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Inhibition of peripheral anion exchanger 3 decreases formalin-induced pain

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ABSTRACT

We determined the role of chloride–bicarbonate anion exchanger 3 in formalin-induced acute and chronic rat nociception. Formalin (1%) produced acute (first phase) and tonic (second phase) nociceptive behaviors (flinching and licking/lifting) followed by long-lasting evoked secondary mechanical allodynia and hyperalgesia in both paws. Local peripheral pre-treatment with the chloride–bicarbonate anion exchanger inhibitors 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid and 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid prevented formalin-induced nociception mainly during phase 2. These drugs also prevented in a dose-dependent fashion long-lasting evoked secondary mechanical allodynia and hyperalgesia in both paws. Furthermore, post-treatment (on day 1 or 6) with 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid reversed established hypersensitivity. Anion exchanger 3 was expressed in dorsal root ganglion neurons and it co-localized with neuronal nuclei protein (NeuN), substance P and purinergic $P2\times3$ receptors. Furthermore, Western blot analysis revealed a band of about 85 kDa indicative of anion exchanger 3 protein expression in dorsal root ganglia of naïve rats, which was enhanced at 1 and 6 days after 1% formalin injection. On the other hand, this rise failed to occur during 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid exposure. These results suggest that anion exchanger 3 is present in dorsal root ganglia and participates in the development and maintenance of short and long-lasting formalin-induced nociception.

1. Introduction

Tissue injury produces a continuous barrage of sensory input to the spinal cord. Primary afferent depolarization is a presynaptic mechanism involved in the modulation of incoming afferent information. It occurs at GABAergic mechanosensitive and nociceptive primary afferents in intraspinal interneurons that are presynaptic to central terminals and depends on γ-amino butyric acid (GABA) release. GABA elicits primary afferent depolarization through voltage-sensitive Na^+ channel inactivation, which causes membrane voltage shunting and suppresses action potential propagation. Accordingly, Ca^{2+} influx and transmitter release decline (Álvarez-Leefmans et al., 1988; Rudomin and Schmidt, 1999). Under normal conditions, low-threshold afferent fibers evoke primary afferent depolarization and presynaptic inhibition of nociceptive afferents reducing pain sensation (Cervero and Laird, 1996; Cervero et al., 2003). However, following tissue injury, nociceptor sensitization occurs. Sensitization enhances primary afferent depolarization to a level that may reach a threshold for generating dorsal root reflexes (Lin et al., 1999; Rees et al., 1995; Willis, 1999). Primary afferent depolarization and dorsal root reflexes are regulated through modulation of the electroneutral Na^+–K^+–$2Cl^-$ 1 cotransporter, which drives intracellular chloride accumulation above its predicted electrochemical equilibrium value (Álvarez-Leefmans et al., 1988; Rocha-González et al., 2008).

It has been described that primary afferent depolarization, dorsal root reflexes and nociception that follow spinal $GABA_A$ receptor activation (Álvarez-Leefmans et al., 2001; Rocha-González et al., 2008), also occurs at the periphery (Carlton et al., 1999). These peripheral effects have been attributed to intracellular chloride accumulation by Na^+–K^+–$2Cl^-$ 1 cotransport activity (Granados-Soto et al., 2005; Rocha-González et al., 2008). However, GABA

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depolarizes immature neurons in Na^+–K^+–$2Cl^-$ 1 cotransporter knock-out mice suggesting the presence of other chloride loaders. Accordingly, in these mice disruption or blockade of the anion exchanger 3 further reduces GABA-induced depolarization (Nickell et al., 2007; Pfeffer et al., 2009). Furthermore, chloride accumulation still occurs after Na^+–K^+–$2Cl^-$ 1 cotransporter blockade and it is completely abated by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (Gonzalez-Islas et al., 2009; Sun et al., 2012). Thus, it is likely that other transporters can contribute to intracellular chloride accumulation above its predicted electrochemical value in primary afferent neurons.

Chloride–bicarbonate anion exchangers (1–3) belong to the SLC4 gene family (Alper et al., 2002). They regulate intracellular pH, cell volume, and contribute to membrane potential difference by eliciting reversible electroneutral intracellular bicarbonate efflux and extracellular chloride influx (Alper et al., 2002). Anion exchanger 3 is expressed in vascular smooth muscle, aorta, kidney, retina (Kobayashi et al., 1994), heart (Linn et al., 1995) and brain (Hentschke et al., 2006). Functional anion exchangers lead to chloride accumulation in chicken embryonic motoneurons, neuronal progenitors, olfactory neurons and dorsal root ganglion neurons (Gallagher et al., 1983; González-Islas et al., 2009; Nickell et al., 2007; Sun et al., 2012). Since anion exchanger 3 is mainly present in brain (Alper, 2009; Hentschke et al., 2006), we hypothesized that this isoform is also expressed in dorsal root ganglia and participates in formalin-induced pain in the rat.

2. Material and methods

2.1. Animals

Experiments were performed on adult female Wistar rats (body weight range, 180–220 g) of 8 to 10 weeks of age. Animals were obtained from our own breeding facilities and had free access to food and drinking water. Female rats were used because in previous experiments performed under the same conditions (Wistar rats, 1% formalin and weight range 180–200 g) we found no significant differences between males and females (unpublished data). Other authors have found differences, but only with other rat strains, animals of greater weight, or using different formalin concentrations (Aloisi et al., 1994; Gaumond et al., 2002). All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983) and EC Directive 86/609/EEC for animal experiments. In addition, the protocol was approved by our local Ethics Committee (Cinvestav, México City, Protocol 455-09). Efforts were made to minimize the number of animals used and their suffering. Rats were euthanized in a CO_2 chamber at the end of an experiment.

2.2. Formalin-induced acute nociception

Acute and tonic nociception was assessed using the 1% formalin test (Wheeler-Aceto and Cowan, 1991). Rats were placed in open observation chambers for 30 min to allow them to acclimate to their surroundings. They were gently restrained while the dorsum of the hind paw was injected with 50 μl of diluted formalin (1%) with a 30-ga needle. The animals were returned to the chambers and nociceptive behavior was then immediately observed. Mirrors were placed in each chamber to enable unhindered observation. Nociceptive behavior was quantified as the number of flinches of the injected paw during 1-min periods every 5 min for up to 60 min after injection (Rocha-González et al., 2005; Wheeler-Aceto and Cowan, 1991). Flinching was readily discriminated and was characterized as both rapid and brief withdrawal or as flexing of the injected paw. We decided to evaluate flinching because it is a simple and reliable parameter of pain behavior and one producing high scores (Wheeler-Aceto and Cowan, 1991). Formalin-induced flinching behavior was biphasic (Rocha-González et al., 2005; Wheeler-Aceto and Cowan, 1991). The initial acute phase (0–10 min) was followed by a relatively short quiescent period, which was then followed by a prolonged tonic response (10–60 min). Rats used in the acute study (1 h) were kept until day 6 after formalin injection and they were also used in the chronic study.

2.3. Formalin-induced long-lasting evoked nociception

The secondary mechanical allodynia and hyperalgesia model was developed by Fu et al. (2001) and Wiertelak et al. (1994) using the formalin test (Dubuisson and Dennis, 1977). We used an adaptation of the model published by Jolivalt et al., 2006. Briefly, rats were placed in testing cages with a wire mesh bottom and allowed to acclimate for 30 min. Then baseline responses to von Frey filaments were recorded immediately before and 6 days after 1% formalin injection. Two von Frey filaments (Stoelting Co, Wood Dale, IL, USA, bending forces of 10 mN [1 g] and 250 mN [26 g]) were applied 10 times in each testing set at the base of the third toe on the plantar surface of both paws. Under normal conditions, a force of 10 mN does not activate cutaneous nociceptors, and cause paw withdrawal in non-injected animals. Accordingly, the occurrence of responses in these rats indicates allodynia. On the other hand, a force of 250 mN or more is considered a noxious stimulus, and hyperalgesia occurs when there is an increased response to this stimulus. Allodynia and hyperalgesia were considered secondary as stimulation with the von Frey filaments were applied to sites different from the formalin injection.

2.4. Immunohistochemistry

Rats were sacrificed with CO_2 delivered via compressed gas cylinder and perfused intracardially with 200 ml of 0.1 M phosphate buffered saline (pH = 7.4 at 4 °C, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) followed by 250 ml of 4% *p*-formaldehyde/12% picric acid in 0.1 M phosphate buffered saline. The lumbar dorsal root ganglia (L4–L6) were removed following perfusion. Tissue was post-fixed for 2 h in the perfusion fixative and cryoprotected for 72 h in a saline solution containing 30% sucrose buffered with 0.1 M phosphate, at 4 °C, and then processed for immunohistochemistry. Frozen dorsal root ganglion sections (16 μm thick) were cut on a cryostat and thaw-mounted on gelatin-coated slides for processing. Preparations were allowed to dry at room temperature for 30 min, washed in 0.1 M phosphate buffered saline three times for 10 min each (3 × 10), blocked with 10% normal donkey serum (Cat # 017-000-121, Jackson Immuno Research, West Grove, PA) in phosphate buffered saline with 0.3% Triton-X 100 for 2 h and then incubated overnight with rabbit-anti anion exchanger 3 (1:200, Cat # HPA040315; Atlas Bioscience, Tucson, AZ) in 1% normal donkey serum and 0.1% Triton-X 100 in 0.1 M phosphate buffered saline at 4 °C. Double immunostaining of chloride–bicarbonate anion exchanger 3 with neuronal nuclei protein (NeuN), substance P and purinergic P2 × 3 receptor was used to identify the cell type that expresses chloride–bicarbonate anion exchanger 3 as NeuN is a neuronal marker (Mullen et al., 1992) while purinergic P2 × 3 receptors (Lucifora et al., 2006; Vulchanova et al., 1998) and substance P (Lawson et al., 1997; Nichols et al., 1999) are markers of non-peptidergic and peptidergic neurons, respectively. Additionally, omission of the primary antibody control was included. Double immunostaining was carried out using the following antibodies: NeuN (mouse; 1:200; Cat # MAB377; Millipore, Billerica, MA), substance P (guinea pig; 1:200; Cat # GP14110; Neuromics, Edina, MN), and purinergic

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