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Full Length Research Paper

Puerarin inhibits acute nociceptive responses via the $P2X_3$ receptor in rat dorsal root ganglia

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The effects of puerarin on acute nociception mediated by the $P2X_3$ receptor in rat dorsal root ganglia (DRG) were studied. Intrathecal injection of adenosine triphosphate (ATP) or α,β-methylene-ATP (α,β-meATP) could potentiate acute nociceptive responses induced by intraplantar injection of ATP or α,β-meATP in rat hindpaw. Intraplantar injection of formalin increased the expression of $P2X_3$ in rat DRG and induced acute nociceptive responses. These induced nociceptive responses were inhibited by intrathecal injection of puerarin, which also decreased the up-regulated expression of $P2X_3$ mRNA and protein in DRG induced by intraplantar injection of formalin. Molecular docking studies revealed that puerarin could interact perfectly with homology-modeled rat $P2X_3$ receptor ($rP2X_3$). $rP2X_3$ can be restricted to binding ATP because of its combination with puerarin (increasing the concentration of free ATP), therefore blocking the $rP2X_3$ channel. Whole cell patch clamp recording showed that puerarin inhibited the potentiation of $P2X_3$ receptor-mediated currents induced by lipopolysaccharide. These results demonstrated that puerarin inhibited nociceptive responses induced by ATP, α,β-meATP, or formalin and its inhibitory effect was mediated by reduction of inflammatory pain-induced up-regulation of $P2X_3$ receptor expression and blockade of ATP binding sites on the $P2X_3$ receptor. Our results suggest that puerarin could decrease acute pain mediated by the $P2X_3$ receptor in the DRG.

Key words: Puerarin, acute nociceptive response, $P2X_3$ receptor, dorsal root ganglia, whole cell patch clamp.

INTRODUCTION

Peripheral administration of purine 2X (P2X) agonists rapidly causes nociceptive behaviors in experimental animals and pain sensation in humans (Andó and Sperlágh, 2013; Burnstock, 2013; Sperlágh et al., 2006). Most of the nociceptive responses to peripheral adenosine triphosphate (ATP) are mediated by the $P2X_3$ receptor, and the $P2X_3$ receptor plays a crucial role in facilitating pain transmission. Animal experiments have demonstrated that a dose-dependent nociceptive response could be observed after the intraplantar injection of α,β-meATP in conscious rats, such as paw lifting, withdrawing, licking, and other pain defensive behaviors (Andó et al., 2010; Cherkas et al., 2012; Ford and Undem, 2013). The number of pain defensive behaviors was significantly reduced after formalin injection into the paws of $P2X_3$-deficient mice (Cockayne et al., 2005; Souslova et al., 2000). It has been shown that $P2X_3$ mRNA and protein expression in the dorsal root ganglia (DRG) were increased, and that ATP-gated currents mediated by the $P2X_3$ receptor from primary sensory neurons were significantly enhanced after neuropathic pain and inflammatory pain stimuli (Borsani et al., 2010; Calvert et al., 2008; Honore et al., 2002; Krimon et al., 2013; Okubo et al., 2010; Tsuda et al.,

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2007; Xu et al., 2012; Yu et al., 2013). These results suggest that ATP and inflammatory substances (such as formalin) are involved in the transmission of pain via activation of the $P2X_3$ nociceptor (Borsani et al., 2010; Fountain, 2013; Li et al., 2013; Nones et al., 2013; Pan et al., 2009).

Radix puerariae (*R. puerariae*) is the dried root of *Pueraria lobata* (Willd.) Ohwi and *Pueraria thomsonii* benth. In China, *R. puerariae* is known as 'Ge Gen', and has been used as a traditional medicine for the management of various diseases including cardiovascular disorders. *R. puerariae* is also known as Kadzu root in the West and contains significant amounts of the isoflavonoid puerarin (PUE) [4*H*-1-benzopyran-4-one, 8-b-D-glucopyranosyl-7-hydroxy-3-(4-hydroxyphenyl), $C_{21}H_{20}C_9$], which is a major active ingredient extracted from the traditional Chinese medicine Ge-gen (*Radix Puerariae*; Rong et al., 1998). The uses of Ge Gen described in pharmacopoeias and in traditional systems of medicine are for the treatment of fever, pain, diabetes mellitus, measles, acute dysentery, or diarrhea, and PUE is widely used in the treatment of cardiovascular diseases in China (Gao et al., 2007; Rong et al., 1998; Zhang et al., 2013). PUE has been shown to possess antioxidant properties such as scavenging reactive oxygen species, increasing superoxide dismutase activity and inhibiting protein nonenzymatic glycation (Guo et al., 2003; Xu, 2003). Both Ge Gen and PUE soup exhibit very similar effects on the inhibition of inflammatory responses and oxidative damage (Peng et al., 2013), suggesting that PUE is the functional active ingredient in anti-nociceptive responses and analgesia. Thus, to understand basic mechanisms underlying PUE inhibition of nociceptive responses, we studied the effects of PUE on acute nociceptive responses in rats. Nociception was induced by both intrathecal and intraplantar injection of ATP/α,β-meATP or intraplantar injection of formalin. The expression levels of $P2X_3$ mRNA and protein after intraplantar injection of formalin were assessed.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (180 to 230 g) were provided by the Center of Laboratory Animal Science of Nanchang University. The animals were housed in plastic cages (five per cage) with room temperatures between 21 and 25°C. Animal use was inspected and approved by the Animal Care and Use Committee of Medical College of Nanchang University. The IASP's ethical guidelines for pain research in animals were followed. All animals were treated in accordance with ARVO Statement for the use of Animals in Ophthalmic and Vision Research in China.

Antibodies and reagents

$P2X_3$ antibody was purchased from Chemicon International Inc. (Temecula, CA). Array slides were obtained from Qiagen (Valencia, CA, USA). β-Actin was from Advanced Immunochemicals (Long Beach, CA). PUE was the product of Kangenbei Pharmaceutical Limited Corporation, China. ATP, α,β-methylen-ATP (α,β-meATP), and LPS were obtained from Sigma-Aldrich (St. Louis, MO). Formalin was obtained from Shanghai Reagent Company (20100300, GB685-79, Shanghai, China). All drugs were dissolved and diluted in 0.9% saline (NS). Other antibodies and reagents are further described in the text.

Animal groups

Animals were randomly divided into three groups and each group was further divided into subgroups as follows and the PUE/NS, NS/NS group for sharing:

Groups of nociceptive behavior induced by α,β-meATP were: NS/α,β-meATP (meaning is "intrathecal injection of NS/intraplantar injection of α,β-meATP", and the following description as the same), PUE/α,β-meATP, α,β-meATP/α,β-meATP, and α,β-meATP+PUE/α,β-meATP. Groups of nociceptive behavior induced by ATP were: NS/ATP, PUE/ATP, ATP/ATP, and ATP+PUE/ATP. Groups of nociceptive behavior induced by formalin were: NS/formalin, PUE/formalin, PUE/NS, and NS/NS.

Observation of rat pain defensive behaviors

Experimental rats were raised in the laboratory for 1 week before being tested. The laboratory temperature was maintained between 21 and 25°C. Rats were placed inside a transparent organic glass box (20×30×30 cm^3) on a stainless steel mesh floor and allowed to acclimate for behavioral experiments. For intrathecal injections, ATP (10 μmol/L), α,β-meATP (1 μmol/L), or different concentrations of PUE were injected into the L5 and L6 space with a trace syringe (18-G needle) after rats were anesthetized with ethylether. The total volume of intrathecal injection was 15 μl for each experiment. A successful injection was indicated by movement (swing) of the animal tail and hind limbs. Rats awoke after 2 to 6 min. For intraplantar injections, ATP (10 μmol/L), α,β-meATP (1 μmol/L), or 2.5% formalin was injected into the left foot with a trace syringe (26G needle). The drugs used for intraplantar injections were diluted to 100 μl of NS and injected at one time. After injection, animals were monitored for 60 min to observe nociceptive responses, such as paw lifting, withdrawing and licking, the occurrence of nociceptive responses from the paw of the injected side were counted. The frequency of lifting, withdrawing and licking the foot/5 min (times/5 min) were measured for estimating the effects of intrathecally applied PUE on nociception induced by α,β-meATP, ATP or formalin injected into rat hindpaw. Each rat was tested only once.

Reverse transcription-polymerase chain reaction (RT-PCR) analyses

Two hours after the intraplantar injection of NS or formalin (2.5%), animals were anesthetized with penthiobarbital sodium (Shanghai Xingya Medical Company, Batch No: 050101), and then ipsilateral L4-L6 DRG were dissected and harvested. The expression of $P2X_3$ mRNA in DRG was detected by RT-PCR. Total RNA was isolated from DRG by the TRIZOL Reagent (Invitrogen) with the guanidinium isothiocyanate method and subjected to DNase I digestion (Pharmacia; 0.1 U/ml, 15 min, 37°C) to eliminate genomic contamination. $P2X_3$ forward and reverse primer sequence genes were 5′-CAACTTCAGGTTTGCCAAA-3′ and 5′-TGAACAGTGAGGGCCTAGAT-3′, respectively, and the size of the product was 519 bp. β-actin, as an internal control, was also amplified using specific primers (forward and reverse sequences were 5′-TAAAGACCTCTATGCCAACACAGT-3′ and 5′-

CACGATGGAGGGGCCGG ACTCATC-3′), with the size of the product being 240 bp. Band densities were measured using the Gel Imaging System software (Junyi Shanghai) and normalized to each β-actin internal control.

Immunohistochemistry for detecting immunoreactivity

Ipsilateral L4-L6 DRG dissection and harvest were the same as described earlier. The isolated DRGs were washed using phosphate-buffered saline (PBS). After fixing with 4% paraformaldehyde (PFA) for 24 h, the ganglia were dehydrated by 20% sucrose for overnight at 4°C, and then ganglia were cut 20 μm in thickness via a cryostat. Immunohistochemical staining was performed using a SP-9001 kit (Beijing Zhongshan Biotech Co). Rabbit anti-$P2X_3$ was obtained from Chemicon International, Inc. (1:2500 dilution in PBS), biotinylated goat anti-rabbit secondary antibody and streptavidin-horseradish peroxidase were obtained from Beijing Zhongshan Biotech Company. The average optical density of $P2X_3$ receptor expression in ganglia was analyzed using an image scanning analysis system (HMIV-2000, Wuhan). Background was determined by average optical density (OD) of ten random areas (from positive cell). Negative control experiments were also conducted to confirm $P2X_3$ receptor expression in the ganglia (figure not shown).

Western blotting analysis

Animals were sacrificed and the collected tissues were quick frozen in tubes on dry ice. DRG was then isolated immediately and rinsed in ice-cold PBS. Ganglia were homogenized by mechanical disruption in lysis buffer and incubated on ice for 50 min. Homogenate was then pelleted at 12000 rpm for 10 min and the supernatant was collected. The quantity of total protein was determined in the supernatant using the Lowry method. After being diluted with sample buffer and being heated to 95°C for 10 min, samples containing equal amounts of protein (20 μg) were separated by SDS-polyacrylamide gel (10%) electrophoresis using a Bio-Rad electrophoresis device, and subsequently transferred onto nitrocellulose (NC) membrane under the same system. The labeled proteins were visualized with enhanced chemiluminescence on high-performance film (Shanghai Pufei Biotech Co). Chemiluminescent signals were collected on autoradiography film, and the band intensity was quantified using AlphaImager 2200 software. The antibodies and their dilutions were: rabbit polyclonal anti-$P2X_3$ (1:1000; Chemicon International Co.), monoclonal β-actin (1:10,000; Advanced Immunochemicals, Long Beach, CA), and secondary antibody (goat anti-rabbit IgG (1:3000, Beijing Zhongshan Biotech Co.). Band densities were normalized to each β-actin internal control.

Homology modeling and molecular docking

The MOE 2012.10 Docking program was used for protein ligand docking calculations (Naim et al., 2007; Sanner, 1999). Docking models the interaction between a ligand and a receptor active site by computer simulation. The technique of docking is to position the ligand in different orientations and conformations within the binding site to calculate optimal binding geometries and energies. MOE's Dock application searches for favorable binding configurations between small- to medium-sized ligands and a not-too-flexible macromolecular target, usually a protein. For each ligand, a number of configurations called poses are generated and scored. The score can be calculated as either a free energy of binding, which takes into account solvation and entropy, or the enthalpic term of the free energy of binding, or a qualitative shaped-based numerical measure. The final top-scoring poses, along with their scores and conformation energies, are written to a database where they are ready for further analysis.

Based on the published crystal structure of the $zfP2X_4$ channel in its closed state (Kawate et al., 2009), the extracellular loop and transmembrane portion of the rat $P2X_3$ receptor ($rP2X_3$) was modeled. The standard modeling techniques implemented in SPDBV4.1.0 (Swiss-PdbViewer) generate a homology model of the $rP2X_3$ (Guex and Peitsch, 1997). Homology modeling was performed with the SWISS-MODEL online server for automated protein homology modeling (Kiefer et al., 2009). Protein Data Bank entry 3I5D, which is believed to represent the closed state of the channel, was used as a template. The sequence of $rP2X_3$ was retrieved from accession number P49654 of the UniProtKB database. Sequence alignment between the template and the model sequence was performed using a modified version of the alignment algorithm. In this approach, alignments are computed by optimizing a function based on residue similarity scores. Structure obtained from homology modeling was verified by PROCHECK (Pontius et al., 1996). PUE (CID 5281807) was downloaded from Pubchem, and prepared by ChemBioDraw Ultra 11.0 and Chimera1.6.1.

Whole cell patch clamp recording

Full details of the electrophysiological methods have been previously reported (Kong et al., 2013). In brief, rats were anesthetized with urethane (1.2 g/kg, i.p.) and DRG neurons were superfused continuously with external solution containing (in millimolar): NaCl 150, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (HEPES) 10, and D-glucose 10 (osmolarity adjusted to 340 mM with sucrose, pH adjusted to 7.4 with NaOH). Cells were patch-clamped in the whole-cell configuration using pipettes with a resistance of 3 to 5 MΩ when filled with the following solution (in millimolar): KCl 140, $MgCl_2$ 2, HEPES 10, EGTA 11, and ATP 5 (pH adjusted to 7.2 with KOH). Cells were held at −60 mV, data were filtered at 1 kHz, and acquired by means of a DigiData 132XInterface and pClamp 10.0 software (Molecular Devices, Sunnyvale, CA, USA). To obtain stable and reproducible $P2X_3$ receptor currents, its synthetic and specific agonist α,β-methylene-ATP (α,β-meATP) was applied with a fast superfusion system, and current peak amplitudes were measured. The drugs were dissolved in external solution and delivered by gravity flow from an array of tubules (500 μm OD, 200 μm ID) connected to a series of independent reservoirs. The distance from the tubule mouth to the cell examined was approximately 100 μm.

Statistical analysis

Statistical analyses of the data were performed using SPSS 17.0. All results were expressed as mean ± standard errorSE. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Fisher post hoc test for multiple comparisons and the unpaired t test for between two groups comparisons, $p < 0.05$ was considered significant. Graphs were prepared using Sigmaplot 11.0 software.

RESULTS

A successful injection was indicated by movement (swing) of the animal tail and hind limbs. No animal deaths in the experimental process.

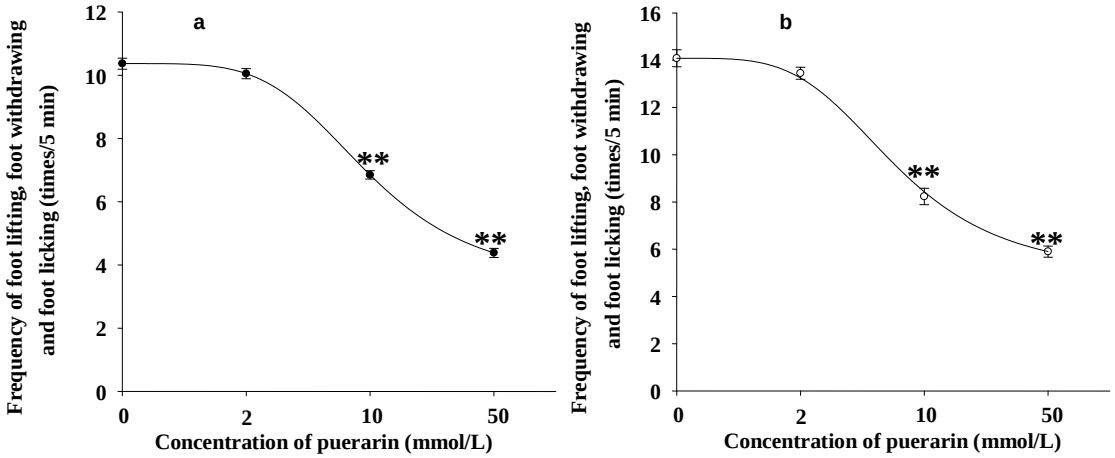

Figure 1. Effects of intrathecal administration of puerarin on acute nociception induced by both intrathecal and intraplantar injection of $P2X_3$ receptor agonist α,β-meATP or ATP. (a) Acute nociception induced by both intrathecal and intraplantar injection of α,β-meATP. (b) Acute nociception induced by both intrathecal and intraplantar injection of ATP. **p < 0.01 compared with no puerarin, n = 5 rats in each group, data are expressed as mean ± SE.

Table 1. Effects of intrathecally applied PUE on nociception induced by α,β-meATP injected into rat hindpaw

Group	Frequency of lifting, withdrawing and licking the foot/5 min
NS/NS	1.25±0.11
PUE/NS	1.37±0.03
NS/α,β-meATP	4.38±0.14***
PUE/α,β-meATP	2.13±0.09
α,β-meATP/α,β-meATP	10.37±0.17***###
α,β-meATP+PUE/α,β-meATP	6.85±0.13@@

The significant difference was denoted as ***p<0.001 compared with the data in NS/NS group or PUE/NS group, ###p<0.001 compared with the data in NS/α,β-meATP group or PUE/α,β-meATP, and @@p<0.01 compared with the data in α,β-meATP/α,β-meATP group; n=5 rats in each group, data shows with mean±SEM.

PUE reduces nociceptive behavior mediated by α,β-meATP

The $P2X_3$ agonist α,β-meATP can induce nociceptive responses in animalsb(Andó and Sperlágh, 2013; Burnstock, 2013). Intrathecal co-administration of PUE (2, 10, 50 mmol/L) and α,β-meATP (1 μmol/L) produced significant and dose-dependent reduction of nociceptive paw lifting, withdrawing and licking behavior in rats injected with α,β-meATP into the hindpaw and potentiated by intrathecal injection of α,β-meATP (1 μmol/L) (Figure 1a).

There was no obvious pain response in NS/NS rats. When NS (15 μl) was intrathecally injected, acute nociception in the rat hindpaw induced by intraplantar injection of α,β-meATP (1 μmol/L; NS/α,β-meATP) was higher than that in the NS/NS group (n = 5 per group, unpaired t test, t_8=17.234, p < 0.001) (Table 1). Both intrathecal injection of PUE (10 mmol/L) and intraplantar injection of α,β-meATP (1 μmol/L) produced less nociceptive paw lifting, withdrawing, and licking behaviors in comparison with rats treated with intrathecal injection of NS and intraplantar injection of α,β-meATP (1 μmol/L; one-way ANOVA, $F_{3,16}$ = 348.479, p < 0.01, n = 5 per group).

Intrathecal injection of α,β-meATP (1 μmol/L) could potentiate the acute nociceptive responses in rat hindpaw induced by intraplantar injection of α,β-meATP (1 μmol/L). The acute nociceptive responses in α,β-meATP/α,β-meATP rats were higher than those in rats treated with intrathecal injection of NS and intraplantar injection of α,β-meATP (n = 5 per group, unpaired t test, t_8 = −42.052, p < 0.001) (Table 1). Nociceptive behaviors in rats treated with intrathecal co-administration of PUE (10 mmol/L) and α,β-meATP (1 μmol/L)/intraplantar injection of α,β-meATP were reduced in comparison with rats

Table 2. Effects of intrathecally applied PUE on nociception induced by ATP injected into rat hindpaw.

Group	Frequency of lifting, withdrawing and licking the foot/5 min
NS/NS	1.25±0.11
PUE/NS	1.37±0.03
NS/ATP	8.22±0.31***
PUE/ATP	5.27±0.16
ATP/ATP	14.08±0.36***###
ATP+PUE/ATP	8.43±0.25@@

The significant difference was denoted as ***p<0.001 compared with the data in NS/NS group or PUE/NS group, ###p<0.001 compared with the data in NS/ATP group or PUE/ATP group, and @@p<0.01 compared with the data of ATP/ATP group; n=5 rats in each group, data shows with mean±SEM.

Table 3. Effects of intrathecally applied PUE on nociception induced by formalin injected into rat hindpaw.

Group	Frequency of lifting, withdrawing and licking the foot/5 min
NS/NS	1.25±0.11
PUE/NS	1.37±0.03
NS/formalin	53.93±0.99**
PUE/formalin	40.8±0.23###

The significant difference was denoted as **p<0.01 compared with the data in NS/NS group, ###p<0.001 compared with the data in NS/formalin group, no difference between NS/NS group and PUE/NS group; n=5 rats in each group, data shows with mean±SEM.

injected with α,β-meATP (α,β-meATP/α,β-meATP; one-way ANOVA, $F_{5,24}$ = 883.653, p < 0.01, n = 5 per group) (Table 1). Our results showed that PUE inhibited nociceptive responses induced by the intrathecal injection of α,β-meATP in rat hindpaw (Table 1).

PUE reduces nociceptive behavior mediated by ATP

ATP can induce the nociceptive behaviors of animals. Intrathecal co-administration of PUE (2, 10, 50 mmol/L) and ATP (10 μmol/L) produced significant and dose-dependent reduction of nociceptive paw lifting, withdrawing and licking behaviors in ATP injected into rat hindpaw potentiated by the intrathecal injection of ATP (10 μmol/L) (Figure 1b).

There was no obvious pain response in rats both treated with intrathecal injection of NS and intraplantar injection of NS. Acute nociception of rats both treated with intrathecal injection of 15 μl NS and intraplantar injection of 100 μl ATP (10 μmol/L) was increased compared with rats in the NS/NS group (n = 5 per group, unpaired t test, t_8 = 20.861, p < 0.001) (Table 2). There was no significant difference between rats treated with intrathecal injection of PUE and intraplantar injection of NS and rats treated with NS/NS (n = 5 per group, unpaired t test, t_8= −1.000, p = 0.347 > 0.05; Table 2).

Intrathecal injection of ATP could potentiate the acute nociceptive responses induced by intraplantar injection of ATP in rat hindpaw. In the intrathecal injection of ATP (10 μmol/L) and intraplantar injection of ATP (10 μmol/L) group, acute nociception was significantly increased in comparison with that in the NS/ATP group (n = 5 per group, unpaired t test, t_8 = −12.281, p < 0.001) (Table 2). Intrathecal co-administration of PUE (10 mmol/L) and ATP (10 μmol/L)/intraplantar injection of ATP, decreased nociceptive behaviors compared with rats intrathecally injected with ATP and intraplantar injected ATP (one-way ANOVA, $F_{5,24}$ = 435.099, p < 0.01, n = 5 per group) (Table 2). No significant difference between the NS/ATP group and ATP+PUE/ATP group was found (one-way ANOVA, $F_{5,24}$ = 435.099, p = 0.52, n = 5 per group) (Table 2). These results indicate that the intrathecal injection of PUE decreased nociceptive behaviors induced by the intrathecal injection of ATP (Table 2).

PUE reduces nociceptive behavior mediated by formalin

After the knockout of the $rP2X_3$, no receptor expression is seen in the DRG, and no spontaneous pain behavior is caused by formalin (Cockayne et al., 2005; Souslova et al., 2000), suggesting that inflammatory substances formalin could produce acute nociceptive responses via the $rP2X_3$. To identify the effect of PUE on acute nociception caused by formalin (2.5%), we compared the acute nociceptive responses of rats in the NS/NS, PUE/NS, NS/formalin, and PUE/formalin groups.

Results showed that acute nociception in rats treated with intrathecal injection NS and intraplantar injection of formalin (NS/formalin) was increased compared with that in rats of other groups (one-way ANOVA, $F_{3,16}$ = 2801.646, p < 0.01, n=5 per group) (Table 3). After both intrathecal injection of PUE (10 mmol/L) and intraplantar injection of formalin, nociceptive paw flinching, licking, and guarding behaviors in rat hindpaw were obviously reduced compared with those in rats treated with intrathecal injection NS and intraplantar injection of formalin (n=5 per group, unpaired t test, t_8 = 13.674, p < 0.001) (Table 3). These aforementioned results indicated that PUE decreased the nociceptive behaviors induced by formalin, which might be mediated via the $rP2X_3$ in rat DRG (Table 3).

RT-PCR demonstrates that PUE decreases the up-regulation of $P2X_3$ mRNA induced by formalin in the DRG

To identify the mechanisms underlying formalin

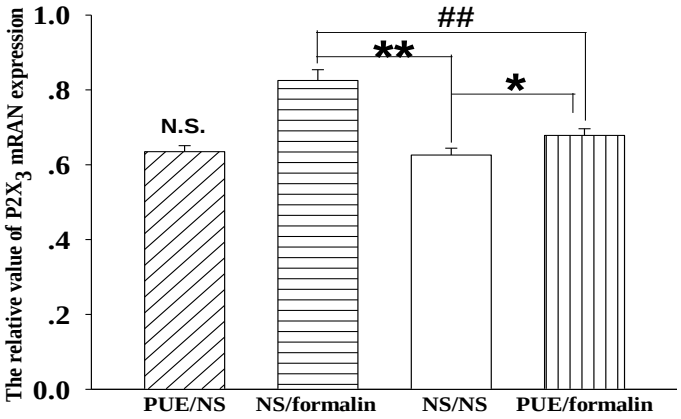

Figure 2. Puerarin reduction of formalin-induced up-regulation of $P2X_3$ mRNA expression in DRG. RT-PCR analysis of DRG showed the up-regulated expression of $P2X_3$ mRNA in the NS/formalin group ($p < 0.01$). Upper panels: representative gels showing RT-PCR products for $P2X_3$ mRNA and β-actin internal control in the same sample. Lower panels: each band density (in arbitrary units) was normalized to its β-actin internal control, and the mean ± SE was shown for the number of independent samples indicated. The expression of $P2X_3$ mRNA in the PUE/formalin group was decreased in comparison with the NS/formalin group ($p < 0.01$). All experiments were conducted in triplicate.
*$p < 0.05$; **$p < 0.01$ compared with the NS/NS group. $^{\#\#}p < 0.01$ compared with the NS/formalin group. N.S. denotes no significant difference between the NS/NS and PUS/NS groups.

involvement in acute nociception, the expression of $P2X_3$ mRNA in rat DRG was further studied using RT-PCR. We studied the effects of PUE on the expression of $P2X_3$ mRNA induced by formalin. On the basis of image analysis, the stain values (average optical density) of $P2X_3$ mRNA expression in NS/NS, PUE/NS, NS/formalin, and PUE/formalin groups were 0.6259 ± 0.0185, 0.6350 ± 0.0161, 0.8253 ± 0.0286, and 0.6783 ± 0.0180, respectively (n = 10 for each group). The stain values of $P2X_3$ mRNA expression in the NS/formalin group were significantly larger than those in the NS/NS, PUE/NS, and PUE/formalin groups (one-way ANOVA, $F_{3,36} = 195.924$, $p< 0.01$, n = 10 per group) (Figure 2). The stain values of $P2X_3$ mRNA expression in the PUE/formalin group were larger than those in the NS/NS group (n = 10 per group, unpaired t test, $t_{18} = -2.296$, $p = 0.034$) (Figure 2). The expression of $P2X_3$ mRNA in the PUE/formalin group was smaller than that in the NS/formalin group (n = 10 per group, unpaired t test, $t_{18} = 13.777$, $p<0.01$) (Figure 2). These results suggest that PUE decreased the up-regulated expression of $P2X_3$ mRNA induced by formalin in rat DRG.

PUE decreases the up-regulation of $P2X_3$ immunoreactivity induced by formalin in the DRG

Immunohistochemistry was used to explore whether PUE affected the increased expression of $P2X_3$ immunoreactivity in DRG after the intraplantar injection of formalin. On the basis of image analysis, the stain values (average optical density) of $P2X_3$ expression in NS/NS, PUE/NS, NS/formalin, and PUE/formalin groups were 0.6337 ± 0.0382, 0.6178 ± 0.0589, 0.8240 ± 0.0639 and 0.7078 ± 0.0381, respectively (n = 10 for each group). The stain values of $P2X_3$ expression in the NS/formalin group were significantly larger than those in NS/NS, PUE/NS, and PUE/formalin groups (one-way ANOVA, $F_{3,36} = 72.332$, $p < 0.01$, n = 10 per group) (Figure 3). The stain values of $P2X_3$ expression in the PUE/formalin group were smaller than those in NS/formalin group (n = 10 per group, unpaired t test, $t_{18} = 5.892$, $p < 0.01$) (Figure 3). These findings further confirmed that the nociception induced by the intraplantar injection of formalin involved the $rP2X_3$ and that PUE inhibited the up-regulated expression of the $rP2X_3$ in the DRG.

Puerarin decreases the up-regulatioin of $P2X_3$ protein in DRG

To identify whether PUE affected the expression of $P2X_3$ protein in rat DRG after the intraplantar injection of formalin, the expression of $P2X_3$ protein in the DRG was further studied using western blotting. Image analysis showed that average optical density of $P2X_3$ protein expression (normalized to β-actin) in NS/NS, PUE/NS, NS/formalin, and PUE/formalin groups was 0.5556 ± 0.0378, 0.5486 ± 0.0371, 0.9727 ± 0.0541, and 0.6854 ± 0.0374, respectively (n = 10 for each group). Optical density of $P2X_3$ protein expression in the NS/formalin group was significantly larger than the NS/NS, PUE/NS, and PUE/formalin groups (one-way ANOVA, $F_{3,36} = 220.533$, $p < 0.01$, n = 10 per group) (Figure 4). No difference in the intensity of $P2X_3$ protein expression was found between the NS/NS and PUE/NS groups (n = 10 per group, unpaired t test, $t_{18} = -0.416$, $p = 0.682$) (Figure 4). The expression of $P2X_3$ protein in the PUE/formalin group was smaller than that in the NS/formalin group (n =10 per group, unpaired t test, $t_{18} = 13.799$, $p < 0.01$) (Figure 4). Consistent with the immunohistochemistry findings, PUE could inhibit the up-regulated expression of $P2X_3$ protein in rat DRG induced by the intraplantar injection of formalin.

Molecular docking of PUE in a homology-modeled $rP2X_3$

The docking experiments revealed that the hydrophilic cavity formed between two adjacent subunits of the homotrimer presumably represented the ATP-binding site. Of the already-studied conserved residues, many were oriented toward the groove of the pocket, indicating that they may bind ATP directly (Lys63, Lys65, Phe171, Thr172, Asn279, Arg281, Lys299) (Kawate et al., 2009).

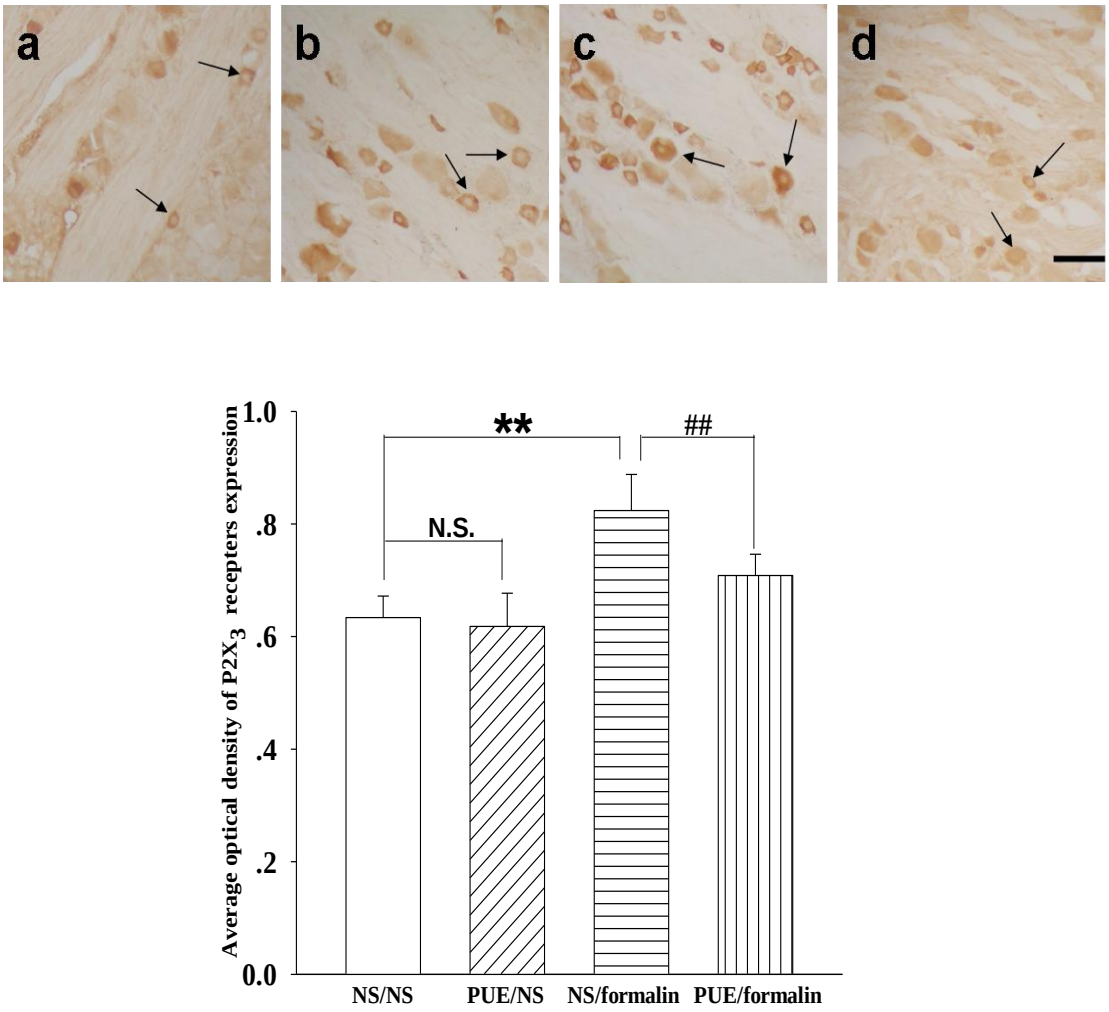

Figure 3. Reduction of $P2X_3$ immunoreactivity by puerarin in rat DRG neurons treated with formalin. $P2X_3$ immunoreactivity in the DRG was quantified (n = 10 each group). Representative results of $P2X_3$ immunoreactivity expression are shown in the upper panel for the NS/NS group (a), PUE/NS group (b), NS/formalin group (c), PUE/formalin group (d). Arrows indicate immunostained neurons; scale bar = 50 μm. Lower panels illustrate the average optical density of $P2X_3$ immunoreactivity expression in each group. The expression level of $P2X_3$ immunoreactivity in PUE/formalin group (d) was lower than the NS/formalin group (c; $p<0.01$). $^{**}p < 0.01$ compared with the NS/NS group; $^{\#\#}p < 0.01$ compared with the NS/formalin group; N.S. = no significant difference.

PUE was shown to be involved in agonist (ATP) binding, which was situated on the opposite sites of the same subunit and was therefore able to form a binding pocket only at the interface of two adjacent subunits (Figure 5a and b). PUE could interact with the protein near the ATP-binding pocket and form hydrogen bonding with Gly66, Gly130, and Arg281 (Figure 5b and c). Interaction energies for the docked-complexes were calculated by MOE 2012.10 as shown in Table 4. The final score of docking between $P2X_3$ and PUE (Kcal/mol) showed that PUE could match and interact perfectly with the $rP2X_3$ (Table 4). The perfect match enabled PUE to interact with residues both deep in the ATP-binding pocket and in the outer sphere.

PUE inhibited the potentiation of $P2X_3$ receptor-mediated currents induced by LPS

The $P2X_3$ receptor is expressed in neurons of the DRG (Burnstock, 2013; Cheng et al., 2013; Noma et al., 2013). $P2X_3$ receptor-mediated currents can be potentiated by LPS (0.5 μg/ml; Franceschini et al., 2013). In this work, the effect of PUE on the potentiation of $P2X_3$ receptor-mediated currents induced by LPS (0.5 μg/ml) was investigated. Figure 6a shows the examples of membrane currents induced by selective $P2X_3$ receptor agonist α,β-meATP (100 μM) to DRG neurons under control conditions, 5 h after LPS (0.5 μg/ml), 5 h after PUE (10 mM) + LPS (0.5 μg/ml), and 5 h after PUE (10 mM). In

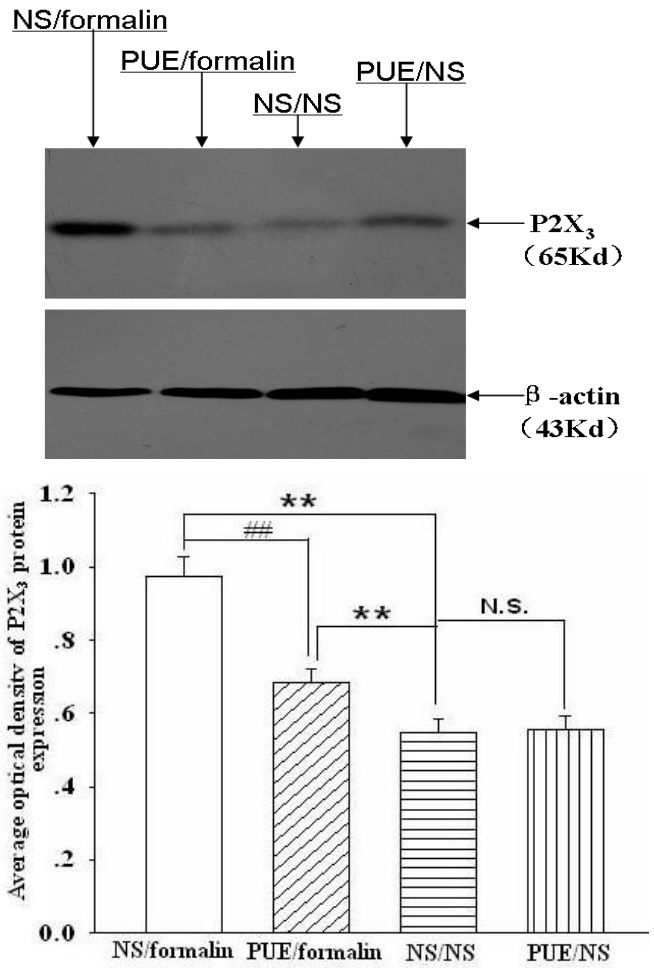

Figure 4. Reduction of $P2X_3$ protein expression by puerarin in rat DRG treated with formalin. The expression levels of $P2X_3$ protein in DRG were measured using western blotting. Representative results are shown in the upper panel. Equal amounts of lysates generated from DRG of each group were electrophoresed under denaturing conditions. The anti-$P2X_3$ antibody recognized a strong band of the expected size (65 kDa). The blot was simultaneously probed for the smaller housekeeping protein β-actin (43 kDa). Lower panel: each band density (in arbitrary units) was normalized to its β-actin internal control. All experiments were conducted in triplicate. **$p < 0.01$ compared with the NS/NS group; $^{##}p < 0.01$ compared with the NS/formalin group. N.S. = no significant difference between the NS/NS vs PUS/NS groups.

In all cases, the agonist application elicited a fast-developing inward current (Figure 6a) that rapidly decayed, because of receptor desensitization, a characteristic typical of currents mediated by $P2X_3$ receptors. When DRG neurons were treated for 5 h with LPS or PUE, a significant potentiation or inhibition of $P2X_3$ receptor-mediated currents was observed ($p < 0.01$) (Figure 6a and b). Nevertheless, when DRG neurons were treated for 5 h with PUE and LPS, the potentiation of α,β-meATP-mediated currents were inhibited as compared with DRG neurons treated for 5 h with LPS ($p > 0.05$).

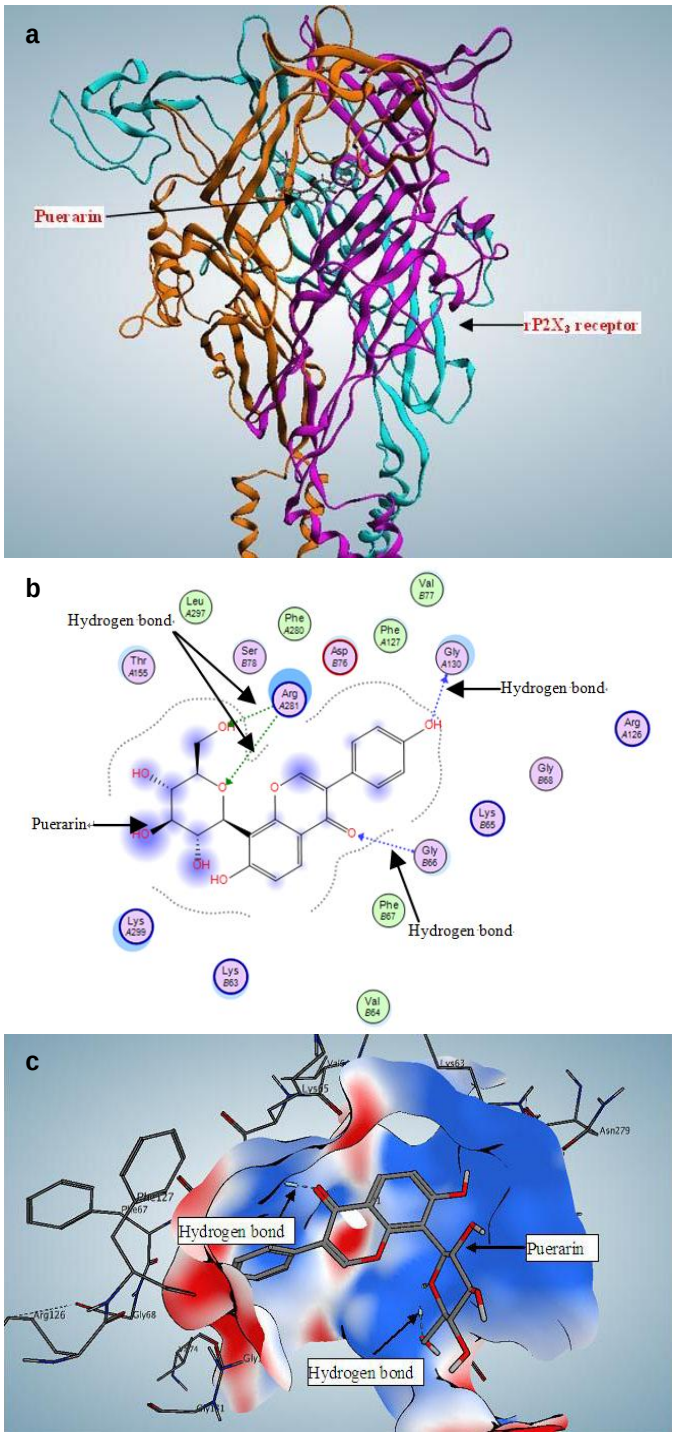

Figure 5. Computer simulation modeling of puerarin docking with the $rP2X_3$ receptor. Molecular docking of puerarin on a homology-modeled $rP2X_3$ receptor was simulated. The groove of the pocket may bind ATP directly (Lys63, Lys65, Phe171, Thr172, Asn279, Arg281, Lys299). ATP-binding sites were located at opposite sites of the same subunit and were therefore able to form a binding pocket only at the interface of two adjacent subunits (Figure 5a, b). Puerarin could interact with $rP2X_3$ receptor protein at the site close to the ATP-binding pocket and form hydrogen bonds with Gly66, Gly130, and Arg281 (Figure 5b, c). The perfect fit enabled the puerarin to interact with residues both deep in the ATP-binding pocket and in the outer sphere.

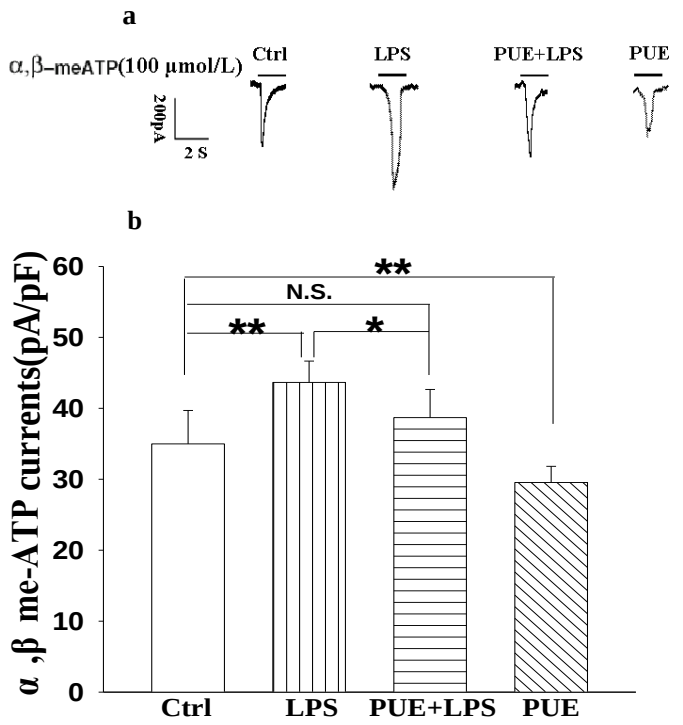

Figure 6. Inhibition of puerarin on the potentiation of $P2X_3$ receptor-mediated currents induced by LPS. Neuronal $P2X_3$ receptor-mediated responses in control conditions and after treatment with LPS or PUE+LPS were observed. (A) Representative examples of currents induced by application of α,β-meATP (100 μM) to DRG neurons in control condition, 5 h after LPS (0.5 μg/ml) or 5 h after PUE (10 mM)+LPS (0.5 μg/ml) application. (B) Histograms show average peak amplitudes of $P2X_3$-mediated currents (Ctrl, n = 32; LPS, n = 36; PUE+LPS, n = 32). *p < 0.05, **p < 0.01, N.S. denotes no significant difference.

Table 4. MOE score of $P2X_3$ and puerarin (Kcal/mol).

S/N	mol	mseq	S
1	puerarin	1	-6.1829
2	puerarin	1	-6.1699
3	puerarin	1	-6.1657
4	puerarin	1	-6.0725
5	puerarin	1	-6.0383
6	puerarin	1	-6.0048
7	puerarin	1	-5.9896
8	puerarin	1	-5.9528
9	puerarin	1	-5.9207
10	puerarin	1	-5.9158
11	puerarin	1	-5.8375
12	puerarin	1	-5.8337
13	puerarin	1	-5.8220
14	puerarin	1	-5.7890
15	puerarin	1	-5.7775
16	puerarin	1	-5.7738
17	puerarin	1	-5.7658
18	puerarin	1	-5.7526
19	puerarin	1	-5.7411
20	puerarin	1	-5.7391
21	puerarin	1	-5.7053

Mol is the research object. How many research objects are shown in MSEQ. S represents the final score.

DISCUSSION

Studies have shown that intraplantar injection of α,β-meATP or ATP produced nociceptive behaviors (such as paw lifting, withdrawing, and licking) and other pain defensive behaviors in conscious rats (Andó et al., 2010; Cherkas et al., 2012; Ford and Undem, 2013). The frequency of these pain defensive behaviors was significantly reduced after formalin injection into the claw of $P2X_3$-deficient mice (Cockayne et al., 2005; Souslova et al., 2000). Activation of the $P2X_3$ receptor was suggested to be involved in signal transmission of pain induced by ATP and inflammatory substances, such as formalin (Borsani et al., 2010; Fountain, 2013; Li et al., 2013; Nones et al., 2013; Pan et al., 2009). Our results showed that nociceptive responses could be induced by the intraplantar injection of ATP or α,β-meATP ($P2X_3$ receptor agonist) in conscious rats and such nociceptive responses were potentiated by intrathecal injection of ATP or α,β-meATP, indicating the nociceptive responses were activated by the $P2X_3$ receptor.

The effective ingredients of *Pueraria lobata* include a variety of flavonoids, such as daidzein, daidzin, PUE, and puerarin-7-xyloside (Peng et al., 2013; Rong et al., 1998), which have been clinically used for cardiovascular and cerebrovascular diseases (Gao et al., 2007; Rong et al., 1998). PUE (a major active ingredient extracted from the traditional Chinese drug called Ge Gen) is widely used for myocardial infarction, coronary heart disease, angina, and other cardiovascular diseases (Gao et al., 2007; Rong et al., 1998; Zhang et al., 2013). As both *Pueraria lobata* and puerarin soup have anti-inflammatory effects (Rong et al., 1998; Zhang et al., 2013), it is highly possible that PUE may have anti-nociceptive effects as well, especially for those nociceptive responses associated with inflammation. In the present study, we have observed that intrathecal injection of PUE inhibited the acute nociception induced by intraplantar injection of α,β-meATP or ATP and strengthened by intrathecal injection of ATP or α,β-meATP in rats. These results suggest that PUE inhibits the nociceptive responses via $P2X_3$ receptors.

It has been reported that neuropathic and inflammatory pain stimuli up-regulate the expression of $P2X_3$ mRNA, and protein in DRG and enhance ATP-gated currents mediated by $P2X_3$ receptor in primary sensory neurons (Borsani et al., 2010; Burnstock, 2013; Cheng et al., 2013; Joseph and Levine, 2012; Krimon et al., 2013; Noma et al., 2013; Prado et al., 2013). It has also been shown that the spontaneous pain behaviors induced by

formalin are significantly reduced in $P2X_3$ receptor knockout mice (Cockayne et al., 2005; Pan et al., 2009; Souslova et al., 2000), and that a variety of pain allergic reactions induced by formalin can be significantly reduced by a specific $P2X_3$ antagonist, A-317491 (Jarvis et al., 2002; Pan et al., 2009). These studies suggest that formalin may injure cells and sensory nerve endings, resulting in release of a large amount of ATP, leading to the up-regulation of $P2X_3$ receptor expression in DRG and ultimately producing nociceptive behavior responses (Calvert et al., 2008; Honore et al., 2002; McGaraughty et al., 2003; Nalepa et al., 2010; Okubo et al., 2010; Xu et al., 2012; Yu et al., 2013). In this study, it was observed that nociceptive behaviors in the NS/formalin group were significantly enhanced compared with those in the NS/NS group. In addition, the expression levels of $P2X_3$ mRNA, and protein in DRG were significantly increased. These results suggested that formalin increases $P2X_3$ expression and strengthens ATP or α,β-meATP-induced paw lifting, withdrawing, and licking and other acute nociceptive responses. When PUE was intrathecally injected, the up-regulated expression of $P2X_3$ mRNA and protein in DRG induced by the intraplantar injection of formalin was significantly reduced compared with the intrathecal injection of NS and intraplantar injection of formalin. Additionally, the nociceptive responses induced by formalin were also significantly decreased. These results indicate that PUE plays a role in the inhibition of formalin-induced acute nociceptive responses by acting on the $P2X_3$ receptor.

Homology modeling of other P2X receptor family members can be generated using the X-ray structure of the closed-state zebrafish (zf) $P2X_4$ receptor as a template (Kawate et al., 2009). Our result for molecular docking of PUE on a homology-modeled $rP2X_3$ indicated that puerarin could block ATP binding sites. As shown in Figure 5, PUE could interact with the $rP2X_3$ protein at the site proximal to the ATP-binding pocket and form hydrogen bonds with Gly66, Gly130, and Arg281. Interaction energies for the docked-complexes were calculated by MOE 2012.10 and are shown in Table 4. A higher value of negative interaction energy was an indicator of more efficient interaction between the $rP2X_3$ and PUE. The $rP2X_3$ can be restricted to binding ATP (increasing the concentration of ATP), because of its combination with PUE and therefore the channel of the $rP2X_3$ is blocked. Taken together, our results indicate that PUE may inhibit transmission of nociceptive information caused by inflammatory substances (ATP or formalin) via down-regulation of the expression levels of $P2X_3$ receptor and blockade of ATP-binding sites of the $P2X_3$ receptor in the DRG.

The $P2X_3$ receptor plays a crucial role in pain transduction (Burnstock et al., 2011). Inflammatory substances, such as LPS, may facilitate the release of ATP, which activated hyper-responsive P2X3 receptor, and then could amplify nociceptive signaling (Franceschini et al., 2013, Leung and Cahill, 2010). Our studies showed that LPS enhanced the $P2X_3$ agonist α,β-meATP-mediated currents, and PUE repressed α,β-meATP -mediated currents. When DRG neurons were treated with PUE and LPS, the up-regulated α,β-meATP currents were decreased. Therefore, these results suggest that PUE can inhibit the α,β-meATP-mediated currents or the potentiation of $P2X_3$ receptor-mediated currents induced by LPS in acute pain.

Conclusions

Our results showed that PUE inhibited $P2X_3$ receptor-mediated acute nociceptive responses by reduction of formalin-induced up-regulation of $P2X_3$ receptor expression and blockade of ATP binding sites of $P2X_3$ receptor in the DRG. Thus, PUE could decrease acute pain mediated by the $P2X_3$ receptor in the DRG.

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