenia or age-related cognitive decline.

Importantly, AUT1 did not affect the firing of PV+FS neurons under normal physiologic conditions, presumably because Kv3 channel function was already optimal, and the firing capacity of the neurons was limited by factors other than Kv3 channels. Consequently, drugs like AUT1 may demonstrate a therapeutic benefit without significant central nervous system side effects.

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Authorship Contributions

Participated in research design: Rosato-Siri, Large.

Conducted experiments: Rosato-Siri, Zambrallo, Mutinelli, Garbati, Benedetti, Aldegheri, Graziani, Virgino.

Contributed new reagents or analytic tools: Alvaro.

Performed data analysis: Rosato-Siri, Zambrallo, Mutinelli, Garbati, Benedetti, Aldegheri, Graziani, Virgino.

Wrote or contributed to the writing of the manuscript: Rosato-Siri, Large.


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