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CaMKIIα and caveolin-1 cooperate to drive ATP-induced membrane delivery of the P2X3 receptor

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The P2X3 receptor plays a vital role in sensory processing and transmission. The assembly and trafficking of the P2X3 receptor are important for its function in primary sensory neurons. As an important inflammation mediator, ATP is released from different cell types around primary sensory neurons, especially under pathological pain conditions. Here, we show that α, β-MeATP dramatically promoted membrane delivery of the P2X3 receptor both in HEK293T cells expressing recombinant P2X3 receptor and in rat primary sensory neurons. α, β-MeATP induced P2X3 receptor-mediated Ca²⁺ influx, which further activated Ca²⁺/calmodulin-dependent protein kinase IIα (CaMKIIα). The N terminus of the P2X3 receptor was responsible for CaMKIIα binding, whereas Thr³⁸⁸ in the C terminus was phosphorylated by CaMKIIα. Thr³⁸⁸ phosphorylation increased P2X3 receptor binding to caveolin-1. Caveolin-1 knockdown abrogated the α, β-MeATP-induced membrane insertion of the P2X3 receptor. Moreover, α, β-MeATP drove the CaMKIIα-mediated membrane coinsertion of the P2X2 receptor with the P2X3 receptor. The increased P2X3 receptors on the cell membrane that are due to Thr³⁸⁸ phosphorylation facilitated P2X3 receptor-mediated signal transduction. Together, our data indicate that CaMKIIα and caveolin-1 cooperate to drive ligand-induced membrane delivery of the P2X3 receptor and may provide a mechanism of P2X3 receptor sensitization in pain development.

Keywords: P2X3 receptor, ATP, membrane delivery, CaMKIIα, caveolin-1

Introduction

An ATP-gated channel, the P2X3 receptor is primarily expressed in the primary sensory neurons located in dorsal root ganglion (DRG) and is responsible for pain processing (Burnstock, 2000; North, 2004; Wirkner et al., 2007). P2X3 receptor function is regulated by intracellular trafficking and post-translational modification of the receptor (Murrell-Lagnado and Qureshi, 2008). Several mediators including calcitonin gene-related peptide (CGRP), nerve growth factor and prostaglandins facilitate the P2X3 receptor-mediated response by either driving the membrane insertion of the receptor or modulating its phosphorylation state at the plasma membrane (Fabbretti and Nistri, 2012).

As an important inflammation mediator, high ATP levels are released from different cell types to the DRGs and the nerve terminals of primary sensory neurons during pain processes (Burnstock, 2000). ATP promotes P2X3 receptor endocytosis and formation of the signaling endosome that is retrogradely transported to the cell body to regulate neuronal function (Chen et al., 2012). A previous study also detected that ATP transiently drives membrane insertion of the P2X3 receptor in transfected HEK293 cells but not in DRG neurons (Vacca et al., 2009). However, ATP-mediated forward trafficking of the P2X3 receptor and its related molecular mechanisms have not been fully clarified.

Importantly, P2X3 receptor activation by ATP phosphorylates Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) in cultured DRG neurons, and this activation has been implicated in neuropathic pain development (Hasegawa et al., 2009). Upregulation of total and active CaMKII levels in DRGs induced by electrical stimulation of the sciatic nerve in vivo promotes P2X3 receptor trafficking to the plasma membrane (Xu and Huang, 2004). Moreover, the P2X3 receptor localizes to lipid rafts (Vacca et al., 2004; Allsopp et al., 2010; Chen et al., 2012) marked by caveolin, which has been implicated in membrane trafficking (Liu et al., 2002). In the present study, we sought to investigate the long-term dynamics of ligand-dependent forward trafficking of the P2X3 receptor and determine the underlying mechanisms. We found that ATP robustly promoted membrane insertion of both native and recombinant P2X3 receptors via CaMKIIα activation. CaMKIIα phosphorylated the P2X3 receptor at Thr³⁸⁸ in a tertiary structure-dependent manner, facilitated the interaction of the P2X3 receptor with caveolin-1, and...
drove the membrane insertion of the P2X3 receptor to enhance its function. We thus propose a novel mechanism of ligand-induced positive feedback to upregulate the P2X3 receptor-mediated response.

**Results**

**α, β-MeATP induces membrane insertion of the P2X3 receptor**

The function of plasma membrane receptors is typically regulated by their spatial and temporal distribution. Both endocytosis and exocytosis control receptor levels on the plasma membrane. It is well documented that agonist exposure promotes the endocytosis of several receptors (Bronfman et al., 2003; Haberstock-Debic et al., 2005). ATP has been reported to potentiate P2X3 receptor endocytosis (Vacca et al., 2009; Chen et al., 2012). However, little is known about the effect of the ligand on the surface levels of the P2X3 receptor, especially in the long term. First, we tested whether forward trafficking of the P2X3 receptor is modulated by the selective agonist of P2X1 and P2X3 receptors α, β-MeATP. We used HEK293T cells, which do not express native P2X receptors, and labeled the membrane receptors with surface biotinylation. Biotinylated receptor levels were compared to total receptor levels in whole cell lysates. The percentage of recombinant P2X3 receptor on the cell membrane was estimated to be <1% in HEK293T cells (Supplementary Figure S1A and B). As controls, the percentages of recombinant P2X2 receptor and native transferrin receptor (TFR) on the cell membrane were 19% and 16% in HEK293T cells, respectively (Supplementary Figure S1A and B). In HEK293T cells expressing P2X3-Myc, treatment with 10 μM α, β-MeATP induced membrane insertion of the P2X3 receptor in a time-dependent manner, with peak levels found at 30–60 min and a return to basal levels within 3 h without a significant effect on the total levels of P2X3 receptor (Figure 1A). This α, β-MeATP-induced membrane delivery of the P2X3 receptor required the persistent presence of ligand in the medium, as a transient treatment with α, β-MeATP for 2 min did not affect the membrane insertion of the P2X3 receptor (Figure 1B). We also found that a 30-min α, β-MeATP treatment immediately followed by a 10-min vehicle incubation recovered the surface levels of the P2X3 receptor (Figure 1B), consistent with the rapid turnover of the P2X3 receptor (Vacca et al., 2009). Pretreatment with the selective antagonist A-317491 for the P2X3 receptor completely abolished α, β-MeATP-induced membrane insertion of the P2X3 receptor (Figure 1C). However, both recombinant P2X1 and P2X2 receptors did not display ligand-induced membrane insertion in HEK293T cells (Figure 1D). Furthermore, in cultured DRG neurons, the percentage of the P2X3 receptor on the cell membrane was ~3% (Supplementary Figure S1A and B). Consistently, treatment with 10 μM α, β-MeATP for 30 min in cultured DRG neurons also induced membrane insertion of the native P2X3 receptor as in HEK293T cells, which was markedly interrupted by A-317491 (Figure 1E). Thus, the P2X3 receptor displays long-term ligand-induced membrane insertion.

Ligand-induced membrane insertion of receptors may derive from the recycling compartment or forward transport from the Golgi complex. Pretreatment with brefeldin A (BFA), which induces Golgi disassembly, not only blocked α, β-MeATP-induced membrane insertion of the P2X3 receptor but also decreased basal membrane levels of this receptor (Figure 1F). This result suggests that α, β-MeATP-induced membrane insertion of the P2X3 receptor derives from the forward transport of newly synthesized receptors.

**CaMKIIα is responsible for α, β-MeATP-induced membrane insertion of the P2X3 receptor**

P2X3 receptor activation induces Ca2+ influx and action potential (North, 2004). In our experiments we defined the downstream signaling pathway that mediates ATP-induced membrane insertion of the P2X3 receptor. Ca2+-free medium prevented α, β-MeATP-induced membrane insertion of the P2X3 receptor in HEK293T cells (Figure 2A), indicating that P2X3 receptor-mediated Ca2+ influx is necessary for this phenomenon. CaMKII and protein kinase C (PKC) are downstream signaling molecules of the P2X3 receptor (Hasegawa et al., 2009; Chen et al., 2012). Pretreatment with the CaMKII inhibitor KN-93 but not the PKC inhibitor BIM completely abolished the α, β-MeATP-induced membrane insertion of the P2X3 receptor (Figure 2B and C). CaMKII is a multifunctional serine/threonine protein kinase that is distributed in the central and peripheral nervous systems (Soderling, 2000; Carlton, 2002; Ichikawa et al., 2004). The α isoform of CaMKII is the most abundant subunit in tissues and is also expressed in DRG neurons (Carlton, 2002).

We demonstrated that α, β-MeATP did not induce receptor membrane insertion in HEK293T cells coexpressing the P2X3 receptor with a kinase-dead CaMKIIα (CaMKIIαK42M, Figure 2D). However, overexpression of CaMKIIα or a constitutively active CaMKIIα (CaMKIIαT286D) elicited a significant increase in P2X3 receptor levels on the cell surface (Figure 2E). We also found that CaMKIIαT286D significantly enhanced total P2X3 receptor levels (Figure 2E), which is consistent with a previous report (Simonetti et al., 2008). Normalized data showed that CaMKIIα activation promoted P2X3 receptor membrane insertion in addition to increasing the synthesis of this receptor protein (Figure 2E). CaMKIIα was expressed at a significantly higher level in DRG neurons than in HEK293T cells (Figure 2F). In cultured DRG neurons, KN-93 pretreatment not only abolished the α, β-MeATP-induced membrane insertion of the P2X3 receptor but also reduced the surface receptor under the basal level (Figure 2G), indicating a physiological role of CaMKII in regulating the membrane delivery of P2X3 receptors. In addition, a 30-min treatment with 1 μM ionomycin, which mediates Ca2+ entry, significantly increased P2X3 receptor membrane insertion in HEK293T cells (Supplementary Figure S2). This effect was inhibited by CaMKIIαK42M overexpression (Supplementary Figure S2), suggesting that intracellular Ca2+ elevation-induced CaMKIIα activation is sufficient to drive the forward transport of the P2X3 receptor. Thus, α, β-MeATP-induced membrane insertion of the P2X3 receptor is mediated by CaMKIIα.

α, β-MeATP-activated CaMKIIα interacts with the P2X3 receptor

CaMKIIα is primarily expressed in isoleucin B3-binding and CGRP-positive DRG neurons (Carlton and Hargett, 2002). To further identify a relationship between CaMKIIα and the P2X3 receptor, we first showed that CaMKIIα was highly expressed in small- and medium-sized DRG neurons positive for the P2X3 receptor in rats (77.2% ± 1.2%, 819 out of 1065 counted neurons; Figure 3A). CaMKIIα phosphorylation at Thr286 is known to be elevated shortly after P2X3 receptor activation in DRG neurons.
Several membrane proteins have been reported to be CaMKIIα substrates (Tan et al., 1994; Omkumar et al., 1996; Roeper et al., 1997). Co-immunoprecipitation experiments showed that the P2X3 receptor interacted with CaMKIIα (Figure 3E), which was potentiated by α, β-MeATP (Figure 3F). We further dissected the P2X3 receptor domain interacting with CaMKIIα. Similar to other P2X subtypes, the P2X3 receptor displays a two-transmembrane topology with the cytoplasmic N and C termini (North, 2002). Considering the different orientation of the N and C termini to the cytoplasmic membrane, we fused them to the type II membrane protein TFR or the type I membrane protein CD8α, respectively (Figure 3G). Co-immunoprecipitation experiments showed that the N but not the C terminus of the P2X3 receptor was responsible for interacting with CaMKIIα (Figure 3G). Importantly, co-immunoprecipitation experiments with native proteins from DRG lysates validated the interaction between P2X3 receptor and CaMKIIα (Hasegawa et al., 2009). In HEK293T cells coexpressing the P2X3 receptor and CaMKIIα, transient CaMKIIα phosphorylation at Thr286 was observed with maximum levels observed at 5 min after α, β-MeATP treatment and recovery to basal levels within 60 min (Figure 3B). Either pretreatment with A-317491 or deprivation of extracellular Ca²⁺ inhibited α, β-MeATP-induced CaMKIIα activation (Figure 3C and D). Thus, α, β-MeATP activates CaMKIIα through P2X3 receptor-mediated Ca²⁺ influx.

Figure 1: Ligand induces membrane insertion of the P2X3 receptor. (A) Surface biotinylation analysis of the P2X3 receptor. HEK293T cells expressing P2X3-Myc were treated with 10 μM α, β-MeATP for different durations followed by cell surface biotinylation/immunoblotting. (B) HEK293T cells expressing P2X3-Myc were treated with 10 μM α, β-MeATP for 2 min plus a 28-min vehicle incubation or for 30 min plus a 10-min vehicle incubation, and subjected to cell surface biotinylation/immunoblotting. (C) HEK293T cells expressing P2X3-Myc were pre-treated with the P2X3 receptor selective antagonist A-317491 for 1 h before α, β-MeATP stimulation and subjected to cell surface biotinylation/immunoblotting. (D) Surface biotinylation analysis of the P2X1 or P2X2 receptor. HEK293T cells expressing P2X1-Myc or P2X2-Myc were treated with 10 μM α, β-MeATP or 100 μM ATP for 30 min, respectively, and subjected to cell surface biotinylation/immunoblotting. (E) Primary cultured DRG neurons were treated with 10 μM α, β-MeATP for 30 min or pre-treated with A-317491 for 1 h before α, β-MeATP stimulation and subjected to cell surface biotinylation/immunoblotting. (F) HEK293T cells expressing P2X3-Myc were pre-treated with BFA for 30 min before α, β-MeATP stimulation and subjected to cell surface biotinylation/immunoblotting. TFR and actin served as internal controls for protein loading. Shown are the mean ± SEM (n = 3–4), *P < 0.05, **P < 0.01, ***P < 0.001.
CaMKII (Figure 3H). Taken together, these results demonstrate that α, β-MeATP-activated CaMKIIα interacts with the N terminus of the P2X3 receptor.

P2X3 receptor phosphorylation at Thr388 is necessary for CaMKIIα-mediated receptor membrane insertion

We explored the effect of CaMKIIα on P2X3 receptor phosphorylation. In HEK293T cells coexpressing CaMKIIα variants with the P2X3 receptor, immunoprecipitation of the P2X3 receptor combined with detection using an anti-phospho-threonine antibody showed that CaMKIIαT286D elevated P2X3 receptor threonine phosphorylation levels (Figure 4A). Substrate phosphorylation by CaMKIIα also occurs at a serine residue (White et al., 1998). The P2X3 receptor serine phosphorylation levels were not obviously changed by CaMKIIαT286D in HEK293T cells (Supplementary Figure S3). Thus, CaMKIIα activation induces P2X3 receptor threonine phosphorylation.

Because CaMKIIα interacts with the P2X3 receptor, the online prediction of the CaMKII phosphosite motif (http://scansite.mit.edu/motifscan_seq.phtml) combined with screening of the minimal recognition motif for phosphorylation by CaMKII (K/RXXS/T) (White et al., 1998) showed a potential phosphosite of the P2X3 receptor (Figure 4B). This motif appears to be conserved across mammalian species and evolutionarily emerged because of the lack of the phosphosite in zebrafish (Figure 4B). We next examined whether Thr388 is involved in ligand-induced membrane insertion of the P2X3 receptor. Typically, threonine is mutated to
Figure 3. α, β-MeATP-activated CaMKIIα interacts with the P2X3 receptor. (A) A representative section of adult rat DRGs labeled with selective antibodies for the P2X3 receptor (green) and CaMKIIα (red). The majority of P2X3 receptor-positive neurons express CaMKIIα (arrows), and a few are negative for CaMKIIα (arrowheads). Scale bar, 30 μm. (B–D) HEK293T cells coexpressing P2X3-Myc and CaMKIIα-GFP were treated with 10 μM α, β-MeATP for different durations (B), pre-treated with A-317491 for 1 h before α, β-MeATP stimulation (C), or treated with 10 μM α, β-MeATP in Ca²⁺-free medium containing EGTA (D), and CaMKIIα activation levels were evaluated by immunoblot analysis of phospho-CaMKIIα (pThr²⁸⁶). Actin served as a loading control. (E and F) HEK293T cells coexpressing P2X3-Myc and CaMKIIα-GFP were treated with 10 μM α, β-MeATP for different durations (F) or not (E). Interaction of the P2X3 receptor with CaMKIIα was detected by immunoprecipitation with a GFP-specific antibody and then subjected to immunoblotting with the indicated antibodies. Shown are the mean ± SEM (n = 4). *P < 0.05, **P < 0.01. (G) A diagram showing that the N and C termini of P2X3 receptor were fused to type II membrane protein transferrin receptor 1 (N-TFR-Myc) and type I membrane protein CD8α (Myc-CD8α-C), respectively (left). Myc-CD8α, Myc-CD8α-C, TFR-Myc or N-TFR-Myc were cotransfected with CaMKIIα-GFP in HEK293T cells. Interaction of CaMKIIα with the N or C terminus of the P2X3 receptor was detected by immunoprecipitation with a GFP-specific antibody and then subjected to immunoblotting with the indicated antibodies (right). (H) Interaction of the native P2X3 receptor with CaMKIIα in DRG lysates was detected by immunoprecipitation with a CaMKIIα-specific antibody and then subjected to immunoblotting with the indicated antibodies.
Aspartic acid and alanine to mimic phosphorylation and non-phosphorylation, respectively. Sometimes, substitution of threonine with alanine mimics the phosphorylation effect possibly due to conformational changes for certain proteins, as previously described (Abramian et al., 2010). We found that substitution of Thr\(^{388}\) with alanine in the P2X3 receptor dramatically potentiated receptor membrane insertion, resembling phosphorylation mimicry (Supplementary Figure S4). In terms of shape and volume, hydrophobic valine roughly resembles threonine and is suitable for mimicking non-phosphorylation (Mori-Konya et al., 2009).

In HEK293T cells, \(\alpha, \beta\)-MeATP treatment did not induce membrane insertion of P2X3\(^{388\beta}\), the non-phosphorylation mutant of the P2X3 receptor (Figure 4C). Importantly, P2X3\(^{388\beta}\), a constitutive phosphorylation mutant of the P2X3 receptor, displayed higher basal levels on the cell membrane compared with the P2X3 receptor, while P2X3\(^{388\beta}\) exhibited substantially lower levels on the cell membrane than the P2X3 receptor (Figure 4D). Neither mutation affected steady receptor expression levels (Figure 4D). Moreover, CaMKII\(^{\alpha T286D}\) did not increase the membrane insertion or the threonine phosphorylation levels of P2X3\(^{388\beta}\) in HEK293T cells (Figure 4E and F). These results indicate that Thr\(^{388}\) in the C terminus is responsible for CaMKII\(^{\alpha}\)-mediated membrane delivery.
phosphorylation and membrane insertion of P2X3 receptor. Because the N terminus of the P2X3 receptor bound to CaM/Kxl (Figure 3G), CaM/Kxl likely phosphorylates the P2X3 receptor in a tertiary structure-dependent manner.

Caveolin-1 is indispensable in α, β-MeATP-induced and CaM/Kxl-mediated membrane insertion of the P2X3 receptor

The P2X3 receptor has been reported to be located in the lipid raft of various cell types (Vacca et al., 2004; Allsopp et al., 2010; Chen et al., 2012). Protein sorting into rafts occurs in the trans-Golgi network (Surma et al., 2012). Caveolin, the main membrane protein in caveoleae, regulates the formation and Golgi exit of caveoleae (Parton et al., 2006). Interaction with caveolin is necessary for the membrane insertion of multiple membrane receptors (Wyse et al., 2003; Syme et al., 2006; Gonzalez et al., 2007). We examined whether caveoleae/caveolin is involved in ATP-induced membrane insertion of the P2X3 receptor. Screening from three different siRNA sequences to knock down endogenous caveolin-1 levels in HEK293T cells showed that caveolin-1 siRNA-2 was the most effective (Figure 5A). However, the functional response of the P2X3 receptor was not affected by caveolin-1 knockdown, as shown by α, β-MeATP-induced activation of extracellular signal-regulated protein kinase (ERK) in HEK293T cells expressing the P2X3 receptor (Supplementary Figure S5). Importantly, caveolin-1 knockdown abrogated α, β-MeATP-induced membrane insertion of the P2X3 receptor (Figure 5B). Thus, caveoleae/caveolin-1 is involved in ligand-induced membrane delivery of the P2X3 receptor.

Co-immunoprecipitation experiments demonstrated that the P2X3 receptor interacted with caveolin-1 in HEK293T cells (Figure 5C). α, β-MeATP treatment facilitated this interaction, which was maintained for a longer time (Figure 5D) than the interaction between P2X3 and CaM/Kxl (Figure 3F). Consistent with this, the phosphorylation mimicry of the P2X3 receptor at Thr^{138} promoted its interaction with caveolin-1, and the non-phosphorylation mimicry significantly attenuated this interaction (Figure 5C). The interaction between P2X3 and caveolin-1 was confirmed through a co-immunoprecipitation experiment with native proteins from the DRG lysates (Figure 5E).

We further found that the cytoplasmic N but not C terminus of the P2X3 receptor was engaged in its binding with caveolin-1.
ATP-induced membrane delivery of the P2X3 receptor

Figure 6 Caveolin-1 is indispensable for α, β-MeATP-induced membrane insertion of the P2X3 receptor. (A) Myc-CD8α, Myc-CD8α-C, TFR-Myc, or N-TFR-Myc was cotransfected with caveolin-1-GFP in HEK293T cells. The interaction of caveolin-1 with the N or C terminus of the P2X3 receptor was detected through immunoprecipitation with a GFP-specific antibody and immunoblotting with the indicated antibodies. (B and C) HEK293T cells expressing P2X3-Myc, P2X3\textsuperscript{(ΔN)-Myc}, P2X3\textsuperscript{(ΔN, T388D)-Myc}, or P2X3\textsuperscript{(ΔC)-Myc} were subjected to cell surface biotinylation/immunoblotting. (D) A diagram mapping caveolin-1 and CaMKII\textalpha binding regions in the N terminus of the P2X3 receptor by alanine scanning mutagenesis. Lysates from HEK293T cells coexpressing caveolin-1-GFP or CaMKII\textalpha-GFP with N-TFR-Myc or various mutants were incubated with a GFP-specific antibody and subjected to coimmunoprecipitation and immunoblotting with the indicated antibodies. (E and F) HEK293T cells expressing P2X3\textsuperscript{FFTYET-AAAAAA}-Myc, P2X3\textsuperscript{FFTYET-AAAAAA, T388D}-Myc, P2X3-Myc, P2X3\textsuperscript{(ΔN)-Myc, P2X3\textsuperscript{(ΔN, T388D)-Myc, or P2X3\textsuperscript{(ΔC)-Myc} were subjected to cell surface biotinylation/immunoblotting. TFR served as an internal control for protein loading. Shown are the mean ± SEM (n = 3 - 4). *P < 0.05, **P < 0.01, ***P < 0.001.

Deletion of the N terminus of the P2X3 receptor abrogated both basal membrane delivery and Thr\textsuperscript{388} phosphorylation-driven membrane insertion, whereas deletion of the C terminus did not affect the basal levels of the P2X3 receptor at the cell membrane (Figure 6B and C). Further mapping of the protein binding regions in the N terminus of the P2X3 receptor identified that the sequence T\textsuperscript{17}KSVVKSWTI\textsuperscript{22} was involved in caveolin-1 interaction, and F\textsuperscript{6}FFTYETKSVVKSWTI\textsuperscript{22} was engaged in CaMKII\textalpha binding (Figure 6D). Substitution of Thr\textsuperscript{388} with aspartic acid significantly increased the membrane delivery of P2X3\textsuperscript{FFTYET-AAAAAA}, which preserved the ability to bind caveolin-1 but eliminated binding to CaMKII\textalpha (Figure 6E). In contrast, the mutant P2X3\textsuperscript{KSWTI-AAAAA, T388D} displayed low surface levels similar to P2X3\textsuperscript{KSWTI-AAAAA}, which bound neither caveolin-1 nor CaMKII\textalpha (Figure 6F). These data indicate that caveolin-1 is required for phosphorylation-driven membrane delivery of the P2X3 receptor and that CaMKII\textalpha is dispensable for forward transport after P2X3 receptor phosphorylation at Thr\textsuperscript{388}.

α, β-MeATP drives CaMKII\textalpha-mediated membrane cotranslocation of the P2X2 and P2X3 receptors

The P2X3 receptor can form a heterotrimeric channel with the P2X2 receptor besides the homotrimeric channel and the assembly of heterotrimeric channels occurs in the endoplasmic reticulum (Murrell-Lagnado and Qureshi, 2008). We first detected that 37.7% ± 1.4% of P2X3 receptor-positive neurons were significantly positive for the P2X2 receptor (554 out of 1449 counted neurons), and 62.2% ± 4.6% of DRG neurons coexpressing P2X2 and P2X3 receptors contained CaMKII\textalpha (328 out of 554 counted neurons) in rat DRGs (Figure 7A). Then, we explored whether P2X2 receptor levels on the cell membrane were affected by ATP when coexpressed with the P2X3 receptor. In HEK293T cells...
Figure 7. α, β-MeATP induces the membrane coinsertion of the P2X3 and P2X2 receptors. (A) A representative section of adult rat DRG triple-labeled with selective antibodies of P2X3 (green), P2X2 (blue), and CaMKIIα (red). The majority of P2X2 and P2X3 receptor-positive neurons express CaMKIIα (arrows). Scale bar, 30 μm. (B) HEK293T cells coexpressing P2X2-Myc and P2X3-Myc were treated with 10 μM α, β-MeATP for 30 min or pre-treated with 10 μM KN-93 for 30 min before α, β-MeATP stimulation and then subjected to cell surface biotinylation/immunoblotting. TFR served as a loading control for surface proteins. Shown are the mean ± SEM (n = 3). *P < 0.05 versus control cells treated with vehicle, **P < 0.05 versus cells treated with α, β-MeATP. (C) HEK293T cells coexpressing P2X2-Myc with P2X3-Myc or P2X3T388D-Myc were subjected to cell surface biotinylation/immunoblotting. Shown are the mean ± SEM (n = 5). *P < 0.05 versus cells coexpressing P2X3-Myc and P2X2-Myc. (D) P2X3-Myc or P2X3T388D-Myc was cotransfected with P2X2-Myc in HEK293T cells. The interaction of P2X2-Myc with P2X3-Myc or P2X3T388D-Myc was detected by coimmunoprecipitation with a P2X3 receptor-specific antibody and then immunoblotting with the indicated antibodies. (E) Primary cultured DRG neurons were treated with 10 μM α, β-MeATP for 30 min and subjected to cell surface biotinylation/immunoblotting. TFR served as an internal control for surface protein loading. Shown are the mean ± SEM (n = 3). **P < 0.01. (F) HEK293T cells expressing the indicated receptors were cross-linked with the non-permeable and irreversible BS3 followed by immunoblotting to detect the homotrimeric and heterotrimeric receptors on the cell membrane.
coexpressing the P2X2 and P2X3 receptors, α, β-MeATP increased the membrane insertion of both P2X3 and P2X2 receptors, and this event was abrogated by KN-93 (Figure 7B). We further found that P2X3T388D drove the membrane insertion of the P2X2 receptor more significantly compared with the P2X3 receptor (Figure 7C). However, P2X3T388D displayed an ability to interact with the P2X2 receptor that was similar to that of the P2X3 receptor (Figure 7D). Importantly, α, β-MeATP treatment for 30 min also facilitated membrane coinsertion of native P2X2 and P2X3 receptors in cultured DRG neurons (Figure 7E). Moreover, we clarified whether the increased P2X2 receptor on the cell membrane assembled with the P2X3 receptor. After cross-linking with the membrane-impermeable and irreversible BS3, a new band with a molecular weight between the homotrimeric P2X2 and P2X3 receptors was observed, corresponding to the heteromeric P2X2/3 receptor with a confined stoichiometry (two P2X3 and one P2X2; Figure 7F). This band had a substantially darker intensity in HEK293T cells coexpressing P2X3T388D and P2X2 than in HEK293T cells expressing P2X3 and P2X2 (Figure 7F). Thus, the increased P2X2 receptor levels driven by P2X3T388D coassembled with P2X3 receptor on the cell membrane. Together, α, β-MeATP increases the membrane coinsertion of P2X2 receptor with P2X3 receptor, which depends on CaMKIIα-mediated Thr388 phosphorylation in the C terminus of the P2X3 receptor.

**Phosphorylation-increased P2X3 receptors on the cell membrane are functional**

As the phosphorylation status of Thr388 affected the membrane insertion of the P2X3 receptor, we explored whether the phosphorylation-increased P2X3 receptor on the cell membrane...
was functional. Our previous study showed that Ca\textsuperscript{2+} influx via the P2X\textsubscript{3} receptor activates ERK (Chen et al., 2012); thus, α, β-MeATP-induced ERK phosphorylation was employed to evaluate the function of the P2X\textsubscript{3} receptor on the cell membrane. In HEK293T cells expressing P2X\textsubscript{3}\textsuperscript{ΔBBD} and P2X\textsubscript{3}\textsuperscript{ΔBBV}, α, β-MeATP treatment for 5 min induced ERK phosphorylation, suggesting normal receptor function for both mutants (Figure 8A). Importantly, α, β-MeATP treatment significantly increased the level of ERK phosphorylation in cells expressing P2X\textsubscript{3}\textsuperscript{ΔBB} compared with cells expressing P2X\textsubscript{3} (Figure 8A and B), indicating that phosphorylation at Thr\textsuperscript{388} of the P2X\textsubscript{3} receptor enhances the P2X\textsubscript{3}-mediated ERK response. However, in HEK293T cells expressing P2X3\textsuperscript{ΔBBV}, α, β-MeATP treatment induced a level of ERK phosphorylation comparable to cells expressing P2X\textsubscript{3} (Figure 8A and B). Taking into consideration that the P2X\textsubscript{3}\textsuperscript{ΔBBV} exhibited substantially lower receptor levels on the cell membrane than the P2X\textsubscript{3} receptor (Figure 4D), the comparable level of ERK phosphorylation mediated by P2X\textsubscript{3}\textsuperscript{ΔBBV} and P2X\textsubscript{3} may be caused by experimental serum starvation-induced downregulation of active CaMKII\textsubscript{α}, leading to a relatively lower level of P2X\textsubscript{3} receptor delivery. Thus, Thr\textsuperscript{388} phosphorylation-increased P2X\textsubscript{3} receptors on the cell membrane magnified the P2X\textsubscript{3} receptor response.

### Discussion

The P2X\textsubscript{3} receptor is involved in pain transduction, and a large amount of ATP is released from cells around primary sensory neurons during pathological pain conditions. Here, we have provided evidence for the presence of ATP-induced long-term membrane insertion of the P2X\textsubscript{3} receptor via the collaboration of CaMKII\textsubscript{α} and caveolin-1 (Figure 8C). CaMKII\textsubscript{α} phosphorylated Thr\textsuperscript{388} in the C terminus of the rat P2X3 receptor in a tertiary structure-dependent manner and therefore facilitated receptor interaction with the lipid-raft resident protein caveolin-1. Caveolin-1 association was dispensable for both basal and ATP-induced membrane insertion of the P2X\textsubscript{3} receptor. Moreover, Thr\textsuperscript{388} phosphorylation-increased membrane delivery of the P2X\textsubscript{3} receptor also promoted coinsertion of the assembled P2X\textsubscript{3} receptor and facilitated receptor function. Our study suggests that forward trafficking of the P2X\textsubscript{3} receptor is dynamically modulated by ligand, which is potentially interrelated to the sensitization of sensory neurons under pathological conditions.

Exocytosis and endocytosis control the receptor levels available on the cell membrane. Here, we found that the P2X\textsubscript{3} receptor underwent constitutive and ligand-driven membrane delivery, which may supply the required constitutive and ligand-driven internalization of this receptor (Vacca et al., 2009; Chen et al., 2012). In HEK293T cells expressing P2X\textsubscript{3} receptors, α, β-MeATP evoked relatively long-term CaMKII\textsubscript{α} activation, with a maximum level at 5 min and continuance beyond 30 min. Membrane insertion of the P2X\textsubscript{3} receptor was relatively delayed with respect to the upstream CaMKII\textsubscript{α} activation. We also showed that a 30-min α, β-MeATP treatment could induce the membrane insertion of native P2X\textsubscript{3} receptors in DRG neurons. The time course of ligand-evoked membrane insertion of the P2X\textsubscript{3} receptor described here is substantially longer than that reported by a previous study (Vacca et al., 2009), in which the authors detected an ATP-induced transient membrane delivery of the P2X\textsubscript{3} receptor with a peak at 2 min and return to basal levels within 10 min. Furthermore, the phenomenon of iomoycin-induced membrane insertion of the P2X\textsubscript{3} receptor provides a possibility that an intracellular calcium elevation activates CaMKII\textsubscript{α} and then promotes membrane delivery of this receptor.

The newly inserted receptors may come from recycling from the intracellular compartment or through de novo synthesis. A previous study showed that treatment with the protein synthesis inhibitor cycloheximide dramatically decreased the levels of both the membrane and total P2X\textsubscript{3} receptors (Vacca et al., 2009). In the present study, BFA treatment significantly decreased both basal and ligand-induced membrane insertion of the P2X\textsubscript{3} receptor. Thus, basal and regulated membrane levels of the P2X\textsubscript{3} receptor are largely dependent on de novo synthesis and forward transport from the ER-Golgi apparatus.

The P2X\textsubscript{3} receptor not only forms homotrimeric receptors but also assembles with the P2X\textsubscript{2} receptor to form the heterotrimeric P2X\textsubscript{2}/3 receptor (North, 2002). The homotrimeric P2X\textsubscript{3} receptor and the heterotrimeric P2X\textsubscript{2}/3 receptor likely mediate the fast-inactivating ATP currents and slow-inactivating ATP currents, respectively (Liu et al., 2001). Complete Freund’s adjuvant (CFA)-induced peripheral inflammation significantly increases both the fast- and slow-inactivating currents in DRG neurons mainly through the upregulation of P2X\textsubscript{3} and P2X\textsubscript{2} receptor expression (Xu and Huang, 2002). In the present study, we found that ligand-induced membrane insertion of the P2X\textsubscript{3} receptor also drove membrane delivery of the coexpressed P2X\textsubscript{2} receptor. Our immunostaining results showed that 38% of P2X\textsubscript{3} receptor-positive DRG neurons were significantly positive for the P2X\textsubscript{2} receptor, which is slightly higher than the in situ hybridization results (Kobayashi et al., 2005; Serrano et al., 2012) and appears to coincide with previous immunohistochemistry results (Vulchanova et al., 1997). We also showed that the increased P2X\textsubscript{2} receptor driven by P2X\textsubscript{3}\textsuperscript{ΔBBV} coassembled with the P2X\textsubscript{3} receptor on the cell surface. Although the levels of P2X\textsubscript{2} receptor alone on the cell membrane is not affected by ligand, the passive membrane insertion of the P2X\textsubscript{2} receptor driven by the assembled P2X\textsubscript{3} receptor could also favor the P2X\textsubscript{2}/3 receptor-mediated response, particularly under pathological conditions that release a large quantity of ATP.

CaMKII\textsubscript{α} has been reported to be involved in the regulated membrane insertion of several receptors such as AMPA and NMDA receptors (Yan et al., 2011; Lismar et al., 2012). The majority of P2X\textsubscript{3} receptor-positive rat DRG neurons contain CaMKII\textsubscript{α}. A previous study has shown that electrical stimulation of the sciatic nerve in vivo drove the membrane insertion of the P2X\textsubscript{3} receptor via upregulation of both total and active CaMKII\textsubscript{α} (Xu and Huang, 2004). In the present study, α, β-MeATP-induced Ca\textsuperscript{2+} influx promoted the membrane insertion of the P2X\textsubscript{3} receptor in phosphorylation-dependent but translation-independent CaMKII\textsubscript{α}. Moreover, iomoycin-induced membrane insertion of the P2X\textsubscript{3} receptor also relied on CaMKII\textsubscript{α} activation. Combined with the result that a constitutively active CaMKII\textsubscript{α} promoted membrane delivery of the P2X\textsubscript{3} receptor, CaMKII\textsubscript{α} activation by extracellular factors is sufficient to induce forward transport of this receptor.

In excitatory neuronal cells, α, β-MeATP-induced depolarization may further activate voltage-dependent calcium channels (VDCC) to increase Ca\textsuperscript{2+} influx (Sokolova et al., 2001). HEK293T cells do not express endogenous VDCC, and α, β-MeATP activates
CaMKIIα only through P2X3 receptor-mediated Ca\(^{2+}\) influx. In DRG neurons, α, β-MeATP activates CaMKIIα through the P2X3 receptor and VDCC-mediated Ca\(^{2+}\) influx, as shown in a previous report (Hasegawa et al., 2009). Generally, the binding site and phosphorylation motif of CaMKII substrates are distributed in a linear sequence (Strack et al., 2000; Ashpole et al., 2012). However, CaMKIIα bound to the N terminus of the P2X3 receptor and phosphorylated Thr\(^{388}\) in the C terminus. Both the short N and C termini of the P2X3 receptor are located in the cytoplasm and are in close proximity, which allows for this unique mode of CaMKIIα-mediated phosphorylation.

Caveolin-1 is an integral membrane protein in the lipid raft/caveolae and is also localized to the Golgi apparatus and transport vesicles derived from the trans-Golgi network (Williams and Lisanti, 2004). Caveolin-1 has been described to regulate caveolae-mediated internalization (Liu et al., 2002; Lajoie and Nabi, 2007) and functions as a chaperon required for the efficient delivery of proteins to the plasma membrane (Wyse et al., 2003; Syme et al., 2006; Gonzalez et al., 2007). The P2X1, P2X4 and P2X7 receptors, located in lipid rafts of different cell types, have been reported to interact with caveolin (Barth et al., 2008; Cristofaro et al., 2012). Here, we found that the P2X3 receptor interacted with caveolin-1, and we dissected the caveolin-1 binding sequence \(^{12}\)KSVVVKSWT\(^{26}\) of the P2X3 receptor, which did not share common characteristics of the canonical caveolin binding motif with rich aromatic residues (Couet et al., 1997). A recent study also demonstrated that protein interaction with caveolin does not always adopt the canonical caveolin binding motif (Byrne et al., 2012).

Caveolin-1 is required for basal and ligand-induced membrane delivery of the P2X3 receptor. Nevertheless, caveolin-1 knockdown did not obviously affect the basal levels of the P2X3 receptor on the cell membrane, which may be attributed to the dual function of caveolin-1 downregulation on P2X3 receptor endocytosis and exocytosis. Moreover, α, β-MeATP treatment facilitated the interaction of P2X3 receptor with caveolin-1, which was maintained for a longer time than that between P2X2 and CaMKIIα. Consistently, a phosphorylation mimic at Thr\(^{388}\) of the P2X3 receptor promoted P2X3 receptor interaction with caveolin-1; however, a non-phosphorylation mimic significantly attenuated interaction between P2X3 receptor and caveolin-1, which parallels the dramatically increased and attenuated levels of the P2X3 receptor on the cell membrane with phosphorylation and non-phosphorylation mimics at Thr\(^{388}\), respectively. With help of the P2X3 mutants which preserved the ability to bind caveolin-1 but eliminated binding to CaMKIIα or bound neither caveolin-1 nor CaMKIIα, we finally propose that caveolin-1 is dispensable for phosphorylation-driven membrane delivery of P2X3 receptor and CaMKIIα is dispensable for forward transport after P2X3 receptor phosphorylation at Thr\(^{388}\).

Materials and methods

Plasmid construction

The P2X2 receptor was amplified from the rat DRG cDNA library with the primers 5′-ATGAATTCATGGCTGCGGCTGCAAGATG-3′ and 5′-ATGGATCGAGGCTCTATCTTCTGCAGC-3′, and inserted into pMyc (with GFP in pEGFP-N3 replaced by Myc). CaMKIIα was amplified from the rat whole brain cDNA library with the primers 5′-ATGAATTCATGGCTACCACCTGCACCCG-3′ and 5′-ATGGATCCATGGCTGCGGCTGCAAGATG-3′, and inserted into pEGFP-N3. For the site mutants from P2X3-Myc and CaMKIIα-GFP, a KOD site-mutagenesis kit (Toyobo) was used. The C terminus of the P2X3 receptor was inserted into the C terminus of a modified Myc-CD8α, which has an extracellular Myc tag, and the N terminus of the P2X3 receptor was inserted into the N terminus of a modified TFR-Myc, which has an extracellular Myc tag (Li et al., 2010). Caveolin-1 was amplified from the rat uterus cDNA library with the primers 5′-AGAATTCATGGCTGCGGCTGCAAGATG-3′ and 5′-ATGGATCGAGGCTCTATCTTCTGCAGC-3′, and inserted into pFlag (with GFP in pEGFP-N3 replaced by Flag) and pEGFP-N3. The P2X3 receptor mutants with N or C terminus deletions were constructed from P2X3-Myc.

Cell culture and transfection

HEK293T cells from the American Type Culture Collection were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum and antibiotics. Transient expression was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. To knock down endogenous caveolin-1, the cells were transfected with 50 nM caveolin-1 siRNA or control scramble siRNA using Lipofectamine 2000. Caveolin-1 siRNAs were designed by RIBOBIO, and the passenger strand sequences were CCAGAAGGCACACAGUU (siRNA-1), GCAUCAUCUUGCAGAAGA (siRNA-2), and GCAGUUGUACACGAUA (siRNA-3). One and a half days after transfection, the cells were or were not serum-starved in DMEM for at least 12 h prior to further experiments.

Preparation of dissociated DRG neurons

Male Sprague-Dawley (SD) rats (body weight, 100–120 g; Shanghai Center of Experimental Animals, Chinese Academy of Sciences, China) were used according to the policies of the Society for Neuroscience (USA) on the use of animals. The experiment was approved by the Committee for the Use of Laboratory Animals and Common Facility, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The rats were anesthetized and sacrificed. The DRGs were dissected and digested with 1 mg/ml collagenase type IA, 0.4 mg/ml trypsin type I and 0.1 mg/ml DNase I (all from Sigma) in DMEM at 37°C for 35 min, and then triturated and cultured in DMEM/F12 (1:1; Invitrogen) supplemented with 1% N2 (Invitrogen) for drug treatment.

Drug treatment of cultured cells

The serum-starved HEK293T cells expressing P2X3-Myc, P2X3-Myc, P2X1-Myc and DRG neurons were treated with 10 μM α, β-MeATP (Sigma), the serum-starved HEK293T cells expressing P2X2-Myc were stimulated with 100 μM ATP (Sigma), and the serum-starved HEK293T cells coexpressing P2X3-Myc with CaMKIIα-GFP or CaMKIIα were treated with 1 μM ionomycin (Sigma) for 30 min. For the inhibition experiments, the cells were pre-treated with 10 μM A-317491 (1 h; Sigma), 5 μg/ml BFA (30 min; Sigma), 5 μM BIM (30 min; Calbiochem) or 10 μM KN-93 (30 min; Sigma) before α, β-MeATP treatment. In the case of extracellular Ca\(^{2+}\)-free conditions, the cells were incubated in Ca\(^{2+}\)-free extracellular solution containing 1 mM EGTA for 20 min and then treated with α, β-MeATP in the same solution. For the detection of ERK activation, the serum-starved HEK293T cells expressing ATGGGGAGCGAGGGAGCGGCG-3′, and inserted into pEGFP-N3.
P2X3-Myc, P2X3T388I-Myc, or P2X3T388V-Myc alone or with scramble siRNA or caveolin-1 siRNA-2 were treated with 10 μM α, β-MeATP for 5 min immediately prior to cell lysis.

Immunohistochemistry
Three male SD rats (body weight ~250 g) were deeply anaesthetized, and the hearts were perfused with 50 ml warm (37°C) saline followed by a warm mixture of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and finally by the same fixative but ice cold for 5 min. The DRGs were dissected, post-fixed in the same fixative for 90 min at 4°C and in 20% sucrose in 0.1 M phosphate buffer for 24 h prior to being cut into 10-μm sections. The sections were incubated with P2X3 (Neuromics) and CaMKIκα (Santa Cruz Biotechnology) antibodies or combined with P2X2 (Chemicon) antibodies in Immunoreaction Enhancer Solution (Toyobo) overnight at 4°C followed by secondary antibodies conjugated with FITC and Cy3 or combined with Cy5 (Jackson ImmunoResearch) at 37°C for 45 min. The sections were then mounted and scanned with Leica SP5 confocal microscopy.

Surface biotinylation
 Cultured DRG neurons or transfected HEK293T cells were biotinylated with 0.25 mg/ml Sulfo-NHS-LC-biotin (Thermo Scientific) in phosphate-buffered saline containing Mg2+/Ca2+ for 45 min at 4°C followed by quenching with glycerin for 20 min. The cells were lysed in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 μg/ml aprotinin, leupeptin, and pepstatin), and the lysates were incubated with NeutrAvidin beads (Thermo Scientific) overnight at 4°C followed by secondary antibodies conjugated with FITC and Cy3 or combined with Cy5 (Jackson ImmunoResearch) at 37°C for 45 min. The sections were then mounted and scanned with Leica SP5 confocal microscopy.

Immunoprecipitation
The cells or rat DRGs were lysed in immunoprecipitation buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 50 mM NaF, 1 mM Na3VO4, 20 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 μg/ml aprotinin, leupeptin, and pepstatin), and the supernatants were incubated with P2X2 (Chemicon) antibodies in Immunoreaction Enhancer Solution (Toyobo) overnight at 4°C followed by secondary antibodies conjugated with FITC and Cy3 or combined with Cy5 (Jackson ImmunoResearch) at 37°C for 45 min. The sections were then mounted and scanned with Leica SP5 confocal microscopy.

Cross-linking of plasma membrane proteins
Transfected HEK293T cells were washed twice in ice-cold PBS. The transfected proteins were cross-linked using 2 mM membrane-impermeable and irreversible BS3 (Thermo Scientific) for 30 min at 4°C. After cross-linking, the cells were lysed with strong RIPA (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 μg/ml aprotinin, leupeptin, and pepstatin), and the proteins were processed for immunoblotting.

Immunoblotting
The cell lysates or beads were incubated in SDS–PAGE loading buffer for 20–30 min at 50°C. The samples were separated on SDS–PAGE, transferred, probed with P2X3 (Neuromics), TFR (Invitrogen), actin (Chemicon), Myc (Proteintech), GFP (Roche), pCaMKIIα, CaMKIIα, caveolin-1, pERK (Santa Cruz), CaMKII (Millipore), phospho-threonine (Cell Signaling Technology), phosphoserine (Sigma), Flag (Abmart), P2X2 (Chemicon), or ERK (Abcam) antibodies and visualized with enhanced chemiluminescence (Amersham Biosciences). The intensity of immunoreactive bands was analyzed using the Image-Pro Plus 5.1 software (Media Cybernetics, Inc.). All experiments were repeated at least three times.

Statistical analyses
Statistical analyses were performed using PRISM (GraphPad Software, Inc.) with the two-tailed and paired Student’s t-test. The significance levels were as follows: *P < 0.05, **P < 0.01, and ***P < 0.001.

Supplementary material
Supplementary material is available at Journal of Molecular Cell Biology online.

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References


