Functional Downregulation of P2X₃ Receptor Subunit in Rat Sensory Neurons Reveals a Significant Role in Chronic Neuropathic and Inflammatory Pain

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The excitation of nociceptive sensory neurons by ATP released in injured tissue is believed to be mediated partly by P2X₃ receptors. Although an analysis of P2X₃ knock-out mice has revealed some deficits in nociceptive signaling, detailed analysis of the role of these receptors is hampered by the lack of potent specific pharmacological tools. Here we have used antisense oligonucleotides (ASOs) to downregulate P2X₃ receptors to examine their role in models of chronic pain in the rat.

ASOs and control missense oligonucleotides (180 μg/d) were administered intrathecally to naive rats for up to 7 d via a lumbar indwelling cannula attached to an osmotic minipump. Functional downregulation of the receptors was confirmed by αβ-methylene ATP injection into the hindpaw, which evoked significantly less mechanical hyperalgesia as early as 2 d after treatment with ASOs relative to controls. At this time point, P2X₃ protein levels were significantly downregulated in lumbar

L4 and L5 dorsal root ganglia. After 7 d of ASO treatment, P2X₃ protein levels were reduced in the primary afferent terminals in the lumbar dorsal horn of the spinal cord.

In models of neuropathic (partial sciatic ligation) and inflammatory (complete Freund's adjuvant) pain, inhibition of the development of mechanical hyperalgesia as well as significant reversal of established hyperalgesia were observed within 2 d of ASO treatment. The time course of the reversal of hyperalgesia is consistent with downregulation of P2X₃ receptor protein and function.

This study demonstrates the utility of ASO approaches for validating gene targets in *in vivo* pain models and provides evidence for a role of P2X₃ receptors in the pathophysiology of chronic pain.

Key words: dorsal root ganglia; P2X₃; P2X_{2/3}; neuropathic pain; inflammatory pain; antisense oligonucleotide; α,β-methylene ATP

Since the first description of pain produced by the application of the purine ATP into a human blister base preparation (Bleehen and Keele, 1977), there has been accumulating evidence supporting a nociceptive role for extracellular ATP (Burnstock and Wood, 1996; Dunn et al., 2001). ATP released by exocytosis or cell lysis after tissue injury may activate primary sensory afferent neurons via ATP-gated cation channels, termed P2X. Of the seven P2X channel subunits identified to date, P2X₃ subunit-containing receptors (either homomeric or heteromeric combination of P2X₃ and P2X₂ receptors: P2X_{2/3}) are expressed predominantly in nociceptive sensory neurons (Chen et al., 1995; Lewis et al., 1995) and are thought to mediate ATP nociceptive signaling. P2X₃ receptor protein is exported from the soma of dorsal root ganglia (DRG) neurons to peripheral terminals in the skin and viscera (Bradbury et al., 1998) and to central terminals projecting into inner lamina II of the dorsal horn of the spinal cord (Vulchanova et al., 1997, 1998; Llewellyn-Smith and Burnstock, 1998). ATP has been shown to depolarize isolated DRG neurons (Bean et al., 1990) and excite the peripheral terminals of

primary afferent neurons (Dowd et al., 1998; Hamilton and McMahon, 2000; Rong et al., 2000). In the spinal cord, ATP may act at presynaptic P2X₃ receptors, facilitating glutamate release from primary afferents (Gu and MacDermott, 1997; Khakh and Henderson, 1998; Tsuda et al., 1999), as well as at postsynaptic receptors (including P2X₁, P2X₂, P2X₄, P2X₅, and P2X₆ receptors) located on second-order neurons (Fyffe and Perl, 1984; Salter and Henry, 1985; Sawynok and Sweeney, 1989; Li and Perl, 1995; Bardoni et al., 1997; Stanfa et al., 2000).

Investigation of the role of P2X₃ subunit-containing receptor activation *in vivo* is hampered by the lack of selective antagonists. Subplantar application of the agonists ATP and α,β-methylene ATP into the naive rat hindpaw has been shown to elicit thermal hyperalgesia and nocifensive behavior that can be inhibited by selective desensitization with α,β-methylene ATP, implicating a role for a combination of P2X₃, P2X_{2/3}, and P2X₁ receptors (Bland-Ward and Humphrey, 1997).

Evidence for a direct physiological role of P2X₃ homomeric and P2X_{2/3} heteromeric receptors has come from studies on P2X₃ receptor null-mutant mice (Cockayne et al., 2000; Souslova et al., 2000). These mice respond normally to acute noxious, thermal, and mechanical stimuli but display attenuated responses to non-noxious "warming" stimuli and show reduced formalin-induced nocifensive behaviors. In addition, their phenotype includes a marked urinary bladder hyporeflexia (Vlaskovska et al., 2001) and, somewhat unexpectedly, increased hyperalgesia after peripheral inflammation of the hindpaw.

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Little is known of the role of P2X₃ receptors in neuropathic pain, despite evidence that P2X₃ mRNA levels are altered after nerve injury (Novakovic et al., 1999; Bradbury et al., 1998; Decosterd and Woolf, 2000; Tsuzuki et al., 2001). It is conceivable that sympathetic nerves, which sprout into DRG after nerve injury (Ramer and Bisby, 1997), provide a source of ATP that activates P2X₃ receptors in the soma.

Given the lack of specific P2X₃ receptor antagonists, we have used specific antisense oligonucleotides (ASOs) (Dorn et al., 2001) to investigate the effects of reducing the expression levels of P2X₃ receptor subunit-containing receptors in inflammatory and neuropathic pain models in the rat.

MATERIALS AND METHODS

Antisense oligonucleotides. The ASOs against the P2X₃ subunit that were used in this study were characterized essentially as described (Dorn et al., 2001). Briefly, a fully phosphodiester 18-mer with five nucleotides at the 3' and 5' ends modified with 2'-O-(2-methoxyethyl) (MOE) groups was synthesized using phosphoramidite chemistry (Martin and Natt, 2000), HPLC-purified, and characterized by electrospray mass spectrometry and capillary gel electrophoresis. The sequences that were used are as follows: ASO, 5'-ctc caT CCA GCC Gag tga-3', and missense oligonucleotide, (MSO), 5'-cta caG CCA TCC Gcg tga-3'; lowercase letters represent 2'-MOE and uppercase letter represent DNA. *In vitro* experiments confirmed the ability of the P2X₃ ASOs to downregulate P2X₃ receptor mRNA levels (Dorn et al., 2001). P2X₃ ASOs have no effect on P2X₃ receptor mRNA levels (data not shown). The ability to downregulate P2X₃ receptor mRNA levels is lost when more than two to three conservative mutations are introduced to produce the MSO (Dorn et al., 2001).

Animal models. All experiments were performed according to Home Office (United Kingdom) guidelines and with approval of the local Novartis Animal Welfare and Ethics Committee.

Neuropathic pain. The partial sciatic ligation model of neuropathic pain was used as described previously (Seltzer et al., 1990; Fox et al., 2001; Patel et al., 2001). Briefly, male Wistar rats (120–140 gm) were anesthetized, the left sciatic nerve was exposed at mid-thigh level through a small incision, and one-third to one-half of the nerve thickness was tightly ligated with a 7.0 silk suture. The wound was closed with sutures and clips and dusted with antibiotic powder. In sham animals the sciatic nerve was exposed but not ligated, and the wound was closed as before. Mechanical hyperalgesia was assessed by measuring paw withdrawal thresholds of both hindpaws to an increasing pressure stimulus using an Analgesymeter (Ugo-Basile, Milan, Italy). The cutoff was set at 250 gm, and the endpoint was taken as paw withdrawal, vocalization, or overt struggling. Mechanical allodynia was assessed by measuring withdrawal thresholds to non-noxious mechanical stimuli applied with custom-made von Frey hairs to the plantar surface of both hindpaws. Animals were placed individually into wire mesh-bottom cages, with groups of six tested concurrently, and allowed to acclimatize for ~30 min. Von Frey hairs were tested in ascending order of force with a single trial of up to 6 sec for each hair until a withdrawal response was established; cutoff was at 20.6 gm. This was confirmed as the withdrawal threshold by testing a lack of response to hair with the next lowest force. Each animal was tested only once, in random order. The statistical significance of mechanical hyperalgesia and allodynia data obtained from the different experimental animal groups was analyzed using ANOVA followed by Tukey's honest significant difference (HSD) test.

α,β -methylene ATP (0.1 or 1.0 μ M in 10 μ l) was given by intraplantar injection to the contralateral hindpaw on the final day of the experiment, and paw withdrawal thresholds to mechanical stimuli were measured 0.5 and 1.0 hr later.

Inflammatory pain. Male Sprague Dawley rats (200–250 gm) were cannulated as described below, and 24 hr after cannulation, 25 μ l complete Freund's adjuvant (CFA) was injected into one hindpaw. This induces a mechanical hyperalgesia and allodynia that develops after a few hours and lasts up to 7 d. Paw withdrawal thresholds were measured just before CFA injection and then daily for the 7 d of ASO treatment. A 0.1 μ M dose of α,β -methylene ATP was given by intraplantar injection to the contralateral hindpaw on the final day of the experiment. Paw withdrawal thresholds to mechanical hyperalgesia were measured 0.5 and 1.0 hr after administration.

Figure 1. Inhibition of α,β -methylene ATP evoked mechanical hyperalgesia in the hindpaw of naive rats after a 7 d treatment with P2X₃ receptor ASO, MSO, or saline. Paw withdrawal thresholds were measured before cannulation on day 0 and immediately before and 0.5 and 1 hr after administration of 0.1 μ M α,β -methylene ATP. Each point represents the mean $\pm$ SEM from three animals per treatment group. * p < 0.05 compared with saline treatment by ANOVA followed by Tukey's HSD test performed on gram threshold data.

Administration of P2X₃ oligonucleotides. ASOs and MSOs were administered intrathecally via an indwelling cannula that was inserted 24 hr before or 14 d after sciatic nerve ligation or 24 hr before CFA injection. Rats were anesthetized, and an incision was made in the dorsal skin just lateral to the midline and ~10 mm caudal to the ventral iliac spines. A sterile catheter (polyethylene PE10 tubing) was inserted via a guide cannula (20 gauge needle) and advanced 3 cm cranially in the intrathecal space approximately to the L1 level. The catheter, which was inserted subcutaneously in the left or right flank, was then connected to an osmotic minipump (Alzet) delivering P2X₃ receptor ASO, MSO, or saline (1 μ l/h, 7 d). The incision was closed with wound clips and dusted with antibiotic powder. Preliminary experiments determined 180 μ g/d as a maximal tolerated dose. No evidence of neurotoxicity such as paralysis, vocalization, or anatomical damage to the spinal cord was noted at this dose. Delivery of ASOs to the DRG cell bodies was initially confirmed using a fluorescently labeled ASO; an unlabelled version was used in all subsequent experiments.

To assess whether intrathecal cannulation produced any nonspecific damage, a control group of naive animals that were cannulated and received saline was included. Additionally, contralateral thresholds were always measured, which would provide an indication of any nonspecific damage.

Tissue and section preparation. Tissue fixation by perfusion with paraformaldehyde and cryopreservation was performed as described previously (Wotherspoon and Winter, 2000). L4 and L5 DRGs from the left-hand side of naive animals were dissected for *in situ* hybridization. The L4/L5 level of spinal cord was dissected for immunohistochemistry (IHC) analysis. DRG sections were cut to a thickness of 10 μ m using a cryostat (Bright Instrument Co. Ltd., Cambridgeshire, UK) and thaw-mounted onto Superfrost poly-L-lysine-coated slides (VWR International Ltd., Dorset, UK) and air dried. Sections to be analyzed by RNA *in situ* hybridization were stored directly at -80°C . Spinal cord sections were freshly cut as required to 30 μ m and were free floated in PBS before processing for IHC.

Immunohistochemistry in spinal cord. Before IHC, sections were incubated with 10% normal donkey serum for 1 hr to reduce nonspecific staining. Spinal cord sections were incubated in primary antibody for 48 hr. Primary antibodies used were P2X₃ receptor (rabbit anti-rat P2X₃ receptor; Neuromics catalog number RA10109-50; 1:10000 dilution) and calcitonin gene-related peptide (CGRP) (sheep anti-rat CGRP, Affiniti; 1:1000 dilution). All dilutions were in PBT (PBS, 0.1% Triton X-100, and 0.02% sodium azide). Sections were incubated for 2 hr in secondary antibodies: fluorescein (FITC)-conjugated AffiniPure F(ab')₂ fragment donkey anti-rabbit IgG and rhodamine (TRITC)-conjugated AffiniPure F(ab')₂ fragment donkey anti-sheep or anti-guinea pig IgG, diluted in

Figure 2. Molecular analysis of P2X₃ receptor mRNA expression levels in naive rats after a 7 d treatment with P2X₃ receptor ASO, MSO, or saline. *A*, Representative DRG sections from saline-, ASO-, and MSO-treated animals after nonradioactive RNA *in situ* hybridization with a P2X₃ probe. Presence of P2X₃ receptor mRNA is indicated by dark-staining cells. P2X₃ receptor mRNA levels are visibly reduced after ASO but not saline or MSO treatment. Scale bar, 200 μ m. *B*, Quantification of P2X₃ receptor mRNA expression levels in the soma of DRG neurons visualized by *in situ* hybridization. P2X₃ receptor mRNA levels are expressed in arbitrary units. Each bar represents the mean $\pm$ SEM of 24 sections (4 sections per L4 and L5 DRG taken from 3 animals per treatment group). P2X₃ receptor ASO but not saline or MSO treatment decreases P2X₃ receptor mRNA expression levels. *** $p < 0.001$ compared with saline treatment by Kruskal–Wallis ANOVA with Dunn’s multiple comparisons post-test.

PBT at 1:200 (Jackson ImmunoResearch, West Grove, PA). Sections were washed three times for 5 min in PBS before and after the secondary antibody incubations. Sections were mounted in PBS/glycerol (1:3) containing 25 mg/ml diazobicyclo-2,2-octane (Sigma, St. Louis, MO) as an anti-fading agent. Images of antibody-stained sections were captured using a Nikon Eclipse 800 fluorescent microscope attached to a Hamamatsu cooled CCD camera and image capturing system using Image Pro Plus software (Media Cybernetics). The relevant filter blocks that were used depended on the fluorescent marker conjugated to the secondary antibody. Samples from each animal were processed for IHC and analyzed simultaneously (four sections per slide) to allow accurate comparisons. A negative control where the primary antibody was replaced with PBT was included in all experiments. Immunoreactivity was quantified by drawing around the whole section using Image Pro Plus software (Media Cybernetics) and determining the mean fluorescence intensity. CGRP immunoreactivity was used to normalize P2X₃ receptor protein levels in spinal cord sections.

Statistical analysis and data presentation. Data from experimental and control populations were compared statistically using Kruskal–Wallis ANOVA with Dunn’s multiple comparisons post-test.

Western blot analysis of P2X₃ receptors in DRG. L4 and L5 DRG were dissected from naive rats treated with ASO, MSO, or saline and snap-frozen on dry ice. Samples were prepared by crushing in a small pestle and mortar followed by homogenization in 100 μ l lysis buffer [50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, pH 8, 1% Triton, 1% SDS, 1:100 aprotinin-solution, 1 mM PMSF (both Sigma)]. Lysates were passed 10 times through a 0.6 $\times$ 30 gauge needle and centrifuged for 10 min at 14,000 rpm. Fifty micrograms of solubilized proteins from the supernatant fraction were mixed with 3 $\times$ loading buffer (187.5 mM Tris-HCl, pH 6.8, 30% glycerol, 6% SDS, 0.3% Bromophenol blue) subjected to SDS-PAGE through a Bio-Rad 4–20% Tris-glycine gel. Proteins were electrophoretically transferred on to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After blocking for 1 hr with 5% milk powder in 1 $\times$ TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween

20), the blot was incubated overnight with the P2X₃ receptor primary antibody (Neuromics) at a dilution of 1:2000 in TBS-T. The filter was incubated with a horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Pierce (Perbio), Cheshire, UK), diluted 1:2000 in TBS-T, and then developed by enhanced chemiluminescence (ECL, Amersham Biosciences) and detected on film (ECL-Hyperfilm, Amersham Biosciences) according to the manufacturer’s instructions. To check for variation in sample loading, the same blot was then washed and incubated overnight with a 1:2000 dilution of mouse anti-transferrin receptor primary antibody (Zymed, San Francisco, CA), followed by incubation with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce) secondary antibody and then detected using ECL.

P2X₃ receptor mRNA analysis by digoxigenin-labeled RNA *in situ* hybridization. To visualize the level of P2X₃ receptor mRNA in DRG neurons, digoxigenin (DIG)-labeled RNA *in situ* hybridization was performed. A 930 bp (*EagI*–*EcoRV*) fragment was subcloned from a P2X₃ receptor plasmid (nucleotide 583–1513; GenBank accession number X90651) into pBluescript KSII+ (Stratagene). DNA from the subclone (P2X₃–7) was prepared (QiaFilter maxiprep kit, Qiagen) and linearized with *EagI* or *EcoRV* (New England Biolabs, Beverly, MA) for the antisense and sense probes, respectively. DIG-labeled riboprobes were prepared from the linearized plasmids using T3 polymerase (antisense) or T7 polymerase (sense) with a DIG RNA labeling kit (Roche Diagnostics) as instructed. An aliquot (4 μ l) was checked by agarose gel electrophoresis, and the rest was purified using a Quick Spin column (Roche Diagnostics) followed by overnight ethanol precipitation. The dried RNA pellet was resuspended in 50 μ l of 10 mM DTT solution and stored at -80° C. *In situ* hybridization and color development were performed as described previously (Eisenstat et al., 1999). After color development, slides were washed in PBS and mounted as described for the IHC. *In situ* hybridization images were quantified using the method described above, except that optical density was measured.

Figure 3. P2X₃ protein expression levels in the dorsal horn of lumbar spinal cord in naive rats after a 7 d treatment with P2X₃ receptor ASO, MSO, or saline. *A*, Representative images of P2X₃ receptor immunoreactivity in dorsal horn sections of lumbar (L4/L5) spinal cord from animals treated with saline, ASO, or MSO. Red band of P2X₃ immunoreactivity in lamina II is visibly reduced in ASO-treated animals (*top panel*). Coimmunostaining with CGRP (*green*) shows that equivalent regions of each spinal cord were analyzed. Scale bar, 200 μ m. *B*, Quantification of P2X₃ protein expression levels in the dorsal horn of the spinal cord. Protein levels are expressed in arbitrary units. Each bar represents the mean $\pm$ SEM of 12 sections (4 sections per animal and 3 animals per treatment group). *** p < 0.001 compared with saline treatment by Kruskal–Wallis ANOVA with Dunn's multiple comparisons post-test.

RESULTS

Downregulation of P2X₃ receptors in naive animals

ASO treatment of naive animals ($n = 6$) for 7 d resulted in a functional downregulation of peripheral P2X₃ receptors as demonstrated by an abolition of mechanical hyperalgesia evoked by an intraplantar injection of 0.1 μ mol α,β -methylene ATP into the hindpaw (Fig. 1). The same dose of α,β -methylene ATP injected into the hindpaw of animals treated with saline or MSO evoked significant, similar levels of mechanical hyperalgesia (Fig. 1).

Molecular analysis of the P2X₃ receptor ASO-, MSO-, and saline-treated animals ($n = 3$) revealed a downregulation of P2X₃ receptor mRNA in the soma of DRG neurons. Nonradioactive *in situ* hybridization in DRG neurons showed a visible reduction in P2X₃ receptor mRNA signal after treatment with P2X₃ ASO but not saline or MSO (Fig. 2*A*). Quantification of the *in situ* hybridization images ($n = 4$ per L4/L5 DRG per animal) indicated that the P2X₃ receptor mRNA levels were significantly reduced ($p < 0.001$) in P2X₃ receptor ASO-treated animals compared with that of the saline- and MSO-treated groups (Fig. 2*B*).

P2X₃ receptor immunoreactivity in inner lamina II of the dorsal horn of the spinal cord was visibly reduced in P2X₃ receptor ASO-treated animals compared with saline- or MSO-treated groups (Fig. 3*A*). Quantification of P2X₃ receptor immunoreactivity, normalized to that of CGRP (four sections per animal; $n = 3$ per group) (Fig. 3*B*), indicated that P2X₃ receptor protein expression levels in ASO-treated animals were significantly reduced ($p < 0.001$) compared with those of the saline-

and MSO-treated groups. Absolute P2X₃ receptor protein levels that were not normalized to CGRP levels also showed significantly reduced levels in ASO-treated animals compared with MSO- and vehicle-treated animals (data not shown).

Effect of ASO treatment on neuropathic and inflammatory hyperalgesia

The effect of P2X₃ receptor ASO treatment was evaluated in models of neuropathic and inflammatory pain. Intrathecal administration of P2X₃ receptor ASO, initiated 24 hr before partial sciatic nerve ligation, produced a significant inhibition in the development of mechanical hyperalgesia ($p < 0.05$, compared with MSO or saline) within 2 d of surgery that reached 50% inhibition by the final reading on day 7 (Fig. 4*A*). Contralateral paw withdrawal thresholds were not affected by surgery or ASO treatment (Fig. 4*B*).

In a model of established neuropathic hyperalgesia, 7 d of treatment with P2X₃ receptor ASO produced a significant 33% reversal of mechanical hyperalgesia ($p < 0.05$) (Fig. 5*A*) that was evident within 2 d of treatment. Again, there was no effect of ASO treatment on contralateral paw thresholds during the treatment period (Fig. 5*B*).

P2X₃ receptor ASO treatment had no effect on either the development of mechanical allodynia (Fig. 6*A*) or the allodynia in the model of established neuropathic pain (Fig. 6*B*).

In the inflammatory pain model, intrathecal administration of P2X₃ receptor ASO for 7 d, initiated 24 hr before the injection of CFA into the hindpaw, produced a 25% attenuation in the devel-

Figure 4. Effect of P2X₃ receptor ASO, MSO, or saline treatment on development of mechanical hyperalgesia in a rat model of neuropathic pain. Four groups ($n = 6$) of animals were cannulated and infused with P2X₃ receptor ASO, MSO, or saline for 7 d. Twenty-four hours after cannulation, the sciatic nerve was partially ligated in three groups, whereas the fourth saline-treated group was maintained as a control for the effects of cannulation. Mechanical hyperalgesia was assessed on ipsilateral (*A*) and contralateral (*B*) hindpaws before cannulation and then daily until day 7. Each point represents the mean $\pm$ SEM from six animals per treatment group. * $p < 0.05$ compared with neuropathic saline-treated group by ANOVA followed by Tukey's HSD test performed on gram threshold data.

opment of mechanical hyperalgesia ($p < 0.05$) (Fig. 7*A*). Contralateral withdrawal thresholds were not affected by ASO treatment (Fig. 7*B*). The development of mechanical allodynia was not reduced by P2X₃ receptor ASO treatment (data not shown).

Antisense treatment results in incomplete receptor knockdown

In all of the above investigations, functional downregulation of P2X₃ receptors was assessed in the contralateral "untreated" hindpaw using an injection of α,β -methylene ATP (0.1 and 1 μ M) on the final day of the experiment (Fig. 8). These doses of α,β -methylene ATP were chosen selectively to activate P2X₃ and P2X_{2/3} receptors on the basis of previous published studies (Tsuda et al., 2000). In experiments examining the development of neuropathic pain, ASO but not MSO or saline treatment abolished the mechanical hyperalgesia evoked by 0.1 μ M α,β -methylene ATP (Fig. 8) and attenuated (66% relative to predose)

Figure 5. Effect of P2X₃ receptor ASO, MSO, or saline treatment on established mechanical hyperalgesia in a rat model of neuropathic pain. The sciatic nerve was partially ligated in three groups of six animals. On day 13, all three groups were cannulated and treated with P2X₃ receptor ASO, MSO, or saline for 7 d. An additional saline group was set up on the same day as a control for cannulation. Mechanical hyperalgesia was assessed on ipsilateral (*A*) and contralateral (*B*) hindpaws before surgery and then daily from days 13–20. Each point represents the mean $\pm$ SEM from six animals per treatment group. * $p < 0.05$ compared with neuropathic saline treatment group by ANOVA followed by Tukey's HSD test performed on gram threshold data.

the mechanical hyperalgesia evoked by 1 μ M α,β -methylene ATP (Fig. 8). Similarly, ASO but not MSO or saline treatment abolished the mechanical hyperalgesia evoked by a 0.1 μ M dose of α,β -methylene ATP on established neuropathic pain and the development of inflammatory hyperalgesia (data not shown).

In all of the pain models, mechanical hyperalgesia was significantly reversed 2 d after ASO treatment. To assess whether P2X₃ receptor ASO treatment could cause a functional downregulation of P2X₃ receptor that might directly account for this relatively fast reversal, α,β -methylene ATP-induced mechanical hyperalgesia was evaluated in naive rats after treatment with ASO for 2 d. A significant reduction (45% relative to predose) in 1 μ M α,β -methylene ATP-induced mechanical hyperalgesia was observed in ASO-treated relative to saline- or MSO-treated rats (Fig. 9*A*). Furthermore, immunoblot analysis of the DRG from these naive rats showed a visible downregulation of P2X₃ receptor after treatment with ASO relative to MSO or saline treatment for 2 d (Fig. 9*B*).

Figure 6. Effect of P2X₃ receptor ASO, MSO, or saline treatment on the development and maintenance of mechanical allodynia in a rat model of neuropathic pain. *A*, Mechanical allodynia was assessed in the ipsilateral and contralateral (data not shown) hindpaws of animals described in Figure 4 before cannulation and then daily until day 6. Each point represents the mean $\pm$ SEM from six animals per treatment group. No significant difference was detected on the development of mechanical allodynia. *B*, Mechanical allodynia was assessed in the ipsilateral and contralateral (data not shown) hindpaws of animals described in Figure 5 before surgery and then daily from days 13–20. Each point represents the mean $\pm$ SEM from six animals per treatment group. No significant difference was detected on the maintenance of mechanical allodynia.

DISCUSSION

The aim of the present study was to investigate the effects of downregulating P2X₃ receptors in rat models of chronic inflammatory and neuropathic pain using ASO technology. We have demonstrated that P2X₃ receptor oligonucleotides, previously characterized extensively by Dorn et al. (2001), can be reproducibly delivered to lumbar DRG and spinal cord neurons via an indwelling intrathecal cannula attached to an osmotic minipump. We observed no signs of neurotoxicity from the surgical procedure such as paralysis, vocalization, or gross anatomical changes in the spinal cord. In addition, there was no evidence of neurotoxicity from the chemical composition [phosphodiester flanked by 2'-O-(2-methoxyethyl) groups] or the dose (180 μ g/d) of oligonucleotides administered.

Figure 7. Effect of P2X₃ receptor ASO, MSO, or saline treatment on the development of mechanical hyperalgesia in the complete Freund's adjuvant (CFA) model of inflammatory pain. Four groups ($n = 6$) of animals were cannulated and infused with P2X₃ receptor ASO, MSO, or saline for 7 d. Twenty-four hours after cannulation, three groups received an intraplantar stimulus of CFA into the hindpaw, and the fourth saline group was maintained as a control for cannulation. Mechanical hyperalgesia was assessed on ipsilateral (*A*) and contralateral (*B*) hindpaws before cannulation and then daily until day 7. Each point represents the mean $\pm$ SEM from six animals per treatment group. * $p < 0.05$ compared with CFA saline treatment group by ANOVA followed by Tukey's HSD test performed on gram threshold data.

Delivery of P2X₃ receptor ASO, but not MSO or saline, for 7 d produced significant reduction of P2X₃ message in the DRG as well as P2X₃ receptor protein in inner lamina II of the dorsal horn of the spinal cord in naive animals. This was consistent with the reduced mechanical hyperalgesia evoked by intraplantar administration of α,β -methylene ATP into the hindpaw at this time point, demonstrating a functional downregulation of P2X₃ receptors in peripheral terminals of sensory neurons.

In the Seltzer et al. (1990) model of neuropathic pain, ASO treatment significantly reduced the development of mechanical hyperalgesia and partially reversed established mechanical hyperalgesia. Similarly, ASO treatment inhibited the development of CFA-induced mechanical hyperalgesia. These ASO effects were evident only in the ipsilateral paws in the pain models, whereas no significant changes in nociceptive indices were observed in the contralateral paws or indeed in any MSO- or saline-treated groups. Although oligonucleotide treatment of neuropathic and inflammatory animals was maintained for up to 7 d, a partial

Figure 8. Inhibition of α,β -methylene ATP evoked mechanical hyperalgesia in the contralateral hindpaw of neuropathic rats after a 6 or 7 d treatment with P2X₃ receptor ASO, MSO, or saline. On the final experimental day, α,β -methylene ATP was administered by intraplantar injection to the contralateral hindpaws of the animals described in Figure 4. Paw withdrawal thresholds were determined before and 0.5 hr after α,β -methylene ATP administration. Doses of 0.1 or 1.0 μ mol were used. In each case the mean $\pm$ SEM of the mean paw withdrawal thresholds of $n = 6$ per treatment group are shown. In all cases ASO treatment results in a significant inhibition of the development of α,β -methylene ATP-evoked mechanical hyperalgesia. * $p < 0.05$ compared with saline treatment group by ANOVA followed by Tukey's HSD test performed on gram threshold data. However, the 100% inhibition seen in the ASO-treated group at the 0.1 μ mol dose is reduced to 66% relative to predose values at a higher 1.0 μ mol dose.

reversal of mechanical hyperalgesia in these models was observed as early as 2 d after ASO application. We were able to demonstrate a reduction in P2X₃ receptor protein expression in DRG as well as a functional downregulation of P2X₃ receptor in the periphery, measured by injecting α,β -methylene ATP into the hindpaw, after 2 d of ASO administration in naive animals. Taken together, these data are consistent with the hypothesis that the rapid reversal of mechanical hyperalgesia, observed in the pain models, can be directly attributed to the downregulation of P2X₃ receptor.

It is notable that at the maximum ASO dose administered (180 μ g/d), significant but incomplete reversal of mechanical hyperalgesia in either the neuropathic or the inflammatory pain models is observed. This could reflect a partial role of P2X₃ receptors in nociceptive signaling, or it may be the result of incomplete receptor "knockdown." ASO treatment does not commonly produce a total protein knockdown (Hallbook et al., 2000; Yoshimura et al., 2001). Incomplete receptor downregulation is consistent with the residual P2X₃ message and receptor in DRG and spinal cord immunoreactivity observed in ASO-treated animals. Incomplete downregulation of P2X₃ receptors is also consistent with the observed reduction, but not abolition, of mechanical hyperalgesia evoked after an intraplantar injection of 1 μ mol α,β -methylene ATP into the contralateral hindpaw. This dose is similar to concentrations of ATP reached in injured tissue. In inflammatory pain states, persistent and augmented ATP responses are observed, which suggests that significant endogenous ATP levels contribute to the pathophysiology of pain (Hamilton and McMahon, 2000).

It has been reported previously (Tsuda et al., 2000) that intraplantar injection of α,β -methylene ATP into the hindpaw of naive rats elicits nocifensive behavior (paw licking), which is attributed to activation of peripheral P2X₃ receptors, as well as mechanical allodynia, which was attributed to heteromeric P2X_{2/3} receptor activation. Although P2X₃ ASO treatment is predicted to downregulate all receptors containing P2X₃ subunits, including P2X₃ homomers and P2X_{2/3} heteromers (and formally P2X_{1/3} homomers) (Torres et al., 1999; Petruska et al., 2000a), we observed no reversal on mechanical allodynia in either neuropathic

or inflammatory pain states. One possible explanation for this observation is that P2X₃ receptors do not mediate this modality in neuropathic and inflammatory pain states. It has been proposed that specific subpopulations of DRG afferents, in particular the A-fiber low-threshold mechanoreceptors, are involved in gen-

Figure 9. *A*, Inhibition of α,β -methylene ATP evoked mechanical hyperalgesia in the hindpaw of naive rats after a 2 d treatment with P2X₃ receptor ASO, MSO, or saline. Paw withdrawal thresholds were measured before cannulation on day 0 and immediately before and 1 hr after administration of 1 μ mol α,β -methylene ATP. Each point represents the mean $\pm$ SEM from six animals per treatment group. * $p < 0.05$ compared with saline treatment by ANOVA followed by Tukey's HSD test performed on gram threshold data. *B*, P2X₃ receptor protein levels in DRG are reduced in ASO relative to MSO or saline groups after 2 d of treatment in naive rats. L4 and L5 DRG were isolated and pooled from naive rats and evaluated by Western blot analysis. Immunoblots were reprobated for transferrin to control for protein loading. Representative results are shown for three saline-, MSO-, and ASO-treated animals.

eration of allodynic behavior (Matzner and Devor, 1994; Campbell, 2001; Kim et al., 2001). Indeed, immunohistochemical techniques show that P2X₃ receptors are not localized to these neurons (Bradbury et al., 1998), suggesting that these receptors may not have a role in mechanical allodynia. However, an equally likely possibility for our observations is that there was insufficient downregulation of P2X₃ subunits to elicit a reversal of mechanical allodynia.

Taken together, the findings in the present study show, for the first time, that P2X₃ ASO treatment produces a partial functional downregulation of peripheral P2X₃ receptors and a reduction in P2X₃ receptors in the DRG and the primary afferent terminals of the spinal cord that are associated with a significant, but partial, inhibition of mechanical hyperalgesia in models of inflammatory and neuropathic pain. Previous studies using P2X₃ receptor null-mutant mice (Cockayne et al., 2000; Souslova et al., 2000) did not investigate the effects of the absence of these receptors on neuropathic pain. They did report, however, that these mice show hypersensitivity to an inflammatory stimulus, which is clearly different from our data. The reason for this disparity is unclear. Cook and McCleskey, (2000) have suggested previously that the increased response to the inflammatory stimulus in the null-mutant mice may have been a compensatory artifact of the gene knock-out approach. Although Souslova et al., (2000) studied other P2X mRNA levels in the knock-out mice, protein and functional receptor levels were not examined and neither were the levels of other transcripts that may have contributed to the nociceptive mechanism.

In conclusion, our data indicate that homomeric P2X₃ and/or heteromeric P2X_{2/3} receptors play a significant role in both neuropathic and inflammatory pain processing. We would propose that small molecule P2X₃ receptor antagonists would have a beneficial role in alleviating neuropathic, inflammatory, and possibly mixed pain conditions such as osteoarthritis and cancer pain. Moreover, specific P2X₃ receptor antagonists are also likely to reveal the extent of the role of homomeric P2X₃ and heteromeric P2X_{2/3} receptors in these pain states.

Additionally, this work validates the use of ASO technology for dissecting the role of receptor subunits in cases where no selective pharmacological tools are available.

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