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Acidic pH facilitates peripheral αβmeATP-mediated nociception in rats: Differential roles of P2X, P2Y, ASIC and TRPV1 receptors in ATP-induced mechanical allodynia and thermal hyperalgesia

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ABSTRACT

Peripheral ischemia is commonly associated with an increase in tissue ATP concentration and a decrease in tissue pH. Although *in vitro* data suggest that low tissue pH can affect ATP-binding affinities to P2 receptors, the mechanistic relationship between ATP and low pH on peripheral nociception has not been fully examined. This study was designed to investigate the potential role of an acidified environment on intraplantar αβmeATP-induced peripheral pain responses in rats. The mechanical allodynia (MA) produced by injection of αβmeATP was significantly increased in animals that received the drug diluted in pH 4.0 saline compared to those that received the drug diluted in pH 7.0 saline. Moreover, animals injected with αβmeATP (100 nmol) in pH 4.0 saline developed thermal hyperalgesia (TH), which did not occur in animals treated with αβmeATP diluted in pH 7.0 saline. To elucidate which receptors were involved in this pH-related facilitation of αβmeATP-induced MA and TH, rats were pretreated with PPADS (P2 antagonist), TNP–ATP (P2X antagonist), MRS2179 (P2Y1 antagonist), AMG9810 (TRPV1 antagonist) or amiloride (ASIC blocker). Both PPADS and TNP–ATP dose-dependently blocked pH-facilitated MA, while TH was significantly reduced by pre-treatment with MRS2179 or AMG9810. Moreover, amiloride injection significantly reduced low pH-induced facilitation of αβmeATP-mediated MA, but not TH. These results demonstrate that low tissue pH facilitates ATP-mediated MA via the activation of P2X receptors and ASICs, whereas TH induced by ATP under low pH conditions is mediated by the P2Y1 receptor and TRPV1, but not ASIC. Thus distinct mechanisms are responsible for the development of MA and TH under conditions of tissue acidosis and increased ATP.

1. Introduction

ATP is recognized as a potent extracellular nociceptive molecule because it can act on ionotropic P2X and metabotropic P2Y receptors located on primary afferent neurons (Shao et al., 2007). The mRNAs or subunit proteins of all 7 subtypes of cloned mammalian P2X receptors (P2X1–P2X7) have been found in the central and peripheral nervous systems. Of these subtypes, P2X3 and P2X4 receptors are expressed selectively in neurons of the trigeminal ganglion (TG) and dorsal root ganglia (DRG) that are involved in the development of peripheral sensitization and/or nociception (Chizh and Illes, 2001; Kage et al., 2002; Tsuda et al., 2009). In addition, several behavioral studies support the existence of functional P2X receptors on peripheral nociceptors (McGaraughty et al., 2003; Lu et al., 2008). Intraplantar injections of ATP, as well as αβmeATP have been shown to evoke nocifensive behavior in rats (Hamilton et al., 1999). Similarly, P2Y receptors are comprised of 8 subtypes, which are also found in primary afferent neurons. In particularly, P2Y1 and P2Y2 receptors are typically expressed in small DRG neurons that are related to pain sensation (Gerevich and Illes, 2004).

The increase in protons (i.e. acidification, or low pH condition) in peripheral tissues also affects nociception or peripheral sensitization via activation of proton-gated cation channels, including the acid-sensing ion channels (ASICs) and transient receptor potential vanilloid (TRPV1) ion channels (Caterina and Julius, 2001; Leffler

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et al., 2006; Olson et al., 1998; Planells-Cases et al., 2005; Price et al., 2001; Ugawa et al., 2002; Waldmann et al., 1997). In addition, ASICs and TRPV1 are important acid sensors in primary afferent fibers, contributing to acid-induced nociception within a pathophysiologically relevant pH range (Deval et al., 2008; Leffler et al., 2006; Reeh and Kress, 2001; Sluka et al., 2001; Ugawa et al., 2002; Yagi et al., 2006; Yen et al., 2009).

In general, peripheral ischemic conditions are closely associated with both an increase in extracellular ATP and an elevated proton concentration (i.e. a decrease in tissue pH) (Issberner et al., 1996; Jones et al., 2004; Sutherland et al., 2000). Recently we have reported that the activation of peripheral ASICs and P2X receptors contributed to the development of mechanical allodynia (MA) under a thrombus-induced peripheral ischemic condition in rats (Seo et al., 2010). Moreover, it was previously shown that localized tissue acidosis enhances pain perception via an action on ATP-gated ion channels on mammalian sensory neurons (Li et al., 1996). In this regard, Li et al. demonstrated that extracellular protons regulated the function of P2X receptors by modulating the affinity of the ATP-binding site (Li et al., 1996, 1997). Furthermore, extracellular protons have been shown to significantly potentiate the agonist potency of recombinant P2Y4 receptors, indicating the functional potentiation of P2Y receptors by protons (Wildman et al., 2003). However, the mechanisms that contribute to the effect of low pH on extracellular ATP-induced nociception need to be better defined. In addition, the specific P2 receptor subtypes involved in this pH enhancing effect on ATP-mediated *in vivo* pain sensation remain to be delineated.

Therefore, this study was designed to investigate the facilitatory role of an acidified environment on the pain responses produced by intraplantar injection of the ATP congener, αβmeATP in rats. Moreover, one of the goals of this study was to determine which P2 receptor subtype is involved in αβmeATP-induced pain and its facilitation by low tissue pH. In order to examine this, we used PPADS (a non-selective P2X, P2Y receptor antagonist), TNP–ATP (a selective P2X receptor antagonist) or MRS2179 (a selective P2Y1 receptor antagonist). In addition, the involvement of ASICs and TRPV1 in the low pH-induced facilitation of αβmeATP-mediated pain was investigated using amiloride (an ASICs blocker) and AMG9810 (a TRPV1 antagonist), respectively.

2. Materials and methods

2.1. Animals

Experiments were performed on male Sprague-Dawley rats (300–400 g), provided by The Laboratory Animal Center of Seoul National University (SNU). All procedures on animals were approved and reviewed by the SNU Animal care and Use Committee and performed according to guidelines established by NIH (NIH publication No. 86-23, revised 1985). Animals were housed in a standard environment consisting of a 12 h light/dark cycle, a constant room temperature (maintained between 20 and 25 °C), and 40–60% humidity. Food and water were given *ad libitum* throughout the investigation.

2.2. Intraplantar drug administration into rat hindlimb paw

All drugs were prepared in normal (pH = 7.0) or pH-adjusted (pH = 4.0) saline. Although the pH 7.4 is known as a normal pH level in tissue, the sterile saline detected the neutral (pH 7.0) was used in this study because most of studies have used this sterile saline as a control vehicle of intraplantar or subcutaneous drug injection (Bhatia et al., 2005; Cheng et al., 2008). In addition, the acidic pH saline was adjusted pH 4.0 because several studies have also examined the actual pH level in the plantar tissue, and they indicated that the measured tissue pH was considerably higher than solution pH level before injection (Hamamoto et al., 1998; Sluka et al., 2001). The pH 4.0 saline was adjusted with 1 N HCl solution (Skyba et al., 2005; Sluka et al., 2001). Rats were then briefly anesthetized with Gerolan®, inhalation anesthetic (Choongwae Pharma Corp., Republic of Korea) in an acylic chamber and injected intraplantarly into the central sole region of the hind paw with 50 μl of αβmeATP (Sigma, St. Louis, MO, USA) or they were pretreated with 30 μl a P2 antagonist, ASIC blocker or a TRPV1 receptor antagonist followed 30 min later by αβmeATP as described below. The animals were then tested behaviorally for their responses to both mechanical and thermal stimulations as described below. The control group received an intraplantar injection of the appropriate vehicle for each drug. Animals were randomly assigned to experimental groups and subsequent drug treatment and behavioral analysis were performed blindly.

2.3. Assessment of mechanical allodynia

The number of paw withdrawal responses to a normally innocuous mechanical stimuli produced by using a von Frey filament of 4.0 g (North Coast Medical, Morgan Hill, CA) was measured as previously described (Roh et al., 2004; Seo et al., 2008). Briefly, rats were placed on a metal mesh grid under a plastic chamber, and von Frey filaments were applied from underneath the metal mesh flooring to the central sole region of hind paw for 10 trials at approximately 10-s intervals. The number of paw withdrawal responses to each set of 10 stimuli was then counted. The data resulting from the mechanical allodynic behavioral testing for each experimental and control group are presented as the percentage of withdrawal response frequency (WRF, %). Mechanical allodynia (MA) produced in response to application of a von Frey filament of 4.0 g was measured at 0.5, 1, 2 and 4 hours after the injection of αβmeATP.

2.4. Assessment of thermal hyperalgesia

To assess nociceptive responses to heat stimuli, we measured paw withdrawal response latency (WRL) using a standard plantar thermal test apparatus (Series 8, Model 390, IITC Life Science Inc., Woodland Hills, CA, USA; Hargreaves' test) as previously described (Seo et al., 2008). Briefly, rats were placed in a plastic chamber with a glass floor and allowed to acclimate for 10 min before testing. Behavioral room temperature was maintained constantly between 26 and 28 °C. A radiant heat source was positioned under the glass floor beneath the hind paw to be tested and withdrawal latency was measured by using a photoelectric cell connected to a digital clock. The intensity of the light source was calibrated to produce a withdrawal response within 10–12 s in normal animals. The test was repeated at each time point and a mean withdrawal latency was calculated. A cut-off time of 20 s was used to protect the animal from excessive tissue damage. Thermal hyperalgesia (TH) was measured at 15, 30 and 60 min after the injection of αβmeATP.

2.5. Drugs

The purinoceptor ligands, pyridoxalphosphate-6-azophenyl-2′, 4′-disulfonic acid (PPADS) tetrasodium salt (a non-selective P2 purinergic antagonist), 2′,3′-O-(2,4,6-Trinitrophenyl)adenosine-5′-triphosphate (TNP-ATP) triethylammonium salt (a $P2X_1$, $P2X_3$ and $P2X_{2/3}$ antagonist), 2′-Deoxy-N^6-methyladenosine 3′,5′-bisphosphate (MRS2179) tetrasodium salt (a competitive antagonist at $P2Y_1$ receptors) and (2E)-N-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide (AMG9810; a competitive vanilloid TRPV1 receptor antagonist) were purchased from Tocris (Ellisville, MO). α,β-Methyleneadenosine 5′-triphosphate (αβmeATP) lithium salt was purchased from Sigma (St. Louis, MO). PPADS, TNP–ATP, MRS2179 were dissolved in pH 7.0 saline, respectively. AMG9810 was dissolved in 10% ethyl alcohol. This 10% ethyl alcohol (pH 7.0) was used as a vehicle control for AMG9810. A 30 μl volume of the above drugs was intraplantarly injected for each experiment.

2.6. Statistical analysis

Data are presented as the mean ± SEM. Behavioral data were statistically tested using a repeated measures 2-way analysis of variance (ANOVA) to determine the overall effect of drugs. For posthoc analysis, Bonferroni's multiple comparison test was subsequently performed to determine significant differences among groups (GraphPad Prism, San Diego, CA). A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. αβmeATP-evoked MA and its facilitation by low pH condition

Intraplantar administration of αβmeATP (10, 30, 100 nmol) diluted in pH 7.0 saline dose-dependently increased paw withdrawal response frequency (%) to innocuous mechanical stimuli (MA) at both the 30 min and 1-hour time points post-injection. This mechanical hypersensitivity to intraplantar αβmeATP was absent by the 2-hour post-injection time point (Fig. 1A, $^{*}p < 0.05$ and $^{***}p < 0.001$ as compared to those in the pH 7.0 saline alone injected group). While intraplantar injection of pH 4.0 saline alone did not produce an allodynic response, it significantly enhanced αβmeATP (30 and 100 nmol)-induced MA (Fig. 1B, $^{*}p < 0.05$ as compared to those in the pH 4.0 saline alone injected group). In this regard area under curve (AUC) data showed that pH 4.0 saline had