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CENTRAL SENSITIZATION OF NOCICEPTIVE NEURONS IN RAT MEDULLARY DORSAL HORN INVOLVES PURINERGIC P2X7 RECEPTORS

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Abstract—Central sensitization is a crucial process underlying the increased neuronal excitability of nociceptive pathways following peripheral tissue injury and inflammation. Our previous findings have suggested that extracellular adenosine 5'-triphosphate (ATP) molecules acting at purinergic receptors located on presynaptic terminals (e.g., P2X2/3, P2X3 subunits) and glial cells are involved in the glutamatergic-dependent central sensitization induced in medullary dorsal horn (MDH) nociceptive neurons by application to the tooth pulp of the inflammatory irritant mustard oil (MO). Since growing evidence indicates that activation of P2X7 receptors located on glia is involved in chronic inflammatory and neuropathic pain, the aim of the present study was to test *in vivo* for P2X7 receptor involvement in this acute inflammatory pain model. Experiments were carried out in anesthetized Sprague–Dawley male rats. Single unit recordings were made in MDH functionally identified nociceptive neurons for which mechanoreceptive field, mechanical activation threshold and responses to noxious stimuli were tested. We found that continuous intrathecal (i.t.) superfusion over MDH of the potent P2X7 receptor antagonists brilliant blue G and periodated oxidized ATP could each significantly attenuate the MO-induced MDH central sensitization. MDH central sensitization could also be produced by i.t. superfusion of ATP and even more effectively by the P2X7 receptor agonist benzoylbenzoyl ATP. Superfusion of the microglial blocker minocycline abolished the MO-induced MDH central sensitization, consistent with reports that dorsal horn P2X7 receptors are mostly expressed on microglia. In control experiments, superfusion over MDH of vehicle did not produce any significant changes. These novel findings suggest that activation of P2X7 receptors *in vivo* may be involved in the development of central sensitization in an acute inflammatory pain model.

Key words: ATP, benzoylbenzoyl ATP, brilliant blue G, minocycline, mustard oil, periodated oxidized ATP.

Central sensitization is a crucial process underlying the increased neuronal excitability of nociceptive pathways. It is reflected in increases in nociceptive neuronal spontaneous activity, mechanoreceptive field (RF) size and responses to noxious mechanical stimuli and a decrease in mechanical activation threshold following peripheral tissue injury and inflammation, and has been implicated in the development and maintenance of persistent pain (for review, see Dubner and Basbaum, 1994; Sessle, 2005; Woolf and Salter, 2006; Ren and Dubner, 2008; Chiang et al., 2011). We have developed an acute inflammatory pain model (Chiang et al., 1998) whereby a glutamatergic-dependent central sensitization (lasting for >40 min) can be induced in brainstem nociceptive neurons of trigeminal subnucleus caudalis (also termed the medullary dorsal horn, MDH) by application to a molar tooth pulp of the inflammatory irritant and small-fibre excitant mustard oil (MO), a transient receptor potential ankyrin 1 (TRPA1) agonist. In this acute pulpitis pain model, we have also recently provided evidence indicating that glial cells as well as extracellular adenosine 5'-triphosphate (ATP) molecules acting at several purinergic receptor subunits may be involved in MDH central sensitization (Chiang et al., 2005, 2007, 2008, 2010; Xie et al., 2007). Growing evidence indicates that extracellular ATP molecules and glia-neuron interactions are involved in mechanisms underlying the development of chronic inflammatory and neuropathic pain (for review, see Ji et al., 2009; McMahon and Malcongio, 2009; Milligan and Watkins, 2009; Chiang et al., 2011). There are seven P2X receptor subunits in the spinal cord (North, 2002; Burnstock, 2008) and numerous studies have shown the involvement in chronic inflammatory and neuropathic pain models of especially P2X7 receptors and P2X4 receptors which are primarily located in microglia (Chessell et al., 2005; Tsuda et al., 2009; for review, see Carroll et al., 2009; Inoue and Tsuda, 2009; Surprenant and North, 2009), but less clear is whether they are involved in acute inflammatory pain (Watkins et al., 2001; Hua et al., 2005; Ledeboer et al., 2005; Qin et al., 2006; Hughes et al., 2007).

In our previous studies using the acute pulpitis pain model, we have shown that the broad spectrum P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2,4-

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Abbreviations: ANOVA, analysis of variances; BBG, brilliant blue G; BzATP, 2′3′-o-(4-benzoylbenzoyl) adenosine 5′-triphosphate (ATP); DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; MDH, medullary dorsal horn; MO, mustard oil; NS, nociceptive-specific; oATP, periodate oxidized ATP; PBS, phosphate-buffered saline; PPADS, pyridoxal-phosphate-6-azophenyl-2,4-disulfonic acid; RF, mechanoreceptive field; RM ANOVA, repeated measures analysis of variances; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl)-ATP; TRPA1, transient receptor potential ankyrin 1.

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disulfonic acid (PPADS), a non-selective antagonist of purinergic receptors including P2Y2 and P2Y4 receptor subunits and most P2X receptor subunits (Ralevic and Burnstock, 1998), can completely block the MO-induced central sensitization in MDH. In contrast, the high affinity, selective P2X receptor antagonist 2′,3′-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP), which antagonizes P2X1, P2X3 and heteromeric P2X2/3 subunits with IC_{50} values of 6, 0.9 and 7 nM respectively and has a 1000-fold selectivity over P2X2, P2X4 and P2X7 subunits (Lewis et al., 1998; Virginio et al., 1998; North and Surprenant, 2000), only partially blocks the MDH central sensitization (Chiang et al., 2005). Furthermore, our recent studies have revealed that suramin alone only attenuates the MO-induced MDH central sensitization, whereas its co-application with the P2X7 receptor antagonist brilliant blue G (BBG) can completely block the central sensitization (Chiang et al., 2009). Suramin is a non-selective P2 purinergic receptor antagonist of all P2X receptor subunits as well as most P2Y receptor subunits, and also blocks calmodulin binding to recognition sites and G protein coupling to G protein-coupled receptors, but is less potent at P2X4 and P2X7 receptor subunits (Beindl et al., 1996; Klinger et al., 2001; Soto et al., 1996; for review, see North and Surprenant, 2000; von Kügelgen, 2006; Wu et al., 2004); and BBG is a potent P2X7 receptor antagonist (Chu et al., 2010; Fulgenzi et al., 2008; Gunosewoyo et al., 2007; Jarvis and Khakh, 2009). These findings raise the possibility that P2X7 receptors may also be involved in central sensitization in acute inflammatory pain states, and thus this electrophysiological study was designed to test *in vivo* for the P2X7 receptor involvement in this acute inflammatory pain model.

Data have been partly reported in abstract form (Li et al., 2008; Chiang et al., 2009, 2010; Itoh et al., 2009).

EXPERIMENTAL PROCEDURES

Detailed descriptions of most of the methods have been previously reported (Chiang et al., 1998, 2007; Xie et al., 2007), so the following focuses on methodologies that we have not previously described.

Animals

Male adult rats (275–420 g) were anesthetized by i.p. α-chloralose (50 mg/kg; Fisher Scientific Co., Toronto, ON, Canada)/urethane (1 g/kg; Sigma-Aldrich, Toronto, ON, Canada). The right maxillary first molar pulp was exposed and covered with a saline-soaked cotton pellet, and the dorsal surface of the caudal medulla was surgically exposed. The rat then received a continuous i.v. infusion of a mixture of 70% urethane solution (0.2 g/ml) and 30% pancuronium solution (2 mg/ml) at a rate of 0.3–0.4 ml/h and was artificially ventilated throughout the whole experimental period. Heart rate, percentage expired CO_2, and rectal temperature were continuously monitored and maintained at physiological levels of 333–430 beats/min, 3.5–4.5%, and 37–37.5 °C, respectively. All surgeries and procedures were approved by the University of Toronto Animal Care Committee in accordance with the regulations of the Ontario Animal Research Act (Canada).

Electrophysiological recordings and stimulation procedures

The activity of single neurons was recorded by a tungsten microelectrode (5–15 MΩ) in histologically verified sites in MDH (Lateral: 1.5–2.0 mm; Posterior: 1.5–2.0 mm referred to the obex). Responses to stimulation of the orofacial region were amplified and displayed on oscilloscopes and also led to an analogue-to-digital converter (CED 1401 plus; Cambridge Electronic Design, Cambridge, Cambridgeshire, UK) connected to a personal computer. Data were analyzed off-line with Spike 2 software (Cambridge Electronic Design, Cambridge, Cambridgeshire, UK).

Mechanical (brush, pressure and pinch) and noxious thermal (radiant heat, 51–53 °C) stimuli were applied to classify nociceptive-specific (NS) neurons in the deep laminae of MDH that specifically responded to strong mechanical (e.g., pressure or pinch) and/or thermal (radiant heat) stimuli but not to a brush stimulus applied to the neuronal RF (Chiang et al., 1998, 2005, 2007; Xie et al., 2007). Neurons classified as wide dynamic range or low threshold mechanoreceptive were not included in this study. The neuron's spontaneous activity was determined over an initial 1-min recording period, and its cutaneous orofacial RF was determined with non-serrated forceps. Its activation threshold to a mechanical stimulus applied to its RF was assessed by force-monitoring forceps or an electronic von Frey monofilament, and its responses to graded heavy pressure or pinch were determined (25 g, 50 g, 75 g, 100 g, and sometimes 200 g, applied in ascending order, each for 5 s at an interval of $>$45 s). The pressure- or pinch-evoked responses were assessed by summing the number of spikes evoked by each of these graded stimuli.

Superfusion of chemicals

Adenosine 5′-triphosphate (ATP, 30–300 μM), 2′3′-o-(4-benzoylbenzoyl) adenosine 5′-triphosphate (BzATP, 30–300 μM), brilliant blue G (BBG, 1 μM), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 20 μM), [4*S*-(4α,4aα,5aα,12aα)]-4,7-*Bis*(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,10,12,12a, tetrahydroxy-1,11-dioxo-2-naphthacenecarboxamide (minocycline, 500 μM), periodate oxidized ATP (oATP, 100 μM), and 8,8′-{Carbonyl*bis*[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)carbonylimino]}bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt (suramin, 50 μM) were purchased from Sigma-Aldrich Canada Ltd. All chemicals except DPCPX were freshly dissolved in phosphate-buffered saline (PBS, at pH 7.4; Sigma-Aldrich Ltd., Toronto, ON, Canada) before i.t. superfusion. DPCPX was first dissolved in DMSO at 1: 10 as stock solution, and then diluted with PBS to a 20 μM solution for use. All chemicals were taken from stock solutions and freshly dissolved in PBS (vehicle) and continuously superfused onto MDH by means of a micropump (Harvard apparatus Inc, South Natick, MA, USA) at a speed of 0.6 ml/h. The dose of each chemical was chosen based on available related literature and also on our preliminary experiments, in which this dose showed no significant disturbances on baseline values of the neuronal RF and response properties.

Experimental paradigm

To test the effect of P2X7 receptor antagonists, three experimental groups were tested with MO application to the tooth pulp: BBG group, oATP group, and PBS group (as control); each group was comprised of six rats. In all experiments, continuous i.t. superfusion over the exposed ipsilateral medulla of either oATP or BBG or PBS started soon after surgery and was maintained throughout the observation period. Following isolation and identification of a stable NS neuron in MDH 2 h after surgery, two assessments of neuronal properties were carried out for the identified NS neuron at an interval of 10 min and were taken as baseline measures. Thereafter, the saline-soaked cotton pellet which covered the