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THE CONTRIBUTION OF PERIPHERAL 5-HYDROXYTRYPTAMINE2A RECEPTOR TO CARRAGEENAN-EVOKED HYPERALGESIA, INFLAMMATION AND SPINAL FOS PROTEIN EXPRESSION IN THE RAT

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Abstract—The present study was conducted to test the hypothesis that the peripheral 5-hydroxytryptamine (5-HT)2A receptor is involved in inflammatory hyperalgesia and production of noxious stimulus-induced neuronal activity at the level of the spinal cord dorsal horn. Intraplantar (i.pl.) injection of carrageenan dramatically reduced paw withdrawal latency to noxious heat (47 °C) and caused paw swelling. Pretreatment with ketanserin, a selective antagonist of 5-HT2A receptor, in the hindpaw produced dose-dependent inhibition of the hyperalgesia (0.5, 3 and 5 μg; i.pl.) with full relief at 5 μg. The drug also moderately reduced carrageenan-induced paw swelling in a dose-dependent manner. Carrageenan induced conspicuous expression of c-*fos*-like immunoreactivity (FLI) in the spinal dorsal horn of segments L4-5. Ketanserin (5 μg) markedly reduced carrageenan-induced FLI in all laminae of the dorsal horn. However, blockade of peripheral 5-HT1A receptors by (*N*-2-[4-(2-methoxyphenyl-1-piperazinyl] ethyl]-*N*-2-pyridinylcyclohexanecarboxamide at maximally effective doses (30 and 100 μg; i.pl.) did not alter carrageenan-induced hyperalgesia, edema or expression of FLI. The present study provided evidence at cellular level that the peripheral 5-HT2A receptor is preferentially involved in the development of thermal hyperalgesia in the carrageenan model of inflammation.

Key words: serotonin, spinal dorsal horn, hyperalgesia, edema, c-Fos protein.

A variety of chemical mediators is released into the injury site during inflammation and injury (Bevan, 1999). Many of these mediators not only maintain activity of primary afferent nociceptors but also enhance nociceptor sensitivity, such that noxious stimuli causes increased pain perception (hyperalgesia) and innocuous stimuli induce pain sensation (allodynia). Serotonin (5-hydroxytryptamine or 5-HT) is one of these mediators (Vinegar et al., 1987; Foon et al., 1976). This chemical is released from platelets, mast cells and endothelial cells into a wound site after tissue injury and inflammation (Parada et al., 2001; Rowley, 1956). 5-HT itself may participate in the mediation of pain as application of 5-HT to the blister base or skin causes pain sensation in humans (Lindahl, 1961; Jensen et al., 1990a,b; Armstrong et al., 1953; Richardson et al., 1985; Orwin and Fozard, 1986). This agent increases nociceptor response to mechanical (Vinegar et al., 1989; Schmelz et al., 2003), thermal (Rang et al., 1991) and chemical (substance P, noradrenaline, prostaglandin E2, bradykinin, etc.; Hong and Abbott, 1994; Khalil and Helme, 1990) stimuli. The importance of peripheral 5-HT in the pathogenesis of pain has been demonstrated in several animal pain models, such as formalin (Abbott et al., 1997), carrageenan (Di Rosa et al., 1971) and complete Freund's adjuvant (CFA)-induced models of inflammation (Okamoto et al., 2002), and arthritis (Pertsch et al., 1993). It has been shown that 5-HT activates C-fiber afferents (Beck and Handwerker, 1974; Herbert and Schmidt, 1992; Grubb et al., 1988), increases the excitability of small-diameter neurons in the dorsal root ganglia (DRG; Cardenas et al., 2001) and releases calcitonin gene-related peptide (Tramontana et al., 1993). These characteristics may underlie the key role of 5-HT in pain associated with tissue injury and inflammation (Holsapple et al., 1980; Khalil and Helme, 1990).

5-HT interacts with multiple subtypes of 5-HT receptors in the periphery to produce nociception, among which the 5-HT2A receptor appears to be pivotal. This notion is based on the findings that 5-HT-induced hyperalgesia and enhancement of pain produced by noradrenaline and prostaglandin E2 are mimicked by 5-HT2A receptor agonists, but not by the agents acting at 5-HT1A or 5-HT3 receptors. Correspondingly, nociceptive responses induced by 5-HT are specifically abolished by 5-HT2A receptor antagonists while blockade of 5-HT1A and 5-HT3 receptors fails to attenuate the 5-HT-induced responses (Grubb et al., 1988; Abbott et al., 1996; Doi-Saika et al., 1997; Tokunaga et al., 1998). Ketanserin, a 5-HT2A receptor antagonist, also profoundly suppresses the 5-HT-induced plasma extravasation in the knee joint model of inflammation (Pierce et al., 1995). Furthermore, the role of 5-HT2A receptors in pain has been demonstrated in several widely used pain models, such as the formalin test (Abbott et al., 1997), CFA

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Abbreviations: CFA, complete Freund's adjuvant; DMSO, dimethylsulphoxide; DRG, dorsal root ganglia; FLI, c-*fos*-like immunoreactivity; i.pl., intraplantar or intraplantarly; *m*-CPG, 1-(*m*-chlorophenyl)-biguanide; PB, phosphate buffer; PBS, phosphate-buffered saline; PWL, paw withdrawal latency; WAY-100635, (*N*-2-[4-(2-methoxyphenyl-1-piperazinyl] ethyl]-*N*-2-pyridinylcyclohexanecarboxamide; 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamine)tetralin.

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model of inflammation (Okamoto et al., 2002) and arthritis (Pertsch et al., 1993). In addition, the 5-HT2A receptor is involved in the development of hyperalgesia and edema induced by adenosine (Sawynok et al., 1997).

Despite increasing evidence that peripheral 5-HT2A receptors are involved in the pathological process of nociception, the precise contribution of this receptor in nociceptive processing remains unclear. It has been demonstrated that activation of peripheral 5-HT2A receptor contributes to 5-HT-evoked activity of spinal dorsal horn neurons (Doi-Saika et al., 1997). However, these results do not necessarily indicate that 5-HT2A receptors mediate noxious stimulus-evoked neuronal activity at the spinal level in inflammatory pain states, which involves the release and interaction of a variety of inflammatory mediators. To assess the role of peripheral 5-HT2A receptors in the development of inflammatory pain and in the central processing of nociceptive response, we examined the effects of intraplantar (i.pl.) administration of the 5-HT2A receptor antagonist ketanserin on hyperalgesia, inflammation and expression of c-*fos*-like immunoreactivity (FLI) in the spinal cord dorsal horn in the carrageenan model of inflammation.

EXPERIMENTAL PROCEDURES

Experimental animals

Male Sprague–Dawley rats (Fuzhou Animal Center, Fuzhou, China), weighing 230–300 g, were used. The rats were maintained in a controlled environment with food and water *ad libitum*. All experiments were performed in a soundproof room from 9:00 to 17:00 h (the light cycle). Rats were acclimatized to the experimental room and habituated to handling and behavioral testing every day for 4–5 days. The behavioral studies were conducted using blind testing protocols and were repeated by different observers. Animals were used only once and were always carefully handled throughout the experiment to minimize behavioral stress and suffering. All procedures were approved by the Institutional Animal Care and Use Committee at the University and conducted according to the guidelines for the treatment of animals of the International Association for the Study of Pain (Zimmermann, 1983). Efforts were made to reduce the number of animals used.

Behavioral study of pain sensitivity

Rats received a s.c. injection of carrageenan (2% in saline, 100 μl) in the right hindpaw. Drugs or vehicle were administered i.pl. (50 μl) 5 min before carrageenan injection and testing was performed at 1, 2, 2.5 and 3 h. Hyperalgesic responses to heat were determined as described before (Wisden et al., 1990; Abbadie et al., 1994; Buritova and Besson, 2000). Briefly, rats were placed in a device that held the body without restraining the head or legs. Noxious thermal stimulation was carried out by immersing the hind paw up to the ankle joint into a slowly stirred water bath at 47 °C while gently holding the site proximal to the stimulated part with a hand. The time that elapsed before the rat withdrew its paw was recorded as the paw withdrawal latency (PWL). The water temperature was adjusted to 47 °C as this temperature produced an average baseline PWL of 7–8 s in naive rats. The PWL for any test time point was measured three times at 1.5–2 min intervals and the mean values were calculated. Baseline latency was measured 10 min before drug or vehicle administration and was determined by averaging five measurements.

Assessment of inflammation

The magnitude of inflammatory response to carrageenan was evaluated by measuring the thickness of the dorsal–ventral paw using a vernier micrometer (Nakayama et al., 2002). Studies of carrageenan-induced hyperalgesia, peripheral edema extent and spinal c-Fos neurons were performed in the same rat, allowing correlation of these parameters.

Immunohistochemistry for c-Fos protein

To analyze the induction of c-Fos immunoreactivity, rats were deeply anesthetized with sodium pentobarbital (65 mg/kg, i.p.; Shenggong Chemicals, Shanghai, China) 3 h after carrageenan injection with various pretreatment. Animals were perfused through the heart with 200 ml of phosphate-buffered saline (PBS; 0.05 M; pH 7.4), followed by 500 ml of fresh cold fixative (4% paraformaldehyde in 0.1 M phosphate buffer or PB; pH 7.4). The lumbar spinal cord (L4–L5) was removed from the vertebral canal and post-fixed in the same fixative at 4 °C for 24 h. The tissue was then cryoprotected in 30% sucrose PBS for at least 2 days at 4 °C and then kept in PBS at 4 °C until sectioning. Transverse sections were cut at 40 μm on a sliding microtome and collected at intervals of 120 μm in cold PBS. Sections were processed by a free-floating slice immunohistochemistry procedure. The sections were rinsed twice in PBS, incubated in 0.3% hydrogen peroxide for 30 min, and washed four times in PBS. After immersion in PBS containing 2% normal goat serum and 0.3% Triton X-100 for 30 min at room temperature, the sections were incubated for 20 h at 4 °C with rabbit anti-FLI polyclonal antibody (1:5000 in PBS containing 2% normal rabbit serum; catalog number SC-52; does not react with Fos B, Fra-1 and Fra-2; Santa Cruz Biotechnology, CA, USA). The tissue was then washed three times in PBS and transferred to a goat anti-rabbit biotinylated secondary IgG complex (1:200 in 10% goat serum in PBS; Vector, Burlingame, CA, USA) for 2 h at room temperature followed by exposing to avidin–biotin horseradish peroxidase complex (1:100; Vectastain ABC-Elite kit; Vector) for 1 h at room temperature. After the final wash with PBS, the chromogen was developed with 0.01% hydrogen peroxide and 0.05% diaminobenzidine. Tissue sections were thoroughly rinsed with PB, mounted from distilled water onto gelatin-coated slides, air dried, dehydrated in a series of graded alcohols, cleared in xylene, and coverslipped.

To verify the specificity of immunostaining, some sections from three rats were treated following the above protocol but incubated in 2% normal rabbit serum rather than in the primary antibody. In this case no immunolabeling was observed.

Data analysis

Sections were visually scanned and photographed using a bright-field microscope. The individual sections were printed and overlaid with an acetate sheet on which the distribution of c-*fos* immunoreactive neurons was plotted. For the quantification of FLI-labeled neurons, each section of the spinal dorsal horn was divided into three regions of interest according to the cytoarchitectonic organization of the spinal cord (Molander et al., 1984): superficial layer (laminae I–II), nucleus proprius (laminae III–IV) and deep dorsal horn (laminae V–VI). Six sections with highest density from each animal were selected for quantification of FLI. The terms Fos-LI and Fos-positive neurons are meant to be synonymous with Fos-positive neuronal nuclei. All FLI neurons in defined areas on the both sides were counted manually by an observer blinded to the treatment conditions. For each rat, the number of FLI neurons in each region was determined by averaging the counts made in the six sections and expressed as the mean±S.E.M. of these values in all the rats in that treatment