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Evidence for the participation of peripheral α_5 subunit-containing $GABA_A$ receptors in $GABA_A$ agonists-induced nociception in rats

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ABSTRACT

The activation of $GABA_A$ receptor by γ-amino butyric acid (GABA) in primary afferent fibers produces depolarization. In normal conditions this depolarization causes a reduction in the release of neurotransmitters. Therefore, this depolarization remains inhibitory. However, previous studies have suggested that in inflammatory pain, GABA shifts its signaling from inhibition to excitation by an increased GABA-induced depolarization. The contribution of peripheral α_5 subunit-containing $GABA_A$ receptors to the inflammatory pain is unknown. The purpose of this study was to investigate the possible pronociceptive role of peripheral α_5 subunit-containing $GABA_A$ receptors in the formalin test. Formalin (0.5%) injection into the dorsum of the right hind paw produced flinching behavior in rats. Ipsilateral local peripheral pre-treatment (−10 min) with exogenous GABA (0.003–0.03 μg/paw) or common $GABA_A$ receptor agonists muscimol (0.003–0.03 μg/paw), diazepam (0.017–0.056 μg/paw) or phenobarbital (1–100 μg/paw) significantly increased 0.5% formalin-induced nociceptive behavior. The pronociceptive effects of GABA (0.03 μg/paw), muscimol (0.03 μg/paw), diazepam (0.056 μg/paw) and phenobarbital (100 μg/paw) were prevented by either the $GABA_A$ receptor antagonist bicuculline (0.01-0.1 μg/paw) or selective α_5 subunit-containing $GABA_A$ receptor inverse agonist L-655,708 (0.017-0.17 μg/paw). The α_5 subunit-containing $GABA_A$ receptor protein was expressed in dorsal root ganglion (DRG) and dorsal spinal cord of naïve rats. The formalin injection did not modify α_5 subunit-containing $GABA_A$ receptor expression. Overall, these results suggest that peripheral α_5 subunit-containing $GABA_A$ receptors play a pronociceptive role in the rat formalin test.

1. Introduction

Activation of neuronal γ-amino butyric acid (GABA) receptors typically results in hyperpolarization, and thus GABA is considered as the main inhibitory neurotransmitter in the central nervous system. However, there is evidence that stimulation of $GABA_A$ receptors may also lead to primary afferent depolarization (PAD) (Rudomin and Schmidt, 1999; Kullmann et al., 2005). GABA-mediated PAD produces voltage-sensitive Na^+ channel inactivation, which, along with membrane voltage shunting, either suppresses or shortens action potential propagation or duration. Accordingly, Ca^{2+} influx and transmitter release decline (Álvarez-Leefmans et al., 1998; Rudomin and Schmidt, 1999; Price et al., 2009). Furthermore, PAD is accompanied by low-threshold outward K^+ efflux, which offsets this response (Gold et al., 1996). Under normal conditions, low-threshold afferent fibers evoke PAD and presynaptic inhibition of nociceptive afferents reduces pain

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sensation (Cervero and Laird, 1996; Cervero et al., 2003). However, PAD can not only cause presynaptic inhibition, but may under certain conditions also give rise to action potentials (Barron and Matthews, 1938; Willis, 1999). They occur when PAD reaches the threshold for generating spike activity. Then, these action potentials may propagate in an orthodromic and antidromic direction. The centripetally conducted action potentials excite the neurons normally driven by nociceptors and evoke pain and hyperalgesia. The centrifugally conducted action potentials release substances such as peptides in peripheral tissues (e.g., joints and skin), inducing neurogenic inflammation and hyperalgesia (Sluka et al., 1995; Lin et al., 1999; Willis, 1999; Zeilhofer et al., 2012a).

$GABA_A$ receptors are heteropentameric ligand-gated chloride channels, most of which are composed of α, β, and γ subunits (Olsen and Sieghart, 2008). To date, 19 subunits have been cloned including α (1–6), β (1–3), γ (1–3), δ, ε, θ, λ and ρ (1–3) (Farrant and Nusser, 2005). $GABA_A$ receptors are the main sites of action for a numerous class of drugs including anxiolytics, anticonvulsants, benzodiazepines, barbiturates and alcohol. α_5 Subunit-containing $GABA_A$ receptors can be localized in both synaptic and extrasynaptic sites (Serwanski et al., 2006; Hines et al., 2012). Extrasynaptic axonal $GABA_A$ receptors have a high sensitivity to GABA allowing ambient concentrations of GABA to modulate neuronal excitability in neurons of peripheral and central nervous system (Farrant and Nusser, 2005), and they are present in myelinated axons of peripheral nerves (Morris et al., 1983; Zeilhofer et al., 2012a). Previous reports have demonstrated that α_5 subunit-containing $GABA_A$ receptors are predominantly extra-synaptic (Farrant and Nusser, 2005; Delgado-Lezama et al., 2013; Loeza-Alcocer et al. 2013) and they co-localize with CGRP-positive primary afferent terminals (Zeilhofer et al., 2012b) suggesting their participation in nociception. Based on these considerations, in this study we investigated the possible pronociceptive participation of the α_5 subunit-containing $GABA_A$ receptors in formalin-induced nociception.

2. Material and methods

2.1. Animals

The experiments were carried out in female Wistar rats (body weight 180–200 g) of 8–10 weeks of age. Female rats were used based on the fact that previous experiments in our conditions (Wistar rats, formalin concentration 0.5% and weight range 180–220 g) have not shown significant differences between males and females (unpublished data). Other authors have found differences only with other rat strains, greater weight or different formalin concentrations (Gaumond et al., 2002). Animals were obtained from our own breeding facilities and had free access to food and drinking water before the experiments. All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983) and were approved by our local Ethics Committee. In addition, every effort was made to minimize pain and suffering in animals and the number of rats used was the minimal required to obtain significant statistical power.

2.2. Induction and measurement of nociceptive activity

Nociception was assessed with the formalin test (Dubuisson and Dennis, 1977; Wheeler-Aceto and Cowan, 1991). The rats were placed in open acrylic observation chambers for 30 min to allow them to acclimatize to their surroundings. Then, they were removed for formalin administration. Rats were gently retrained while the dorsum of the hind paw was injected with 50 μL of diluted 0.5% formalin with a 30-gauge needle. The rats were returned to the chambers and the nociceptive behavior was assessed immediately after formalin administration. Mirrors were placed in each chamber to enable unhindered observation. Nociceptive behavior was quantified as the numbers of flinches of the injected paw during 1-min periods every 5 min, up to 60 min after injection. Flinching was readily discriminated and was characterized as 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). It is known that formalin-induced nociceptive behavior occurs in two phases (Wheeler-Aceto and Cowan, 1991; Granados-Soto et al., 2010; Cervantes-Durán et al., 2012). The initial acute phase (0–10 min) was followed by a relatively short quiescent period, which was then followed by a prolonged tonic response (15–60 min). At the end of the experiment, the rats were sacrificed in a CO_2 chamber.

2.3. Western blot

Rats were sacrificed by decapitation. The lumbar segment of the spinal cord as well as ipsilateral and contralateral dorsal root ganglia (L4–L6) were excised, placed on ice-cold isotonic saline solution and cleaned from the surrounding tissue. The dorsal and ventral parts of the spinal cord were gently marked unilaterally by a scalpel incision to enable the ipsilateral (injured) and contralateral (uninjured) sides to be identified. Excised tissues were dropped into liquid nitrogen for 1 min and then stored in a freezer (−70 °C). Tissues were homogenized in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 5 mM EDTA, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A and 0.1% Triton X-100 during 30 min at 4 °C). After that, they were centrifuged and the supernatant fraction was used to measure protein concentration by the Bradford's method (Bio-Rad, Hercules, CA). Sixty micrograms of protein were resolved by denaturing by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (PVDF). The membranes were blocked with 5% non-fat milk in phosphate-buffered saline at pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) with Tween 0.05% and they were incubated with goat anti-α_5 subunit-containing $GABA_A$ receptors (1:500, Santa Cruz Biotechnology Inc, Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibody (1:2000, Invitrogen, Life Technologies, Carlsbad, CA) was applied for detecting the primary antibody. The signal of the α_5 subunit-containing $GABA_A$ receptors was detected using an enhanced chemiluminescence detection system according to the manufacturer´s instructions (Millipore, Billerica, MA). Blots were stripped and incubated with a monoclonal antibody directed against β-actin, which was used as an internal control to normalize α_5 subunit-containing $GABA_A$ receptors protein expression levels.

2.4. Drugs

GABA (γ-amino butyric acid), muscimol (5-(aminomethyl)-2,3-dihydro-1,2-oxazol-3-one), diazepam (7-chloro-1-methyl-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-2-one), phenobarbital (5-ethyl-5-phenyl-1,3-diazinane-2,4,6-trione), bicuculline ((6R)-6-[(5S)-6-methyl-7,8-dihydro-5H-[1,3]dioxolo[4,5-g]isoquinolin-5-yl]-6H-furo[4,3-g][1,3]benzodioxol-8-one), muscimol (3-hydroxy-5-aminomethyl-isoxazole) and L-655,708 (ethyl (S)-11,12,13,13a-tetrahydro-7-methoxy-9-oxo-9H-imidazo[1,5-a]pyrrolo[2,1-c][1,4]benzodiazepine-1-carboxylate) were purchased from Sigma-Aldrich (St. Louis, MO). All of them were diluted in 20% dimethyl sulfoxide (DMSO). Formalin was diluted in saline.