THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

CELLULAR DISTRIBUTION OF HCN2 ION CHANNEL IMMUNOREACTIVITY IN THE SPINAL DORSAL HORN OF RATS IN CONTROL AND INFLAMMATORY PAIN CONDITIONS

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DEBRECEN, 2010.

INTRODUCTION

The complexity of functions of the nervous system are attained by a fine-tuned interplay between intrinsic neuronal properties and synaptic interconnections. Intrinsic electrophysiological characteristics of neurons are defined by the type, density and distribution of voltage- and ligand-gated ion channels attached to the plasma membrane. The conductance of ion channels is regulated by the membrane potential of the plasma membrane or variable chemical substances (ligands). The direction and magnitude of the flow of ionic currents through the ion channels are controlled by the electrochemical gradient of the specific ions.

The majority of voltage-gated cation channels, e. g. various Na^+- and K^+-channels, is activated by depolarization. Interestingly, one group of them, unlike the other voltage-gated channels, is activated at membrane potentials negative to resting potential, namely by hyperpolarization, and its inward cation current slowly depolarizes the plasma membrane. This inward current with unusual properties was initially discovered by independent laboratories in amphibian photoreceptors and in mammalian heart in the 1970s (Bader és mtsai., 1979; Brown és mtsai., 1979). The ionic current registered in photoreceptors was named as hyperpolarization-activated current (I_h), while the current measured in sinoatrial node and Purkinje-fibers of mammalian heart was referred to as „funny" current (I_f) (DiFrancesco, 1981). Halliwell and Adams (1982) confirmed the presence of this current also in the central nervous system, in hippocampal pyramidal neurons, and due to its queer characteristics and unknown role it was named as „queer" current (I_q). Later the current was described in numerous regions of the peripheral and central nervous system, and distinguishing it from the current that was previously discovered in the heart (I_f), it was referred to as hyperpolarization-activated current (I_h) in the brain (Hille, 1992).

Another interesting feature of ion channels mediating hyperpolarization-activated current is that they can bind cyclic nucleotides, primarily cAMP. The binding of cAMP shifts the steady-state activation of I_h towards more positive membrane potentials. Taking this

characteristic feature of the channels also into account, nowadays they are referred to as **h**yperpolarization-activated and **c**yclic **n**ucleotide-gated cation channels (**HCN**).

Cloning of genes encoding HCN channel proteins, four different isoforms (HCN1-4) were identified. The isoforms differ from each other in their activation kinetics and the extent of cAMP dependency. The functional HCN channels form tetrameric ion channels composing of four subunits.

The expression of HCN channel isoforms and hyperpolarization-activated current (I_h) was first described in the heart, but later it was also examined thoroughly in numerous regions of the peripheral and central nervous system, including cells in the spinal dorsal root ganglia (DRG). From the four subunits, mRNA-s and proteins of HCN1-, 2-, and 3-isoforms were identified in the DRG.

Although the expression pattern and the role of HCN channels are well known in the cell bodies of dorsal root ganglion cells, we do not know whether HCN proteins can be transported to the central axon terminals of primary sensory neurons. It would also be interesting to know which type of primary afferents can transport HCN proteins, and if functional HCN channels are inserted into the cell membranes of central axon terminals of primary afferents what roles they can play in the spinal processing of somatosensory information.

MAJOR AIMS

At the beginning of our investigations, there were no data available in the literature concerning the expression of HCN ion channel proteins in the central axon terminals of primary afferents. Thus, we did not know whether I_h currents may play a role in the regulation of synaptic transmission between primary afferent and secondary spinal sensory neurons.It was also unknown, how the expression of HCN channels changes in primary sensory neurons during chronic inflammatory pain.

Therefore, the major aims of our studies were the followings:

1. Do HCN ion channel isoforms, in addition to perikarya of the dorsal root ganglion cells, express in the central axon terminals of primary sensory neurons in the spinal dorsal horn of rats?
2. Which type of primary afferents express HCN2 ion channel proteins, and what are the postsynaptic targets of HCN2-expressing primary afferents in the superficial spinal dorsal horn in rodents?
3. What can be the role of HCN2 ion channels in neural transmission between axon terminals of nociceptive primary afferents and secondary spinal sensory neurons?
4. Does the spinal expression of HCN2 proteins change in chronic inflammatory pain?

MATERIALS AND METHODS

Animals and preparation of tissue sections

Experiments were carried out on 42 adult, nine 3-week-old male rats (Wistar-Kyoto), and 3 GAD65-eGFP transgenic adult mice. The animals used for immunohistochemical examinations were deeply anesthetized with sodium pentobarbital (50 mg/kg, i. p.) and transcardially perfused first with Tyrode's solution, followed by a fixative containing (a) 4% paraformaldehyde, or (b) 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid. After the fixation, the lumbar spinal segments (L3-L5) and lumbar dorsal root ganglia were removed (3 animals), postfixed in the same fixative overnight, and immersed in 10 and 20% sucrose solution until they sank. To enhance reagent penetration, the removed spinal segments and dorsal root ganglia were freeze-thawed in liquid nitrogen, sectioned at 50-60 µm on a Vibratome and extensively washed in 0.1 M phosphate buffer.

In three rats 2 weeks prior to the transcardial fixation, the lumbar spinal cord was exposed by laminectomy and the L2-S1 spinal dorsal roots were cut unilaterally under deep sodium pentobarbital anaesthesia. Two weeks after the dorsal rhizotomy, the animals were sacrificed and transcardially fixed as described above.

Nine adult male rats used for the inflammatory pain model were divided into three experimental groups. Experimental group 1: 100 µl complete Freund's adjuvant (CFA) diluted with physiological saline has injected into the plantar surface of the right hind paw, experimental group 2: 100 µl physiological saline has injected into the plantar surface of the right hind paw. Experimental group 3: animals without any treatment were used as controls. Before and after CFA injection, all animals were tested for withdrawal responses to gradually increasing mechanical stimuli applied onto the plantar skin, and on the 4-th day following CFA injection the animals were transcardially perfused. The L3-L5 spinal segments were removed, from which 60 µm thick free-floating serial sections were cut as described above.

Behavioral test for demonstrating the development of mechanical allodynia

To investigate the expression of HCN2 ion channels under inflammatory pain condition, we used the Freund's adjuvant-induced pain model as a widely used experimental model of inflammatory pain.

Responsiveness of the paws of the animals to mechanical stimuli was measured daily, for 3 days before and after the CFA injection using a „Dynamic Plantar Aesthesiometer" (Ugo Basile). Rats were placed upon a network platform and the hind paws of the animals were

stimulated mechanically, with an increasing force by a metal filament (0-50 g). The force at which paw withdrawal occured was detected. The mechanical withdrawal threshold for both hind paws of the rats was measured before and after CFA injection.

Immunoperoxidase histochemistry

To demonstrate spinal expression of the HCN2 ion channel and c-Fos proteins, we used indirect, DAB-based immunoperoxidase method. Free-floating sections of the lumbar spinal cord of naive and treated animals were first kept in 20% normal goat serum (NGS) for 50 minutes, and were washed in 0.01 M TPBS (pH = 7.4) solution containing 1% NGS. The sections were first incubated with an antibody against HCN2 (1:400) or c-Fos (1:8000) raised in rabbit for 2 days at 4 ºC. Following extensive washing, the sections were transferred into biotinylated goat anti-rabbit (b-GAR) IgG (1:200) for 5-6 h. After repeated washes, they were treated with an avidin-biotinylated horseradish peroxidase complex (ABC, 1:100) overnight at 4 ºC, and the immunoreaction was completed with a diaminobenzidin (DAB) chromogen reaction. Sections were mounted on gelatin-coated glass slides, dehydrated in ethanol and covered with Permount neutral medium.

The specificity of the primary antibody (anti-HCN2, Alomone Labs.) was tested by preadsorption of the HCN2 peptide to the antibody. Sections were incubated according to the immunocytochemical procedure by using the antibody against HCN2 treated with the HCN2 peptide as primary serum. Under these conditions, specific immunostaining was completely abolished. To check the specificity of the secondary antibody, sections were treated with the primary antibody was replaced with normal rabbit serum (NRS), 1:100). In these sections no peroxidase reaction was observed.

Sections immunostained for HCN2 were digitized by a Nikon Eclipse 800 light microscope equipped with a Spot camera. Quantitative analysis of the HCN2 immunostaining was performed by Image J software, and the cross-sectional surface area of the spinal dorsal horn that was immunostained for HCN2 was measured in the medial, intermediate and lateral regions, and in the total medio-lateral extent of the superficial spinal dorsal horn.

To obtain a quantitative view of c-Fos immunoreactivity in the spinal dorsal horn, c-Fos-positive cells in the superficial dorsal horn of sections of L4 spinal segment were first drawn with a camera lucida, then three-dimensional reconstruction of the distribution of these cells was performed by a Neurolucida. During the quantitative analysis, the c-Fos-immunoreactive cells were counted in the full medio-lateral extension of the superficial dorsal horn and in its three regions mentioned earlier.

Preembedding nanogold immunohistochemistry

In order to investigate subcellular distribution of HCN2 proteins in the immunoreactive axon terminals, electron microscopic examinations were carried out. Following extensive washes in 0.1 M phosphate buffer (PB) and treatment with 1% sodium borohydride ($NaBH_4$) for 30 min, free-floating sections of the lumbar spinal cord from animals fixed with 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid were first incubated with anti-HCN2 antibody raised in rabbit (1:400) for 2 days at 4 ºC. The sections were then transferred into a solution of goat anti-rabbit IgG conjugated to 1-nm gold particles (GAR-Au, 1:100) for 6 h at room temperature. After repeated washes, the sections were post-fixed for 10 min in 2.5% glutaraldehyde, and the gold labelling was intensified with a silver enhancement reagent. Sections were treated with 1% osmium-tetroxide (OsO_4) for 45 min, then dehydrated and flat embedded into Durcupan ACM resin on glass slides. Dorsal horns form the sections showing the best immunostaining were cut, and re-embedded. Ultrathin sections (60 nm) were cut, collected on Formvar-coated single-slot nickel grids, and counterstained with uranyl acetate and lead citrate.

Immunofluorescent staining for confocal microscopy

To demonstrate presynaptic markers and postsynaptic targets of HCN2-immunoreactive nociceptive primary afferent terminals, double and triple immunofluorescent stainings were performed on sections from the lumbar spinal cord and dorsal root ganglia. In order to identify axons with different origin and their postsynaptic targets, the following markers were used:

(1) markers characteristic of nociceptive primary afferents: calcitonin gene-related peptide (CGRP), isolectin-B4 (IB4) binding and substance P (SP)

(2) markers of intrinsic excitatory axon terminals of the spinal dorsal horn: vesicular glutamate transporter 1, 2, and 3 (VGluT1, 2, 3)

(3) markers of excitatory interneurons: neurokinin1 receptor (NK1-R), calbindin D28k (CaB), μ-opioid receptor (MOR) and subunit 2 of the AMPA-type glutamate receptor (GluR2)

(4) marker for inhibitory interneurons: isoform 65 of glutamic acid decarboxylase (GAD 65 in GAD65-eGFP transgenic mice).

Fifty μm thick free-floating sections of the lumbar spinal cord and DRGs were first kept in 20% normal goat or horse serum (NGS, NHS) for 50 min. Following washes, sections were then incubated with a mixture of primary antibodies for 2 days at 4 ºC. For double and

triple immunostaining, besides the anti-HCN2 raised in rabbit (1:200) antibodies against the markers described earlier were used. Thereafter, the sections were transferred into the appropriate mixtures of secondary antibodies that were conjugated to fluorescent dyes for 5-6 h at room temperature. After repeated washes, the sections were mounted on glass slides and covered with Vectashield.

Due to the extensive colocalization between immunoreactivities for HCN2 and SP, the specificity of the primary antibodies were tested on some sections by treating the anti-HCN2 and anti-SP with purified HCN2 peptide and SP.

The immunofluorescent sections were digitized by using Olympus Fluoview FV1000 laser scanning confocal microscope. The colocalization of HCN2 with presynaptic markers was quntitatively analysed using Neurolucida for Confocal software. The analysis was performed in 1 µm thick confocal optical images obtained from three animals (3 images/animal), on which 100 axon terminals in laminae I-IIo of the spinal dorsal horn that showed immunoreactivity for a marker were randomly selected. The selected terminals were then examined to determine whether they were also immunoreactive for the other marker. In the inflammatory pain model, the colocalization between HCN2 and SP was analysed using a standard square grid that was put onto the confocal images, and the terminals showing single or double immunostainings over the edges of the grid were counted. The quantitative measurement was carried out in 10 confocal images obtained from 3 control and 3 CFA-injected animals, respectively.

Electrophysiological experiments

To investigate the role of the HCN2 ion channels in the synaptic transmission between nociceptive primary afferent terminals and spinal sensory neurons, electrophysiological experiments were carried out on 3-week-old rats. Under deep isofluorane anesthesia the animals were decapitated, the lumbar spinal cord was removed and put into oxygenated artificial cerebrospinal fluid (pH 7.4). Blocks of the lumbar spinal cord with dorsal roots attached were embedded into agar and sectioned at 400-600 µm on a Vibratome. Slices with dorsal roots attached were recorded in a recording chamber, which was constantly perfused with artifical cerebrospinal fluid.

Neurons located in laminae I-II were identified with a Zeiss Axioskop FS microscope equipped with a X40 water immersion objective, differential interference contrast filter and infrared camera system. The dorsal root was introduced into a stimulating electrode, while single cells in laminae I-II were recorded using whole-cell patch-clamp recordings in a

current-clamp mode. Patch pipettes with a resistance of 4-6 MΩ and an Axoclamp ID amplifier were used for the recording. Dorsal roots were stimulated at 0.2 Hz with 0.1-ms-long current pulses, with an amplitude of 0.5-2.0 mA, while spontaneous and evoked excitatory postsynaptic potentials (EPSP) as well as action potentials were rerecorded from cells that received monosynaptic inputs from C and/or Aδ primary afferents. Failure rates of synaptic transmission were calculated for each 10 consecutive dorsal root stimulations by counting the numbers of stimulations that failed to evoke EPSP on the postsynaptic neuron. After recording the spontaneous and evoked EPSPs in control condition, postsynaptic potentials of cells in laminae I-II were recorded applying HCN channel blocker (ZD7288, 10 μM). Finally, under continuous recording, ZD7288 was washed out of the bath.

Data were digitized (Digidata 1320), recorded on an IBM computer and analysed by using Pclamp, Origin and Whole Cell Program and Electrophysiology Data Recorder software packages.

Western blot analysis

The analysis was performed on 15 adult male rats, from which 9 rats received diluted CFA-injection into the plantar surface of the right hind paw. Four days after the injection, the spinal dorsal horns at the level of L4 lumbar segments were dissected under overanaesthesia with diethyl-ether. Six naive rats were used as controls.

The removed dorsal horns were processed in two ways: 1) membrane extracts, and 2) total tissue extracts were prepared. During membrane preparation the tissue samples were first sonicated in 20mM TRIS lysis buffer (pH 7.4) containing protease inhibitor (pH 7.4), then the tissue suspension was centrifuged at 50 000 g for 90 minutes at 4 ºC. The supernatant was discarded and the pellet was resuspended in the lysis buffer (20 mM TRIS, 137 mM NaCl, pH 7.4) supplemented with 2% Triton-X 100, then the suspension was centrifuged again at 50 000 g for 55 min at 4 ºC. The other part of the tissue samples was pulverized in liquid nitrogen and solubilised in 20 mM TRIS solution containing 2% TRITON-X 100 and protease inhibitors, then the cellular debris were removed by centrifugation (16 000 g, 10 min, 4 ºC).

The supernatants obtained from both the total tissue and membrane extracts were dissolved in reducing sample buffer (35 μg protein/lane) and run on 10% SDS-polyacrylamide gels according to the method of Laemmli (1970). The separated proteins were electrophoretically transferred onto PVDF membrane, and the membranes were treated with TTBS solution containing 10% normal bovine serum (NBS) then incubated with anti-HCN2 raised in rabbit (1:1000) and loading control antibody (anti-β-tubulin raised in mouse, 1:4000)

for 2 h at room temperature. After repeated TTBS washes, the membranes were transferred into a solution containing biotinylated anti-rabbit IgG or anti-mouse IgG (1:200) and then were transferred into a solution containing ABC complex (1:100). The immunostaining was visualized with a DAB chromogen reaction. Immunostained bands were digitized with the GelCapture software. The optical densities of the bands were measured with the GelQuant software, and the density values obtained for HCN2 were normalized to density values acquired for loading control β-tubulin.

Statistical analysis

From quantitative data, mean values and standard error of the means (SEM) were calculated, and the observed differences were statistically evaluated using a one-way ANOVA test and a paired t-test for the electrophysiological experiments. The different levels of significance were indicated with asterisks (* $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$).

RESULTS

HCN2 immunoreactivity in the spinal dorsal horn

We found darkly stained punctate profiles in laminae I-IIo of the superficial spinal dorsal horn of rats. The immunostaining for HCN2 was punctate, similar to the labeling of axon terminals. In some cases the immunostained puncta were aligned in elongated rows that either run parallel with the border between the gray and white matters in lamina I, or extended down to deeper laminae of the dorsal horn. In contrast to the strong immunolabelling in laminae I-IIo, the deeper laminae of the dorsal horn were free of immunostaining. We have never observed any somatic or dendritic immunolabeling in the lumbar spinal dorsal horn. Dorsal rhizotomy at the level of L2-S1 spinal segment completely abolished the immunostaining from the dorsal horn of L4 spinal segment, indicating that the immunoreactive puncta in laminae I-IIo may represent terminals of nociceptive primary afferents.

Co-localization between HCN2 and markers of peptidergic and non-peptidergic primary afferents

To demonstrate whether HCN2 protein is transported by peptidergic or non-peptidergic nociceptive primary afferents to the spinal dorsal horn, we examined the colocalization of HCN2 with CGRP immunoreactivity and IB4 binding using triple immunofluorescent labeling. CGRP is known as a marker of peptidergic nociceptive primary afferents (Lawson, 1995), and IB4 binding is the most characteristic feature of non-peptidergic nociceptive primary afferents (Gerke and Plenderleith, 2002).

In agreement with previous results, CGRP-immunoreactive axon terminals were found in laminae I-IIo, whereas profiles showing IB4 binding were exclusively confined to lamina IIi. Accordingly, HCN2 immunoreactivity displayed a substantial colocalization with CGRP immunoreactivity in laminae I-IIo of the spinal dorsal horn, whereas HCN2 was almost completely segregated from IB4 binding in the spinal dorsal horn.

Interestingly, almost all neurons in the DRG were stained for HCN2, consequently all neurons that showed immunoreactivity for CGRP or IB4 binding were also stained for HCN2.

Subcellular distribution of HCN2 proteins in axon terminals of peptidergic nociceptive primary afferents

Ultrastructure of neuronal elements immunostained for HCN2 was examined using preembedding nanogold immunohistochemical method in the superficial spinal dorsal horn. Silver particles labelling HCN2 molecules were recovered exclusively in axon terminals with a diameter of 0.5-2.5 µm, glial processes and dendrites or cell bodies of neurons did not show HCN2 immunolabelling. The immunoreactive axon terminals were found in both non-glomerular and glomerular synaptic arrangements. In synaptic glomeruli, the immunoreactive axons always represented the central axon terminals. Immunoreactive axon terminals were packed with clear spheroid vesicles among which dense core vesicles were scattered, characteristic of peptidergic axon terminals.

Investigating the subcellular distribution of particles labelling HCN2, most immunogold particles were distributed in the axoplasm, but some of them were attached to the plasma membrane of axon terminals. The membrane-associated silver particles were found at the intracellular face of the plasma membrane, and were always distributed on extrasynaptic membrane, although some of them were located in close vicinity to synaptic appositions.

Colocalization of HCN2 ion channels with vesicular glutamate transporters and substance P

To investigate presynaptic properties, particularly possible neurotransmitters of HCN2-immunoreactive peptidergic nociceptive primary afferent terminals we used double-staining immunofluorescent protocols. In order to demonstrate glutamate content of HCN2-positive axon terminals, we studied their immunoreactivity for vesicular glutamate transporters (VGluT1-3) in the superficial spinal dorsal horn. Since substance P (SP) is also released as neurotransmitter by some groups of peptidergic nociceptive primary afferents, therefore colocalization of SP with HCN2 was also examined.

In agreement with previous results, we observed punctate terminal immunostaining for all three VGluTs in the spinal dorsal horn (Varoqui és mtsai., 2002). In laminae I-IIo of the dorsal horn, VGluT2 immunostaining was particularly dense, whereas VGluT3 labeling was much weaker, and VGluT1 immunoreactivity was very sparse and confined to deeper laminae of the dorsal horn. We found that VGluT-immunoreactive puncta were almost completely segregated from HCN2-containing terminals, and only 6% of the investigated HCN2-immunoreactive terminals colocalized with VGluT2.

In accordance with previous results, immunostaining for SP was very intense in the superficial spinal cord and confined to laminae I-IIo, similar to the distribution of axon terminals immunostained for HCN2. In addition, HCN2 and SP immunoreactivity showed a substantial colocalization (92%).

Postsynaptic targets of HCN2-immunoreactive primary afferents

It has been reported that peptidergic nociceptive primary afferents project to both excitatory and inhibitory interneurons in laminae I-II of the spinal dorsal horn (Hayes and Carlton, 1992, Wang et al., 2000). To identify postsynaptic targets of HCN2-positive peptidergic nociceptive primary afferents, we utilized double- and triple-staining immunofluorescent protocols using the following markers: NK1-R, calbindin D28k, MOR, GluR2 subunit of AMPA-type glutamate receptor, and GAD65-eGFP.

Confirming results of previous observations we found NK1-R-immunoreactive perikarya in laminae I and III-IV of the dorsal horn. Dendrites of NK1-R-positive neurons in lamina I branched within this lamina, while dendrites arising from NK1-R-immunoreactive perikarya in laminae III-IV extended towards the superficial dorsal horn and arborized there. HCN2-positive axon terminals formed close contacts with somata and dendrites of NK1-R-immunoreactive neurons in lamina I, and made serial appositions with NK1-R-positive dendrites arising from the deeper laminae.

In agreement with previous results, calbindin D28k- and GluR2-immunoreactive excitatory neurons were found in large numbers in laminae I and II, whereas neurons stained for MOR were confined to lamina II. The HCN2-immunoreactive terminals formed close appositions with somata and dendrites of CaB- and MOR-positive neurons, and somata of GluR2-immunoreactive neurons.

To investigate connections of HCN2-expressing nociceptive primary afferents with inhibitory spinal interneurons, we utilized spinal cord sections obtained from GAD65-eGFP transgenic mice. Although a number of GFP-labeled neurons were found in the superficial spinal dorsal horn, close appositions between these neurons and HCN2-positive terminals were only occasionally found. Most of the contacts were revealed on either the somata or primary dendrites of GFP-labeled inhibitory neurons.

Effect of HCN channel blocker (ZD7288) on synaptic transmission between C/Aδ primary afferents and neurons in laminae I-II of the spinal dorsal horn

ZD7288 is the most commonly used selective antagonist of HCN channels, which totally blockes I_h in a concentration of 10 μM (Harris and Constanti, 1995; Pal et al., 2003). In order to investigate the function of HCN2 ion channels expressed in nociceptive primary afferent terminals, we tested the efficacy of synaptic transmission between nociceptive primary afferents and secondary sensory neurons by applying ZD7288 onto spinal cord slices.

We made whole-cell patch-clamp recordings from 30 spinal neurons, of which 8 received monosynaptic inputs from C and/or Aδ primary afferents. The stimulated primary afferents were identified on the basis of their conduction velocity (C fiber: 0.3-0.8 m/s, Aδ fiber: 3.8-8 m/s). Of the eight investigated neurons, six received monosynaptic inputs from C fibers, one was monosynaptically excited by Aδ fibers, whereas one received monosynaptic inputs from both types of primary afferents.

All of the investigated neurons exhibited spontaneous excitatory postsynaptic potentials (sEPSP) with an amplitude that varied in the range 0.5-10.0 mV. The application of ZD7288 at a concentration of 10 μM caused a marked depression in the amplitude (0.5-4.0 mV) but only a slight reduction in the numbers of sEPSPs. After washing out the antagonist, the control values concerning both the frequency and amplitude recovered without any substantial delay.

Electrical stimulation of both C and Aδ fibers evoked excitatory postsynaptic potentials on the recorded neurons with varying reliability. The average failure rate was 34 ± 10% under control circumstances. By application of ZD7288, the reliability of synaptic transmission dropped and the number of trials that failed to evoke EPSPs on the recorded neurons increased. The failure rate increased only slightly at the beginning of ZD7288 application (46 ± 15.5%, $p = 0.21$), later it became significantly higher (74.7 ± 14.5%, $p = 0.0051$). The effect of ZD7288 was reversible and the failure rate returned to control values during the wash-out period (39.4 ± 9.8%).

Development of mechanical allodynia in CFA-induced inflammatory pain condition

Inflammation of the hind paw induced by subcutan injection of complete Freund's adjuvant (CFA) is the most commonly used model of inflammatory pain (Iadarola et al., 1988; Millan et al., 1988). During our experiments the development of mechanical allodynia was investigated in this pain model. Pressure in the range of 35-40 g exerted on the plantar surface of the hind paw of control animals evoked paw withdrawal responses, which was

considered to be the basic values of the responsiveness to mechanical stimuli. After CFA injection, the paw withdrawal threshold of the ipsilateral hind paw of CFA-injected animals gradually decreased, and dropped to 35% of basic values on the third experimental day (12.4 ± 0.9 g, $p = 0.004$). The paw withdrawal threshold values of the ipsilateral hind paw slightly decreased on the first and second experimental day in animals that received physiological saline injection, but the values returned to the basic level on the third experimental day. A great deal of experimental evidence show that the nociceptiv responsiveness peaks on the third day after CFA injection, therefore the behavioral tests were performed for 3 days after CFA injection.

Expression of c-Fos protein in the superficial spinal dorsal horn of rats in CFA-induced inflammatory pain condition

One of the members of „immediate early genes" (IEG) the c-Fos gene encodes a nuclear protein (c-Fos protein) with 62 kDa weight, and the immunoreactivity of this protein has been used as an indicator of neuronal activity for a long time (Hunt et al., 1987; Sagar et al., 1988). So we tested the activity of neurons in the spinal dorsal horn using c-Fos immunoreactivity.

Although mechanical allodynia was developed by the unilateral inflammation only on the side ipsilateral to CFA injection, the numbers of c-Fos-positive neurons were significantly elevated in both sides of the spinal dorsal horns. Quantitative analysis of c-Fos-immunoreactive neurons revealed that the numbers of c-Fos-positive cells substantially increased in the medial, intermediate and lateral regions and in the total length of medio-lateral extent of the superficial spinal dorsal horn ipsilateral to CFA injection. Interestingly, we also found a marked increase in the numbers of c-Fos-immunoreactive cells in the superficial spinal dorsal horn contralateral to the site of CFA injection, although this increment was much lower than in the ipsilateral dorsal horn.

Expression of HCN2 protein in the lumbar spinal dorsal horn in CFA-evoked inflammatory pain

To study how CFA-evoked inflammation of the right hind paw changes HCN2 protein expression in the superficial dorsal horn at the level of L4 spinal segment, we carried out the following quantitative analyses in control and CFA-injected animals: 1) we measured the cross-sectional surface area of the spinal dorsal horn immunoreactive for HCN2, 2) we determined the numbers of axon terminals immunoreactive for HCN2 and SP and the

colocalization between them, and 3) we measured the amount of HCN2 protein. Quantitative data obtained from CFA-treated animals were compared with similar data from control animals.

The crossectional surface areas of the superficial spinal dorsal horn immunoreactive for HCN2 were measured on light microscopic images following filtering the background staining out using Image J software. Comparing the sizes of crossectional surface areas immunoreactive for HCN2 in control and CFA-injected animals, we observed no substantial difference in the dorsal horn.

Quantitative analysis of the HCN2-immunoreactive axon terminals showed that the numbers of HCN2-positive terminals, with the exception of the lateral region of the dorsal horn, where no substantial difference was found, significantly increased in the dorsal horn ipsilateral to CFA injection. We also observed a slight elevation in the numbers of immunoreactive terminals in the medial and intermedier regions of the superficial spinal dorsal horn contralateral to the site of CFA injection. Quantitative analysis of axon terminals immunoreactive for SP showed that the numbers of immunoreactive terminals in the dorsal horn of CFA-injected animals ipsilateral to CFA injection were significantly higher than the control values in all regions of the dorsal horn, especially in the intermedier region.

Investigating the numbers of double-stained, HCN2- and SP-immunoreactive axon terminals, we observed that with the exception of the lateral region, the numbers of double-stained terminals substantially elevated in the ipsilateral superficial dorsal horn of CFA-treated animals. The numbers of the double-labeled terminals were also increased in the contralateral dorsal horn, but in a lesser amount, than in the ipsilateral superficial dorsal horn. The colocalization between the HCN2 and SP was almost the same in the ipsilateral (82-86%) and contralateral (83-88%) superficial dorsal horn of animals that received CFA injection, as well as in control animals (81-84%).

We investigated the quantity of HCN2 proteins with a Western blot analysis in total tissue extract and membrane fraction of the spinal dorsal horn of control and CFA-injected animals. After a densitometric measurement of the HCN2 immunoreactive Western blot bands, the measured values were normalized to the optical density of β-tubulin which was used as a loading control. We found that the relative quantity of HCN2 protein significantly elevated both in the total tissue extract and the membrane fraction of the ipsilateral dorsal horn of CFA-injected animals, compared to the values obtained from the control animals.

DISCUSSION

Expression of HCN2 ion channels in central axon terminals of peptidergic nociceptive primary afferents in the superficial spinal dorsal horn

HCN channel proteins and hyperpolarization-activated currents (Ih) have previously been identified in cell bodies and peripheral axons of dorsal root ganglion cells (Mayer and Westbrook, 1983; Grafe et al., 1997; Moosmang et al., 2001 However, nothing was known about the expression of HCN ion channel proteins on central axon terminals of primary sensory neurons in the spinal dorsal horn. So here we demonstrated for the first time, that HCN2 channel protein is also expressed in the central axon terminals of some primary sensory neurons. Although, in situ hybridization and immunocytochemical experiments confirmed that HCN2 is expressed by all DRG neurons in the rat (Chaplan et al., 2003), we found immunoreactive axon terminals in the superficial spinal dorsal horn exclusively in laminae I-IIo. The distribution of HCN2 immunoreactivity and its extensive colocalization with CGRP and segregation from IB4 binding indicate that the central transport and expression of HCN2 protein in spinal terminals is the strongest in peptidergic C and Aδ-type nociceptive primary afferents that terminate in laminae I-IIo of the dorsal horn. This highly restricted spinal occurrence of HCN2 immunoreactivity suggests that different types of DRG neurons transport HCN2 protein to their central axon terminals to a variable extent. It is likely that selective axon transport mechanism can be responsible for the central transport of HCN2 protein, and this mechanism allows some proteins travelling from the cell bodies exclusively to either the central or the peripheral axon terminals. Although the molecular mechanism of this phenomenon is unknown, the molecular apparatus governing the central transport of HCN2 proteins may exist only in C and Aδ primary sensory neurons.

Segregation between HCN2 and VGluT immunoreactivity in the spinal dorsal horn

Glutamate, as one of the most prevalent neurotransmitter, is known to be released from nociceptive C and Aδ primary afferents during sensory neurotransmission. Due to its ubiquitous appearance in neural proteins the morphological verification of the glutamate content of axon terminals is very difficult, therefore vesicular glutamate transporters that are responsible for the transport of glutamate into synaptic vesicles are nowadays used to identify glutamatergic neurons. Until now, three members of VGluTs (VGluT1-3) have been cloned, and their distribution has been described in the brain and spinal cord (Herzog et al., 2001, Takamori et al., 2002; Todd et al., 2003). These previous results are in agreement with our

observations concerning the spinal expression of VGluTs in laminae I-II of the dorsal horn. However, it was unexpected that we found a very low level of colocalization between VGluT2 and HCN2 immunoreactivity, while the VGluT1 and VGluT3 completely segregated from the HCN2 immunolabeling. The lack of colocalization between HCN2 and VGluTs suggests that besides the known three VGluTs, an additional vesicular glutamate transporter may be expressed in nociceptive primary afferents.

Colocalization between HCN2 and SP immunoreactivity in the superficial spinal dorsal horn

The intensive colocalization of HCN2 with CGRP and the almost complete segregation of HCN2 from IB4 binding in the superficial spinal dorsal horn indicates that HCN2 is primarily expressed in peptidergic nociceptive primary afferents. In addition to CGRP, various population of peptidergic nociceptive primary afferents may release other neuropeptides, including substance P (SP) that is known to play a crucial role in the transmission of nociceptive signals from primary afferents to secondary sensory neurons (Willis and Coggeshall, 2004). On the basis of the extensive colocalization between HCN2 and SP immunoreactivity we suggest that I_h mediated by HCN2 channels may modulate SP-mediated pain processing from nociceptive primary afferents to secondary sensory neurons at the level of the first synaptic contact along the pain pathways.

Postsynaptic targets of HCN2-immunoreactive axon terminals in the superficial spinal dorsal horn

It has been well established that nociceptive primary afferents terminate on both excitatory and inhibitory interneurons in the superficial spinal dorsal horn (Carlton and Hayes, 1990; Wang et al., 2000). The peptidergic nociceptive primary afferent terminals in non-glomerular synaptic arrangement have never been found to establish synaptic contacts with GABA-immunoreactive dendrites in the rat spinal dorsal horn. However, it has been reported that 28% of C1 glomerular terminals of peptidergic nociceptive terminals formed synaptic contacts with GABAergic dendrites (Bernardi et al., 1995). Since most axon terminals of HCN2-expressing peptidergic nociceptive primary afferents was found in non-glomerular arrangement and only a small proportion of them belonged to the C1 glomerular type, it appears to be understandable that we only occasionally found contacts between HCN2-positive terminals and GAD65-eGFP-expressing spinal neurons. Thus, it is likely that peptidergic nociceptive primary afferents expressing HCN2 channel protein establish synaptic

contacts preferentially with excitatory neurons and contact inhibitory spinal neurons only occasionally in the spinal dorsal horn of rodents.

It has been reported that SP-containing primary afferents establish asymmetric synaptic contacts with neurons expressing NK1-Rs and form non-synaptic contacts, close appositions with MOR-immunoreactive neurons in the superficial spinal dorsal horn of rats (Spike et al., 2002; Todd et al., 2002). Although we do not have any electron microscopic evidences, the high degree of colocalization between HCN2 and SP may suggest that HCN2-containing primary afferents also establish synaptic and non-synaptic contacts with neurons expressing NK1-Rs and MORs, respectively. It has previously been identified that NK1-R-positive neurons have been identified as projection neurons and MOR-immunoreactive neurons are generally regarded as a subpopulation of excitatory interneurons. Thus, we may postulate that presynaptic HCN2 channel mechanisms modulate the transmission of nociceptive signals from SP-containing primary afferents to both projection neurons and excitatory interneurons in the superficial spinal dorsal horn.

Effects of I_h mediated by HCN2 ion channels on synaptic transmission between C/Aδ primary afferents and neurons in the superficial spinal dorsal horn

A remarkable property of many excitatory synapses in the central nervous system is their ability to undergo activity-dependent, long-lasting increases in synaptic strength. This is referred to as long-term potentiation (LTP) in many brain regions or long-term facilitation (LTF) in the spinal dorsal horn. LTF of synaptic interaction between nociceptive primary afferents and secondary spinal interneurons is believed to play a substantial role in the development of central sensitization, a functional state of nociceptive neural circuits that leads to enhanced pain processing (Ji et al., 2003).

It has been reported that activation of HCN ion channels induces synaptic facilitation at the crayfish neuromuscular junction, and LTP of the presynaptic mossy fibers of the hippocampus is also influenced by the activation of I_h (Beaumont and Zucker, 2000; Mellor et al., 2002). These previous observations are confirmed by our electrophysiological results. Application of the selective antagonist (ZD7288) of HCN channels onto neurons that were recorded in spinal cord slices reduced the number of monosynaptic EPSPs, suggesting that I_h may increase the reliability of synaptic transmission from primary afferents to secondary sensory neurons and thus may play a role in the presynaptic modulation of pain transmission in the spinal dorsal horn.

The question is raised how might the activation of I_h increase the reliability of synaptic transmission and lead to long-term facilitation. One possible explanation is that activation of HCN channels by causing depolarization of the presynaptic terminal may evoke the opening of voltage-gated Ca^{2+} channels. Calcium entry into the terminal during repetitive stimulation activates calcium-calmodulin-sensitive adenylyl cyclase and thus c-AMP level is elevated that further increases I_h currents that result in an even stronger depolarization and in slowing calcium channel deactivation. So the activation of I_h for prolonged periods of time, due to the enhanced calcium influx, can facilitate neurotransmitter release.

Bilateral changes in the superficial spinal dorsal horn during Freund's adjuvant-induced (CFA) unilateral inflammation of the hind paw

One of the striking results in CFA-induced unilateral inflammation of the hind paw was that the inflammation evoked unilateral mechanical allodynia but bilateral neurochemical changes in the superficial spinal dorsal horn. In addition to the upsurge of HCN2 and SP immunoreactivity in the dorsal horn ipsilateral to CFA injection, we also found a slight elevation in the numbers of immunoreactive terminals in the contralateral dorsal horn. We found similar changes concerning the number of c-Fos-immunoreactive cells. Although the explanation of the contralateral spinal reactions to unilateral CFA injection is far from being clear, one may assume that multiple factors may contribute to the development of this phenomenon.

Since unilateral experimental interventions cause bilateral responses in the spinal dorsal horn, the comparison of data obtained from the ipsilateral and contralateral dorsal horn without using real control values obtained from naive animals may result in various misinterpretations of experimental results.

Presynaptic HCN2 channels on central terminals of nociceptive primary afferents in inflammatory pain

A significant body of molecular and pharmacological evidence confirms that Ih mediated by HCN channels play a significant role in spontaneous, ectopic activity of the injured primary afferent neurons, which leads finally to pain sensation (Chaplan et al., 2003; Luo et al., 2007). Recent results indicate that HCN2 ion channel mechanisms may have some role in the development of central sensitization of pain processing neural circuits in chronic inflammatory pain conditions. Cho et al. (2009) have recently demonstrated that levels of HCN proteins in the trigeminal ganglion are increased and the proportion of dura projecting

neurons that are immunoreactive for HCN2 more than doubles 3-4 days after an inflammation of the dura mater. It has also been reported that application of ZD7288, a specific HCN channel blocker, reversed thermal hypersensitivity and mechanical allodynia of the hind paw of rats in a dose dependent manner (Dunlop et al, 2009; Jiang et al., 2008). Comparing our results to previous observations, it appears to be likely that the over-expression of HCN2 channel proteins in the superficial spinal dorsal horn may provide at least a partial explanation for the analgesic effect of ZD7288 application.

The close collaboration between SP-mediated synaptic transmission and presynaptic HCN2 channel mechanisms is also reinforced by results received in CFA-induced inflammatory pain condition. The elevation of HCN2 immunoreactivity generated by CFA-evoked inflammation of the hind paw was paralleled with the increase of SP immunoreactivity. In addition, the co-localization between HCN2 and SP immunoreactivity was also remarkably high after CFA injection, like in the control animals, suggesting that central axon terminals of nociceptive primary afferents that increased their SP expression in response to CFA-evoked inflammation also increased their HCN2 expression. This remarkably strong coupling between SP and HCN2 expression indicates that HCN2 ion channel mechanisms may play a role in SP-mediated spinal pain processing not only in naive animals but also in chronic inflammatory pain.

SUMMARY

Hyperpolarization-activated currents (I_h) mediated by hyperpolarization-activated cyclic nucleotide-gated cation channels (HCN) play an important role in shaping up the electrical properties of neurons and fine-tuning of synaptic transmission. Utilizing immunocytochemical methods, here we demonstrated that – in addition to perikarya – HCN ion channel protein isoform 2 (HCN2) is also expressed by central axon terminals of some population of dorsal root ganglion (DRG) neurons in laminae I-IIo of the spinal dorsal horn of rats. With precise neurochemical identification of primary afferents terminating in laminae I-IIo, we demonstrated that HCN2 immunoreactivity is shown by peptidergic nociceptive primary afferent terminals. With further characterization, we also demonstrated that HCN2-positive axon terminals display also a strong immunoreactivity for substance P. Investigating the postsynaptic targets of HCN2-positive nociceptive primary afferents, it was revealed that these afferents primarily form close appositions with dendrites and perikarya of putative excitatory interneurons that are immunoreactive for neurokinin1 receptor, calbindin, GluR2 subunits of AMPA type glutamate receptors and μ-opioid receptor, but they sparsely come also into contact with GAD65-positive inhibitory interneurons. Our electrophysiological studies confirmed that HCN2 ion channels expressed by axon terminals of nociceptive primary afferents are functional and their activation increase the reliability of synaptic transmission from nociceptive primary afferents to secondary sensory neurons and thus may play an important role in the presynaptic modulation of nociceptive synaptic transmission.

In chronic inflammatory pain state evoked by intraplantar injection of complete Freund's adjuvant into the hindpaw of experimental animals, the number of peptidergic nociceptive primary afferent terminals immunoreactive for HCN2 channel protein was substantially increased. It was also found that in the majority of axon terminals, showing elevated HCN2 channel protein expression in chronic inflammation, substance-P immunoreactivity was also enhanced. This finding suggests that presynaptic mechanisms modulated by I_h currents may primarily play a role in synaptic mechanisms mediated by substance-P release.

With the description of spinal expression of HCN2 ion channels and their role in nociceptive transmission, our results contributed to a more exhaustive understanding of the morpho-functional properties of nociceptive neural circuits in the spinal dorsal horn and may initiate further studies that may lead to the development of analgesic drugs inhibiting HCN ion channel mechanisms.

PUBLICATION LIST

Publications on which the doctoral thesis is based

Antal, M., **Papp, I.**, Niyazi, B., Veress, G. & Vereb, Gy. 2004. Expression of hyperpolarization-activated and cyclic nucleotide-gated cation channel subunit 2 in axon terminals of peptidergic nociceptive primary sensory neurons in the superficial spinal dorsal horn of rats. *Eur. J. Neurosci*., 19, 1336-1342. **IF.: 3.820**

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Papp, I., Holló, K. & Antal, M. Plasticity of hyperpolarization-activated and cyclic nucleotide-gated cation channel subunit 2 expression in the spinal dorsal horn in inflammatory pain. *Eur. J. Neurosci*. (Submitted)

Number of independent citations: 15

Cumulative impact factor: 7.529

Conference abstracts related to the doctoral thesis

Papp, I., Veress, G., Niyazi, B., Vereb, Gy., Antal, M.: Expression of hyperpolarization-activated and cyclic nucleotide-gated cation channel subunit 2 in axon terminals of peptidergic nociceptive primary sensory neurons in the superficial spinal dorsal horn of rats. IBRO International Workshop, Budapest, 2004, *Clinical Neurosci*., 57. 1. 2004.

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