European Journal of Pharmacology ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

Biophysical characterization of $K_V3.1$ potassium channel activating compounds

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ARTICLE INFO

Article history:
Received 2 September 2014
Received in revised form
1 March 2015
Accepted 14 March 2015

Keywords:
Potassium channels
$K_v3.1$ channel activator
Fast-spiking interneurons
GABAergic interneurons

ABSTRACT

The effect of two positive modulators, RE1 and EX15, on the voltage-gated K^+ channel $K_v3.1$ was investigated using the whole-cell patch-clamp technique on HEK293 cells expressing $K_v3.1a$. RE1 and EX15 increased the $K_v3.1$ currents in a concentration-dependent manner with an EC_{50} value of 4.5 and 1.3 μM, respectively. However, high compound concentrations caused an inhibition of the $K_v3.1$ current. The compound-induced activation of $K_v3.1$ channels showed a profound hyperpolarized shift in activation kinetics. 30 μM RE1 shifted $V_{1/2}$ from 5.63 ± 0.31 mV to -9.71 ± 1.00 mV and 10 μM EX15 induced a shift from 10.77 ± 0.32 mV to -15.11 ± 1.57 mV. The activation time constant (Tau_{act}) was reduced for both RE1 and EX15, with RE1 being the fastest activator. The deactivation time constant (Tau_{deact}) was also markedly reduced for both RE1 and EX15, with EX15 inducing the most prominent effect. Furthermore, subjected to depolarizing pulses at 30 Hz, both compounds were showing a use-dependent effect resulting in a reduction of the compound-mediated effect. However, during these conditions, RE1- and EX15-modified current amplitudes still exceeded the control condition amplitudes by up to 200%.

In summary, the present study introduces the first detailed biophysical characterization of two new $K_v3.1$ channel modifying compounds with different modulating properties.

1. Introduction

Voltage-gated potassium channels (K_v) form a family of transmembrane ion channels which are voltage-sensitive and selective for potassium ions. They are essential for maintaining cell homeostasis and for generation and propagation of electrical signals in neurons (Lenz et al., 1994; Ho et al., 1997; Sekirnjak et al., 1997; Rudy et al., 1999; Davies, 2009). Among K^+ channels, members of the Shaw-type K^+ channel, K_v3, have unique biophysical properties that enables firing of brief action potentials at very high frequencies (Weiser et al., 1995; Chow et al., 1999; Rudy et al., 1999; Rudy and McBain, 2001; Macica et al., 2003). One of the subtypes of the K_v3 subfamily, $K_v3.1$, is extensively expressed throughout the CNS (Lenz et al., 1994; Sekirnjak et al., 1997; Chow et al., 1999; Rudy et al., 1999; Li et al., 2001), most markedly in fast-spiking (FS) parvalbumin-positive GABAergic interneurons, which are characterized by their ability to sustain high frequency firing (Weiser et al., 1995; Sekirnjak et al., 1997; Chow et al., 1999; Rudy and McBain, 2001). $K_v3.1$ channels are localized in the somatic, axonal and presynaptic-terminal membranes, and to a smaller extent in the dendrites (Weiser et al., 1995; Rudy et al., 1999; Ozaita et al., 2002; Davies, 2009). Due to these expression patterns, and their unique biophysical properties, $K_v3.1$ channels are believed to be important for action potential duration and fast-firing properties in neurons and thus for neurotransmitter release, thereby affecting synaptic transmission (Lenz et al., 1994; Porcello et al., 2002; Brooke et al., 2004; Goldberg et al., 2005; Espinosa et al., 2008; Davies, 2009).

The voltage-gated K_v3 channels are functionally different from other K^+ channels. $K_v3.1$ potassium channels display a high activation threshold (positive to -20 mV) and very rapid activation and deactivation kinetics (Martina et al., 1998; Chow et al., 1999; Rudy and McBain, 2001; Davies, 2009). These properties of $K_v3.1$ are presumably an important contributing factor for the fast after-hyperpolarization (fAHP) required for high frequency firing observed in $K_v3.1$-expressing interneurons (Martina et al., 1998; Wang et al., 1998). Hence, it is hypothesized that modifying $K_v3.1$ activity can affect the excitability of the FS interneurons (Weiser et al., 1995; Susan Cochran and Yoshitika, 2003; Brown et al., 2012, 2013).

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http://dx.doi.org/10.1016/j.ejphar.2015.03.061
0014-2999/

Indeed, both pharmacological inhibition and gene deletion of $K_v3.1$ channels results in broader action potentials (measured as larger AP half-width) (Goldberg et al., 2005; Pedroarena, 2011; Alvaro et al., 2012; Large et al., 2012; Barry et al., 2013). Thus, $K_v3.1$ activating compounds are theorized to affect the morphology of the action potential, leading to a decreased action potential half-width and thereby a shortening of the action potential duration. This would increase the impact of the fAHP, resulting in increased spiking frequency in interneurons (Weiser et al., 1995; Alvaro et al., 2012). The exact effect will, however, be determined by the precise biophysical properties of the activating compound. An *in silico* approach has previously been applied to address possible effects of $K_v3.1$ channel activation: In this study, the action potential duration was found to be more narrow and the refractory period shorter, which caused a sustained high-frequency firing (Kanemasa et al., 1995).

In the present study, the effect of two compounds, patented by Autifony Therapeutics (AUT), reference 1 (RE1) and example 15 (EX15), are studied on stably expressed Kv3.1 channels in monoclonal HEK293 cells(Alvaro et al., 2012). We report that RE1 and EX15 modulate the $K_v3.1$ currents in a concentration- and voltage-dependent manner, the effect of EX15 showing use-dependent characteristics.

2. Materials and methods

2.1. Stable monoclonal HEK293-cells

The monoclonal cell line was constructed by transiently transfecting a pcDNA3-h$K_v3.1$a plasmid, custom-made by Biomatik, into HEK293-cells using the Lipofectamine reagent (Lifetechnologies). HEK293-cells were cultured in DMEM growth medium supplemented with 10% fetal bovine serum (Sigma),100 μg/ml penicillin and 100 μg/ml streptomycin and kept in humidified 95% air/5% CO_2 environment at 37 °C (Thermo scientific, Australia). Following 2 weeks selection with 500 μg/ml Geneticin, colonies were pooled into a polyclonal culture. To develop a monoclonal cell-line, cells were diluted in a 1:1.000.000 range and sown on a 96 well plate (BG Falcon) containing growth medium. After 2 days, the wells were screened for single colonies of cells. The current obtained from each cell-line was tested using the whole-cell patch-clamp technique. The chosen cell-line exhibited current amplitudes around 10 nA at +40 mV.

2.2. Electrophysiological recordings

The $K_v3.1$ currents were measured using the whole-cell configurations of the patch-clamp technique. Currents were monitored with the patch-clamp amplifier HEKA EPC9 (HEKA elektronik, Germany). The software used was Pulse 8 (V. 8.08, HEKA elektronik, Germany) and MasterFit (V. 2 × 65, HEKA elektronik, Germany). Micropipettes were fabricated from glass capillary tubing (Scandidact), using a (Sutter instrument p-97; CA, USA) puller and had a tip resistance of 1.5–3 MΩ when filled with the internal pipette solution. Series resistance (R_S) was compensated by 80% and was always kept ≤5 MΩ. The membrane resistance (R_m) was ≥500 MΩ. All experiments were performed at room temperature.

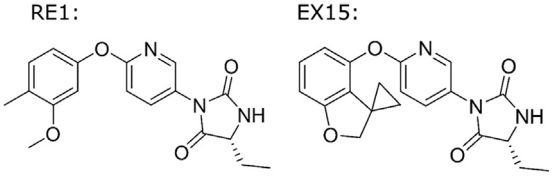

Fig. 1. The molecular structure of reference 1 (REF1) and example 15 (EX15) (Alvaro et al., 2012).

2.3. Solutions and drugs

The internal micropipette solution contained (in mM): 140 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 2 EGTA, 10 HEPES and 1 ATP (Na^+ salt). The pH level was adjusted to 7.4 with KOH and filtered at 0.22 μm. The free calcium is calculated to be 3 nM (Patton). This solution was stored at −20 °C in micro-centrifuge tubes and thawed immediately before use.

The external solution contained the following (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES. The solution was titrated with KOH to a pH of 7.4.

The two compounds, RE1 and EX15, were supplied by Lundbeck A/S. Structures are depicted in Fig. 1. The compounds were applied to the extracellular solution. A final concentration of less than 0.33% DMSO was maintained in all concentrations of assay compounds. This concentration of DMSO had no effect on $K_v3.1$ currents.

2.4. Data analysis

For analysis, Igor Pro 4 (V. 4.04, WaveMetrics, Inc, USA) and GraphPad Prism 5 (V. 5.01, GraphPad Software, Inc, USA) were used. Current–voltage (*I–V*) curves were fitted with a Boltzmann function, $G/G_{max}=1/(1+e\widehat{(}(-(V-V_{1/2}))/k))$, where V represents the test potential, $V_{1/2}$ is the potential at which the conductance was half-maximal, k is the slope factor, G is the conductance, and G_{max} is the maximal conductance. When testing RE1, the *I–V* curves were obtained by injecting 200 ms depolarizing currents, thus gradually increasing the membrane potential from −80 mV to +40 mV, 10 mV at a time. The *I–V* curves for EX15 were similarly conducted by measuring the current generated at membrane potentials between −60 mV and +40 mV. The currents were normalized to cell size (pA/pF). Deactivation protocols were designed to start at a −80 mV holding potential followed by a depolarizing step of +20 mV and a repolarizing step ranging from −120 mV to +20 mV with 10 mV increments. The activation- and deactivation curves were fitted to a single exponential function to obtain the activation- and deactivation time constants (Tau_{act} and Tau_{deact}).

Concentration–response curves were constructed by fitting the activation curves measured during depolarizing pulses of −10 mV to a nonlinear regression function "log(agonist)vs. response" with the equation: $Y=Bottom+(Top-Bottom)/(1+10\widehat{(}log(EC_{50}-X))$. EC_{50} is the concentration resulting in 50% effect of the maximum current amplitude. To measure and compare the activation- and deactivations kinetics and use-dependency of RE1 and EX15, a concentration of 7–8 times the EC_{50} was used (corresponding to 30 μM RE1 and 10 μM EX15).

All data are depicted as mean ± S.E.M. The two-way analysis of variance (ANOVA), followed by a Bonferroni posttest, was used to evaluate the statistical significance of the observed differences. One-way ANOVA, followed by Dunnet posttest, was used to evaluate significance in the obtained half maximal activation values ($V_{1/2}$). Paired *t*-test was used when comparing mean of responses. Statistical significance was considered at $P<0.05$.

3. Results

3.1. Concentration dependent activation

Fig. 2A illustrates the activation time course of $K_v3.1$ channels in the presence of 30 μM RE1 and 10 μM EX15. The current

Please cite this article as: Taskin, B., et al., Biophysical characterization of $K_V3.1$ potassium channel activating compounds. Eur J Pharmacol (2015), http://dx.doi.org/10.1016/j.ejphar.2015.03.061