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Characterization of hyperpolarization-activated currents in deep dorsal horn neurons of neonate mouse spinal cord *in vitro*

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ABSTRACT

Emerging evidence suggests that blockade of hyperpolarization-activated current (I_h) produces analgesia acting at peripheral sites. However, little is known about the role of this current in central pain-processing structures. The aim of the present work was to characterize the I_h in deep dorsal horn neurons and to assess the role of the current in the transmission of somatosensory signals across spinal circuits.

To these purpose *in vitro* preparations of the spinal cord from mice pups were used in combination with whole cell recordings to characterize the current in native neurons. Extracellular recordings from sensory and motor pathways were performed to assess the role of the current in spinal somatosensory processing. Cesium chloride and ZD7288 were used as current blockers.

Most deep dorsal horn neurons showed a functional I_h that was blocked by ZD7288 and cesium. I_h blockade caused hyperpolarization, increased input resistance and potentiation of synaptic responses. Excitatory effects of I_h blockade on synaptic transmission were confirmed in projecting anterolateral axons and ventral roots. I_h modulation by cAMP produced a rightward shift in the voltage dependency curve and blocked excitatory effects of ZD7288 on sensory pathways.

Results indicate that I_h currents play a stabilizing role in the spinal cord controlling transmission across sensory and motor spinal pathways via cellular effects on input resistance and excitability. In addition, results suggest that current modulation may alter significantly the role of the current in somatosensory processing.

1. Introduction

Hyperpolarization-activated currents (I_h) were first detected in cardiac muscle cells and then reported in neurons from different areas of the nervous system (Pape, 1996; Robinson and Siegelbaum, 2003). The I_h is a slowly activating and non-inactivating mixed current carried by Na^+ and K^+ ions that, contrary to most ionic conductances, activates with hyperpolarization reducing the extent of hyperpolarization and stabilizing the membrane potential (Pape, 1996; Robinson and Siegelbaum, 2003). Homo- or hetero- tetrameric assemblies of HCN subunits 1 to 4 yield functional channels that represent the molecular correlates of I_h (Wahl-Schott and Biel, 2009). This current is sensitive to cesium ions and it is blocked by organic compounds like ZD7288 or ivabradine (Robinson and Siegelbaum, 2003). In some neurons, I_h generates an excitatory inward current at rest affecting membrane potential, membrane resistance and intrinsic excitability (Aponte et al., 2006; Chen, 2004; Gasparini and DiFrancesco, 1997; George et al., 2009; Lamas, 1998; Park et al., 2007). In thalamic neurons and cardiac cells, the I_h seems to contribute to maintain pacemaker activity (Pape, 1996). These effects of the I_h on basic membrane properties can affect synaptic transmission and network behavior in a number of ways.

Several investigations suggest a prominent role of the I_h in somatosensory processing, especially after peripheral injury (Emery et al., 2011; Wickenden et al., 2009). The expression of HCN channels has been reported in dorsal root ganglion neurons, nerves and peripheral terminals affecting the transmission of signals through afferent fibers (Chaplan et al., 2003; Luo et al., 2007; Tu et al., 2004). It has also been shown that i.p. administration of the channel blocker ZD7288 attenuates signs of pain in animals after neuropathic treatments (Chaplan et al., 2003; Jiang et al., 2008; Lee et al., 2005; Luo et al., 2007; Takasu et al., 2010).

In the spinal cord, the presence of H-like currents in dorsal and ventral horn neurons was suggested by the presence of anomalous rectification in response to hyperpolarizing current injections

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(Lopez-Garcia and King, 1994; Takahashi, 1990; Yoshimura and Jessell, 1989). More recently this current has been identified in superficial dorsal horn neurons, especially in islet cells from substantia gelatinosa (Grudt and Perl, 2002; Melnick, 2008). Expression of HCN channels has been reported in the neuropil and cell bodies of the spinal cord (Antal et al., 2004; Milligan et al., 2006; Santoro et al., 2000; Tu et al., 2004). However a detailed study on the function of I_h in spinal neurons is still lacking.

Here we have used the hemisected *in vitro* spinal cord preparation of mice pups, a wide range of electrophysiological techniques and the I_h blockers ZD7288 and cesium to carry out a detailed study on the role of I_h at controlling dorsal horn neuron excitability and spinal processing of somatosensory and somatomotor information. Results obtained in untreated animals indicate that the I_h acts on the spinal cord as a stabilizer of neuronal excitability and synaptic integration. However this role may change in sensory areas of the cord after current modulation by cAMP.

2. Materials and methods

Experiments were performed in B6CBA mice pups (6–13 days-old) of either sex weighing between 3.5 and 8.2 g. Experiments were designed following European Union and Spanish Government regulations and the experimental protocols were approved by the University of Alcala Ethics Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.1. In vitro spinal cord preparation and dorsal root stimulation

Animals were anaesthetized with urethane (2 g/kg i.p.) and their spinal cords extracted following a rostrocaudal laminectomy (Rivera-Arconada and Lopez-Garcia, 2010). After extraction and still under anesthesia, animals were killed by cervical dislocation. The cord was hemisected in ice cooled cerebrospinal fluid (ACSF, in mM was: NaCl 127, KCl 1.9, KH_2PO4 1.5, $MgSO_4$ 1.3, $CaCl_2$ 2, $NaHCO_3$ 22, glucose 10, pH 7.4). One hemisection was placed in a recording chamber and maintained in oxygenated artificial cerebrospinal fluid at room temperature. For extracellular experiments entire cords were used and the hemisection procedure was skipped.

A lumbar dorsal root (L4–L5) was placed in a tight fitting glass suction electrode and electrically stimulated to activate afferent fibers. Electrical stimuli routinely consisted of (1) a series of pulses of 200 μs duration and increasing intensities to recruit progressively the different afferent types within the root and (2) repetitive stimuli (series of 15 stimuli at 1 Hz) at intensity sufficient to activate C-fibers (i.e. 200 μs and 200 μA) (Martinez-Gomez and Lopez-Garcia, 2005).

2.2. Whole cell recordings

Electrodes were pulled from borosilicate glass tubing (Harvard apparatus ltd, UK) with internal filament using a horizontal puller (Sutter instruments, Novato, CA, USA). Pipette resistances were within the range of 6–9 MΩ when filled with an intracellular solution consisting of (mM): KCl 30, EGTA 3, HEPES 40, $MgCl_2$ 2, potassium acetate 95, $CaCl_2$ 0.5, Na_2-ATP 3, Na-GTP 0.3 (pH 7.4). In a set of experiments 1 mM adenosine 3′,5′-cyclic monophosphate (cAMP) was added to the intracellular solution to study the modulation of voltage dependent activation of the I_h.

In some experiments biocytin (5 mg/ml) was added to the intracellular solution to obtain basic data on the morphology of recorded neurons and their situation within the spinal cord. Biocytin was electrophoresed into neurons using continuous hyperpolarizing current (−0.5 to −0.8 nA for 15 min) and processed following a procedure previously described (Rivera-Arconada and Lopez-Garcia, 2005). Electrical signals were amplified with a Multiclamp 700A (Molecular Devices Inc., USA) and digitized at 10 kHz for offline analysis with Spike 2 or Signal (CED, UK).

2.2.1. Voltage clamp recordings

Series resistances below 40 MΩ were routinely obtained and not compensated (mean estimated voltage error <4 mV at −130 mV).

Hyperpolarization-activated currents were studied from a holding of −50 mV using 1.5 s duration step pulses at 10 mV decrements to a final potential of −130 mV. I_h amplitude was measured as the difference between instantaneous and steady-state current (Pape and McCormick, 1989). The maximum voltage step to −130 mV was used to calculate current density dividing current intensity by cell capacitance. Time constant of current activation at −130 mV was calculated by fitting to a single o double exponential function. For voltage dependent activation studies, I_h was measured as the instantaneous current in a voltage step to −130 mV from different test potentials between −60 and −130 mV (Stieber et al., 2005). Reversal potential was calculated as the intersection between extrapolated curves obtained measuring the instantaneous current in voltage steps between −60 and −110 mV from a holding of −50 mV (before I_h activation) and the tail current at potentials between −100 and −140 mV after activation of the current with a step to −130 mV (Lamas, 1998).

2.2.2. Current clamp recordings

Depolarizing and hyperpolarizing intracellular current pulses of 500 ms injected via the recording pipette were applied in increments of 16 pA at 2 s intervals to study the intrinsic excitability and the input resistance of neurons. For negative pulses the voltage change at the end of the 500 ms pulse and the maximum voltage change during the first 200 ms were measured. The ratio between these values was calculated to obtain a reference for the amount of depolarizing sag in each neuron (sag ratio). Neurons with sag ratios ≥1 in pulses of −200 pA were classed as no sag neurons.

The number of action potentials in response to depolarizing pulses was counted as indication of intrinsic excitability. ZD7288-induced membrane potential hyperpolarization was compensated when necessary by constant positive current injection through the electrode. Current–voltage curves were constructed measuring the steady-state membrane potential at the end of the current command step. Input resistance was calculated as the slope of the curve using negative current pulses of low intensity (≤100 pA) to avoid underestimation due to I_h activation. Membrane time constant was calculated fitting voltage responses to small intensity hyperpolarizing current pulses to a single exponential function. Cell capacitance was calculated dividing the time constant by the input resistance (Golowasch et al., 2009).

Responses to dorsal root stimulation were quantified in terms of number of action potentials and integrated area of the EPSPs with a cut-off at 4 s from stimulus artifact. Only neurons exhibiting overshooting action potentials were fully characterized and included for analysis.

2.2.3. Miniature excitatory post-synaptic current (mEPSC) and spontaneous inhibitory post-synaptic currents (sIPSCs) recordings

For mEPSC recordings neurons were held at −70 mV to minimize inhibitory events and maintained in the continuous presence of 0.5 μM tetrodotoxin (TTX) to block action potentials and synaptic transmission. Inhibitory currents were recorded at 0 mV to avoid excitatory events and using a cesium based intracellular fluid (Zhang et al., 2010) consisting in (mM): Cs_2SO_4 120, NaCl 5, $MgCl_2$ 1, EGTA 0.5, HEPES 10, Na-ATP 2, Na-GTP 0.1 (pH 7.2).

The analysis of mEPSCs and sIPSCs was performed on single events of amplitude ≥ four times the Root Mean Square (RMS) of the original baseline signal.

2.3. Extracellular recordings from ventral roots

Recordings from ventral roots (L4–L5) were obtained using tight fitting glass suction electrodes. Responses to electrical stimulation of the dorsal root were recorded from the corresponding ventral root using AC recordings (Rivera-Arconada et al., 2004). Spike counts were used to quantify responses to dorsal root stimuli.

2.4. Extracellular recordings from anterolateral axons

Extracellular recordings from anterolateral axons were obtained with suction microelectrodes filled with ACSF in the entire spinal cord as previously described (Martinez-Gomez and Lopez-Garcia, 2005). Electrode tips were placed close to the anterolateral white matter contralateral to the stimulated dorsal root, at upper thoracic segments. Experiments were run only when single units were clearly identified and discriminated on the basis of spike amplitude and shape using Spike 2 sorting analysis. Responses of anterolateral axons to dorsal root stimulation were quantified as the number of spikes elicited by the stimulus.

2.5. Drugs and chemicals

The I_h blocker 4-Ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride (ZD7288) and tetrodotoxin (TTX) were purchased from Tocris Bioscience (UK). Cesium chloride, 8-Bromoadenosine 3′,5′-cyclic monophosphate sodium salt, all synaptic receptor blockers, components for the ACSF and the intracellular solutions were purchased from Sigma–Aldrich (Spain).

Drugs were dissolved in ultrapure water or DMSO as concentrated stocks and diluted in ACSF to the final concentration just prior to use (concentration of solvents less than 0.1%).

2.6. Data analysis

All statistical analysis and curve fittings were performed using GraphPad Prism 4.0 (GraphPad Software, USA). Data is presented as mean ± SEM unless otherwise stated.

Voltage dependent activation curves for I_h were fitted to a Boltzmann sigmoid curve of the type: $I/I\max = \text{Bottom} + (\text{Top}-\text{Bottom})/(1 + \exp((V_{50} - V)/\text{Slope}))$.

Linear regression analysis was employed to extrapolate the curves for reversal potential estimation.

Frequency distribution analysis was performed to analyze frequency and amplitude of mEPSC and sIPSC.