Selective Calcium-Dependent Inhibition of ATP-Gated P2X3 Receptors by Bisphosphonate-Induced Endogenous ATP Analog ApppI

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ABSTRACT

Pain is the most unbearable symptom accompanying primary bone cancers and bone metastases. Bone resorptive disorders are often associated with hypercalcemia, contributing to the pathologic process. Nitrogen-containing bisphosphonates (NBPs) are efficiently used to treat bone cancers and metastases. Apart from their toxic effect on cancer cells, NBPs also provide analgesia via poorly understood mechanisms. We previously showed that NBPs, by inhibiting the mevalonate pathway, induced formation of novel ATP analogs such as ApppI [1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) triphosphoric acid diester], which can potentially be involved in NBP analgesia. In this study, we used the patch-clamp technique to explore the action of ApppI on native ATP-gated P2X receptors in rat sensory neurons and rat and human P2X3, P2X2, and P2X7 receptors expressed in human embryonic kidney cells. We found that although ApppI has weak agonist

activity, it is a potent inhibitor of P2X3 receptors operating in the nanomolar range. The inhibitory action of ApppI was completely blocked in hypercalcemia-like conditions and was stronger in human than in rat P2X3 receptors. In contrast, P2X2 and P2X7 receptors were insensitive to ApppI, suggesting a high selectivity of ApppI for the P2X3 receptor subtype. NBP, metabolite isopentenyl pyrophosphate, and endogenous AMP did not exert any inhibitory action, indicating that only intact ApppI has inhibitory activity. Ca^{2+} -dependent inhibition was stronger in trigeminal neurons preferentially expressing desensitizing P2X3 subunits than in nodose ganglia neurons, which also express nondesensitizing P2X2 subunits. Altogether, we characterized previously unknown purinergic mechanisms of NBP-induced metabolites and suggest ApppI as the endogenous pain inhibitor contributing to cancer treatment with NBPs.

Introduction

Patients with primary bone cancers or bone metastases from breast and prostate cancer experience a high level of pain, which remains intractable in many cases (Mantyh, 2014; Maier et al., 2015; van den Beuken-van Everdingen et al., 2016).

Bisphosphonates are among the most specific bone cancer treatments available to date (Lin, 1996; Kavanagh et al., 2006; Russell, 2011; Debiais, 2013; Coleman et al., 2014). Bisphosphonates are divided into two main groups: 1) simple bisphosphonates without a nitrogen group and 2) the most promising third-generation nitrogen-containing bisphosphonates (NBPs) (Frith et al., 2001; Rogers et al., 2011). NBPs also have an antinociceptive effect (Kavanagh et al., 2006; Fujita et al., 2009), which contributes to their therapeutic effect (Abe et al.,

2011; Maier et al., 2015). However, the mechanisms of NBP analgesia remain unclear. We previously showed that inhibition of farnesyl-diphosphate synthase by the NBP zoledronate (Mönkkönen et al., 2006; Rääkkönen et al., 2009, 2010, 2011) induces formation of the ATP analog ApppI [1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) triphosphoric acid diester], which can produce a toxic effect on osteoclasts and tumor cells (Lehenkari et al., 2002; Green, 2004; Mönkkönen et al., 2006). However, the interaction of ApppI with the nociceptive system has not yet been characterized. Since ApppI structurally resembles the antinociceptive diadenosine polyphosphates (Viatchenko-Karpinski et al., 2016), we investigated the effects of the NBP-induced derivative ApppI on the activity of nociceptive receptors P2X2, P2X3, and P2X7.

P2X3 receptors implicated in neuropathic (McGaraughty et al., 2003; Chen et al., 2005) and inflammatory pain (Cockayne et al., 2000; Souslova et al., 2000) are almost exclusively expressed in nociceptive neurons (Chen et al., 1995; Lewis et al., 1995). Notably, they are expressed in terminals of C- and Aδ-fibers

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ABBREVIATIONS: A-317491, 5-[(3-phenoxyphenyl)methyl-[(1S)-1,2,3,4-tetrahydronaphthalen-1-yl]carbamoyl]benzene-1,2,4-tricarboxylic acid; ApppI, 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) triphosphoric acid diester; GFP, green fluorescent protein; HEK, human embryonic kidney; IPP, isopentenyl pyrophosphate; NBP, nitrogen-containing bisphosphonate; NMR, nuclear magnetic resonance; TMSB, Trizma-maleate sucrose buffer.

innervating the bone (Hukkanen et al., 1992; Mach et al., 2002; Kaan et al., 2010; Falk et al., 2012; Zhao and Levy, 2014). There is abundant evidence of the involvement of P2X3 receptors in cancer pain (Kaan et al., 2010; Falk et al., 2012; Hansen et al., 2012; Wu et al., 2012; Burnstock and Di Virgilio, 2013). Interestingly, unlike in rodent models, the P2X3 receptor subtype in human nociceptive neurons of the dorsal root ganglion does not express heterotrimeric functional receptors with P2X2 subunits (Serrano et al., 2012), meaning that homomeric P2X3 is the main receptor involved in nociception in bone cancer. The P2X3 and P2X2/3 receptor antagonist A-317491 (5-[(3-phenoxyphenyl)methyl-[(1S)-1,2,3,4-tetrahydronaphthalen-1-yl]carbamoyl]benzene-1,2,4-tricarboxylic acid) attenuated bone cancer-induced pain in mice transiently but had no effect in the late stage of the malignant process (Hansen et al., 2012). The increased expression of P2X3 receptors in calcitonin gene-related peptide-containing nerve fibers during tumor growth also suggests a role for ATP receptors in cancer-related pain (Gilchrist et al., 2005). Another study also found that P2X3 receptors are functionally upregulated in the dorsal root ganglia in a rat model of bone cancer (Wu et al., 2012).

Growing evidence suggests that cancer-related bone pain is associated with osteoclast activation (Luger et al., 2005; Nagae et al., 2006) and Ca^{2+} misbalance. In some pathologic conditions such as parathyroid hormone-related protein-mediated hypercalcemic crisis, serum Ca^{2+} reaches hypercalcemic levels that increase from a normal level of approximately 2 mM up to 3.5 mM (Rahil and Khan, 2012; Fijorek et al., 2014). In addition, malignancy-associated hypercalcemia is often associated with headache (Stewart, 2005; Basso et al., 2011; Hu et al., 2013; Goldner, 2016; Tagiyev et al., 2016). Notably, serum Ca^{2+} levels can be elevated in malignancy (to 4 mM or more in the most severe cases) (Goldner, 2016); however, in the clinic, hypercalcemia decreased 24–48 hours after NBP treatment and the serum Ca^{2+} level was normalized for 60%–90% of patients after 4–7 days of NBP treatment (Stewart, 2005; Endres, 2012).

Extracellular Ca^{2+} plays an important role in P2X3 signaling (Cook and McCleskey, 1997; Cook et al., 1998). Thus, elevated Ca^{2+} accelerates resensitization of these receptors (Cook and McCleskey, 1997; Cook et al., 1998; Giniatullin et al., 2003), making them functionally more active. In contrast, Ca^{2+} -free solution prolongs the ATP-evoked inactive desensitized P2X3 receptor state (Cook et al., 1998; Giniatullin et al., 2003).

Here, we show strong Ca^{2+} -dependent inhibition of the pronociceptive P2X3 receptors by low nanomolar concentrations of ApppI. Our data predict that the antinociceptive action of ApppI should be largely enhanced when the concentration of Ca^{2+} decreases in response to NBP treatment. Thus, we propose that ApppI generated by cancer treatment with NBPs (e.g., zoledronate) can contribute to pain relief via the selective inhibition of ATP-gated P2X3 receptors in the sensory neurons.

Materials and Methods

All animal use protocols were in accordance with recommendations of the Federation for Laboratory Animal Science Associations and were approved by the local institutional animal care and use committees. Wistar rats (P10-12 and P35-38) were obtained from the Animal Facilities of the University of Eastern Finland (Kuopio, Finland). Experiments were designed to minimize the number of animals used in research.

Cell Culture and Transfection

Trigeminal and nodose ganglia cells from Wistar male rats (P10-12) were prepared as described previously (Simonetti et al., 2006; Li and Schild, 2007; Yegutkin et al., 2016) with minor modifications. Briefly, after decapitation, trigeminal and nodose ganglia were isolated and dissociated in an enzymatic cocktail containing collagenase, trypsin, and DNAase (Sigma-Aldrich, St. Louis, MO) added to F-12 media (Gibco/Invitrogen, Grand Island, NY) under continuous mixing (900 rpm) in an Eppendorf ThermoMixer (Eppendorf, Hamburg, Germany) at 37°C for 15 minutes. After dissociation, cells were resuspended in fresh F-12 media, centrifuged at 1000 rpm for 5 minutes at room temperature, and plated on coverslips coated with poly(L-lysine) (Sigma-Aldrich). Cells were maintained in F-12 GlutaMAX medium containing 10% fetal bovine serum (Gibco) and 100 U/ml penicillin and streptomycin at 37°C in a 5% CO_2 atmosphere in a humidified incubator. Cells were used for experiments 48 hours after plating.

Human embryonic kidney HEK293 and HEK293T cell cultures were maintained as reported previously (Fabbretti et al., 2004; Viatchenko-Karpinski et al., 2016) in Dulbecco's modified Eagle's medium (Gibco/Invitrogen) supplemented with 10% fetal bovine serum and antibiotics. Cells were passaged at 80% confluence. HEK cells were transiently transfected in a 12-well plate (10^5 cells per well) using FugeneHD (Promega, Madison, WI) (Jindrichova et al., 2011). Twelve hours after transfection, cells were replated on coverslips coated with poly(L-lysine) (Sigma-Aldrich). Transfection was performed with cDNA encoding rat rP2X3 tagged with green fluorescent protein (GFP) (rP2X3-GFP), rP2X2-GFP, and rP2X7-GFP and human hP2X3, hP2X2, and hP2X7 (GFP added separately). Sufficient receptor expression was observed in 24 hours for recombinant rP2Xs and 48–72 hours for the hP2Xs homolog when cells were used for electrophysiological experiments.

Electrophysiological Recording and Solutions. Whole-cell patch-clamp recordings from HEK293 cells and trigeminal and nodose ganglia neurons were performed using a HEKA-10 amplifier with HEKA PatchMaster software at a holding potential of -70 mV (HEKA Elektronik, Holliston, MA). Glass electrodes (3–6 M Ω) filled with intracellular solution containing 130 mM CsCl, 5 mM MgCl₂, 10 mM HEPES, 5 mM EGTA, 0.5 mM CaCl₂, 2 mM Mg-ATP, 0.5 mM Na-GTP, and 5 mM KCl, pH 7.2, adjusted by CsOH were used for the current recordings. During the experiment, cells were superfused (2 ml/min) with basic extracellular solution containing 148 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 HEPES, and 10 D-glucose, pH 7.4, adjusted by NaOH.

Drug Delivery. P2X receptors were activated by fast agonist application (exchange time of approximately 30 milliseconds) using the rapid solution exchange system (RSC-200; Bio-Logic Science Instruments, Seyssinet-Pariset, France) synchronized with a HEKA amplifier. Application tubes were placed approximately 100–150 μm from cell recordings. Stocks of α,β -meATP, ATP, ApppI, isopentenyl pyrophosphate (IPP), and zoledronate were prepared in ultrapure water and diluted to the final concentrations before the experiment. The duration of agonist applications (α,β -meATP, ATP, and ApppI) was typically 2 seconds unless indicated otherwise. ApppI, IPP, zoledronate, and AMP inhibitory activity was tested by applying the compounds for 120 seconds between the test pulses.

Synthesis of ApppI and IPP

The ATP analog ApppI was synthesized as previously reported (Weisell et al., 2015). The purity of ApppI was $\geq 95\%$, as determined from ^1H and ^{31}P nuclear magnetic resonance (NMR) spectra. The purified ApppI tris(triethylammonium) salt was highly stable based on the ^1H and ^{31}P NMR spectra in samples stored in D₂O for approximately 1.5 years at room temperature. IPP was prepared from pyrophosphate tris(tetrabutylammonium) salt and isopentenyl tosylate (Kao et al., 2005; Weisell et al., 2015). The purity of IPP was $\geq 95\%$ according to ^1H and ^{31}P NMR spectra (Weisell et al., 2015).

Phosphate Enzymatic Histochemistry

Rat hemiskulls were prepared as previously described (Yegutkin et al., 2016). Briefly, the skull was cut along the sagittal suture into two halves. The brain was gently removed, leaving the dura mater intact and attached to the skull cavity. Hemiskulls were then placed in an aerator with 5% CO₂/95% O₂ artificial cerebrospinal fluid containing 5 mM KCl, 119 mM NaCl, 18 mM glucose, 2.7 mM CaCl₂, 0.5 mM MgCl, 1.1 mM NaH₂PO₄, and 30 mM NaHCO₃, pH 7.4. A modification of the lead nitrate [Pb(NO₃)₂] method was used to evaluate ApppI and ATP degradation (Mercier et al., 2012). In brief, after 30 minutes of washing in aerated artificial cerebrospinal fluid, hemiskulls were washed with Trizma-maleate buffer (40 mM Trizma maleate; Sigma-Aldrich) and then preincubated for 45 minutes in Trizma-maleate sucrose buffer (TMSB) (with 0.25 M sucrose, pH 7.4) containing the alkaline phosphatase inhibitor levamisole (2 mM; Sigma-Aldrich). For the next 40 minutes, hemiskulls were incubated with ATP or ApppI (both 300 μM) in TMSB-buffered solutions with freshly added 2 mM Pb(NO₃)₂ after washing in Trizma-maleate buffer. The same amount of incubation medium was used for blank specimens. Samples treated with 0.5% (NH₄)₂S (Sigma-Aldrich) formed the lead orthophosphate precipitates, which presented as brown deposits. After three washings in TMSB, stained meninges were carefully removed from hemiskulls and mounted on glass slides coated with poly(lysine) (Polysine; Thermo Fisher Scientific, Waltham, MA). Slides were mounted with Aquatex mounting medium (Merck, Kenilworth, NJ). An Olympus BX60 microscope with an Olympus DP71 camera with a PlanC ×4/0.10 or UPlanFL ×10/0.30 objective (Olympus, Tokyo, Japan) was used to acquire images. All images were taken with standard settings for further analysis with CorelDRAW Graphics Suite software (version 17; Corel, Ottawa, ON, Canada).

To quantify histochemical images, signal intensity was evaluated in each stained sample from the region of the interest located in similar areas around meningeal vessels with the same diameter (approximately 60–95 μm).

ATP Luminescence Assay

ATP release from the trigeminal ganglion cells (obtained from P10–12 rats) was measured with the ATPlite Luminescence Assay System (catalog no. 6016941; PerkinElmer, Waltham, MA). Cells were cultured in a 24-well plate for 48 hours. Growth medium was then changed with fresh medium (control) or with fresh medium containing 100 μM zoledronate. After 24 hours of treatment, cells were washed and the bathing medium was changed with basic salt solution containing 152 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4. The supernatant was collected and used for the ATP assay. The assay was performed following the ATPlite Assay Kit protocol instructions using white polystyrene 96-well Costar plates (Corning Inc., Corning, NY). Luminescence was measured with a POLARstar Optima microplate reader (BMG Labtech GmbH, Ortenberg, Germany).

Statistical Analysis

We used HEKA FitMaster software for patch-clamp data analysis. Further analysis was performed using Origin 8.5 (OriginLab, Northampton, MA). The paired two-tailed *t* test was used to assess significant differences. Dose-response fitting was performed using the Hill function. Data are presented as means ± S.E.M. *P* < 0.05 was accepted as significant.

Results

Agonist Activity of ApppI

First, we investigated the agonist activity of ApppI on rat recombinant P2X3 receptors expressed in HEK cells compared

with responses induced by the full agonist of P2X3, α,β-meATP. We found that ApppI was able to activate P2X3 receptors only in relatively high concentrations. Figure 1 illustrates the typical responses of two agonists that were both applied for 2 seconds in a 10-μM concentration (Fig. 1B) and the dose-response curve for agonist activity of ApppI (EC₅₀ = 16.4 ± 0.9 μM, *n* = 7; Fig. 1C). One distinguishing property of the P2X3 receptor is the fast (millisecond range) desensitization in the continuous presence of the agonist (Chen et al., 1995; Lewis et al., 1995; Cook and McCleskey, 1997; Jiang et al., 2003). In our experiments, both α,β-meATP and ApppI induced desensitizing currents (Fig. 1B). However, the time for recovery from desensitization of P2X3 receptors was longer in the case of

Fig. 1. Agonist activity of ApppI on P2X3 receptors. (A) Chemical structures of ApppI synthesized and used for the experiments. The chemical structure of ApppI was constructed using ChemBioDraw Ultra software. (B) Example of rP2X3 receptor-mediated currents induced by application of 10 μM α,β-meATP and 10 μM ApppI to rP2X3-transfected HEK293 cells. (C) Dose-response curve of ApppI action on rP2X3 receptors (EC₅₀ = 16.37 ± 0.94 μM, *n* = 7). (D) Example of P2X3 receptor-mediated desensitizing currents induced by paired application of 10 μM ApppI, α,β-meATP.

ApppI. Thus, the paired-pulse protocol with variable intervals (5–120 seconds) indicated that the signal activated by ApppI recovered only to $68.4\% \pm 1.2\%$ in 2 minutes ($n = 4$; Fig. 1D), whereas recovery in this interval was almost complete for α,β -meATP (Fig. 1B). In summary, membrane currents activated by $10\ \mu\text{M}$ ApppI were nearly 10 times lower in amplitude than currents induced by a similar concentration of α,β -meATP (Fig. 1A). This indicated that ApppI is only a partial agonist of P2X3 receptors.

ApppI-Induced Inhibition of P2X3 Receptors

We then evaluated the ability of ApppI to induce a high-affinity desensitization of P2X3 receptors leading to inhibitory and thus antinociceptive effects (Giniatullin et al., 2008; Viatchenko-Karpinski et al., 2016). Therefore, we tested the different concentrations of ApppI to determine its inhibitory potency on rP2X3 receptors. In the control, a paired application of $10\ \mu\text{M}$ α,β -meATP for 2 seconds with a 120-second interval produced membrane currents with similar amplitude (Fig. 2A). We found that even $10\ \text{nM}$ ApppI applied for 120 seconds between two α,β -meATP test pulses was able to inhibit P2X3 receptor-mediated currents to $36\% \pm 8\%$ of control values ($P = 0.0013$, $n = 10$; Fig. 2B).

ApppI applied at the higher 100-nM concentration inhibited test currents to $15.4\% \pm 3.9\%$ of control values ($P = 0.02$, $n = 4$), whereas $1\ \mu\text{M}$ ApppI reduced the α,β -meATP-induced currents to $7.1\% \pm 3.9\%$ ($P = 0.03$, $n = 4$). Using the same protocol, we also tested the inhibitory potency of $10\ \text{nM}$ ApppI when P2X3 receptors are activated by their natural agonist ATP (Fig. 2C). We found, that $10\ \text{nM}$ ApppI significantly inhibited ATP-induced P2X3 currents to $42.2\% \pm 4\%$ ($P = 0.018$, $n = 12$; Fig. 2D), which was very similar to results obtained with α,β -meATP. Pooled data for the inhibitory action of ApppI on α,β -meATP- and ATP-evoked currents are shown in Fig. 2E.

Furthermore, to characterize the inhibitory action of ApppI, we constructed the dose-response curve for α,β -meATP in the control and in the presence of $10\ \text{nM}$ ApppI. We found that $10\ \text{nM}$ ApppI strongly decreased currents induced by different concentrations of α,β -meATP, indicating reduced efficacy without significant changes in affinity. Thus, the EC_{50} for α,β -meATP-induced currents was $1.10 \pm 0.03\ \mu\text{M}$ in the control ($n = 14$) and $1.16 \pm 0.02\ \mu\text{M}$ in the ApppI-treated group ($n = 5$, $P > 0.05$; Fig. 2F). These results indicated that ApppI is a noncompetitive antagonist, consistent with the desensitizing mechanism of its inhibitory action.

Fig. 2. Inhibitory potency of ApppI on P2X3 receptors. (A) Example of current induced by $10\ \mu\text{M}$ α,β -meATP applied for 2 seconds with an interval of 120 seconds. Notice the full recovery of the second current. (B) Example of the inhibitory action of $10\ \text{nM}$ ApppI applied for 120 seconds between two α,β -meATP applications. (C) Example of current induced by $20\ \mu\text{M}$ ATP applied for 2 seconds with an interval of 120 seconds. (D) Example of the inhibitory action of $10\ \text{nM}$ ApppI applied for 120 seconds between two ATP applications, which demonstrates similar rates of ApppI inhibition for ATP and α,β -meATP-induced rP2X3 currents. (E) Histograms showing the inhibitory effect of different ApppI concentrations on α,β -meATP-induced currents (open white circles, $7.1\% \pm 3.9\%$, *, $P = 0.03$; $15.4\% \pm 3.9\%$, *, $P = 0.02$; and $36\% \pm 8\%$, **, $P = 0.0013$, respectively; $n = 4$ –10) and the inhibitory effect of $10\ \text{nM}$ ApppI on ATP-stimulated (black circles) rP2X3 currents (*, $P = 0.018$, $n = 12$). (F) Dose-response curves of α,β -meATP alone (black line, $\text{EC}_{50} = 1.10 \pm 0.03\ \mu\text{M}$, $n = 14$) and in the presence of $10\ \text{nM}$ ApppI (gray line, $\text{EC}_{50} = 1.16 \pm 0.02\ \mu\text{M}$, $n = 5$) on rP2X3 receptors. α,β , α,β -meATP.

Ca²⁺ Dependence of the Inhibitory Action of ApppI

Since bone cancer is often associated with disturbances of Ca²⁺ homeostasis, we tested the action of ApppI in different concentrations of extracellular Ca²⁺. We found that in a low concentration (0.2 mM) of extracellular Ca²⁺, 10 nM ApppI had the strongest depressant action on rP2X3 receptors (to 12% ± 3% of control, $P = 0.0002$, $n = 14$; Fig. 3, A and C). Lower inhibition was observed in 4 mM Ca²⁺ (to 68% ± 8%, $P = 0.035$, $n = 4$; Fig. 3C). In sharp contrast, in a high concentration (10 mM) of extracellular Ca²⁺ (which corresponds to severe hypercalcemia), we did not observe significant current suppression by ApppI (86% ± 10%, $P > 0.1$, $n = 7$; Fig. 3, B and C).

Selectivity of ApppI Action on P2X3 Subtype

To determine the specificity of ApppI action on pronociceptive P2X3 receptors, we tested the action of this agent on rP2X2 receptors expressed in HEK293 cells. We did not detect any agonist activity for the relatively high (10 μM) concentration

Fig. 3. Ca²⁺-dependent inhibitory action of ApppI on rP2X3 receptors. (A) Example of the inhibitory action of 10 nM ApppI in low (0.2 mM) extracellular Ca²⁺. (B) Example of ApppI (10 nM) application in high (10 mM) extracellular Ca²⁺. (C) Histograms showing the Ca²⁺-dependent action of 10 nM ApppI on rat recombinant rP2X3 receptors (***, $P = 0.0002$, $n = 14$; **, $P = 0.0013$, $n = 10$; *, $P = 0.035$, $n = 4$; and $P > 0.1$, $n = 7$, respectively, for 0.2, 2, 4, and 10 mM extracellular Ca²⁺). $\alpha\beta$, α,β -meATP.

of ApppI on rP2X2 receptors, although we did observe a large response with 10 μM ATP ($n = 4$; Fig. 4A). We also did not observe any inhibitory effect of ApppI on rP2X2 receptors ($n = 4$; Fig. 4B). Likewise, we observed neither an agonist nor antagonist (inhibitory) action of ApppI on rP2X7 receptors ($n = 4$; Fig. 4, C and D). These data suggest that ApppI acts specifically on P2X3 receptors. We further investigated the possibility that ApppI interferes with the hP2X7 and hP2X2 receptor subtypes. We used similar protocols as for rP2X2 and rP2X7 receptors to evaluate both the agonist and inhibitory action of ApppI. We used 1 mM ATP for stronger stimulation of the hP2X7 receptors (Fig. 4, G and H). As with

Fig. 4. Testing ApppI action on P2X2 and P2X7 receptors. (A) Example of ATP-activated rP2X2 receptor-mediated currents and the absence of the agonist effect of 10 μM ApppI on this receptor type ($n = 4$). (B) Test of the inhibitory action of 1 μM ApppI applied for 120 seconds on rP2X2 receptors ($n = 4$). Notice that even this relatively high concentration of ApppI was unable to inhibit non-desensitizing rP2X2 currents. (C) Example of ATP-activated hP2X2 receptor-mediated currents and the absence of the agonist effect of 10 μM ApppI on this receptor type ($n = 4$). (D) Absence of the inhibitory action of 1 μM ApppI applied for 120 seconds on hP2X2 receptors ($n = 7$). (E) Example of ATP-activated rP2X7 receptor-mediated currents showing the absence of the agonist effect of 100 μM ApppI on this receptor type ($n = 4$). (F) Test of the inhibitory action of 1 μM ApppI applied for 120 seconds on rP2X7 receptors. Notice no effect of ApppI ($n = 4$). (G) Example of ATP-mediated (1 mM) hP2X7 receptor currents showing the absence of the agonist effect of 100 μM ApppI on this receptor type ($n = 6$). (H) Experimental trace demonstrating no effect after 1 μM ApppI application for 120 seconds on hP2X7 receptors ($n = 8$). $\alpha\beta$, α,β -meATP.

the rat receptors, we found neither an agonist nor inhibitory effect of ApppI on hP2X7 and hP2X2 receptors (Fig. 4, C, D, G, and H), again suggesting that ApppI is selective for P2X3 subunit-containing receptors in both rats and humans.

We compared currents activated by α,β -meATP in trigeminal and nodose ganglia because the nodose neurons, responsible for the visceral pain, express preferentially heteromeric P2X2/3 subunits that produce slow currents with little desensitization (Viatchenko-Karpinski et al., 2016). Trigeminal ganglion nociceptors take part in bone innervations; in particular, they supply the cranial periosteum (Zhao and Levy, 2014). We found that fast desensitizing currents activated with α,β -meATP in trigeminal ganglion neurons were potently inhibited by 10 nM ApppI (to $44\% \pm 4\%$, $P = 0.0007$, $n = 17$; Fig. 5A). In contrast, slow currents in nodose ganglion neurons were almost insensitive to this treatment ($90.4\% \pm 3.4\%$, $P > 0.1$, $n = 8$; Fig. 5B). These data also suggest the selectivity of ApppI for homomeric P2X3 receptors.

Consistent with the data from rat recombinant P2X3 receptors, we observed a strong Ca^{2+} -dependent inhibitory action of ApppI on native rP2X3 receptors in trigeminal neurons. Thus, in a low Ca^{2+} concentration (0.2 mM), the test currents were suppressed by 10 nM ApppI to $9.0\% \pm 4.1\%$ ($P = 0.05$, $n = 7$); in the physiologic concentration of Ca^{2+} (2 mM), α,β -meATP currents were suppressed only to 44% of the control value. In conditions mimicking hypercalcemia (10 mM), we detected no inhibitory action of ApppI on trigeminal neurons ($87.7\% \pm 4.9\%$, $P > 0.5$, $n = 10$; Fig. 5C).

ApppI Action on Human P2X3 Homolog

For translational aspects and to explore whether this action is conserved in different species, it was interesting to study the inhibitory effect of low nanomolar ApppI on hP2X3 receptors. Interestingly, we found a very strong depressant effect of 10 nM ApppI in the physiologic Ca^{2+} concentration in hP2X3 receptors compared to the rat homolog (depression to $2.7\% \pm 1.0\%$, $P = 0.02$, $n = 8$; Fig. 6, A and C). Such a strong effect of low concentrations was consistent with the potential role of endogenous ApppI in human sensory neurons. Next, as with rP2X3 receptors, we characterized ApppI inhibition of hP2X3 receptors in conditions mimicking hypercalcemia (modeled with 10 mM Ca^{2+}). As expected, the inhibitory effect of 10 nM ApppI was almost fully eliminated in this hypercalcemia-like condition ($84\% \pm 4\%$, $n = 14$, $P > 0.05$; Fig. 6, B and C). Thus, we confirmed the strong Ca^{2+} dependence of ApppI inhibition in both rP2X3 and hP2X3 receptors.

No Direct Effect of ApppI Metabolites and Zoledronate

To explore whether the inhibitory action of ApppI required the full molecule of ApppI or whether it was due to ApppI metabolites that could exert their own physiologic effect, we tested the action of IPP and AMP on P2X3 receptors. The same protocol of drug application as for ApppI was used for this aim. However, administration of 1 μM IPP and 1 μM AMP on P2X3 receptors did not produce any inhibitory effect (Fig. 7, C and D). Next, we investigated whether zoledronate (Mönkkönen et al., 2006), an inducer of ApppI, can directly inhibit P2X3 receptors. We found that 10 μM zoledronate did not affect the function of P2X3 receptors (Fig. 7E), suggesting a lack of direct action of this NBP on the P2X3 receptor (Fig. 7F). However, using the ATPlite Luminescence Assay System, we found that

Fig. 5. Comparing ApppI inhibition in trigeminal and nodose ganglia neurons. (A) Example of α,β -meATP current inhibition by 10 nM ApppI in trigeminal ganglia neurons. (B) Example of the lack of inhibitory effect of 10 nM ApppI on currents in nodose ganglia neurons. (C) Histograms presenting Ca^{2+} -dependent inhibition by 10 nM ApppI of native P2X receptors in trigeminal and nodose ganglion neurons. (*, $P = 0.050$, $n = 7$; **, $P = 0.0007$, $n = 17$; and $P > 0.5$, $n = 10$ for the trigeminal ganglion in 0.2, 2, and 10 mM Ca^{2+} , respectively; $P > 0.1$, $n = 8$ for nodose ganglion neurons). α,β , α,β -meATP.

zoledronate was able to induce the release of purines from cells to the extracellular medium. Thus, 100 μM zoledronate applied for 24 hours to the trigeminal neuronal culture significantly increased the concentration of extracellular ATP from 0.70 ± 0.26 nM to 3.2 ± 0.4 nM ($P = 0.018$, $n = 12$ and 6, respectively; Fig. 7G), suggesting a similar release of endogenous ApppI.

ApppI Biodegradation

Because many endogenous polyphosphates are quickly degraded in the live tissues by multiple nucleotidases (Yegutkin et al., 2008, 2016), we next investigated the intensity of ApppI hydrolysis in rat meninges. For that purpose, we used the lead nitrate method, based on the generation of lead orthophosphate precipitation in the course of nucleotidase activity (Yegutkin et al., 2008), which we recently optimized for application in transparent rat meninges (Yegutkin et al., 2016). We performed this labeling to evaluate the speed of

Fig. 6. ApppI inhibition of the hP2X3 subtype. (A) Example of the inhibitory action of 10 nM ApppI in physiologic (2 mM) extracellular Ca^{2+} on α,β -meATP-activated currents in hP2X3 receptors. (B) Example of ApppI (10 nM) application in high (10 mM) extracellular Ca^{2+} on α,β -meATP-activated currents in hP2X3 receptors. To note, the Ca^{2+} -dependent decrease in ApppI inhibition on hP2X3 replicates the effect on the rP2X3 subtype. (C) Histograms showing statistics on the inhibition by 10 nM ApppI and recovery of the hP2X3-induced currents in 2 mM and 10 mM extracellular Ca^{2+} , respectively ($2.7 \pm 1.0\%$ versus control, **, $P = 0.02$, $n = 8$, open white circles; $84 \pm 4\%$, $n = 14$, $P > 0.5$, black circles). α,β , α,β -meATP.

ApppI degradation compared with ATP. Precipitates were clearly visualized in the meninges as brown deposits around the main arteries (Yegutkin et al., 2016), which is the main site for migraine pain generation (Olesen et al., 2009; Zakharov et al., 2015). In comparing the images in Fig. 8, A–C, it is clear that the labeling was very weak in the control (without added purines), stronger with 300 μM ApppI, and very intense in the case of 300 μM ATP, indicating very fast degradation of phosphates from the latter compound. Pooled data are presented for this test in Fig. 8D ($n = 4$ for all cases). These results revealed the relative stability of ApppI versus ATP, suggesting a longer effect of the former on P2X3 receptors.

Fig. 7. Lack of the inhibitory effect of AMP, IPP, or zoledronate on P2X3 receptors. (A and B) Chemical structures of IPP and zoledronate synthesized and used for the experiments. Chemical structures of IPP and zoledronate were constructed using ChemBioDraw Ultra software. (C–E) Examples of the action of 1 μM AMP, 1 μM IPP, or 10 μM zoledronate on α,β -meATP-activated currents. (F) Histograms represent the average effects of AMP, IPP, and zoledronate on P2X3 receptors ($P \geq 0.9$, $n = 10$; $P \geq 0.8$, $n = 11$; and $P \geq 0.8$, $n = 13$, respectively). (G) The histogram showing ATP-luminescent assay data obtained from trigeminal neurons. Notice that the extracellular ATP concentration was significantly increased after treatment with 100 μM ZOL for 24 hours (0.70 ± 0.26 nM, $n = 12$ in the control; 3.2 ± 0.4 nM, $n = 6$ after treatment; **, $P = 0.018$). α,β , α,β -meATP; ZOL, zoledronate.

Discussion

The main finding of this study is that the NBP-induced ATP analog ApppI has a very strong and selective inhibitory effect on pronociceptive P2X3 receptors. Relative stability and slow resensitization of receptors interacting with this compound favor the inhibitory action of endogenous ApppI. We also suggest that reducing the level of extracellular Ca^{2+} during bone tumor treatment with NBPs should promote the antinociceptive effect of ApppI on P2X3 receptors.

Low Agonist and High Antagonist Activity of ApppI on P2X3 Receptors

Here, we show the strong inhibition of pain transducing P2X3 receptors by a low nanomolar concentration of ApppI in

Fig. 8. Comparison of nucleotide hydrolysis of ApppI and ATP. (A–C) Images of meninges treated with vehicle (A), 300 μM ApppI (B), and 300 μM ATP (C), respectively. The activity of nucleotidases generating phosphate due to polyphosphate hydrolysis was visualized using lead nitrate and diammonium sulphide ($n = 5$). (D) Histogram representing statistics of the signal intensity of the vessels colored after phosphate staining with added substances (control: 28.4 ± 2.4 , $n = 4$; ATP: 221.1 ± 8.7 , $***$, $P < 0.0001$, $n = 4$; ApppI: 59.6 ± 6.3 , $**$, $P = 0.0033$, $n = 4$).

trigeminal neurons and transfected HEK293 cells. Our pharmacological testing with a wide range of ApppI concentrations indicated this ATP analog to be only a weak and partial agonist of ATP-gated P2X3 receptors. Indeed, even in saturating concentrations, the currents activated by ApppI were approximately 20% of the currents induced by the full agonist of P2X3 receptors (α, β -meATP). In contrast, ApppI demonstrated the ability to inhibit P2X3 receptors in impressively low nanomolar concentrations. Thus, the concentration of ApppI needed to inhibit this receptor type is approximately 10,000 times lower than the concentration required for activation. ApppI inhibition is most likely due to high-affinity desensitization (Sokolova et al., 2006), which is also

observed with ATP (Sokolova et al., 2006; Giniatullin and Nistri, 2013). However, ATP is a full P2X3 agonist with limited stability, whereas the stability of ApppI appears to be much higher, a property suggesting that it is able to induce prolonged inhibitory antinociceptive action. In a previous study, we characterized synthetic Ap_nA -analogs with comparable inhibitory action on hP2X3 receptors and antinociceptive action in vivo (Viatchenko-Karpinski et al., 2016); however, ApppI presents even better characteristics as a stronger inhibitory antinociceptive agent.

Selectivity of ApppI Action

High selectivity is essential for a potential analgesic agent. This study reveals that the action of ApppI on the P2X3 receptor is highly selective and acts as such. Whereas the ApppI metabolite AMP can act on adenosine A1 receptors (Rittiner et al., 2012) and IPP is known to inhibit transient receptor potential TRPV3 and TRPA1 channels (Bang et al., 2011), they did not show any activity on the P2X3 receptor in our study. Notably, as the other indicator of selectivity, ApppI did not show any agonist or antagonist activity on either human or rP2X2 and rP2X7 receptor subtypes. Interestingly, the action of ApppI on hP2X3 was even stronger than on the rat homolog.

We also found that the inducer of ApppI, the NBP zoledronate, did not exert any direct effect on the pronociceptive P2X3 receptors, indicating that this bisphosphonate might produce an analgesic effect indirectly through generation of endogenous ApppI.

Ca²⁺ Dependence of ApppI Action and the Role of P2X3 Receptors in Cancer Pain

One specific property of ApppI activity found in this study was that the inhibition of P2X3 receptors was dramatically dependent on the level of extracellular Ca²⁺. Importantly, the level of Ca²⁺ is increased in many bone cancers (Stewart, 2005; Basso et al., 2011; Coleman et al., 2014), whereas the concentration of this cation is decreased to a normal level during NBP anticancer treatment (Dodwell et al., 1992; Stewart, 2005; Basso et al., 2011). In this study, we showed that ApppI inhibition of P2X3 receptors is almost absent in a high level of extracellular Ca²⁺ but is very pronounced at low concentrations of this cation. Importantly, the strong inhibition of hP2X3 receptors was observed in physiologic concentrations of extracellular Ca²⁺ but was eliminated in hypercalcemia-like conditions. Thus, we suggest that the painful stimuli resulting from activation of P2X3 receptors

Fig. 9. Schematic presentation of the potential action of NBP and ApppI on P2X3-mediated bone cancer pain. (A) Presentation of the cancer-related local bone environment and the potential role of P2X3 receptors in mediating bone cancer pain. Notice the ATP release from cancer cells, associated hypercalcemia, and Ca²⁺-dependent persistent activation of painful P2X3 receptors in the cancer state. (B) Cancer treatment with NBP leads to less bone resorption and a lower level of extracellular Ca²⁺, which is taken up into osteoblasts. All of this promotes the inhibitory action of NBP-induced ApppI on the pronociceptive P2X3 receptors, resulting in pain relief. Red lines indicate pronociceptive effects, whereas black lines show antinociceptive effects.

in bone cancer tissues (Hukkanen et al., 1992; Kaan et al., 2010; Falk et al., 2012; Burnstock and Di Virgilio, 2013; Zhao and Levy, 2014) are amplified by high levels of extracellular Ca^{2+} (Giniatullin et al., 2003; Petrenko et al., 2011; Giniatullin and Nistri, 2013). During NBP treatment, NBP-induced endogenous ApppI, assisted by normalized (reduced from hypercalcemia) Ca^{2+} concentrations, could inactivate P2X3 receptors. The schematic presentation of these findings is depicted in Fig. 9. ATP-activated P2X3 receptors, the most common type of nociceptors expressed in sensory neurons (Chen et al., 1995; Lewis et al., 1995; Simonetti et al., 2006), are activated by ATP released from multiple cellular sources, including cancer cells (Fig. 9A). Elevated Ca^{2+} , resulting from bone cancer, helps to keep P2X3 receptor activity at a high level. We propose that cancer treatment with NBP not only generates endogenous ApppI but also sensitizes sensory neurons to ApppI inhibition via a reduced level of extracellular Ca^{2+} (Fig. 9B). Importantly, previous studies showed that antagonists of P2X3 receptors were able to decrease bone pain (Mach et al., 2002; Falk et al., 2012), which supports the proposed purinergic mechanism for pain relief.

This study clarifies previously unexplored mechanisms of antinociception induced by NBP and proposes the analgesic role of the endogenous ATP analog generated during bone cancer treatment. We suggest that pain relief derived from NBPs includes ApppI-induced inactivation of the P2X3 receptors, further amplified by a lowered level of extracellular Ca^{2+} . The high efficiency of ApppI in the inactivation of pain receptors can help to design new types of antinociceptive agents to manage painful bone cancer and bone metastases.

Authorship Contributions

Participated in research design: Määttä, Mönkkönen, Giniatullin.

Conducted experiments: Ishchenko, Shakirzyanova, Giniatullina, Skorinkin.

Contributed new reagents or analytic tools: Turhanen.

Performed data analysis: Ishchenko, Shakirzyanova, Giniatullina.

Wrote or contributed to the writing of the manuscript: Ishchenko, Shakirzyanova, Giniatullina, Skorinkin, Bart, Turhanen, Määttä, Mönkkönen, Giniatullin.

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