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Kv2 dysfunction after peripheral axotomy enhances sensory neuron responsiveness to sustained input

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ABSTRACT

Peripheral nerve injuries caused by trauma are associated with increased sensory neuron excitability and debilitating chronic pain symptoms. Axotomy-induced alterations in the function of ion channels are thought to largely underlie the pathophysiology of these phenotypes. Here, we characterise the mRNA distribution of Kv2 family members in rat dorsal root ganglia (DRG) and describe a link between Kv2 function and modulation of sensory neuron excitability. Kv2.1 and Kv2.2 were amply expressed in cells of all sizes, being particularly abundant in medium-large neurons also immunoreactive for neurofilament-200. Peripheral axotomy led to a rapid, robust and long-lasting transcriptional Kv2 downregulation in the DRG, correlated with the onset of mechanical and thermal hypersensitivity. The consequences of Kv2 loss-of-function were subsequently investigated in myelinated neurons using intracellular recordings on *ex vivo* DRG preparations. In naïve neurons, pharmacological Kv2.1/Kv2.2 inhibition by stromatoxin-1 (ScTx) resulted in shortening of action potential (AP) after-hyperpolarization (AHP). In contrast, ScTx application on axotomized neurons did not alter AHP duration, consistent with the injury-induced Kv2 downregulation. In accordance with a shortened AHP, ScTx treatment also reduced the refractory period and improved AP conduction to the cell soma during high frequency stimulation. These results suggest that Kv2 downregulation following traumatic nerve lesion facilitates greater fidelity of repetitive firing during prolonged input and thus normal Kv2 function is postulated to limit neuronal excitability. In summary, we have profiled Kv2 expression in sensory neurons and provide evidence for the contribution of Kv2 dysfunction in the generation of hyperexcitable phenotypes encountered in chronic pain states.

Introduction

Chronic neuropathic pain is associated with profound changes in the anatomy and function of sensory neurons. One of the most extensively documented, but not well understood, consequences of direct nerve injury in animal models and human subjects is the subsequent increase of sensory neuron excitability, primarily manifested as spontaneous discharge and increased responsiveness to stimulation (Kajander and Bennett, 1992; Liu et al., 1999; Study and Kral, 1996; Zhang et al., 1997). This injury-mediated hyperexcitability is thought to underlie poorly managed chronic symptoms in patients, such as spontaneous pain and hypersensitivity to stimulation.

Voltage-gated potassium (Kv) channels play a vital role in neuronal function by regulating resting membrane potential and controlling the waveform and frequency of APs (Hille et al., 1999). Indeed, injury-induced Kv dysfunction is linked to reduction of associated currents, augmented sensory neuron excitability and pain phenotypes (Chien et al., 2007; Everill and Kocsis, 1999; Tan et al., 2006; Tsantoulas et al., 2012). Accordingly, Kv blocker application to the DRG induces neuronal firing (Kajander et al., 1992), while Kv openers restrict neuronal excitability and relieve pain symptoms (Blackburn-Munro and Jensen, 2003; Dost et al., 2004; Mishra et al., 2012; Roza and Lopez-Garcia, 2008).

In many neurons, delayed rectifying currents due to Kv2 conductance (Guan et al., 2007; Malin and Nerbonne, 2002; Murakoshi and Trimmer, 1999) are a key modulator of excitability by facilitating AP repolarisation and inter-spike hyperpolarisation during repetitive firing (Blaine and Ribera, 2001; Johnston et al., 2010; Malin and Nerbonne, 2002). The Kv2 family consists of the Kv2.1 and Kv2.2 subunits (Frech et al., 1989; Hwang et al., 1992; Swanson et al., 1990). In the central nervous system (CNS) Kv2.1 features activity-dependent localisation and function

Abbreviations: AP, action potential; APD50, AP half width; AHPD50, after-hyperpolarization half width; ATF3, activating transcription factor 3; CGRP, calcitonin gene-related peptide; CNS, central nervous system; DRG, dorsal root ganglion; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IB4, isolectin B4; IHC, immunohistochemistry; IR, input resistance; ISH, *in situ* hybridization; Kv channel, voltage-gated potassium channel; NF200, neurofilament 200; RP, refractory period; ScTx, stromatoxin-1; SNT, spinal nerve transection.

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(Misonou et al., 2004; O'Connell et al., 2010) and has a paramount role in regulating somatodendritic excitability, especially during high frequency input (Du et al., 2000; Misonou et al., 2005). Additional Kv2.1 functional diversity is achieved through interaction with modulatory Kv subunits (Bocksteins et al., 2012; Hugnot et al., 1996; Kerschensteiner and Stocker, 1999; Kramer et al., 1998; Sano et al., 2002; Stocker et al., 1999; Vega-Saenz de Miera, 2004) and auxiliary proteins (Leung et al., 2003; Peltola et al., 2011), while some studies have also proposed non-conducting roles (Deutsch et al., 2012; Feinshreiber et al., 2010; O'Connell et al., 2010; Pal et al., 2003; Redman et al., 2007). Although there is substantially less knowledge on Kv2.2, the high degree of conservation between the two subunits suggests common characteristics. Indeed, Kv2.2 mediates membrane hyperpolarization during trains of APs (Johnston et al., 2008; Malin and Nerbonne, 2002) and can associate *in vitro* with modulatory Kv subunits in a similar fashion to Kv2.1 (Fink et al., 1996; Hugnot et al., 1996; Salinas et al., 1997a, 1997b).

Despite the recognised prominent role of Kv2 channels in shaping CNS excitability, no expressional or functional profiling in the periphery has been performed yet. As a result, the Kv2 involvement in sensory neuron excitability and in pain processing in particular remains unknown. Here, we characterized the Kv2 distribution in the DRG and examined the effect of nerve injury on Kv2 expression and function. In addition, we investigated whether pharmacological Kv2 modulation can recapitulate excitability changes linked to chronic pain states.

Methods

Animals and surgery

Adult male Wistar rats (200–250 g, Harlan Labs) were used in all experiments. All animal procedures conformed to institutional guidelines and the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. Experimental neuropathy was induced by L5 spinal nerve transection (SNT, n = 8), using the method previously described (Kim and Chung, 1992). Briefly, a small incision on the skin overlaying left side L5–S1 was made and the vertebral transverse processes were exposed after retraction of the paravertebral musculature. The L6 transverse process was partially removed using bone rongeurs and the L5 spinal nerve was identified, tightly ligated and sectioned 1–2 mm distal to the ligature. The wound was cleaned with saline and the overlying muscles and skin were sutured. For dorsal rhizotomy (n = 3), a hemilaminectomy was performed at the cervical level and the central processes of three consecutive DRGs (C5–C7) were identified and cut with fine iridectomy scissors. The wound was cleaned with saline and sutured at both muscle and skin levels. Animals were allowed to recover in a temperature-regulated chamber before returned to the home cage.

Behavioural studies

Behavioural experiments were performed by a single experimenter, blinded to the identity of surgery the animals received. All tests were conducted in a quiet, temperature controlled room (22 °C). Animals were allowed to acclimatize for 15 min or until exploratory behaviour ceased before testing commenced. Mechanical allodynia was assessed using a von-Frey filament connected to a Dynamic Plantar Aesthesiometer (Ugo Basile). Each rat was placed in a ventilated plexiglass cage (22 × 16.5 × 14 cm) upon an elevated aluminium screen surface with 1 cm mesh openings. An actuator filament (0.5 mm diameter) under computer control delivered a linear stimulation ramp of 2.5 g/s to the plantar surface of the hind paw. Withdrawal thresholds were averaged over three consecutive tests with at least 5 min intervals in between measurements. A cut-off of 50 g was imposed to avoid the risk of tissue damage. Thermal response latencies were determined using the method previously described (Hargreaves et al., 1988). Briefly, each animal was placed into a clear ventilated plexiglass cage (22 × 16.5 × 14 cm) with a glass floor. A thermal challenge from a calibrated (190 mW/cm^2) radiant light source was applied to the hindpaw until a withdrawal reflex was recorded. Withdrawal latencies were averaged over three consecutive tests, at least 5 min apart from each other. A cut-off of 20 s was imposed to prevent the possibility for tissue damage.

Tissue preparation for histology

When tissue was destined for *in situ* hybridization (ISH), all preparation steps were carried out using ribonuclease (RNAse)-free or diethylpyrocarbonate (DEPC, Sigma)-treated reagents and equipment to minimize mRNA degradation. Rats were transcardially perfused under pentobarbitone anaesthesia with heparinized saline followed by fixation with freshly made 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. DRGs were removed and post-fixed in the perfusion fixative for 2 h. Tissue was then equilibrated in 20% sucrose in 0.1 M phosphate buffer (pH 7.4) at 4 °C overnight, embedded in O.C.T. compound. Tissue was cut at 8 μm thickness on a cryostat, and sections were thaw-mounted onto Superfrost Plus glass slides (VWR).

Immunohistochemistry

When combined with *in situ* hybridization, immunohistochemistry (IHC) was performed first using RNAse-free or DEPC-treated materials and antibody solutions were supplemented with 100 U/ml RNasin Plus ribonuclease inhibitor (Promega). For IHC, sections were incubated overnight at RT with the appropriate primary antibody solution in PBS supplemented with 0.2% Triton X-100 and 0.1% NaN_3 (PBS-Tx-Az). Primary antibodies used in this study were mouse anti-β3tubulin (1:2000, Promega), rabbit anti-ATF3 (1:200, Santa Cruz Biotechnologies), rabbit anti-CGRP (1:2000, Sigma), mouse anti-NF200 (1:500, Sigma) and rabbit anti-glial fibrillary acidic protein (rabbit anti-GFAP, 1:1000, DakoCytomation). Secondary antibodies were added for 4 h and were donkey anti-mouse AlexaFluor 488 and donkey anti-rabbit AlexaFluor 546 (1:1000, Invitrogen). IB4 detection was performed by using biotin-conjugated IB4 (1:200, Sigma) and AMCA Avidin D (1:400, Vector Labs).

In situ hybridization

ISH was performed using 34-nucleotide long probes, as previously described in detail (Michael et al., 1997). Probe sequences were Kv2.1: tctggtttcttcgtggagagtcccaggagttcca, and Kv2.2: catccaaaggtctatccccac gagttcccaagca, complementary to bases 1954–1987 and 2650–2683 of kcnb1 (NM_013186.1) and kcnb2 (NM_054000.2) mRNAs, respectively. Probes were radioactively end-labelled with ^{35}S-dATP (Perkin-Elmer) and unincorporated nucleotides were removed with a Sephadex G50 DNA chromatography column (GE Healthcare). Following prehybridization treatments (acetylation in 0.1 M triethanolamine/0.025 M acetic anhydride, dehydration in graded alcohols, chloroform dilipidation, ethanol rehydration), probe was added on sections overnight at 37 °C. The hybridization buffer composition was 2× Denhardt's solution (Sigma), 20× standard saline citrate, 50% deionised formamide, 10% dextran sulphate (Pharmacia Biotech), 100 μg/ml poly A (Sigma), 100 μg/ml sheared salmon sperm DNA (Boehringer), 20 μg/ml tRNA (Sigma) and 20 mM DTT. The following day, slides were washed in salt solutions with increasing stringencies to remove unspecific labelling (2 × 15 min in 2× SSC/β-ME at RT, 2 × 15 min in 1× SSC at 50 °C, 1 × 15 min in 0.2× SSC at 50 °C, 2 × 20 min in 1× SSC at RT, 0.1× SSC), dehydrated and air-dried. Slides were dipped in autoradiographic emulsion (LM1, GE Healthcare), stored away at 4 °C in sealed boxes with silica gel and developed after 3–4 weeks using developer (Kodak D19, 2.5 min), stop (0.5% acetic acid, Sigma) and fix (25–40% sodium thiosulphate, 2 × 5 min, BDH) solutions. Unless combined with IHC, slides were counterstained with 0.1% Toluidine blue (Sigma) and coverslipped with DPX mounting medium (BDH). As a control, a