Involvement of ATP in Noxious Stimulus-Evoked Release of Glutamate in Rat Medullary Dorsal Horn: A Microdialysis Study

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Abstract

Our electrophysiological studies have shown that both purinergic and glutamatergic receptors are involved in central sensitization of nociceptive neurons in the medullary dorsal horn (MDH). Here we assessed the effects of intrathecal administration of apyrase (a nucleotide degrading enzyme of endogenous adenosine 5-triphosphate [ATP]), a combination of apyrase and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, an adenosine A1 receptor antagonist), or 2,3-O-2,4,6-trinitrophenyl-adenosine triphosphate (TNP-ATP, a P2X1, P2X3, P2X2/3 receptor antagonist) on the release of glutamate in the rat MDH evoked by application of mustard oil (MO) to the molar tooth pulp. *In vivo* microdialysis was used to dialyse the MDH every 5 min, and included 3 basal samples, 6 samples after drug treatment and 12 samples following application of MO. Tooth pulp application of MO induced a significant increase in glutamate release in the MDH. Superfusion of apyrase or TNP-ATP alone significantly reduced the MO-induced glutamate release in the MDH, as compared to vehicle. Furthermore, the suppressive effects of apyrase on glutamate release were reduced by combining it with DPCPX. This study demonstrates that application of an inflammatory irritant to the tooth pulp induces glutamate release in the rat MDH *in vivo* that may be reduced by processes involving endogenous ATP and adenosine.

Keywords

pain; excitatory amino acids; trigeminal dorsal horn; adenosine; tooth pulp inflammation; purinergic receptors

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6. Conflict of Interest

The authors declare they have no conflicts of interest

1. Introduction

Central sensitization, which is considered an important neuroplastic process underlying the development and maintenance of pain states, is a sustained hyperexcitability of dorsal horn nociceptive neurons that can be evoked by peripheral injury or inflammation (Chiang et al. 1998, 2005). We have previously shown that injection of the inflammatory irritant mustard oil (MO) into the rat molar tooth pulp or other orofacial tissues (e.g., temporomandibular joint [TMJ], masticatory muscle) induces central sensitization in the rat medullary dorsal horn [MDH] (also termed the trigeminal subnucleus caudalis) (Chiang et al. 1998, 2005). The MDH central sensitization can be blocked by application of glutamatergic antagonists to the MDH, consistent with findings that MO injection into the TMJ (Bereiter and Benetti 1996) induces glutamate release in the rat MDH.

We have also shown that MDH central sensitization can be modulated by agents acting at purinergic P2X receptors (Chiang et al. 2005; Hu et al. 2002), in part by regulating the presynaptic release of glutamate (Jennings et al. 2006). These purinergic agents include apyrase (an ATP degrading enzyme), and 2,3-O-2,4,6-trinitrophenyl-ATP (TNP-ATP), a selective antagonist to P2X1, P2X3 and P2X2/3. These findings are consistent with studies using knockout mice (Cockayne et al. 2005), antisense oligonucleotide technologies (Oliveira et al. 2009), selective antagonists (Oliveira et al. 2009) and agonists (Bland-Ward and Humphrey, 2000) that have indicated that ATP is co-released with neuro- and gliotransmitters such as glutamate, and contributes to the development of inflammatory hyperalgesia through activation of P2X receptors.

The current study has utilized *in vivo* microdialysis to determine if MO application to the rat molar pulp induces release of glutamate in MDH, and also to examine purinergic processing in MDH by determining the effects of intrathecal (i.t.) application of apyrase alone or in combination with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; adenosine A1 receptor antagonist), or TNP-ATP on MO-induced glutamate release in MDH. DPCPX with apyrase was used to block the effect of endogenous adenosine which is produced by the metabolism of ATP by ectonucleotidases and produces inhibitory neuromodulation by the activation of adenosine A1 receptors.

2. Materials and Methods

2.1 Animal preparation

The experiments were performed in anesthetized male Sprague-Dawley rats (300–370 g, Charles River), housed in groups of 3–4, on a 12:12 h light dark cycle; food and water were available *ad libitum*. The methods used for animal preparation were similar to those previously described in detail (Chiang et al. 1998, 2005; Hu et al. 2002). In brief, adult rats were anesthetized with urethane and α -chloralose (1 g/kg and 50 mg/kg, respectively, i.p.), and immobilized after anesthesia by intravenous (i.v.) pancuronium 1 mg/ml (initial dose, 0.3 ml), followed by a continuous intravenous infusion of a mixture of 70% urethane (0.2 g/ml) and 30% pancuronium (1 mg/ml) at a rate of 0.4–0.5 ml/h. They were artificially ventilated throughout the whole experimental period. Heart rate, percentage expired CO₂,

and rectal temperature were constantly monitored and maintained at physiological levels of 333–430 beats/min, 3.5–4.2%, and 37–37.5°C, respectively.

To expose the pulp of the right maxillary first molar, an occlusal cavity was prepared with a dental drill (Rotex 780) and immediately filled with a small piece of cotton pellet soaked with normal saline which subsequently was carefully removed from the molar pulp cavity and replaced with a segment of dental paper soaked with 0.2 µl of a 0.94 µg/µl solution of MO (allyl isothiocyanate, 95%; Aldrich Chemical, USA) to the pulp, at the appropriate time during dialysate sample collection as described below. The cavity was promptly sealed with Cavit (ESPE, 3M, Germany) to prevent MO leaking out of the tooth, and to ensure the chemical's sustained action on pulp afferents. 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).

2.2 Placement of medullary microdialysis fibre

After exposing the pulp, the head of the rat was fixed in a stereotaxic apparatus and the neck muscles dissected and the dura mater opened to expose the obex, which served as a reference point for the lateral and anterior-posterior coordinates of an *in vivo* rat microdialysis probe (1 mm active length, 0.3 mm diameter, crescent type; Bio-analytic Systems, West Lafayette, IN), which was inserted and held by a stereotaxic needle holder in a perpendicular direction into the right MDH (L:1.4 mm; P:1.4 mm relative to obex). Artificial cerebrospinal fluid (CSF, see below) was infused through the probe fibre at a flow rate of 2 µl/min, and phosphate-buffered saline (PBS) was superfused (i.t.) over the exposed MDH at a rate of 0.6 µl/hr. The artificial CSF contained 154.7 mM Na⁺, 2.9 mM K⁺, 1.1 mM Ca²⁺, 0.82 mM Mg²⁺ and 132.49 mM Cl⁻, and was bubbled with 95% carbon dioxide and 5% oxygen at the start of the experiment in order to adjust pH to 7.4.

2.3 Microdialysis sample collection

After a 1 h stabilization period, microdialysis samples were collected in the anesthetized rats (n=6, each group) every 5 min at a flow rate of 2 µl/min; these included 3 basal samples, 6 samples after treatment with either continuous 90-min intrathecal medullary superfusion (i.t.) of apyrase alone (30 units/ml), or a combination of apyrase (30 units/ml) with DPCPX (20 µM), or TNP-ATP alone (10 µM) or vehicle (PBS, as control), and 12 samples following application of MO to the exposed molar pulp. The drug doses were chosen because these doses have been documented to be effective in blocking MO-induced MDH central sensitization (Chiang et al. 2005). Dialysis was performed using an infusion pump (BioAnalytical Systems, West Lafayette, IN), and samples were sorted in a fraction collector kept at 4°C. Samples were stored at -80°C until assayed by high performance liquid chromatography (HPLC) technique. At the end of the sample collection, the anesthetized rats were quickly perfused following a standard fixation protocol for subsequent histological examination to confirm the site of the probe fibre in the MDH (Chiang et al. 1998).

2.4 Estimation of glutamate concentration by HPLC assay

Glutamate content in each sample was determined using gradient, reverse-phase HPLC with fluorescent detection as we have performed previously (Coderre et al., 2005). In brief, pre-

column derivatization of glutamate in samples was carried out in the autoinjector of the HPLC system (Waters Alliance 2690, Waters Corp., Milford, MA). Samples of 10 μ L microdialysis perfusate were incubated with 10 μ L of derivatizing reagent consisting of o-phthalaldehyde (OPA-incomplete reagent) and 2 mercaptoethanol 0.4% (v/v) (both Sigma, St. Louis, MO, USA) for 1.5 min and then injected into the HPLC system. The system was fitted with a C18 ODS Supelco column (15 cm $\times$ 4.6 mm, 5 μ m particle size, ODS Supelco, Bellefonte, PA). The derivatized samples were eluted with a mobile phase at a flow rate of 1 ml/min. The mobile phase contained 70 mM sodium acetate (pH 6.95), 1.5% of tetrahydrofuran, and 11% methanol. An isocratic elution was maintained for 8.5 min to obtain a clear separation of glutamate. The temperatures of the column heater and sample chamber were kept at 37°C and 5°C, respectively. The eluted derivatives were assayed with a fluorescent detector (Waters 474, Waters Corp., Milford, MA) at excitation and emission wavelengths of 330 and 450 nm, respectively. Peak area was used to calculate the concentration of each analyte, and quantification of glutamate was determined from a standard curve derived using external standards. The sensitivity of the assay for glutamate was 1 ng/ml in the sample. The accuracy and precision of the estimation method was acceptable as the inter-day and intra-day assay coefficients were 5.42% and 4.31%, respectively. The recovery of glutamate from the probe was determined in an *in vitro* trial as 36%.

2.5 Statistical analysis

The baseline glutamate concentrations were normalized. All values, post-drug and post-MO application to the tooth pulp, were expressed as mean $\pm$ SEM of percentage change from baseline. Data from the test groups, including apyrase alone, a combination of apyrase and DPCPX, or TNP-ATP alone were compared to those from the PBS control group by two-way repeated measures (RM) ANOVA followed by Fisher's least squared difference post-hoc comparisons. One-way RM ANOVA was used for post-MO values compared with baseline values in each group.

3. Results

3.1 Increase in glutamate release in MDH induced by MO application to tooth pulp

Compared with baseline and the post-drug period, extracellular glutamate release in the MDH was increased significantly during the first 10 min ($\dagger p < 0.05$ and $\dagger\dagger p < 0.01$, RM ANOVA, $n=6$; Fig. 1) following MO application and then returned to baseline level, whereas treatment with PBS itself (starting 30 min prior to MO application) did not significantly affect baseline values.

3.2 Effects of superfusion of apyrase on MO-induced glutamate release in MDH

Continuous superfusion of apyrase lowered glutamate levels, 10 to 30 min after apyrase administration, and significantly attenuated the subsequent MO-induced glutamate release as compared to PBS-treated control rats ($n=6$ per group, * $p < 0.05$ ** $P < 0.01$, Fig. 1A).

3.3 Effects of superfusion of apyrase and DPCPX combination on MO-induced glutamate release in MDH

Compared with PBS treatment, the continuous superfusion of the combination of DPCPX and apyrase attenuated the MO-induced glutamate release, but unlike apyrase alone, the superfusion of DPCPX and apyrase did not attenuate the baseline glutamate level in the post-drug period (n=6 per group, * $p < 0.05$, ** $p < 0.001$, Fig. 1B).

3.4 Effects of superfusion of TNP-ATP on MO-induced glutamate release in MDH

Compared to vehicle treatment, continuous superfusion of TNP-ATP significantly reduced the MO-induced glutamate release, as well as the glutamate baseline level during the post-drug period (Fig. 1C) (n=6 per group, * $p < 0.05$, ** $p < 0.001$, Fig. 1C).

4. Discussion

The present *in vivo* microdialysis study has shown that i.t. superfusion of apyrase alone, or a combination of DPCPX with apyrase, or TNP-ATP alone significantly reduced the extracellular release of glutamate in the MDH induced by application of the inflammatory irritant MO to the rat molar pulp. The findings thus suggest that ATP and purinergic receptors may be crucially involved in the MDH glutamate release induced in acute inflammatory pain conditions. The findings also are consistent with our published findings on the suppressive effects of these agents on MDH central sensitization induced by MO application to the rat molar pulp (Chiang et al. 2005; Hu et al. 2002). The pattern of response to MO application was characterized by a rapid increase in the glutamate concentrations in the dialysate samples within the first 10 minutes, followed by a slow return to baseline levels over the subsequent 10–40 min period, consistent with the time course of MDH central sensitization in our acute tooth pulp inflammatory model (Chiang et al. 1998, 2005; Hu et al. 2002). Similarly, glutamate levels in the spinal dorsal horn increased when formalin, a nociceptive stimulus, was applied to the hind paw in the rat (Coderre et al. 2005).

In the present study, basal (i.e., pre-MO) glutamate levels, as well as MO-induced glutamate release, in the MDH were suppressed by i.t. superfusion of either apyrase or TNP-ATP, while only the MO-induced increases in glutamate release were reduced by apyrase + DPCPX. Apyrase is an ATP-diphosphohydrolase which metabolizes extracellular ATP to form adenosine, which is a potent presynaptic and postsynaptic inhibitor. DPCPX is an inhibitor of the A1 adenosine receptor, and TNP-ATP is a selective antagonist of P2X1, 3 and 2/3 receptors. There is evidence that ATP acts presynaptically on P2X3 receptors to regulate glutamate release onto MDH neurons (Chiang et al. 2005; Hu et al. 2006). Thus, our findings of suppression of MO-evoked glutamate release by apyrase or TNP-ATP could be explained by these two purinergic modulators blocking presynaptic P2X3 receptors causing a reduced presynaptic release of glutamate (Jennings et al. 2006). Basal (pre-MO) glutamate release may also depend on a tonic activation of P2X3 receptors by endogenous ATP due to nociceptive inputs evoked by the surgery required for insertion of the microdialysis probe. Our results suggest that the strong effects of apyrase on basal glutamate release also depend on the effects of adenosine at A1 receptors, since the adenosine A1 receptor antagonist DPCPX restricted the effects of apyrase to the period during MO-

induced increased glutamate release. Commonly, ATP is rapidly converted into extracellular adenosine that has been shown to reduce glutamatergic neurotransmission through its actions at A1 receptors (Choi et al. 2001). These various findings collectively suggest that A1 as well as P2X receptors may be involved in modulating evoked glutamate release in the MDH, with A1 receptors critical to the effects of apyrase on basal post-surgical release of glutamate, and P2X receptors critical to the effects of apyrase on MO-evoked glutamate release. However, TNP-ATP produced reductions in basal glutamate release that were as effective, if not more effective, than apyrase. We expect these pronounced effects of TNP-ATP are because the agent antagonises the excitatory effects of ATP at P2X3 receptors, but does not affect the established inhibitory effects of ATP at P2Y receptors (Gerevich & Illes, 2004), or potential further inhibitory effects of adenosine acting at A1 receptors.

We have previously shown the involvement of P2X3 receptors as well as glutamatergic receptors in the MO-induced central sensitization in MDH nociceptive neurons (Chiang et al. 1998, 2005; Hu et al. 2002). Further support comes from other observations: 1) Endogenous ATP, acting at P2X3 and P2X2/3 receptors, contributes to the development of inflammatory hyperalgesia in the TMJ (Teixeira et al. 2010) and cutaneous tissues (Oliveira et al. 2009), as well as tooth pulp (Chiang et al. 2005; Hu et al. 2002); 2) Allodynia evoked in rats after spinal nerve ligation can also be attenuated by treatment with TNP-ATP (Tsuda et al. 2003); 3) Intraperitoneal administration of TNP-ATP blocks visceral nociception in the abdominal constriction assay (Honore et al. 2002); and 4) Intrathecal administration of TNP-ATP attenuates hyperalgesia in mice induced by the P2X3 agonist $\alpha\beta$ -meATP, as well as the pronociceptive effect of hind paw formalin and capsaicin injections (Tsuda et al. 1999). In addition, ionotropic purinergic P2X3 receptors are expressed in nerve fibers of the dental pulp (Jiang and Gu, 2002), consistent with a role for ATP signaling in dental nociception.

Glia also play an important role in nociceptive mechanisms, such as central sensitization, and involve purinergic processes. Microglia for example express both ionotropic (P2X) and metabotropic (P2Y) purinergic receptors, and application of ATP to spinal cord slices triggers the activation of spinal microglia (Tsuda et al. 2003). And astroglia, by means of the astroglial glutamate-glutamine shuttle and purinergic receptors, can also influence glutamatergic neurotransmission in the MDH. Thus, it is possible that some of the effects described in this study may also be related to interactions with glial purinergic and glutamatergic receptor mechanisms.

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References

- Bereiter DA, Benetti AP. Excitatory amino acid release within spinal trigeminal nucleus after mustard oil injection into the temporomandibular joint region of the rat. *Pain*. 1996; 67:451–459. [PubMed: 8951941]
- Bland-Ward PA, Humphrey PP. P2X receptors mediate ATP-induced primary nociceptive neurone activation. *J Auton Nerv Syst*. 2000; 81:146–151. [PubMed: 10869713]

- Chiang CY, Park SJ, Kwan CL, Hu JW, Sessle BJ. NMDA receptor mechanisms contribute to neuroplasticity induced in caudalis nociceptive neurons by tooth pulp stimulation. *J Neurophysiol.* 1998; 80:2621–2631. [PubMed: 9819268]
- Chiang CY, Zhang S, Xie YF, Hu JW, Dostrovsky JO, Salter MW, Sessle BJ. Endogenous ATP involvement in mustard-oil-induced central sensitization in trigeminal subnucleus caudalis (medullary dorsal horn). *J Neurophysiol.* 2005; 94:1751–1760. [PubMed: 15901761]
- Choi IS, Cho JH, Jang IS. A1 receptors inhibit glutamate release in rat medullary dorsal horn neurons. *Neuroreport.* 2001; 22:711–715.
- Cockayne DA, Dunn PM, Zhong Y, Rong W, Hamilton SG, Knight GE, Ruan HZ, Ma B, Yip P, Nunn P, McMahon SB, Burnstock G, Ford AP. P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP. *J Physiol.* 2005; 567:621–639. [PubMed: 15961431]
- Coderre TJ, Kumar N, Celeste D, Lefebvre A, Yu JSC. Evidence that gabapentin reduces neuropathic pain by inhibiting the spinal release of glutamate. *J Neurochem.* 2005; 94:1131–1139. [PubMed: 16092950]
- Gerevich Z, Illes P. P2Y receptors and pain transmission. *Purinergic Signal.* 2004; 1:3–10. [PubMed: 18404394]
- Honore P, Mikusa J, Bianchi B, McDonald H, Cartmell J, Faltynek C, Jarvis MF. TNP-ATP, a potent P2X3 receptor antagonist, blocks acetic acid-induced abdominal constriction in mice, comparison with reference analgesics. *Pain.* 2002; 96:99–105. [PubMed: 11932066]
- Hu B, Chiang CY, Hu JW, Dostrovsky JO, Sessle BJ. P2X receptors in trigeminal subnucleus caudalis modulate central sensitization in trigeminal subnucleus oralis. *J Neurophysiol.* 2002; 88:1614–1624. [PubMed: 12364492]
- Jennings EA, Christie MJ, Sessle BJ. ATP potentiates neurotransmission in the rat trigeminal subnucleus caudalis. *NeuroReport.* 2006; 17:1507–1510. [PubMed: 16957598]
- Jiang J, Gu J. Expression of adenosine triphosphate P2X3 receptors in rat molar pulp and trigeminal ganglia. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2002; 94:622–626. [PubMed: 12424458]
- Oliveira MC, Pelegrini-da-Silva A, Tambeli CH, Parada CA. Peripheral mechanisms underlying the essential role of P2X3, 2/3 receptors in the development of inflammatory hyperalgesia. *Pain.* 2009; 141:127–134. [PubMed: 19081189]
- Teixeira JM, Oliveira MC, Nociti FH Jr, Clemente-Napimoga JT, Pelegrini-da-Silva A, Parada CA, Tambeli CH. Involvement of temporomandibular joint P2X3 and P2X2/3 receptors in carrageenan-induced inflammatory hyperalgesia in rats. *Eur J Pharmacol.* 2010; 645:79–85. [PubMed: 20558155]
- Tsuda M, Ueno S, Inoue K. Evidence for the involvement of spinal endogenous ATP and P2X receptors in nociceptive responses caused by formalin and capsaicin in mice. *Br J Pharmacol.* 1999; 128:1497–504. [PubMed: 10602329]
- Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K. P2X4 receptors induced in spinal microglia gate tactile allodynia after injury. *Nature.* 2003; 424:729–730. [PubMed: 12917663]

Highlights

MO application to the molar tooth pulp induces glutamate release in MDH. Apyrase or TNP-ATP significantly reduced the MO-induced glutamate release in the MDH, and the apyrase effects were reduced by DPCPX.

Fig. 1.

Effect of A) apyrase alone, B) DPCPX and apyrase, or C) TNP-ATP pretreatment on glutamate release from medullary dorsal horn (MDH) induced by mustard oil (MO) application to the molar tooth pulp in anesthetized rats ($n=6$ per group). Each graph (A, B and C) shows basal, post-drug and post-MO glutamate levels for groups of rats treated with the 3 test drugs or PBS (as vehicle control). The first arrow indicates the start of intrathecal (i.t.) medullary superfusion of test drugs, apyrase (30 units/ml), a combination of apyrase (30 units/ml) and the adenosine A1 antagonist DPCPX 20 μ M), TNP-ATP (10 μ M), or vehicle (PBS, 0.6 μ l/hour) at -30 min, and the second arrow indicates MO application to the tooth pulp at 0 min. In A-C, RM-ANOVA revealed a significant main effect of time, $F(3, 20) = 12.82$, $p < 0.0001$, drug $F(20, 400) = 2.19$, $p = 0.0024$, and a drug X time interaction, $F(60, 400) = 1.72$, $p = 0.0014$. Post-hoc comparisons (Fisher's test) indicated that MO application to the pulp significantly increased glutamate concentration at 5 and 10 min as

compared to baseline ($\dagger p < 0.05$ and $\dagger\dagger p < 0.01$). However, pretreatment with all test drugs attenuated the MO-induced glutamate release compared to the PBS controls ($*p < 0.05$, $**p < 0.01$, A–C). Apyrase significantly reduced glutamate levels at one time point during the pretreatment period (at -10 min pre-MO), and also reduced MO-evoked glutamate release between 5 and 40 min post-MO, as compared to PBS control treatment ($*p < 0.05$, $**p < 0.01$). A combination of apyrase and DPCPX significantly reduced the MO-induced glutamate levels (at 5–15 and 30 min post-MO) as compared to PBS control treatment, but did not affect glutamate levels during the pretreatment period (B). TNP-ATP significantly reduced the glutamate levels both during the entire pretreatment and post-MO periods (C).