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Noradrenaline Stimulates ATP Release From DRG Neurons by Targeting β_3 Adrenoceptors as a Factor of Neuropathic Pain

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Noradrenaline (NA), released in association with sympathetic nerve sprouting into the dorsal root ganglion (DRG) after peripheral nerve injury, may enhance neuropathic pain. ATP serves as a pain mediator; however, NA-regulated ATP mobilizations in the DRG is far from understanding. In the present study, we analyzed ATP mobilizations in acutely dissociated rat DRG neurons by recording single-channel currents through P2X receptor channels as an ATP biosensor in an outside-out patch-clamp configuration and by monitoring real-time enzymatic NADPH fluorescent imaging, and examined the role for β_3 adrenoceptors in allodynia using an in vivo rat model. We show here that NA stimulates ATP release from DRG neurons as mediated via β_3 adrenoceptors linked to G_s protein involving PKA activation, to cause allodynia. This represents a fresh regulatory pathway for neuropathic pain relevant to noradrenergic transmission in the DRG.
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Noradrenaline (NA), a neurotransmitter released from postganglionic sympathetic nerve terminals, exhibits a wide range of actions via adrenoceptors such as α_1 (α_{1a}, α_{1b}, and α_{1d}), α_2 (α_{2a}, α_{2b}, α_{2c}, and α_{2d}), and β (β_1, β_2, β_3, and β_4) receptors. Sympathetic nerve sprouting into the dorsal root ganglion (DRG) is suggested to occur after peripheral nerve injury (Mclachlan et al., 1993; Kim et al., 2001), suggesting the implication of noradrenergic transmission in nociception. Peripheral nerve axotomy, alternatively, induces hyperexcitation of DRG neurons via a sympathetic pathway (Chung and Chung, 2002), causing neuropathic pain such as hyperalgesia and allodynia. Interestingly, an increase in the mRNAs for α_{1b}, α_{2a}, α_{2b}, and β_2 adrenoceptors is obtained with the ipsilateral DRG neurons after sciatic nerve injury, where NA potentiates slow type of ATP-evoked currents in DRG neurons via α_1 adrenoceptors involving protein kinase C (PKC) activation (Maruo et al., 2006). This may account for amplification of pain through noradrenergic transmission. Blockade of sympathetic nerves or α_1 adrenoceptors is shown to relieve neuropathic pain (Dunn, 2000a,b). NA, thus, appears to serve as a regulator or modulator for neuropathic pain.

ATP mediates communications between neurons and neurons or between neurons and astrocytes via ATP receptors such as ionotropic P2X receptors and metabotropic P2Y receptors (Koizumi et al., 2003; Fields and Burnstock, 2006). ATP receptors are divided into the two major classes of the receptors; ionotropic P2X receptors ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$) and metabotropic P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$). Several lines of evidence have shown that ATP receptors expressed in DRG neurons mediate a variety of pain (Chen et al., 1995; Lewis et al., 1995; Cockayne et al., 2000; Hamilton and McMahon, 2000; Tsuda et al., 2000, 2002; Ueda et al., 2000; Bao et al., 2003; Moriyama et al., 2003; North, 2004; Burnstock, 2006); P2X receptors for acute, inflammatory, and neuropathic pain, $P2X_3$ receptor for spontaneous pain and heat hyperalgesia, $P2X_{2/3}$ receptor for acute mechanical allodynia, and $P2Y_2$ receptor for transient receptor potential vanilloid-receptor-1-mediated thermal hypersensitivity. The ATP receptor agonist ATP is not released from presynaptic terminals in the DRG, since no synapse is present in DRG neurons. Then, where ATP comes from in the DRG is a big question. In addition, it remains unknown whether NA regulates ATP release in the DRG, to cause neuropathic pain.

To address these questions, we analyzed molecular ATP mobilizations by two different methods and assessed the implication of noradrenergic transmission in neuropathic pain using a rat in vivo model. We show here that NA stimulates ATP release from DRG neurons as mediated via β_3 receptors linked to G_s protein involving PKA activation, as a factor of neuropathic pain.

Materials and Methods

Animal care

All procedures have been approved by the Animal Care and Use Committee at Hyogo College of Medicine and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Preparation of DRG cells

The DRGs in the L4–L6 segment were isolated from Wistar rats (male, 6 weeks) and incubated 0.3% (w/v) collagenase at 37°C for 30 min. Then, cells were mechanically dissociated and plated on poly-L-lysine-coated glass cover slips. Experiments were carried out within 12–24 h after preparation.

Single-channel current recording

Outside-out patches were made from acutely dissociated DRG neurons. Single-channel currents were monitored with an Axopatch-200A amplifier (Axon Instruments, Inc., Sunnyvale, CA) in a recording chamber continuously superfused with an extracellular solution [in mM; 135 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 5 *N*-2-hydroxyethyl piperazine-*N*′-2-ethansulfonic acid (HEPES),

Additional Supporting Information may be found in the online version of this article.

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and 10 glucose, pH 7.3] containing 6,7-dinitroquinoxaline-2,3-dione (DNQX) (20 μM) and DL-2-amino-5-phosphonopentanoic acid (DL-AP5) (100 μM) at a flow rate of 2 ml/min at 21–22°C. Single-channel currents evoked by ATP were recorded in the extracellular solution in the presence of DNQX (20 μM) and DL-AP5 (100 μM), and those evoked by glutamate in the presence of DL-AP5 (100 μM). The patch electrode-filling solution was as follows: (in mM) 135 Cs-gluconate, 5 CsCl, 2 $MgCl_2$, 5 ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 5 HEPES, 5 MgATP, and 1 Na_2GTP, pH 7.2. Currents were analyzed using pClamp software (version 9.2, Axon Instruments, Inc.). The slope conductance was calculated from the current amplitude (I)/voltage (V) relation. NA was bath applied with an air-pressure injector (PicoPump PV 820, World Precision Instruments, Stevenage, UK).

Construction and transfection of siRNA

Three cocktails of the siRNAs were used to silence the rat β_3 adrenoceptor gene (B-Bridge International, Inc., Sunnyvale, CA; Table 1). The non-specific siRNAs were constructed as a negative control (B-Bridge International, Inc.; Table 1). Acutely dissociated neurons were transfected with siRNAs together with an enhanced green fluorescence protein (eGFP)-expression vector using a Nucleofector kit (Amaxa GmbH, Cologne, Germany). Single-channel recording, single-cell quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR), and enzymatic fluorescent imaging analysis were carried out 24 h after transfection.

Single-cell quantitative real-time RT-PCR

An eGFP-positive single DRG neuron was picked up with a glass capillary containing 18 μl of lysis buffer [1× first strand buffer, 0.5 mg/ml bovine serum albumin, RNase inhibitor, 0.6% NP40, 0.6% Tween-20, anti-sense primer for β_3 adrenoceptor, anti-sense primer for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), dNTP, and 1 mg/ml yeast tRNA], incubated at 70°C for 1 min to break the cell membrane. Cell lysate was incubated in the presence of DNase I at 37°C for 30 min to remove genomic DNAs and at 70°C for 10 min to inactivate DNase I. Then, the sample was incubated in a DTT solution containing SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) at 55°C for 40 min followed by at 70°C for 15 min. The gene expression was amplified by double PCRs. Initially, PCR was carried out in the reaction solution containing 10× PCR buffer, dNTPs, oligonucleotide, dimethyl sulfoxide [final concentration, 5% (v/v)], and 5 U of Taq polymerase (Fermentas, St. Leon-Roth, Germany; final volume, 20 μl) with a PCR Thermal Cycler Dice (Takara, Otsu, Japan) programmed as follows: first step, 98°C for 4 min, the ensuring 40 cycles, 98°C for 1 sec, 65°C for 15 sec, and 72°C for 30 sec. Subsequently, real-time PCR was performed using the first PCR products with a SYBR Green real-time PCR Master Mix (Toyobo, Osaka, Japan) and the Applied Biosystems 7900 real-time PCR detection system (ABI, Foster City, CA) programmed as follows: first step, 98°C for 4 min; the ensuring 40 cycles, 98°C for 1 sec, 65°C for 15 sec, and 72°C for 30 sec. The expression level of the rat β_3 adrenoceptor mRNA was normalized by that of GAPDH mRNA. RT-PCR was carried out using primers described in Table 1.

Enzymatic fluorescent imaging analysis for ATP

ATP was indirectly monitored with real-time enzymatic NADPH fluorescent imaging (Corriden et al., 2007). Glucose and ATP (1:1) are converted into glucose-6-phosphate (G6P) and ADP by hexokinase (HK). Subsequently, glucose-6-phosphodehydrogenase (G6PDH) reduces nicotinamide adenine dinucleotide phosphate ($NADP^+$) to NADPH, a product with fluorescence, in parallel with a reaction from G6P to 6-phosphogluconolactone. ATP mobilizations, therefore, can be monitored by detecting NADPH fluorescent signals. Acutely dissociated rat DRG neurons were incubated in an extracellular solution (in mM; 145 NaCl, 5 KCl, 2.4 CaCl2, 10 glucose, 10 HEPES, pH7.4) containing HK (150 μg/ml), G6PDH (30 μg/ml), and NADP+ (50 μM). The fluorescent signal of NADPH produced in reaction with ATP released from a neuron was detected at an excitation of 365 ± 10 nm and an emission of over 400 nm using an UV laser light, visualized with an intensified charge coupled device camera (ARGUS-50/CA, Hamamatsu Photonics, Hamamatsu, Japan), and analyzed with an image-processing software (AQUACOSMOS, Hamamatsu Photonics).

Assessment of tactile allodynia using a rat model

Under anesthesia with pentobarbital sodium (50 mg/kg, i.p.) the spinal nerve from the L5 segment on the left side for Wistar rats (male, 5 weeks) was exposed and ligated at distal to the DRG. For unlesioned group, operation was done without ligation after exposure of the spinal nerve from the L5 segment on the left side.

Four weeks later, rats were transferred in a clear plastic, mesh-bottomed cage for 30 min to habituate to environments. Then, mechanical pain stimuli were applied to left-sided plantar surface of the hind-paw through below the mesh floor using calibrated von Frey filaments (0.02–26 g; North Coast Medical, Morgan Hill, CA), before and after intraperitoneal injection with vehicle or SR59230A. The hind-paw withdrawal threshold was measured by the up-down method (Chaplan et al., 1994).

Results

Spontaneous ATP release from DRG neurons

In the immunocytochemical analysis using an antibody against microtubule-associated protein 2 (MAP2) for neurons, S-100 for Schwann cells, fibronectin for fibroblasts, and glial fibrillary acidic protein (GFAP) for satellite cells, we identified each cell type in acutely dissociated DRG cells (Supplementary Fig. 1A–D).

An outside-out patch was made from an acutely dissociated DRG neuron, and P2X receptor-mediated single-channel

TABLE 1. Oligonucleotide sequence for siRNA construction and PCR primers

Name	Oligonucleotide sequence
siRNA	
β_3 adrenergic receptor	5′-GAGGCAACCUGCUGGUAAUTT-3′ and 5′-AUUACCAGCAGGUUGCCUCTT-3′ 5′-GAGUGUUCGUCGUAGCUAATT-3′ and 5′-UUAGCUACGACGAACACUCTT-3′ 5′-CGACCAAGUUUGAGGUUUUTT-3′ and 5′-AAAACCUCAAACUUGGUCGTT-3′
Non-specific siRNA	5′-ATCCGCGCGATAGTACGTA-3′ 5′-TTACGCGTAGCGTAATACG-3′ 5′-TATTCGCGCGTATAGCGGT-3′
PCR primers	
β_3 adrenergic receptor (NM_013108)	Sense: 5′-CAGGCAGAACTCACCGCTCAACAG-3′ Anti-sense: 5′-CCTAAGCATCCAGCACAAACATAATAAGC-3′
GAPDH (BC059110)	Sense: 5′-CCTTCCGTGTTCCTACCCCCAAT-3′ Anti-sense: 5′-CCTCTCTCTTGCTCTCAGTATCCTTGCT-3′

currents were recorded by approaching the patch-electrode tip close to DRG cells; if ATP is released from a cell, single-channel currents should be evoked by activating P2X receptors (Supplementary Fig. 2). When the patch-electrode tip was brought within 5 μm close to a neuron without satellite cells, single-channel currents were evoked; we call here the currents "neuron-evoked single-channel currents (NeSCCs)" (Fig. 1A), while no current was induced when a distance between the patch-electrode tip and a neuron was over 5 μm (Fig. 1B). NeSCCs were abolished by pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (10 μM), an inhibitor of P2X receptors (Fig. 1A), suggesting that P2X receptors present in outside-out patches are activated in response to ATP released from DRG neurons. The slope conductance of NeSCCs was 37.7 ± 1.1 pS, that was well consistent with the conductance (37.6 ± 2.1 pS) for ATP (100 μM)-evoked single-channel currents in outside-out patches from DRG neurons, distinct from the conductance (14.1 ± 1.8 pS) for glutamate (1 mM)-induced single-channel currents (Fig. 2A,B). This further supports the note that P2X receptors are responsible for NeSCCs. Consequently, these results provide evidence for spontaneous ATP release from DRG neurons. This also indicates that ATP, released from DRG neurons, activates its own P2X receptors expressed in DRG neurons.

When the patch-electrode tip was positioned near to Schwann cells or fibroblasts, single-channel currents were never evoked (Fig. 1C,D), which excludes the possibility for ATP release from Schwann cells or fibroblasts. Approaching the patch-electrode tip to a neuron enclosed with satellite cells induced single-channel currents with the slope conductance of 37.6 ± 2.2 pS at the event frequency of 0.9 ± 0.4/sec, similar to the conductance and the frequency (1.0 ± 0.6/sec) for a neuron alone without satellite cells (Fig. 1E). This suggests that ATP released from a neuron leaks out through gaps between satellite cells, although there is no direct evidence that satellite cells do not release ATP, since we could not isolate satellite cells alone.

Regulation of ATP release from DRG neurons via β_3 adrenoceptors

Evidence has pointed to the participation of noradrenergic transmission in nociception (Mclachlan et al., 1993; Dunn, 2000a,b; Kim et al., 2001; Chung and Chung, 2002; Maruo et al., 2006). This prompted us to assess the effect of NA on ATP release from DRG neurons. Bath-application with NA (100 μM) to a neuron significantly increased the frequency of NeSCCs with the slope conductance of 37.8 ± 1.0 pS (Fig. 3A,C), and the currents were blocked by PPADS (10 μM; Fig. 3A). An increase in the frequency was inhibited by propranolol (10 μM), a broad inhibitor of β adrenoceptors, while it was not affected by prazosin (1 μM), an inhibitor of α_1 adrenoceptors, and yohimbine (2 μM), an inhibitor of α_2 adrenoceptors (Fig. 3B,C). Notably, SR59230A (100 nM), a selective inhibitor of β_3 adrenoceptors, but not CGP20712 (300 nM), an inhibitor of β_1 adrenoceptors, and ICI118551 (50 nM), an inhibitor of β_2 adrenoceptors, abolished the NA effect (Fig. 3B,C). These results indicate that NA increases the frequency of NeSCCs via β_3 adrenoceptors, that is, NA stimulates ATP release from DRG neurons via β_3 adrenoceptors.

To obtain further evidence for this, we examined the effect of NA on NeSCCs using DRG neurons transfected with the siRNA silencing the β_3 adrenoceptor-targeted gene. In the single-cell quantitative real-time RT-PCR analysis, expression of the β_3 adrenoceptor mRNAs was significantly reduced for acutely dissociated rat DRG neurons transfected with the siRNA (Fig. 4A). When applied to a neuron transfected with the

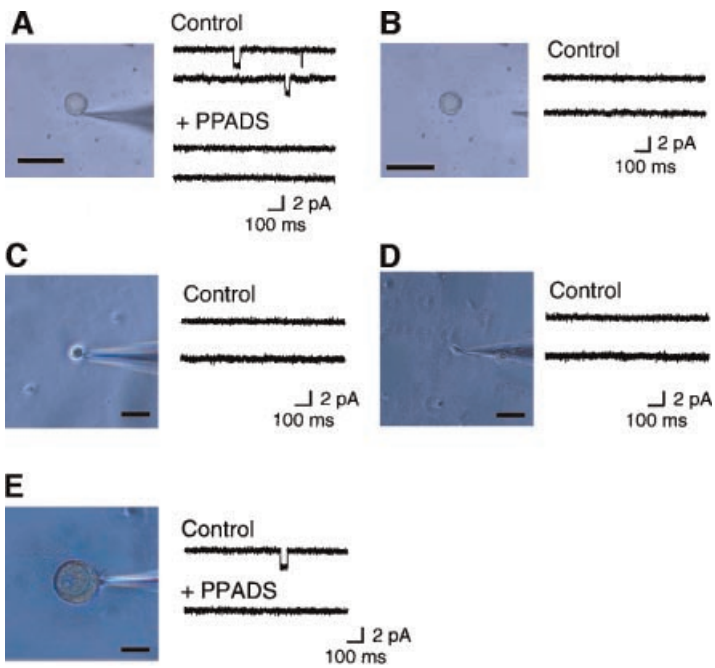

Fig. 1. NeSCCs are evoked in response to ATP released from a DRG neuron. Outside-out patches were made from acutely isolated DRG neurons, and the patch-electrode tip was brought within 5 μm close to a neuron without satellite cells (A), a Schwann cell (C), a fibroblast (D), and a neuron enclosed with satellite cells (E) or positioned over 5 μm far from a neuron (B) in the presence and absence of PPADS (10 μM). In cases of (A) and (E), single-channel currents were evoked, that is abolished in the presence of PPADS, while no current was induced in the remaining cases. The holding potential was −70 mV. Bars in the photos: 50 μm.

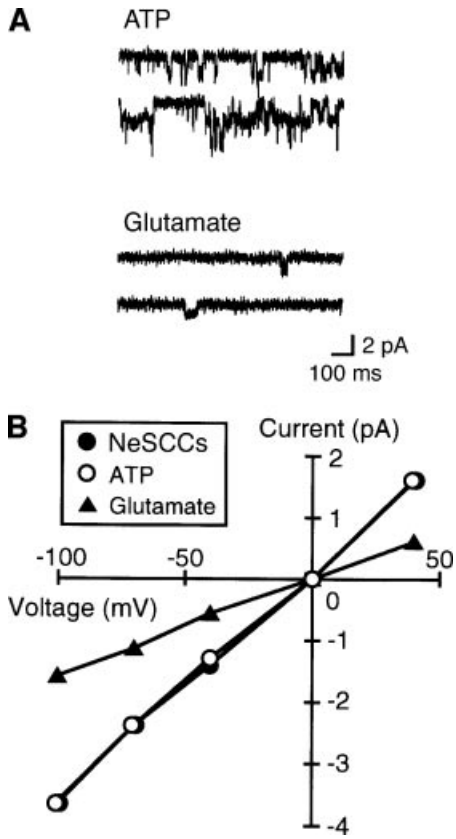

Fig. 2. NeSCCs are produced through P2X receptor channels. A: Outside-out patches were made from acutely isolated DRG neurons, and single-channel currents were evoked by bath-application with ATP (100 μM) or glutamate (1 mM). The holding potential was −70 mV. B: Typical current (I)/voltage (V) relations for NeSCCs (n = 8 independent patches), ATP-induced single-channel currents (n = 5 independent patches), and glutamate-induced single-channel currents (n = 4 independent patches) are shown in the graph.

negative-control siRNA, NA (100 μM) significantly increased the frequency of NeSCCs (Fig. 4B). The NA effect, on the other hand, was never found in a neuron with knocking down β_3 adrenoceptors (Fig. 4B). This confirms that NA stimulates ATP release from DRG neurons via β_3 adrenoceptors.

We subsequently monitored ATP mobilizations with a real-time enzymatic NADPH fluorescent imaging method in acutely dissociated rat DRG neurons. NA (100 μM) enhanced an NADPH signal in acutely dissociated rat DRG neurons (Fig. 5A). If HK and G6PDH react to intracellular ATP, then the fluorescent signal should be increased. No increase in the fluorescent signal, however, was found by adding those enzymes to extracellular solution for neurons without NA stimulation (data not shown). This implies that the NADPH signals detected here are reactions to extracellular ATP released but not intracellular ATP. NA-induced NADPH signal rise was significantly inhibited by propranolol (10 μM; Fig. 5A) or SR59230A (100 nM; Fig. 5B). Moreover, no enhancement in the NADPH signal was found by silencing the β_3 adrenoceptor-targeted gene (Fig. 5C). These results further support the note that NA exerts its stimulatory action on ATP release from DRG neurons via β_3 adrenoceptors.

NA (100 μM)-induced increase in the frequency of NeSCCs was prevented by H-89 (1 μM), an inhibitor of PKA, while the

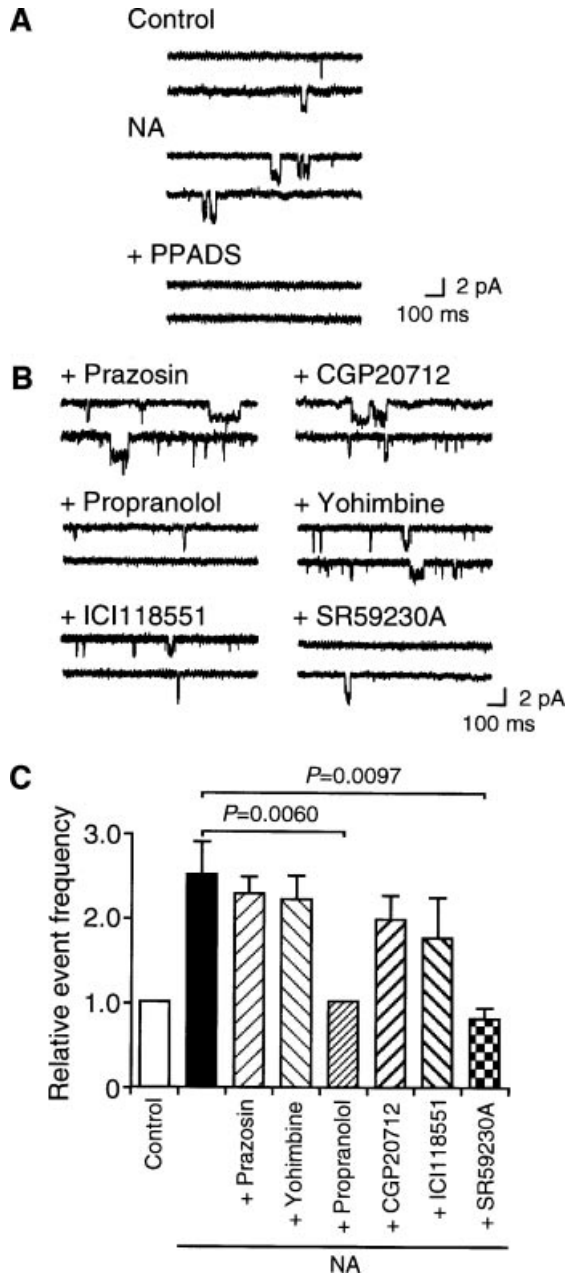

Fig. 3. NA increases the frequency of NeSCCs via β_3 adrenoceptors. Outside-out patches were made from acutely isolated DRG neurons, and NeSCCs were recorded at a holding potential of −70 mV. A: NeSCCs were monitored before and after bath-application with NA (100 μM) in the presence and absence of PPADS (10 μM). B: NeSCCs were assayed before and after bath-application with NA (100 μM) in the absence and presence of prazosin (1 μM), yohimbine (2 μM), propranolol (10 μM), CGP20712 (300 nM), ICI118551 (50 nM), or SR59230A (100 nM). C: In the graph, the event frequency (mean ± SEM) was normalized by regarding the frequency of NeSCCs before application with NA (control) as 1 (n = 4–8 independent patches). *P*-values, one-way analysis of variance (ANOVA).

PKC inhibitor GF109203X (100 nM) had no effect (Fig. 6A,B). This, in the light of the fact that β_3 adrenoceptor is linked to G_S protein involving adenylate-cyclase-catalyzed cAMP production followed by PKA activation (Sato et al., 2005), indicates that NA stimulates ATP release from DRG neurons under the control of PKA activated via β_3 adrenoceptors linked to G_s protein.

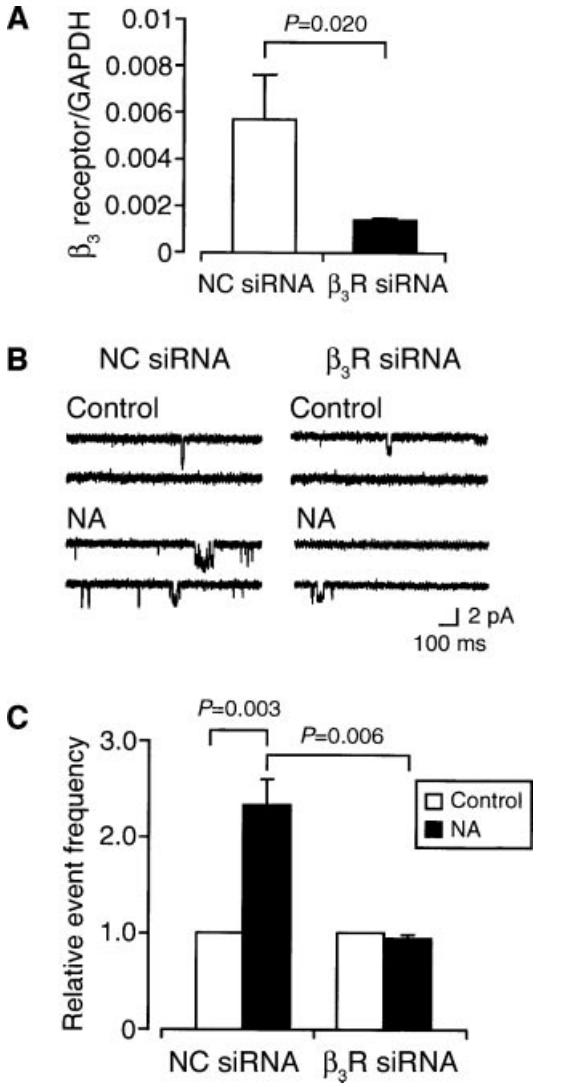

Fig. 4. NA-induced increase in the frequency of NeSCCs is prevented by knocking down β_3 adrenoceptors. Acutely dissociated DRG neurons were transfected with the negative control siRNA (NC siRNA) or the siRNA silencing the β_3 adrenoceptor-targeted gene (β_3R siRNA), together with eGFP expression vector to identify transfected neurons. A: Single-cell quantitative real-time RT-PCR was performed 24 h after transfection. Expression of the β_3 adrenoceptor mRNA was normalized by expression of the GAPDH mRNA. In the graph, each value represents the mean (±SEM) ratio of the β_3 receptor mRNA/the GAPDH mRNA (n = 5 independent experiments). *P*-values, one-way ANOVA. B: NeSCCs were monitored before and after bath-application with NA (100 μM) in neurons with NC siRNA or β_3R siRNA at a holding potential of −70 mV. C: In the graph, the event frequency (mean ± SEM) was normalized by regarding the frequency of NeSCCs before application with NA (control) as 1 (n = 5 independent patches). *P*-values, one-way ANOVA.

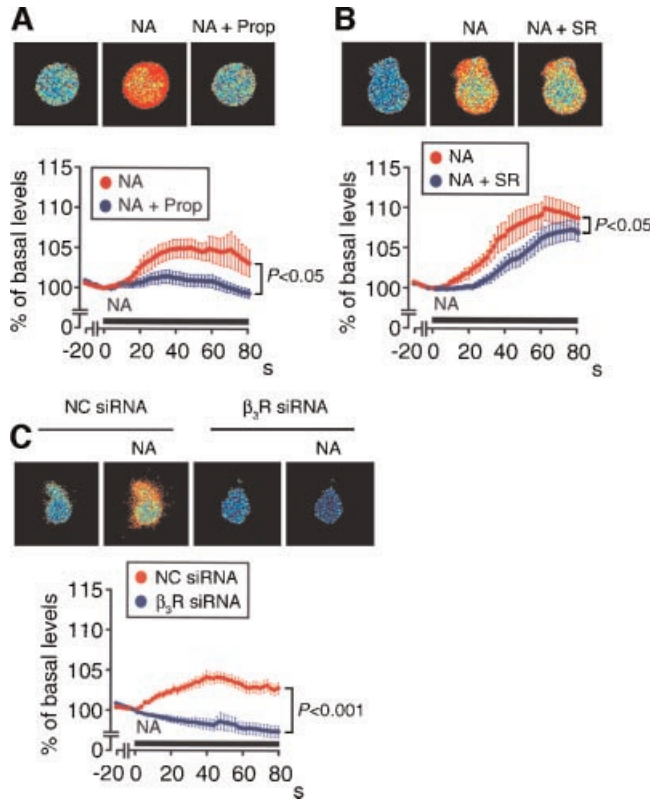

Fig. 5. NA stimulates ATP release from DRG neurons via β_3 adrenoceptors. In the real-time enzymatic fluorescent imaging analysis for ATP, NADPH signals were monitored in acutely dissociated rat DRG neurons before and after bath-application with NA (100 μM) in the presence and absence of propranolol (Prop) (10 μM; n = 12 independent neurons) (A) or SR59230A (SR) (100 nM; n = 13 independent neurons) (B). In a different set of experiments, NADPH signals were assayed in neurons with NC siRNA or β_3R siRNA before and after bath-application with NA (100 μM; n = 13 independent neurons) (C). In the graphs, each point represents the mean (±SEM) percentage of basal NADPH intensities before treatment with NA. *P*-values, *F*-test.

Suppression of allodynia by inhibiting β_3 adrenoceptors

We finally assessed the implication of β_3 adrenoceptors in neuropathic pain with a rat model. Mechanical pain stimuli were applied to left-sided plantar surface of the hind-paw using a von Frey filament 4 weeks after non-ligation and ligation of the spinal nerve from the L5 segment on the left side. For unlesioned group (Sham), the hind-paw withdrawal threshold was approximately 14 g (Fig. 7). For lesioned group, the threshold reduced to nearly 2 g (Fig. 7), indicating that rats with lesion have an allodynia. Intraperitoneal injection (i.p.) with vehicle or SR59230A at doses of 1 mg/kg (~2.8 μM), 5 mg/kg (~14 μM), and 10 mg/kg (~28 μM) had no effect on the reduced threshold (Fig. 7). In contrast, SR59230A [50 mg/kg (~138 μM), i.p.] significantly reversed the reduced threshold (Fig. 7). This implies that allodynia is relieved by inhibiting β_3 adrenoceptors; in other words, β_3 adrenoceptors are implicated in the development of allodynia.

Discussion

ATP receptors are abundantly expressed in DRG neurons and regulate nociception (Chen et al., 1995; Lewis et al., 1995; Cockayne et al., 2000; Hamilton and McMahon, 2000; Tsuda et al., 2000, 2002; Ueda et al., 2000; Bao et al., 2003; Moriyama et al., 2003; North, 2004; Burnstock, 2006). For general synaptic transmission, the ATP receptor agonist ATP is co-released with other neurotransmitters from presynaptic terminals. For neuron–astrocyte communications, ATP is released from astrocytes in response to neurotransmitters released from neurons or to mechanical stimulation (Koizumi et al., 2003; Fields and Burnstock, 2006). ATP is also originated from

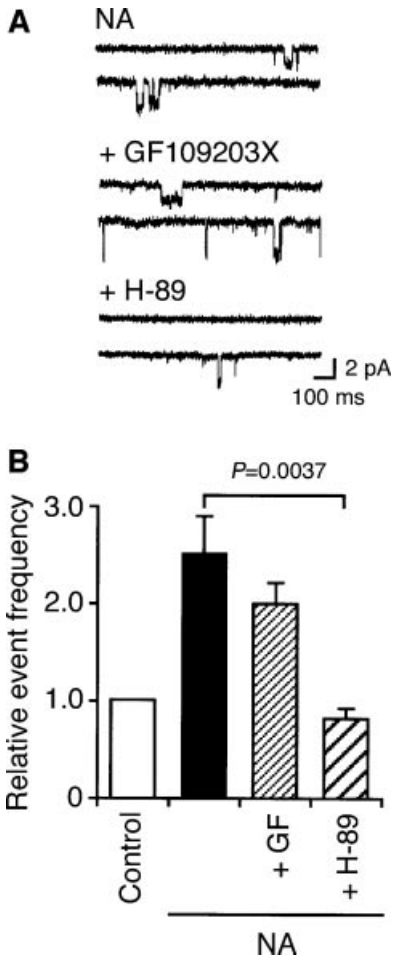

Fig. 6. NA increases the frequency of NeSCCs under the control of PKA. A: Outside-out patches were made from acutely isolated DRG neurons, and NeSCCs were recorded before and after bath-application with NA (100 μM) in the presence and absence of H-89 (1 μM) or GF109203X (GF; 100 nM) at a holding potential of −70 mV. B: In the graph, the event frequency (mean ± SEM) was normalized by regarding the frequency of NeSCCs before application with NA (control) as 1 (n = 4 independent patches). *P*-values, one-way ANOVA.

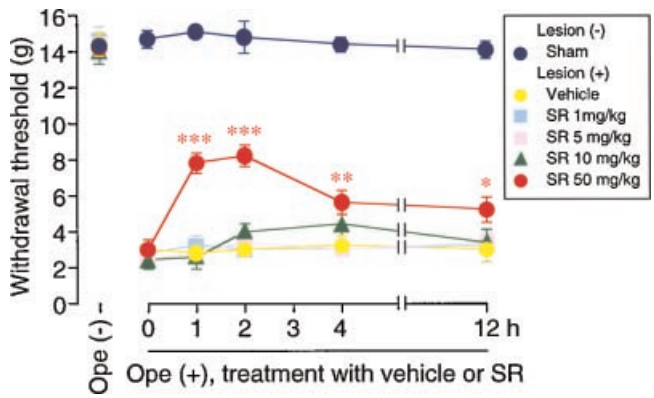

Fig. 7. An β_3 adrenoceptor inhibitor suppresses allodynia. To assess the extent of allodynia, the hind-paw withdrawal threshold was measured by the up-down method. Before operation, it was confirmed that there is no difference in the withdrawal threshold between rats used here [Ope (−)]. Mechanical pain stimuli with a von Frey filament were applied to left-sided plantar surface of the hind-paw for rats with non-operation and non-injection (Sham) or with operation [Ope (+)] that are divided into two groups; rats undergoing non-ligation [Lesion (−)] and ligation of the spinal nerve from the L5 segment on the left side [Lesion (+)], before and after intraperitoneal injection with vehicle (Vehicle) or SR59230A (SR). Each point represents the mean (±SEM) threshold from 10 independent rats. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ as compared with that for Vehicle, one-way ANOVA.

damaged cells and tissues. The origin of ATP in the DRG, however, remained unknown. To address this question, we had attempted to monitor ATP released from cultured DRG cells with a high-performance liquid chromatography, but in fail. We, therefore, devised to utilize P2X receptors as a biosensor for ATP and succeeded in its molecular monitoring. An outside-out patch was made from an acutely dissociated DRG neuron, and single-channel currents were evoked by approaching the patch-electrode tip close (within 5 μm) to a neuron, with the slope conductance similar to that for ATP-induced single-channel currents, that was blocked by the P2X receptor inhibitor PPADS. This confirms spontaneous ATP release from DRG neurons. This also indicates that P2X receptors expressed in DRG neurons are activated by ATP released from DRG neurons.

NA, released in association with sympathetic nerve sprouting into the DRG after peripheral nerve injury, may contribute to development of neuropathic pain (Mclachlan et al., 1993; Dunn, 2000a,b; Kim et al., 2001; Chung and Chung, 2002; Maruo et al., 2006). In the present study, NA significantly increased the frequency of NeSCCs that was blocked by the broad inhibitor of β adrenoceptors, propranolol, and the selective inhibitor of β_3 adrenoceptors, SR59230A. Moreover, the NA effect was never obtained with neurons transfected with the siRNA silencing the β_3 adrenoceptor-targeted gene. In the real-time enzymatic fluorescent imaging analysis for ATP, NA enhanced an NADPH signal in DRG neurons, and the enhancement was inhibited in the presence of propranolol or SR59230A and by knocking down β_3 adrenoceptors. Taken together, these results indicate that NA stimulates ATP release from DRG neurons via β_3 adrenoceptors.

NA did not increase the frequency of NeSCCs in the presence of the PKA inhibitor H-89, indicating that NA increases ATP release from DRG neurons in a PKA-dependent manner. This further supports the note that the NA-induced ATP release is mediated via β_3 adrenoceptors linked to G_S protein involving adenylate-cyclase-catalyzed PKA activation (Sato et al., 2005). It is presently unknown how ATP is released from DRG neurons in response to NA. NA still increased the frequency of NeSCCs in the presence of latrunculin B (10 μM), to inhibit vesicular transport by preventing actin polymerization, or botulinum toxin A (1000 U/ml), an inhibitor of vesicular exocytosis (data not shown), indicating that NA engages non-vesicular ATP release from DRG neurons. The NA effect was blocked by the inhibitor of cystic fibrosis transmembrane conductance regulator (CFTR), CFTRinh-172 (5 μM) (data not shown). This suggests that NA might stimulate ATP efflux through CFTR. To address this question, we are currently carrying out further experiments.

In the analysis using an allodynia rat model, the hind-paw withdrawal threshold was markedly reduced. SR59230A (50 mg/kg, i.p.), an inhibitor of β_3 adrenoceptors, significantly reversed the reduced threshold, indicating that allodynia is suppressed by inhibiting β_3 adrenoceptors. This also suggests that NA stimulates non-vesicular ATP release from DRG neurons as mediated via β_3 adrenoceptors after peripheral nerve injury, to enhance activity of ATP receptors including P2X receptors expressed in the DRG, which triggers allodynia.

β_3-Adrenoceptor, thus, could be a target of treatment of neuropathic pain.

In summary, the results of the present study demonstrate that NA stimulates ATP release from DRG neurons as mediated via β_3 adrenoceptors linked to G_s protein involving PKA activation, thereby causing allodynia. This represents a novel regulatory pathway for neuropathic pain relevant to noradrenergic transmission.

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