Endogenous ATP Involvement in Mustard-Oil-Induced Central Sensitization in Trigeminal Subnucleus Caudalis (Medullary Dorsal Horn)

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Chiang, C. Y., S. Zhang, Y. F. Xie, J. W. Hu, J. O. Dostrovsky, M. W. Salter, and B. J. Sessle. Endogenous ATP involvement in mustard-oil-induced central sensitization in trigeminal subnucleus caudalis (medullary dorsal horn). J Neurophysiol 94: 1751–1760, 2005. First published May 18, 2005; doi:10.1152/jn.00223.2005. Central sensitization represents a sustained hypersensitive state of dorsal horn nociceptive neurons that can be evoked by peripheral inflammation or injury to nerves and tissues. It reflects neuroplastic changes such as increases in neuronal spontaneous activity, receptive field size, and responses to suprathreshold stimuli and a decrease in activation threshold. We recently demonstrated that purinergic receptor mechanisms in trigeminal subnucleus caudalis (Vc; medullary dorsal horn) are also involved in the initiation and maintenance of central sensitization in brain stem nociceptive neurons of trigeminal subnucleus orals. The aim of the present study was to investigate whether endogenous ATP is involved in the development of central sensitization in Vc itself. The experiments were carried out on urethane-α-chloralose anesthetized and immobilized rats. Single neurons were recorded and identified as nociceptive-specific (NS) in the deep laminae of Vc. During continuous saline superfusion (0.6 ml/h) over the caudal medulla, Vc neuronal central sensitization was readily induced by mustard oil application to the tooth pulp. However, this mustard-oil-induced central sensitization could be completely blocked by continuous intrathecal superfusion of the wide-spectrum P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2, 4-disulphonic acid tetra-sodium (33–100 μM) and by apyrase (an ectonucleotidase enzyme, 30 units/ml). Superfusion of the selective P2X1, P2X3, and P2X2/3 receptor antagonist 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (6–638 μM) partially blocked the Vc central sensitization. The two P2X receptor antagonists did not significantly affect the baseline nociceptive properties of the Vc neurons. These findings implicate endogenous ATP as an important mediator contributing to the development of central sensitization in nociceptive neurons of the deep laminae of the dorsal horn.

INTRODUCTION

Central sensitization represents a sustained hypersensitive state of dorsal horn nociceptive neurons that can be evoked by peripheral inflammation or injury to nerves and tissues. Although N-methyl-D-aspartate (NMDA) and non-NMDA receptors are thought to be fundamental mechanisms underlying central sensitization (Ren and Dubner 1999; South et al. 2003; Woolf and Thompson 1991), recent studies have also implicated purinergic receptor mechanisms (for review, see Burnstock 2000; Chizh and Illés 2000; Khakh et al. 2001; North and Surprenant 2000). The role of P2X receptors at peripheral sites has been extensively studied (for review, see Chizh and Illés 2000; Dunn et al. 2001; Hamilton and McMahon 2000; Jacobson et al. 2002; North 2003). There has also been some limited identification of their role at central sites involved in nociceptive transmission and spinal dorsal horn sensitization, although some inconsistencies exist (for review, see Gu 2003; Salt 2004; Salter and Sollevi 2001). While neurons cannot be functionally identified in vitro preparations, in vitro studies have reported that neuronal excitatory postsynaptic currents in both superficial and deep spinal dorsal horn are modulated by local application of ATP or P2X receptor agonists and antagonists (Li et al. 1998; Nakatsuka and Gu 2001; Nakatsuka et al. 2002, 2003), and it has been proposed that ATP acts through presynaptic mechanisms to increase the release of glutamate and neuropeptides from the primary afferents (Gu 2003; Gu and MacDermott 1997; MacDermott et al. 1999). Earlier electrophysiological in vivo studies in the spinal dorsal horn had shown that some wide dynamic range neurons as well as most low-threshold mechanoreceptive neurons can be excited by local ATP application (Fyffe and Perl 1984; Salter and Henry 1985; for review, see Salter et al. 1993). More recent studies have reported that intrathecal application of the selective P2X1 receptor agonist βγ-methylene ATP (βγ-meATP), but not the selective P2X1, P2X3, and P2X2/3 receptor agonist αβ-methylene ATP (αβ-meATP), produces facilitation of C-fiber-evoked responses of WDR neurons in the spinal dorsal horn in normal rats, whereas intrathecal application of the wide spectrum P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2, 4-disulphonic acid tetra-sodium (PPADS) can attenuate these responses after carrageenan-induced inflammation (Stanfa et al. 2000). Behavioral studies in rodents however have reported that intrathecal αβ-meATP, but not βγ-meATP, elicits thermal hyperalgesia that can be prevented by intrathecal pretreatment with the selective P2X1, P2X3, and P2X2/3 receptor antagonist 2′,3′-O-2,4,6-trinitrophenyl-ATP (TNP-ATP) or PPADS, whereas intrathecal TNP-ATP or PPADS or another nonelective antagonist (suramin) can attenuate nociceptive behavior associated with formalin-induced

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inflammation (Driessen et al. 1994; Tsuda et al. 1999a,b). Other studies have reported that behavioral responses to noxious mechanical and thermal stimuli are unaltered in P2X<sub>3</sub>-knockout animals, whereas formalin-induced nociceptive behavior is attenuated, suggesting that P2X<sub>3</sub> receptors are involved in certain types of inflammatory-related pain but not in acute nociceptive processing (Cockayne et al. 2000; Souslova et al. 2000). These various findings collectively suggest that P2X receptors are important in nociceptive behavior and that P2X<sub>3</sub> receptors in particular may be involved in some inflammatory pain conditions, although there is some disagreement on the involvement of P2X<sub>3</sub> receptors in acute nociceptive processing in the spinal cord.

In the trigeminal system, we have also recently demonstrated that NMDA receptor mechanisms are involved in the initiation of central sensitization produced by application of the small-fiber excitant and inflammatory irritant mustard oil (MO) to the tooth pulp in brain stem nociceptive neurons of the trigeminal subnucleus caudalis (Vc) and the more rostral subnucleus oralis (Vo) (Chiang et al. 1998, 2003a; Park et al. 2001). Furthermore, the central sensitization in Vo could be blocked by intrathecal application to the Vc of TNP-ATP or the synaptic transmission blocker CoCl<sub>2</sub> and mimicked by intrathecal application of α,β-meATP to the Vc (Chiang et al. 2002; Hu et al. 2002), suggesting that P2X receptor mechanisms within Vc are involved in the development of central sensitization in Vo through ascending connections that have been documented from Vc to Vo (e.g., Greenwood and Sessle 1976; Jacquin et al. 1990; Young and King 1972). So far, there has been no in vivo studies specifically to examine if P2X agonists and antagonists can modulate central sensitization of functionally identified nociceptive neurons in Vc itself. Therefore the aims of the present study were to test whether intrathecal continuous superfusion over Vc of P2X receptor antagonists, TNP-ATP or PPADS, can attenuate the MO-induced central sensitization in Vc itself and whether similar intrathecal superfusion of apyrase, an ATP degrading enzyme (Zimmermann 2000), can also attenuate this MO-induced central sensitization in Vc.

Some data have been briefly presented in abstract form (Chiang et al. 2003b; Zhang et al. 2004).

**M E T H O D S**

**Animal preparation**

The experiments were performed in 34 anesthetized rats. The methods used for animal preparation, stimulation, and neuronal recording and classification were similar to those described previously in detail (Chiang et al. 1998; Hu et al. 2002) and so will only be briefly outlined here. Male Sprague-Dawley adult rats (300–380 g) were anesthetized by single intraperitoneal injection of a mixture of α-chloralose (50 mg/kg) and urethane (1 g/kg). Then a tracheal cannula was inserted, and the left external jugular vein was cannulated. 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. After the rat was placed in a stereotaxic apparatus, the caudal medulla was surgically exposed, and the overlying dura and subarachnoid membrane were removed. Just before the recording session, a supplemental dose of urethane (200–300 mg/kg iv) was administered, and the rat was then immobilized with intravenous pancuronium bromide [initial dose, 0.3 ml of 1 mg/ml solution, followed by a continuous intravenous infusion of a mixture of 70% urethan solution (0.2 g/ml) and 30% pancuronium solution (1 mg/ml) at a rate of 0.4–0.5 ml/h] and artificially ventilated throughout the whole experimental period. A deep level of anesthesia was confirmed periodically by the lack of spontaneous movements and responses to pinching the paw when pancuronium-induced muscle paralysis was allowed to wear off. Heart rate, percentage expired CO<sub>2</sub>, 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. 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).

**Recording and stimulation procedures**

Single neuronal activity was recorded extracellularly by means of an epoxy resin-coated tungsten microelectrode. As the microelectrode was advanced with a rostral inclination of 23° into the right caudal medulla, 1.4–2.0 mm lateral to the midline and 1.5–2.0 mm behind the obex, natural stimuli (see following text) were applied to the orofacial tissues to search for Vc nociceptive neurons receiving an orofacial sensory input. Neuronal activity was amplified, displayed on oscilloscopes and also led to a window discriminator connected to an A/D converter (Cambridge Electronic Design, CED 1401 plus, UK) and a personal computer. Data were analyzed off-line with Spike 2 software (Cambridge Electronic Design, UK).

A wide range of mechanical (brush, pressure, and pinch) and noxious thermal (radiant heat, 51–53°C) stimuli was applied to the orofacial region to classify each neuron as low-threshold mechanoreceptive (LTM), wide dynamic range (WDR), or nociceptive-specific (NS) (Chiang et al. 1998, 2002). Consistent with our earlier findings (Chiang et al. 1994, 1998), we observed that a majority of neurons in lamina IV respond to tactile stimulation alone (LTM) or both tactile and pinch stimuli (WDR), whereas most neurons (NS neurons) in lamina V and adjacent reticular formation responded only to firm pressure or pinch. Only NS neurons in the deep laminae of Vc were tested with the purinergic agents (see following text).

The level of spontaneous activity of a NS neuron was determined over an initial 1-min recording period. As mentioned in our previous studies (Chiang et al. 1998, 2002; Hu et al. 2002; Park et al. 2001), the cutaneous perioral facial mechanoreceptive receptive field (RF) of each NS neuron was determined through the use of a blunt probe and a pair of nonserrated forceps. A burst response consisting of at least two spikes during each stimulus trial was accepted as the criterion for the RF boundary of the neuron tested. Noxious stimulation was used sparingly so as to avoid damage to the skin and peripheral sensitization. A deep nociceptive input was considered to occur only if application of a blunt probe to the skin overlying muscle or bone evoked a neuronal response at a mechanical threshold > 5 g, but no response could be evoked by the wide range of mechanical pinch or thermal stimuli applied to the skin itself (Chiang et al. 1994; Yu et al. 1993). The activation threshold to a mechanical stimulus applied to the orofacial RF was assessed by a pair of force-monitoring forceps (with an attached strain gauge that monitored force levels ≤ 600 g/mm²) or an electronic von Frey monofilament. The gradual increase in the mechanical force and the responses of the tested neuron were monitored and recorded simultaneously by the use of the Spike2 program (CED 1401 plus, UK). The NS neuronal responses to graded pressure by means of electronic von Frey monofilament applications or graded mechanical pinch stimuli with a force-monitoring forceps (25, 50, 75, 100, and even 200 g, applied in ascending order, each for 5 s at an interval of > 45 s) applied to the neuronal orofacial RF were determined as previously described (Chiang et al. 1998, 2002; Hu et al. 2002; Park et al. 2001). The pinch- or pressure-evoked responses of a given neuron were assessed by summing the number of spikes evoked by each of these four graded stimuli during application of the stimulus.
The chemicals used included MO (allyl isothiocynate, 95%; Aldrich Chemical), PPADS (10, 33, 100 μg/ml, equivalent to 17, 55, 167 μM, were used; Research Biochemicals International, Natick, MA), TNP-ATP (5 or 500 μg/ml, equivalent to 6.4 or 638 μM, respectively; Molecular Probes, Eugene, OR), apyrase grade VI (30 units/ml; Sigma-Aldrich, St. Louis, MO). All drugs except MO were freshly dissolved in buffered saline at pH 7.4 (Sigma-Aldrich).

Experimental paradigm

P2X RECEPTOR ANTAGONIST EXPERIMENTS. Four groups (saline/MO, PPADS/MO, and TNP-ATP/MO low dose and TNP-ATP/MO high dose) of animals were studied, and a standard assessment of neuronal spontaneous activity, mechanical activation threshold, orofacial RF size, and pinch- or pressure-evoked responses was performed periodically throughout the experiment. After baseline values of neuronal properties were obtained, either PPADS (PPADS/MO group) or TNP-ATP (TNP-ATP/MO low or high groups) or buffered saline (saline/MO group as a control) was continuously superfused (intrathecally) over the ipsilateral Vc (at a rate of 0.6 ml/h). Ten minutes after the commencement of the superfusion, another assessment of neuronal properties was carried out. At 20 min, the saline-soaked cotton pellet was carefully removed from the molar pulp cavity and replaced with a segment of dental paper point soaked with MO (0.2 μl). The cavity was promptly sealed with Cavite (ESPE, 3M, Germany) to prevent MO leaking out of the tooth and to ensure the chemical’s sustained action on pulp afferents. Three minutes after MO application, the standard assessment of neuronal properties was repeated at 10-min intervals throughout a 50-min observation period.

EXTRACELLULAR ATP-DEGRADATION EXPERIMENTS. Another group (apyrase/MO group) of animals received continuous intrathecal superfusion of apyrase followed by MO application to the molar pulp. A similar protocol as used in the P2X antagonist experiments (see preceding text) was used, except that the apyrase intrathecal superfusion started 30 min before MO application to the pulp and hence one more assessment of neuronal properties was carried out before MO application. The decision to start the MO application at 30 min was based on preliminary experiments carried out on five NS neurons with continuous superfusion of apyrase (30 units/ml it) alone. Soon after the start of the apyrase superfusion, there were transient changes in threshold and pinch/pressure-evoked responses (74 ± 14 and 138 ± 55% of control, respectively, means ± SE; P < 0.05, paired t-test), but not in RF size; importantly, these changes only appeared at the 10-min time point after the superfusion started and did not persist over the remaining superfusion period lasting ~1 h.

Histological and statistical analyses

Recording sites were marked by electrolytic lesions (anodal current of 8 μA for 13 s) and verified histologically (e.g., see Fig. 1). All values were presented as means ± SE. Statistical analyses were based on the normalized data (in percentage). Differences between the baseline value and values at different postdrug (i.e., MO, P2X receptor antagonist, apyrase) or postsaline time points in each group were treated by repeated-measures ANOVA (RM ANOVA) or RM ANOVA on ranks, followed by Dunnett’s test. Differences between the different drug group and the saline group were treated by two-way RM ANOVA followed by Bonferroni t-test. Values associated with drug dose-response curves were treated by ANOVA. The level of significance was set at P < 0.05.

In the saline/MO group (n = 6), only one neuron had baseline activity (0.07 Hz), and continuous intrathecal superfusion of saline did not affect its firing; however, the subsequent MO application produced an immediate response (>25 Hz) followed by sustained high firing (~4 Hz) lasting 25 min in that neuron and also produced a transient response (0.02–0.04 Hz) in two other neurons.

In the TNP-ATP/MO (5 μg/ml; n = 6), PPADS/MO (33 μg/ml; n = 6), and apyrase/MO (30 units/ml; n = 6) groups, none of these NS neurons tested had baseline activity. Continuous intrathecal superfusion of these chemicals did not evoke any spontaneous activity. In the TNP-ATP/MO low-dose group, MO application produced an immediate response (~18 Hz for 3 min) followed by low level of firing (0.1–2.8 Hz) for 30 min in one neuron and produced a transient response (0.02–15 Hz) in another two neurons. With the higher dose of MO, PPADS/MO, and TNP-ATP/MO (5 μg/ml, equivalent to 17, 55, 167 μM, were used; Research Biochemicals International, Natick, MA), TNP-ATP (5 or 500 μg/ml, equivalent to 6.4 or 638 μM, respectively; Molecular Probes, Eugene, OR), apyrase grade VI (30 units/ml; Sigma-Aldrich, St. Louis, MO). All drugs except MO were freshly dissolved in buffered saline at pH 7.4 (Sigma-Aldrich).

RESULTS

Forty-two functionally identified NS neurons responding to ipsilateral orofacial noxious stimulation were studied. Nearly all of the recording sites were in the deep laminae of Vc (Fig. 1).

Spontaneous activity

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TNP-ATP (500 μg/ml it), five of the six neurons tested had no baseline activity, and the subsequent MO application produced a short-lasting and weak response (<0.6 Hz) in three neurons and a sustained high firing rate (±18 Hz) in one neuron. In both the PPADS/MO and apyrase/MO groups, the subsequent MO application did not evoke any spontaneous activity in any of these neurons.

**Orofacial RF size**

All six NS neurons in the saline/MO group had at baseline an ipsilateral pinch/pressure RF (2.0 ± 0.5 cm²) that involved both maxillary and ophthalmic divisions (5 had a dumbbell-like-shaped RF involving the periorbital region, nose and vibrissal pad; 1 neuron had a periorbital RF). MO application to the pulp produced a significant, long-lasting increase in RF size in all neurons tested: their RFs not only expanded 100–200% beyond the original RF but also reached the perioral region and medial face. Compared with the baseline value, the mean RF size increased significantly throughout the 50-min observation period after MO application (P < 0.001, RM ANOVA), with its peak at 18 min (P < 0.05, Dunnett’s test) as shown in Fig. 2, and Table 1. In addition, soon after MO application, a novel tactile RF appeared for 10–30 min in three of the six neurons.

The 6 NS neurons in the low-dose (5 μg/ml) TNP-ATP/MO group had at baseline an ipsilateral RF with a mean size of 1.7 ± 0.4 cm²: the RF of these neurons involved both maxillary and ophthalmic divisions (3 had a dumbbell-shaped RF covering the periorbital region and vibrissal pad; the other 3 had a RF covering the nose and/or vibrissal pad). As shown in Fig. 2 and Table 1, TNP-ATP continuous superfusion itself did not affect the neuronal RF size, but the subsequent MO application produced a small but significant increase in RF size of these neurons (P < 0.01, RM ANOVA); however, no novel tactile RF appeared in any of these neurons. Even in the higher-dose (500 μg/ml) TNP-ATP group, this MO-induced RF expansion still occurred (P < 0.005, RM ANOVA) and was associated with a transient appearance of a novel tactile RF in two neurons.

The six NS neurons tested in the PPADS/MO group had a mean baseline ipsilateral RF size of 1.2 ± 0.2 cm²: the RF of five neurons involved the maxillary division (the vibrissal pad in 3 and the perioral region in 2); the RF in the remaining one neuron included both maxillary and ophthalmic divisions. As shown in Fig. 2 and Table 1, neither PPADS superfusion itself nor the subsequent MO application significantly affected RF sizes (P > 0.3, RM ANOVA). Also, no novel tactile RF appeared in any of these neurons.

The six NS neurons tested in the apyrase/MO group had a mean baseline RF size of 1.3 ± 0.3 cm²; all their RFs involved the maxillary and/or ophthalmic divisions (the vibrissal pad and nose in 3, the perioral region in 2, and the nose in 1). Apyrase superfusion itself did not affect RF size. Thirty minutes later, MO application did not produce any significant changes in RF size (P > 0.3, RM ANOVA; Fig. 2).

The differences in RF size after MO application between the saline/MO group and each of the TNP-ATP/MO, PPADS/MO, and apyrase/MO groups were significant (P < 0.003–0.006, Table 1).

**Mechanical activation threshold**

The six NS neurons tested in the saline/MO group had at baseline a mean threshold of 100 ± 38 g, which was significantly decreased to 50% of control after MO application (P = 0.002, RM ANOVA); the peak decrease occurred at 18 min (P < 0.05, Dunnett’s test; Fig. 3 and Table 1).

The six NS neurons tested in each of the low-dose TNP-ATP/MO, high-dose TNP-ATP, PPADS/MO, and apyrase/MO groups had a mean baseline threshold of 85 ± 17, 94 ± 23, 93 ± 17, and 76 ± 16 g, respectively. Chemical superfusion did not produce any significant changes in threshold values (P > 0.3–0.9, RM ANOVA; Fig. 3). The subsequent MO application produced a significant decrease in threshold in the high dose TNP-ATP group (P < 0.05, ANOVA) but not in the other three groups.

The differences in activation threshold after MO application between the saline/MO group and the PPADS/MO or high dose TNP-ATP group were significant (P < 0.05), whereas those between the saline/MO group and the low dose TNP-ATP/MO or apyrase/MO group were not significant (P > 0.05; Table 1).

**Responses to graded pinch/pressure stimuli**

In the saline/MO group, the mean baseline response to graded pinch/pressure stimuli was 52 ± 15 spikes. The response in five of six NS neurons tested was increased after MO application. The mean response increased significantly to a twofold peak at 18 min and then slowly declined but had not reached baseline levels by 50 min (P = 0.02, RM ANOVA; Fig. 4 and Table 1).
TABLE 1. Effects of intrathecal superfusion of TNP-ATP, PPADS, apyrase or saline onto trigeminal subnucleus caudalis on MO/pulp-induced neuroplastic changes in pinch RF and response properties of nociceptive neurons in adult rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pinch/Pressure RF, cm²</th>
<th>Mechanical Activation Threshold, g</th>
<th>Graded Pinch/Pressure Response, Sum of Spikes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saline/MO experiments</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of NS neurons</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Baseline value</td>
<td>2.0 ± 0.5</td>
<td>100 ± 38.4</td>
<td>52.2 ± 14.8</td>
</tr>
<tr>
<td>Value 10' after saline</td>
<td>1.9 ± 0.5</td>
<td>97.3 ± 35.4</td>
<td>35.7 ± 9.7</td>
</tr>
<tr>
<td>Value 18' after MO</td>
<td>4.9 ± 0.9*</td>
<td>49.5 ± 16.5*</td>
<td>129.5 ± 40.8*</td>
</tr>
<tr>
<td>Value 48' after MO</td>
<td>4.2 ± 0.8*</td>
<td>83.8 ± 30.4</td>
<td>87.3 ± 45.8</td>
</tr>
<tr>
<td><strong>TNP-ATP/MO experiments</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Number of NS neurons</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Baseline value</td>
<td>1.7 ± 0.4</td>
<td>85 ± 16.9</td>
<td>81.3 ± 30</td>
</tr>
<tr>
<td>Value 10' after TNP-ATP</td>
<td>1.5 ± 0.4</td>
<td>84 ± 15</td>
<td>124.7 ± 54.3</td>
</tr>
<tr>
<td>Value 18' after MO</td>
<td>1.9 ± 0.4</td>
<td>60.3 ± 14.8</td>
<td>184.2 ± 53.6</td>
</tr>
<tr>
<td>Value 48' after MO</td>
<td>1.9 ± 0.5</td>
<td>86.2 ± 13.4</td>
<td>62 ± 22.7</td>
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<tr>
<td><strong>PPADS/MO experiments</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of NS neurons</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Baseline value</td>
<td>1.2 ± 0.2</td>
<td>92.5 ± 17.4</td>
<td>60.7 ± 9.9</td>
</tr>
<tr>
<td>Value 10' after PPADS</td>
<td>1.3 ± 0.3</td>
<td>86.2 ± 17.8</td>
<td>75.5 ± 24.9</td>
</tr>
<tr>
<td>Value 18' after MO</td>
<td>1.5 ± 0.4</td>
<td>99.3 ± 32.2</td>
<td>63.0 ± 21.2</td>
</tr>
<tr>
<td>Value 48' after MO</td>
<td>1.6 ± 0.4</td>
<td>91.7 ± 32.7</td>
<td>135.8 ± 55.1</td>
</tr>
<tr>
<td><strong>Apyrase/MO experiments</strong></td>
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<tr>
<td>Number of NS neurons</td>
<td>6</td>
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<tr>
<td>Baseline value</td>
<td>1.3 ± 0.3</td>
<td>76.3 ± 15.6</td>
<td>161.7 ± 27.8</td>
</tr>
<tr>
<td>Value 12' after apyrase</td>
<td>1.3 ± 0.3</td>
<td>68.7 ± 17.2</td>
<td>194 ± 79.8</td>
</tr>
<tr>
<td>Value 25'(%) after apyrase</td>
<td>1.3 ± 0.3</td>
<td>61.2 ± 12.2</td>
<td>180 ± 45.1</td>
</tr>
<tr>
<td>Value 18' after MO</td>
<td>1.3 ± 0.3</td>
<td>58.3 ± 12.9</td>
<td>137.8 ± 37.4</td>
</tr>
<tr>
<td>Value 48' after MO</td>
<td>1.3 ± 0.2</td>
<td>73 ± 11.5</td>
<td>102.8 ± 39.2</td>
</tr>
<tr>
<td>TNP-ATP/MO vs. saline/MO†</td>
<td>F (1, 60) = 11.77; P = 0.006</td>
<td>F (1, 60) = 3.46; P = 0.09</td>
<td>F (1, 60) = 1.37; P = 0.27</td>
</tr>
<tr>
<td>PPADS/MO vs. saline/MO†</td>
<td>F (1, 60) = 11.85; P = 0.006</td>
<td>F (1, 60) = 5.07; P &lt; 0.05</td>
<td>F (1, 60) = 1.64; P = 0.23</td>
</tr>
<tr>
<td>Apyrase/MO vs. saline/MO†</td>
<td>F (1, 60) = 15.38; P = 0.003</td>
<td>F (1, 60) = 4.24; P = 0.06</td>
<td>F (1, 60) = 3.40; P = 0.09</td>
</tr>
</tbody>
</table>

All values are shown as means ± SE; *P < 0.05 for comparison between the baseline value and values at the different time after drug or saline in each group (RM ANOVA followed by Dunnett’s test); †Two-way RM ANOVA results for comparison of all time courses between saline/Mustard oil (MO) and other drug/MO groups.

In the low-dose TNP-ATP/MO group, the mean baseline response was 81 ± 30 spikes, and two of the six NS neurons tested showed increases in responses after TNP-ATP continuous superfusion. The subsequent MO application to the pulp also produced marked increases in responses (190–880% of control) in only three of the six neurons, and the mean response was not significantly different from baseline (P > 0.3, RM ANOVA on ranks; Fig. 4). Similarly, in the high-dose TNP-ATP group, which had a mean baseline response of 66 ± 20 spikes, four of six NS neurons responded markedly to graded pinch stimuli after MO application, but again the mean response was not significantly different from baseline (P > 0.4, RM ANOVA).

The six NS neurons tested in each of the PPADS/MO and apyrase/MO groups had a mean baseline response of 61 ± 10 and 162 ± 28 spikes, respectively. Neither PPADS nor apyrase superfusion nor the subsequent MO application produced significant changes in the neuronal responses to graded noxious mechanical stimuli (P > 0.2–0.9, RM ANOVA on ranks; Fig. 4).

The differences in responses after MO application between these four chemical superfusion groups and the saline/MO group were not significant (P > 0.09, Table 1). Examples of NS neurons in the saline/MO, low dose TNP-ATP/MO, PPADS/MO, and apyrase/MO groups are illustrated in Fig. 5.
inflammation (Cockayne et al. 2000; Souslova et al. 2000; Tsuda et al. 1999b; Vulchanova et al. 2001). In contrast, the baseline nociceptive receptive field and response properties of the Vc nociceptive neurons appeared not to involve ATP-mediated mechanisms, consistent with findings that central P2X receptors are not involved in acute nociception (Cockayne et al. 2000; Souslova et al. 2000; Tsuda et al. 1999b; Vulchanova et al. 2001).

Our finding that the MO-induced Vc central sensitization lasts for 20–40 min is consistent with our several earlier findings of the time course of caudalis central sensitization induced by MO application to tooth pulp, facial skin, masticatory muscle, or temporomandibular joint (Chiang et al. 1998; Hu et al. 1992; Yu et al. 1993). We found in the present study that the application to Vc of PPADS, TNP-ATP, and apyrase prior to the tooth pulp application of MO prevented the occurrence of Vc central sensitization. This effect could be due to antagonism of induction or maintenance of central sensitization, or both, and other experimental designs would be needed to differentiate between induction and maintenance. In addition, it should be noted that the MO-induced central sensitization occurred in many nociceptive neurons independent of their being activated by MO. This is consistent also with our earlier findings (e.g., Chiang et al. 1998) and implies that the ATP-mediated Vc central sensitization does not necessarily require direct activation of the neuron under study.

PPADS is a wide-spectrum P2X receptor antagonist, whereas TNP-ATP is a potent selective P2X1, P2X3, and P2X2/3 receptor subtype antagonist (Burgard et al. 2000; Khakh et al. 2001; Nörenberg and Illes 2000; North 2003; North and Surprenant 2000; Virginio et al. 1998). In spinal cord slice preparations, α,β-meATP induces a long-lasting glutamate release from the neurons in lamina V that can be antagonized by PPADS (10 μM) but not by TNP-ATP (1 μM) (Nakatsuka and Gu 2001; Nakatsuka et al. 2003). Furthermore, some dorsal root ganglion cells were found to be resistant to TNP-ATP as well as to capsaicin but sensitive to PPADS, and selective removal of P2X3-expressing afferent terminals by the targeting toxin saporin-conjugated isoleucine B4 did not affect P2X-mediated long-lasting modulation in lamina V neurons (Nakatsuka et al. 2003). Similarly, Tsuda et al. (1999a) reported that both PPADS and TNP-ATP (intrathecal) reduced inflammatory pain in a behavioral model, although the TNP-ATP effect was much less potent compared with PPADS. These findings suggest that P2X3 receptors are not involved in sensory processing in lamina V (Nakatsuka et al. 2003; Suzuki et al. 2003; for review see Chizh and Illés 2000; Gu 2003) and are consistent with our in vivo observations that PPADS was much more effective than TNP-ATP in counteracting the development of central sensitization in NS neurons of the deep laminae of Vc. Nevertheless, in our study, some of these NS neurons were found to be affected by TNP-ATP; a possible explanation is that TNP-ATP might initially act on the superficial laminae where it blocks dense P2X3 and P2X2/3 receptor subtypes and then through interneuronal connections indirectly influences NS neurons in the deep laminae. This may also explain our previous finding (Hu et al. 2002) that the MO-induced central sensitization in Vc can be significantly blocked by TNP-ATP intrathecal application onto Vc; i.e., that superficial layers rather than deeper layers of Vc provide input to and influence the activity of nociceptive neurons in the Vc.
through pathways documented to ascend from Vc to Vo (e.g., Greenwood and Sessle 1976; Jacquin et al. 1990; Young and King 1972). Further in vivo studies are needed to determine if neurons in the superficial laminae of Vc are affected by P2X agonists and antagonists.

In the spinal dorsal horn, the P2X subtypes 1, 2, and 4–6 are expressed intrinsically, and the P2X3 subtype is exclusively expressed in the dorsal root and trigeminal ganglion cells and their central terminals (Vulchanova et al. 1998). The P2X2, P2X3, and P2X6 subtypes are the most abundant in the dorsal horn (in lamina II in particular), and P2X1 and P2X5 are localized predominantly in the central and ventral gray matter (Collo et al. 1996; Vulchanova et al. 1998). However, only P2X4 and P2X6 subtypes appear to be expressed in Vc (Collo et al. 1996). Although the effect of TNP-ATP on P2X6 subtypes has not been tested to date, several recent in vitro studies have confirmed that the P2X1–4 subtypes are sensitive to TNP-ATP and only the P2X4 subtype is relatively resistant to PPADS (Bo et al. 1995; Buell et al. 1996; Chizh and Illes 2000; Tsuda et al. 2003; Virginio et al. 1998; for reviews see Khakh et al. 2001; Norenberg and Illes 2000; North and Surprenant 2000). Furthermore, Tsuda et al. (2003) have demonstrated that allodynia evoked in the rat spinal nerve ligation model can be eliminated by pretreatment with TNP-ATP, but not with PPADS, which might indicate that the P2X4 subtypes are particularly involved in allodynia; furthermore, they found that P2X4 subtypes were upregulated in microglia in the superficial dorsal horn (for review, see Salter 2004). Because we have documented in our tooth inflammatory model that PPADS is much potent than TNP-ATP in reducing central sensitization in
PPADS has been reported to antagonize P2Y1 and P2Y2 receptors plays an important role in this model. On the other hand, because findings of transient changes in neuronal activation threshold, and responses to graded mechanical stimuli. Data were taken from the 18 min after MO application to the tooth pulp. Note that in the PPADS/MO group, 3 different doses were applied: 10 μg/ml, n = 3; 33 μg/ml, n = 6; 100 μg/ml, n = 4; in the Saline/MO group, n = 6. There were significant differences in all 3 measures of neuronal properties between either the 33 or 100 μg/ml dose group and the Saline/MO group (as a control) (P < 0.02–0.001, ANOVA). *Value compared with the saline group, P < 0.05, Bonferroni t-test.

The deep laminae of Vc, it is unlikely that the P2X3 subtype plays an important role in this model. On the other hand, because PPADS has been reported to antagonize P2Y1 and P2Y2 receptors in spinal astrocytes (Fam et al. 2000; Gallagher and Salter 2003; Wildman et al. 2003) and P2Y1 receptors in the dorsal root ganglion (Gerevich et al. 2004; for review, see Kennedy et al. 2003; Liu and Salter 2005), the possibility cannot be discounted of P2Y receptor involvement in the MO-induced neuroplastic changes that we have documented in Vc.

Apyrase is an ATP-diphosphohydrolase that catalyzes the hydrolysis of phosphoanhydride bonds of nucleoside tri- and di-phosphates in the presence of divalent cations. This enzyme has broad substrate specificity for nucleotides and has been widely used in in vitro neurobiological studies (Nourizad et al. 2003; Zimmermann 2000). The degradation of ATP in the CNS includes several steps: from ATP to ADP, to AMP, and then to adenosine. It has been reported that P2Y1 receptors are preferentially activated by ADP, which facilitates intercellular spread of Ca2+ waves among astrocytes and hence promotes ATP and excitatory amino acid release (Fam et al. 2000; Gallagher and Salter 2003; Kimelberg 2004; Mongin and Kimelberg 2002; for review, see Kennedy et al. 2003; Liu and Salter 2005); however, a recent study has reported that P2Y1 receptor activation decreases glutamate release (see Gerevich et al. 2004). P2X and P2Y2 receptors are most sensitive to ATP, and the P2Y1 receptor to ADP (Fam et al. 2000; Gallagher and Salter 2003; North and Surprenant 2000). We used a grade VI of apyrase that contains predominantly ATPase activity with lesser ADPase activity and only trace AMPase activity (Sigma/RBI Catalogue 2003) to minimize degradation of ADP but enhance hydrolysis of ATP. Therefore the expected temporary accumulation of ADP, an intermediate product during ATP degradation by apyrase, may explain our findings of transient changes in neuronal activation threshold and responses to graded pinch/pressure stimuli in the early stages of apyrase superfusion.

Our results showed that apyrase potently attenuates the MO-induced neuroplastic changes. Although this could indicate that either P2X or P2Y2 or both receptors may play a critical role in producing central sensitization (for review, see North and Barnard 1997), our findings with P2X receptor antagonists in the tooth pulp inflammatory pain model suggest that the P2X1,3,6 subtypes may be involved (for review, see Gu 2003; Kennedy et al. 2003), whereas the P2X4 subtype is likely not involved. Because the final end product of ATP degradation is adenosine, which is known as a potent inhibitor acting on purinergic A receptors (for review, see Salter et al. 1993; Salter and Sollevi 2001; Sawynok 1998), the pharmacological action of apyrase may be dichotomized into two components: one is a direct degradation of endogenous ATP that may presynaptically interfere with the initiation of central sensitization, and the other is an inhibition produced by the end-product adenosine that also interferes with the development of central sensitization. Taken together, we speculate that the MO-induced barrage of nociceptive inputs promotes release of endogenous ATP, which in turn through presynaptic mechanisms produces abundant release of glutamate and neuropeptides to develop central sensitization in the medullary as well as spinal dorsal horns (Chiang et al. 2003a; Gu 2003).

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GRANTS

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