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HCN4 SUBUNIT EXPRESSION IN FAST-SPIKING INTERNEURONS OF THE RAT SPINAL CORD AND HIPPOCAMPUS

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Abstract—Hyperpolarisation-activated (I_h) currents are considered important for dendritic integration, synaptic transmission, setting membrane potential and rhythmic action potential (AP) discharge in neurons of the central nervous system. Hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels underlie these currents and are composed of homo- and hetero-tetramers of HCN channel subunits (HCN1–4), which confer distinct biophysical properties on the channel. Despite understanding the structure–function relationships of HCN channels with different subunit stoichiometry, our knowledge of their expression in defined neuronal populations remains limited. Recently, we have shown that HCN subunit expression is a feature of a specific population of dorsal horn interneurons that exhibit high-frequency AP discharge. Here we expand on this observation and use neuroanatomical markers to first identify well-characterised neuronal populations in the lumbar spinal cord and hippocampus and subsequently determine whether HCN4 expression correlates with high-frequency AP discharge in these populations. In the spinal cord, HCN4 is expressed in several putative inhibitory interneuron populations including parvalbumin (PV)-expressing islet cells (84.1%; SD: ±2.87), in addition to all putative Renshaw cells and Ia inhibitory interneurons. Similarly, virtually all PV-expressing cells in the hippocampal CA1 subfield (93.5%; ±3.40) and the dentate gyrus (90.9%; ±6.38) also express HCN4. This HCN4 expression profile in inhibitory interneurons mirrors both the prevalence of I_h sub-threshold currents and high-frequency AP discharge. Our findings indicate that HCN4 subunits are expressed in several populations of spinal and hippocampal interneurons, which are known to express both I_h sub-threshold currents and exhibit high-frequency AP discharge. As HCN channel function plays a critical role in pain perception, learning and memory, and sleep as well as the pathogenesis of several neurological diseases, these findings provide important insights into the identity and neurochemical status of cells that could underlie such conditions.

Key words: HCN channels, spinal cord, hippocampus, interneurons, channelopathy.

INTRODUCTION

Neurons in the central nervous system (CNS) exhibit diverse physiological, morphological and neurochemical properties and these features have been used as defining criteria to help identify functionally distinct populations. For example, the discharge responses of rodent spinal dorsal horn neurons to depolarising current injection is highly variable and can be described as tonic firing, initial bursting, delayed firing or single spiking (Thomson et al., 1989; Grudt and Perl, 2002). Importantly, these action potential (AP) discharge patterns appear to be related to both neurotransmitter phenotype and morphology (Grudt and Perl, 2002; Graham et al., 2004, 2008; Yasaka et al., 2007, 2010). Islet cells, for example, form a morphologically distinct population of inhibitory interneurons in the spinal dorsal horn that typically discharge APs at very high frequencies and express I_h currents (Grudt and Perl, 2002; Yasaka et al., 2007, 2010). Hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels are known to play important roles in both establishing I_h currents and contributing to high-frequency AP discharge patterns in a range of tissues including central and peripheral neurons, cardiac myocytes and taste cells (Moosmang et al., 2001; Stevens et al., 2001; Notomi and Shigemoto, 2004; Baruscotti et al., 2005). Four genes are known to encode for HCN channel proteins and these homologous HCN channel subunits (HCN1–4) can assemble as homomeric or heteromeric tetramer complexes to form channels with differing kinetics and voltage activation profiles (Wahl-Schott and Biel, 2009). Importantly, these properties allow HCN channels to play a critical role in setting resting membrane potential, regulating repetitive AP discharge, and shaping dendritic processing in neuron populations

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Abbreviations: AP, action potential; CB, calbindin; ChAT, choline acetyltransferase; HCN, hyperpolarisation-activated cyclic nucleotide-gated; IaINs, group Ia inhibitory interneurons; PV, parvalbumin; PKCγ, protein kinase C gamma; RCs, Renshaw cells; TSA, tyramide signal amplification.

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involved in learning, memory and pain (Lüthi and McCormick, 1998; Bennett et al., 2000; Dudman and Nolan, 2009; Oswald et al., 2009). More recently, HCN channelopathies have been implicated in the pathophysiology of neurological diseases such as epilepsy and have therefore been proposed as potential targets for future drug therapies (Lewis and Chetkovich, 2011; Reid et al., 2012).

Several studies have surveyed HCN channel subunit expression in the rodent CNS and show that HCN1 and HCN2 are the most widely distributed forms (Moosmang et al., 1999; Santoro et al., 2000; Notomi and Shigemoto, 2004). As a consequence, studies on I_h currents and pacemaker activity in neurons have focused principally on HCN1 and HCN2 subunits despite both HCN3 and HCN4 also being critical for establishing rhythmic firing in certain neuronal populations (Cho et al., 2009; Ying et al., 2011). Furthermore, recent studies show that these subunits are more prevalent in the spinal cord and hippocampus than previously thought, and are expressed in cells that exhibit high-frequency AP discharge (Brewster et al., 2007; Hughes et al., 2012). Despite the documented differences in the biophysical properties of HCN channels in expression systems, little is known about the expression and stoichiometry of these channels in the intact nervous system or how expression patterns are likely to contribute to neuron function. As HCN channelopathies are now being implicated in a number of neurological diseases including epilepsy and chronic pain (Chaplan et al., 2003; Lewis and Chetkovich, 2011), we focus on HCN4 expression in defined subpopulations of neurons in the spinal cord and hippocampus. This strategy will begin to address the paucity of information available on the distribution of this and other HCN subunits in these clinically relevant regions.

HCN channel subunit expression is likely to be an important determinant of membrane properties in CNS neurons and we propose that HCN4 expression in these selected inhibitory interneuron populations is linked to high-frequency AP discharge and the presence of I_h currents (Southan et al., 2000; Dudman and Nolan, 2009). To test this hypothesis, we have used the neurochemical profiles of spinal and hippocampal neurons to first identify populations reported to exhibit either high-frequency AP discharge (e.g. islet cells, Renshaw cells (RCs), Ia inhibitory interneurons, basket cells and oriens-lacunosum moleculare cells) or transient/regular-spiking discharge patterns (motor neurons, pyramidal cells, granule cells) and then determine whether they express immunolabelling for the HCN4 channel subunit.

EXPERIMENTAL PROCEDURES

All experiments were approved by the University's Ethical Review Process Applications Panel and were performed in accordance with the European Community directive 86/609/EC and the United Kingdom Animals (Scientific Procedures) Act 1986.

Fixation and tissue preparation

A total of nine adult male Wistar rats (220–290 g; Harlan UK Ltd., Bicester, UK) were deeply anaesthetised with pentabarbitone and perfused transcardially with 4% depolymerised formaldehyde. The brain and lumbar spinal cord were removed and post-fixed in the same solution for an additional 2 h. Spinal cord (transverse and parasagittal planes) and hippocampal sections (coronal plane) were cut on a Vibratome (60-μm thick) and subsequently incubated in 50% ethanol for 30 min to enhance antibody penetration.

Free-floating spinal cord and hippocampal sections were incubated in either goat anti-parvalbumin (PV), rabbit anti-calbindin (CB) and mouse anti-HCN4 or goat anti-PV, rabbit anti-PKCγ and mouse anti-HCN4 for 72 h. All of these antibodies are available from commercial sources, are widely used and have been fully characterised (see Table 1 for details). HCN4 labelling was visualised using a tyramide signal amplification (TSA) step, whereas immunolabelling for calcium-binding proteins and PKCγ was visualised using species-specific secondary antibodies conjugated to either Alexa 488 or Cy5. Sections were incubated overnight in fluorescent-labelled secondary antibodies and biotinylated donkey anti-mouse. They were subsequently incubated for 3 h in Avidin conjugated to horseradish peroxidase, before carrying out a TSA reaction using a tetramethylrhodamine kit (PerkinElmer Life Sciences, Boston, MA, USA) in accordance with the manufacturer's instructions.

To examine the expression of HCN4 in spinal motor neurons, we first incubated spinal cord sections in mouse anti-HCN4 prior to revealing immunolabelling using the TSA method as described above. These sections were then incubated in a cocktail of mouse anti-gephyrin (7a) and goat anti-choline acetyltransferase (ChAT) before detecting immunolabelling for these markers using secondary antibodies conjugated to Alexa 488 and Cy5 respectively. All primary and secondary antibody cocktails were made up in 0.3 M phosphate-buffered saline with 0.3% Triton X-100. Sections were incubated in primary antibodies for 72 h and in secondary antibodies for 12–18 h.

Neurochemical identification of functionally discrete spinal and hippocampal populations

We have used combinations of different neurochemical markers to determine whether functionally defined neuronal populations in the spinal cord and hippocampus express the HCN4 channel subunit (see Table 2). For example, in the spinal dorsal horn, HCN4-immunolabelling is profuse in lamina II. PV-expressing cells are a prominent group of lamina II inhibitory interneurons and their morphology resembles those of islet cells (Antal et al., 1990; Laing et al., 1994). Their dendritic trees are elongated in the rostro-caudal axis (typically extending over 400 μm) but have relatively limited dorso-ventral spread into adjacent laminae. The axons of islet cells arborise extensively within the volume of the dendritic tree but are mostly confined to lamina II (Gobel, 1975; Grudt and Perl, 2002; Yasaka et al., 2010). Islet cells comprise a physiologically homogeneous population of cells, exhibiting tonic-firing AP discharge patterns and I_h-type subthreshold currents and are therefore likely to express HCN channels (Grudt and Perl, 2002; Yasaka et al., 2007, 2010). Cells that express the gamma isoform of protein kinase C (PKCγ) are also common in the ventral part of lamina II (Polgár et al., 1999). Most of these excitatory interneurons express A-type potassium currents but show heterogeneity in both morphology and AP discharge (Polgár et al., 1999; Hu and Gereau, 2011) and are therefore likely to show different patterns of HCN4 immunolabelling compared to PV cells. In the spinal ventral horn, RCs and group Ia inhibitory interneurons (IaINs) also exhibit high-frequency AP discharge (Renshaw, 1946; Eccles et al., 1954; Mentis et al., 2005) and can be