Lack of evidence for direct phosphorylation of recombinantly expressed P2X2 and P2X3 receptors by protein kinase C

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Abstract P2X3 and P2X2+3 receptors are present on sensory neurons, where they contribute not only to transient nociceptive responses, but also to hypersensitivity underlying pathological pain states elicited by nerve injuries. Increased signalling through P2X3 and P2X2+3 receptors may arise from an increased routing to the plasma membrane and/or gain of function of pre-existing receptors. An obvious effector mechanism for functional modulation is protein kinase C (PKC)-mediated phosphorylation, since all P2X family members share a conserved consensus sequence for PKC, TXR/K, within the intracellularly located N-terminal domain. Contradictory reports have been published regarding the exact role of this motif. In the present study, we confirm that site-directed elimination of the potential phosphor-acceptor threonine or the basic residue in the P+2 position of the TXR/K sequence accelerates desensitization of P2X2 receptors and abolishes P2X3 receptor function. Moreover, the PKC activator phorbol 12-myristate 13-acetate increased P2X3 (but not P2X2) receptor-mediated currents. Biochemically, however, we were unable to demonstrate by various experimental approaches a direct phosphorylation of wild-type P2X2 and P2X3 receptors expressed in both *Xenopus laevis* oocytes and HEK293 cells. In conclusion, our data support the view that the TXR/K motif plays an important role in P2X function and that phorbol 12-myristate 13-acetate is capable of modulating some P2X receptor subtypes. The underlying mechanism, however, is unlikely to involve direct PKC-mediated P2X receptor phosphorylation.

Keywords Phosphorylation · Protein kinase C · P2X receptors · P2X2 · P2X3

Abbreviations
ATP adenosine 5′-triphosphate
αβ-meATP α,β-methylene adenosine 5′-triphosphate
DMEM Dulbecco’s modified Eagle’s medium
DMSO dimethyl sulfoxide
EGFP enhanced green fluorescent protein
HEK293 human embryonic kidney cell line
mGluR1α metabotropic glutamate receptor subtype 1α
PBS phosphate-buffered saline
PBST 0.1% Tween 20 in phosphate-buffered saline
PKC protein kinase C
PMA phorbol 12-myristate 13-acetate
PMSF phenylmethylsulfonyl fluoride
PVDF polyvinylidene fluoride
SF3B1 splicing factor 3b subunit 1

Introduction

P2X receptors constitute an abundant class of ligand-gated ion channels, which respond to extracellular ATP and related nucleotides with the opening of an intrinsic pore permeable to Na⁺, K⁺ and Ca²⁺ [1, 2]. A family encompassing seven P2X subunit genes, designated P2X₁₋₇, has been identified in rodents and mammals. Six of the seven subunit isoforms (P2X₁-P2X₅ and P2X₇) are able to assemble into homotrimERIC receptor channels [3–8] with distinct pharmacological and electrophysiological phenotypes [9]. According to their sensitivity to the synthetic ATP analogue α,β-methylene adenosine 5′-triphosphate (αβ-meATP) and the rate of current desensitization, P2X
receptors are generally subgrouped into at least two categories: (1) rapidly desensitizing and αβ-meATP sensitive (P2X1 and P2X3) and (2) slowly or non-desensitizing and αβ-meATP insensitive (P2X2, P2X4 and P2X7) receptors. The term “non-desensitizing” means that the currents are maintained for at least a few seconds in the continuous presence of agonist. A peculiar phenotype is inherent to heteromeric P2X2/P2X3 (P2X2$\alpha$3) receptors, which feature αβ-meATP sensitivity combined with a non-desensitizing current response.

P2X receptors are found on the surface of a large variety of cells, where they are involved in numerous sensory processes including nociception under both physiological and pathological processes [10–15]. On nociceptive sensory neurons, extracellular ATP acts as a pain-producing neurotransmitter predominantly through homotrimeric P2X3 receptors or heterotrimeric P2X2$\alpha$3 receptors. Conditions and mediators that facilitate P2X3 and P2X2$\alpha$3 receptor-mediated signalling, apparently by potentiating ionic currents, include (1) substance P and bradykinin [16], (2) nerve injury [17] and (3) calcitonin gene-related peptide (CGRP), a potent vasodilator and proinflammatory agent [18]. How this increase in receptor function occurs has not been fully solved. One obvious candidate mechanism is protein kinase C (PKC)-mediated phosphorylation, since all P2X family members share a conserved consensus sequence for PKC-mediated phosphorylation (TXR/K) within the intracellular-phospho-acceptor site [19]. Mutational analysis has further shown that the TXR/K sequence of the P2X1, P2X2 and P2X3 subunits plays an important role in controlling the rate of receptor desensitization [16, 19, 20]. Changes of the rate of desensitization could profoundly influence the efficiency of synaptic transmission and thus contribute to pathological pain states. Overall, there is significant interest in a better understanding of the mechanisms involved in the short-term and long-term regulation of P2X receptors.

The aim of this study was to assess biochemically the conditions in which P2X2 and P2X3 receptors become directly phosphorylated by PKC in a heterologous system. While this work was in progress, a similar study was published for the P2X1 receptor that arrived at essentially the same conclusion, namely that the P2X3 receptor is unlikely to be a direct PKC substrate [21]. Our results complement and extend this finding by demonstrating that the P2X2 receptor also does not serve as a substrate for direct PKC-mediated phosphorylation.

Materials and methods

Materials

The sources of antibodies, enzymes or peptide substrates are specified at the appropriate places in the text. Chemicals not otherwise specified were purchased in the highest available quality from Sigma-Aldrich (Taufkirchen, Germany) or Merck (Darmstadt, Germany).

Peptide-specific antibodies to P2X1, P2X2 and P2X3 subunits

Peptides ATSSTLQLQENMRTS (residues 385–399 of the P2X1 subunit), QQDSTSDPKGLAQL (residues 458–472 of the P2X2 subunit) and VEKQSTDSGAYSIGH (residues 383–397 of the P2X3 subunit) were selected based on their lack of similarity with known proteins and predicted hydrophilicity for raising polyclonal antibodies against the C-terminal end of the rat subunits P2X1, P2X2 and P2X3, respectively. Rabbit polyclonal antibodies were ordered through the Custom Antibody Production Services of Eurogentec (Seraing, Belgium). After 3 months and a total of four immunizations, rabbits were sacrificed by complete bleeding. Peptide-specific antibodies were purified from the serum by affinity columns coupled with the peptides used for immunization. Immunoblotting showed that the antibodies recognized only the receptor against which they were raised, thus demonstrating isoform specificity of these antibodies (results not shown). The antibodies were aliquoted at 0.12–0.16 mg/ml in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.01% thimerosal and stored at −80°C.

cDNA constructs

Plasmids encoding wild-type and N-terminal hexahistidine-tagged (His-tagged) versions of rat P2X1 subunits (rP2X1, GenBank accession no. X80477), P2X2 subunits (rP2X2, GenBank accession no. U14414) or rat P2X3 subunits (rP2X3, GenBank accession no. X90651) in the oocyte expression vector pNKS2 [22] were available from previous studies [3, 8]. Insertion and replacement mutations were introduced by QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA, USA). For protein expression in mammalian cells, the receptor cDNAs were subcloned from the oocyte expression vector pNKS2 into pcDNA3.1. The plasmid His-rP2X2-EGFP-pcDNA3.1 with a C-terminal enhanced green fluorescent protein (EGFP) fusion was generated by eliminating the stop codon in His-rP2X2 and adding the coding sequence of EGFP (Clontech, Palo Alto, CA, USA) in frame. All constructs were verified by restriction analysis and nucleotide sequencing. A pEGFP-
C1 plasmid harbouring the N-terminally EGFP-tagged splicing factor SF3B1 was kindly provided by our colleague Dr. Walter Becker [23].

**P2X receptor expression in Xenopus laevis oocytes**

Capped cRNAs were synthesized from plasmids linearized downstream to the 100 bp long polyA tail provided by the oocyte expression vector pNKS2 [22] and injected at 0.5 μg/μl in 50-nl aliquots into collagenase-defolliculated X. laevis oocytes using a Nanoliter 2000 injector (WPI, Sarasota, FL, USA), as detailed previously [24]. Oocytes were maintained at 19°C in sterile oocyte Ringer’s solution [ORi: 90 mM NaCl, 1 mM KCl, 1 mM CaCl2, 1 mM MgCl2 and 10 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), pH 7.4] supplemented with 50 μM hydroxyethylpiperazine ethanesulfonic acid groups. Two days after cRNA injection, oocytes were washed with oocyte-PBS pH 8.5 (20 mM NaCl, 110 mM NaCl, 1 mM MgCl2) and then incubated for 30 min at ambient temperature with Cy5 dye, which was diluted 200-fold to a final concentration of 50 μg/ml from a dimethyl sulfoxide (DMSO) stock solution. The reaction was terminated by washing the cells with oocyte-PBS, followed by membrane protein extraction with dodecylmaltoside and receptor purification (see below).

**Metabolic [35S]methionine labelling of oocytes**

For metabolic [35S]methionine labelling of oocyte-expressed recombinant proteins, cRNA-injected oocytes and non-injected controls were incubated overnight with L-[35S] methionine (>40 TBq/mmol; PerkinElmer, Boston, MA, USA) at about 100 MBq/ml (0.4 MBq per oocyte) in ORi at 19°C, and then chased for 24 h.

**Surface fluorescence labelling of oocytes**

Selective fluorescence labelling of plasma membrane-bound P2X receptors was achieved by using the amino-reactive fluorescent Cy5 dye (GE Healthcare, Freiburg, Germany), which is membrane impermeant due to its two sulfonic acid groups. Two days after cRNA injection, oocytes were washed with oocyte-PBS pH 8.5 (20 mM Na phosphate, 110 mM NaCl, 1 mM MgCl2), and then incubated for 30 min at ambient temperature with Cy5 dye, which was diluted 200-fold to a final concentration of 50 μg/ml from a dimethyl sulfoxide (DMSO) stock solution. The reaction was terminated by washing the cells with oocyte-PBS, followed by membrane protein extraction with dodecylmaltoside and receptor purification (see below).

**Isolation of recombinant P2X receptors from X. laevis oocytes or HEK293 cells**

His-tagged receptors were purified by Ni2+ nitrilotriacetic acid (NTA) agarose (Qiagen, Hilden, Germany) affinity chromatography from non-ionic detergent extracts of oocytes or HEK293 cells in the presence of iodoacetamide essentially as described [3, 8, 25]. Slight modifications were that buffers were supplemented with ethylenediaminetetraacetate (EDTA)-free Halt™ protease inhibitor cocktail (Pierce, Rockford, IL, USA), 50 mM NaF and 50 mM Na2P2O7 for phosphatase inhibition, and that digitonin was replaced by 0.2% and 0.05% dodecylmaltoside for membrane protein extraction and repetitive washing of resin-bound proteins, respectively. Proteins were released from the washed Ni2+ NTA agarose beads with non-denaturing elution buffer consisting of 250 mM imidazole/HC1 (pH 7.4) and 0.02% dodecylmaltoside, and then kept at 0°C until analysed on the day of purification.

In addition, immunoprecipitation assays were performed using either cRNA-injected Xenopus oocytes or transiently transfected HEK293 cells. Cells were lysed in ice-cold immunoprecipitation lysis buffer containing 150 mM NaCl, 25 mM Tris/HCl pH 7.4, 20 mM NaF, 20 mM Na2P2O7, 10 μl/ml Halt™ protease inhibitor cocktail without EDTA (Pierce, Rockford, IL, USA), 1% sodium dodecyl sulfate (SDS) (w/v) and 50 mM iodoacetamide. For efficient solubilization, lysates were incubated for 30 min on ice with occasional vortexing, and then cleared by centrifugation for 15 min at 16,100 g and 4°C. The cleared SDS extract was diluted 1:1 with immunoprecipitation binding buffer [150 mM NaCl, 20 mM Tris/HCl pH 7.4, 0.4 mM phenylmethyisulfonyl fluoride (PMSF), 0.5% Triton X-100 v/v and 25 mM iodoacetamide], supplemented 1:1,000 with the desired peptide-specific P2X2 or P2X3 antibodies or a polyclonal goat anti-EGFP antibody (Rockland, Gilbertsville, PA, USA) and incubated for 2 h on ice. Immunocomplexes were bound to the protein A Sepharose by overnight head-over-head rotation at 8°C, then washed 5 times with immunoprecipitation binding buffer containing 0.1% Triton X-100 and finally eluted by incubating the beads for 15 min at 56°C in reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

**In vitro PKC phosphorylation assay**

In vitro phosphorylation assays were performed by using P2X receptors as substrates, purified as detailed above. In addition, a selective peptide PKC substrate (RRGRTGRG RRGYYR, Calbiochem-Merck, Darmstadt, Germany, final concentration 150 μM) corresponding to the amino acid sequence 1487-1500 of the hepatitis C virus polyprotein
[26] was used as a positive control. The proteins or the peptide were incubated for 30 min at 30°C with 0.02 unit (0.02 μg) of purified catalytic PKC subunit from rat brain and 50 μM [γ-32P]ATP (10 mCi/ml, Amersham Biosciences GE Healthcare, Freiburg, Germany) in phosphorylation buffer containing 6 mM CaCl2, 10 mM MgCl2, 20 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM mercaptoethanol, 0.05% Triton X-100 (v/v) and 0.1 mg/ml BSA. The reaction was terminated by boiling in reducing SDS-PAGE sample buffer for 30 s. Proteins were resolved by reducing SDS-PAGE and visualized by immunoblotting or phosphorimaging as described below.

In vivo phosphorylation assay

Transiently transfected HEK293 cells were metabolically labelled by a 3-h incubation with 500 μCi/ml of [32P]orthophosphate (Amersham Biosciences GE Healthcare, Freiburg, Germany) in phosphate-free oocyte Ringer solution or in phosphate-free DMEM (Invitrogen, Karlsruhe, Germany) as appropriate. Cells were treated with or without 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Taufkirchen, Germany) or 100 nM calyculin A (Tocris Bioscience, Ellisville, MO, USA) for 10 min at ambient temperature. Non-incorporated radioactivity was removed by rinsing the cells twice with tracer-free medium. Affinity purification or immunoprecipitation was performed as described above followed by SDS-PAGE and immunoblotting or phosphorimaging.

SDS-PAGE

Samples for SDS-PAGE were denatured by incubating in reducing SDS-PAGE sample buffer for 15 min at 56°C and then electrophoresed on a Laemmli SDS-PAGE gel (10% acrylamide) in parallel with [14C]-labelled molecular mass markers (Rainbow™, Amersham Biosciences GE Healthcare, Freiburg, Germany). Cy5-labelled proteins were visualized by scanning the wet SDS-PAGE gel with a fluorescence scanner (Typhoon, GE Healthcare, Freiburg, Germany). For visualization of radio-labelled or non-labelled proteins, SDS-PAGE gels were either fixed, dried and exposed to a PhosphorImager screen, and scanned using a Storm 820 PhosphorImager (Amersham Biosciences GE Healthcare, Freiburg, Germany) or and blotted to polyvinylidene fluoride (PVDF) membranes.

Immunoblotting

Proteins separated by SDS-PAGE were electroblotted onto PVDF membranes. Membranes were blocked for 1 h with 5% non-fat milk in PBS (or, alternatively, with 5% BSA if the phosphothreonine monoclonal antibody was used for immunoblotting), washed 3×5 min with PBST (0.1% Tween 20 in PBS) and incubated for 2 h at 4°C in PBS containing the appropriate primary antibody. Phosphorylated proteins were detected by immunoblotting with a phosphothreonine monoclonal antibody (Cell Signaling Technology, Beverly, MA, USA). Alternatively, the peptide-specific antibodies to P2X2 and P2X3 subunits were used to verify receptor expression levels. Following washing in PBST, an appropriate horseradish peroxidase-conjugated secondary antibody (goat anti-mouse or goat anti-rabbit, Pierce, Rockford, IL, USA) was applied for 1 h in PBS supplemented with 1% non-fat milk. After another 3×5 min washes with PBST, immunoreactive bands were visualized by chemiluminescence imaging using a Fujifilm LAS-3000 system (Tokyo, Japan) and the AIDA Image Analyser Software Version 4.08 (Raytest, Straubenhardt, Germany).

Two-electrode voltage clamp current recordings and electrical capacitance measurements

Two-microelectrode voltage clamp measurements of X. laevis oocytes were performed 2 days after cRNA injection using a Turbo TEC-05 amplifier (npi electronics, Tamm, Germany) interfaced by an INT-20X AD/DA converter (npi electronics, Tamm, Germany) to a personal computer running the CellWorks Lite 5.1 software (npi electronics, Tamm, Germany), as detailed previously [27–29]. Current traces were elicited by 100 μM ATP at 1 to 5-min intervals (Sigma–Aldrich, Taufkirchen, Germany) at a holding potential of −60 mV in nominally Ca2+-free bath medium (designated Mg–ORi) to avoid activation of endogenous Ca2+-activated Cl− channels. ATP was prepared in Mg–ORi at its final concentration and applied by gravity-fed perfusion at a flow rate of 10 ml/min to an oocyte held by the two microelectrodes in the bath chamber (volume ~10 μl). Switching between different perfusion solutions was achieved with electromagnetic valves controlled by the CellWorks Lite 5.1 software. Electrophysiological recordings were analysed using the Origin 6.0 software (Microcal Software Inc., Northampton, MA, USA). Single exponentials were fitted to the decay phase of the macroscopic currents with a nonlinear least squares method. The time constant τ was taken as an estimate of the rate of receptor desensitization.

To monitor PMA-induced changes in the oocyte surface area, depolarizing voltage steps of ΔV = 10 mV were applied from the holding potential to elicit a capacitative transient Icap, the integral of which yields the charge movement Qcap and thus the membrane capacitance Cm = Qcap/ΔV [30–32]. PMA was dissolved in DMSO to a stock solution of 10 mM and stored at −20°C. On the day of the experiment, the stock
solution was pre-diluted to 100 μM in DMSO and then further diluted with Mg-ORi to the final concentration of 100 nM. Vehicle control experiments with a final concentration of 0.1% DMSO did not reveal any effects on P2X2 receptor-mediated currents measured with X. laevis oocytes (results not shown). All measurements were made at room temperature (21–23°C).

Data analysis

Data are shown as means±SEM. The paired Student’s t-test (two-tailed) was used to compare current amplitudes with statistical significance set to $p<0.05$.

Results

Site-directed modification of a putative N-terminal PKC site affects the electrophysiological phenotype of P2X2 and P2X3 receptor channels

First, we tested each of the non-mutated and mutant receptor constructs for expression of an ATP-gated inward current in cRNA-injected X. laevis oocytes. Application of 100 μM ATP induced typical non-desensitizing currents through oocyte-expressed P2X2 receptors (Fig. 1c, left current trace). The P2X2 subunit shares with other P2X subunit isoforms a conserved consensus site for PKC phosphorylation (TXR/K, where T is the phosphorylation site).

Fig. 1 Effect of site-directed modification of a putative PKC motif on currents mediated by ATP-activated P2X2 and P2X3 receptors. a Schematic model of the transmembrane topology of the rat P2X3 subunit illustrating the N-terminal position of the12TTK14 sequence. b Alignment of intracellular N-terminal amino acid sequences of the seven P2X subunit isoforms reveals a highly conserved consensus motif, TXR/K. c Typical current traces elicited by applying 10-s pulses of 100 μM ATP to oocytes expressing the indicated wild-type or mutant P2X2 receptors. d Typical current traces elicited by applying 10-s pulses of 100 μM ATP to oocytes expressing the indicated wild-type or mutant P2X3 receptors. Gray areas indicate the duration of ATP application. e All the P2X2 and P2X3 receptors and receptor mutants were expressed efficiently at the cell surface. Intact, healthy oocytes expressing the indicated proteins for 2 days were surface-labelled with the membrane impermeant reactive Cy5 dye and then extracted with dodecylmaltoside. Recombinant proteins were isolated by Ni2+ chelate chromatography and resolved by reducing SDS-PAGE. Shown is a fluorescence scan of an SDS-PAGE gel.
site and X denotes any amino acid; see Fig. 1a, b). Disruption of the conserved N-terminal PKC site by mutating the phosphor-acceptor^18T to A resulted in a rapidly desensitizing channel (Fig. 1c, middle current trace). Also, replacement of 20K by T resulted in a desensitizing phenotype (Fig. 1c, right current trace). Since PKC preferentially phosphorylates threonine (or serine) residues that are close to a C-terminal basic residue, replacement of 20K in the P+2 position by a non-basic residue such as T will destroy the putative PKC site despite preserving the phosphor-acceptor^18T. Virtually identical findings had previously been reported by Boue-Grabot et al. [19]. The average half-times of desensitization (τ) of the^18A and 20T P2X2 mutants were quite similar, with mean τ values (±SEM) of 4.5±0.4 s and 7.3±0.5 s, respectively, of eight oocytes from two females per value.

ATP applied to oocytes expressing the P2X3 receptor elicited a rapidly desensitizing inward current (Fig. 1d, left current trace) as is typical for this P2X receptor subtype. Incorporation of similar mutations, as in the P2X2 subunit in the analogous positions of the P2X3 receptor T^12A or K^14T, completely abolished P2X3 receptor function (Fig. 1d, middle and right current traces). Visualization of plasma membrane-bound fluorescence-labelled P2X receptors by affinity purification combined with SDS-PAGE and fluorescence scanning verified that all the receptors were efficiently exported to the oocyte surface (Fig. 1e).

The PKC activator PMA augments P2X3 receptor-mediated currents, yet leaves P2X2-mediated currents unchanged

Since the mutagenesis data for the P2X2 and P2X3 receptor were consistent with the possibility of a regulatory role for PKC in P2X receptor function, we examined whether the PKC activator PMA was capable of affecting the electrophysiological phenotype of P2X2 or P2X3 receptors. In planning the experiments, we considered that PMA treatment of *X. laevis* oocytes is known to induce a pronounced endocytotic reduction of the cell surface area, as evidenced microscopically and electrophysiologically by the disappearance of virtually all microvilli and a concomitant decrease of the electrical capacitance of the plasma membrane, respectively [31, 32]. Associated with oocyte surface reduction is a proportional internalization of membrane proteins, such as endogenous Na⁺-K⁺ pumps, suggesting that PMA treatment would stimulate internalization of recombinant P2X receptors as well. Re-examination of the time course of the PMA-induced reduction of the oocyte surface area showed that the electrical capacitance did not start to decline significantly before 10 min after PMA application (Fig. 2a). Accordingly, all current recordings were done within a time window of 10 min after PMA application to prevent any contribution of receptor internalization to the PMA effect on P2X receptor-dependent currents.

Figure 2b and c show typical current traces elicited by ATP from P2X2 or P2X3 receptor-expressing oocytes before and after a 10-min exposure to PMA. PMA neither affected the shape nor the amplitude of P2X2 receptor-mediated current (Fig. 2b). However, PMA produced a marked increase of the current amplitude mediated by the P2X3 receptor (Fig. 2c). In one experiment with four oocytes, a statistically significant increase of the P2X3-mediated current amplitude by PMA of 155±42% (SEM) was determined.

P2X2 and P2X3 receptors exist in a non-phosphorylated state in both *X. laevis* oocytes and HEK293 cells

To explain the accelerated desensitization upon site-directed perturbation of the ^18TXK^20 motif, it has been suggested that the wild-type P2X2 receptor is constitutively phosphorylated at ^18T and that this phosphorylation is responsible for the slow desensitization rate [19]. To address this issue, we next examined biochemically whether P2X3...
receptors also exist in a constitutively phosphorylated state; the P2X₂ receptor was also analysed to allow for a direct comparison of the results. Both parent P2X receptors and mutants were purified by Ni²⁺ chelate affinity chromatography from dodecylmaltoside extracts of [³⁵S]methionine-labelled oocytes, resolved by SDS-PAGE, and blotted onto a PVDF membrane. However, probing with a phosphothreonine-specific monoclonal antibody revealed no discernible signal (Fig. 3a) despite the presence of significant amounts of [³⁵S]methionine-labelled P2X₂ and P2X₃ protein in the SDS-PAGE gel (Fig. 3b).

To exclude the possibility that the apparent lack of receptor phosphorylation resulted from an interference between the N-terminal His tag and the PKC site of P2X₃ or P2X₂ receptor subunits, which are separated by only 12 or 20 residues, respectively, we performed analogous experiments with a C-terminally His-tagged P2X₂ receptor, P2X₂-His. As apparent from Fig. 3c, neither N-terminally nor C-terminally His-tagged P2X₂ receptor became phosphorylated, as evidenced by immunoblotting with a

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Fig. 3 Immunoblots show no constitutive phosphorylation of oocyte-expressed P2X₂ or P2X₃ receptors. The indicated wild-type or mutant P2X subunits were purified by Ni²⁺ chelate chromatography from [³⁵S]methionine-labelled X. laevis oocytes and resolved by denaturing SDS-PAGE. a Shown is a representative immunoblot probed with a phosphothreonine-specific monoclonal antibody and a peroxidase-conjugated secondary antibody. No phosphorylation signal could be detected at the SDS-PAGE migration position of P2X₂ and P2X₃ subunits, which are indicated by the adjacent PhosphorImager scan in panel b. b Direct PhosphorImager visualization of metabolically [³⁵S]methionine-labelled parent and mutant P2X subunits resolved by SDS-PAGE of the same samples as in panel a. c Left panel: representative immunoblot of P2X₂ subunits tagged with either an N-terminal or C-terminal hexahistidine sequence (designated His-P2X₂ or P2X₂-His, respectively) and probed with a phosphothreonine-specific monoclonal antibody and a peroxidase-conjugated secondary antibody. No phosphorylation signal could be detected at the SDS-PAGE migration position of P2X₂ subunits, which were visualized by immunoblotting with the P2X₂ subunit polyclonal antibody (middle panel) or PhosphorImager scanning of incorporated [³⁵S]methionine (right panel).
phosphor-specific anti-threonine antibody (Fig. 3c). The presence of P2X2 receptor protein was verified by immunoblotting and metabolic labelling with [35S]methionine (Fig. 3c, middle and right panel).

Boue-Grabot et al. [19] have demonstrated phosphorylation of P2X2 receptors expressed in HEK293 cells. To address the possibility that P2X receptor phosphorylation is host cell specific, we analysed HEK293 cells transiently transfected with P2X2 or P2X3 expression plasmids. P2X receptor proteins were isolated by immunoprecipitation and resolved by reducing SDS-PAGE. The efficient expression of the P2X3 receptor or the C-terminally EGFP-tagged P2X2 receptor in HEK293 cells was verified by immunoblotting with subunit bands migrating at 54 kDa or 83 kDa (27 kDa contributed to the P2X2 subunit by the EGFP tag), respectively (Fig. 4b). However, phosphorylated P2X2 or P2X3 subunits could not be detected by immunoblotting with a phosphothreonine-specific antibody (Fig. 4a). In contrast, the PKC-phosphorylated splicing factor SF3B1, which was expressed in HEK293 cells as a positive control substrate and immunoprecipitated with an EGFP antibody, was efficiently phosphorylated under identical conditions, assuring that the assay was able to detect phosphorylated proteins (Fig. 4a, lane 1). Taken together, these results suggest that both P2X2 and P2X3 receptors exist constitutively in a non-phosphorylated state in both X. laevis oocytes and HEK293 cells.

PMA-activated PKC does not drive the phosphorylation of P2X2 or P2X3 receptors

A majority of protein kinases are quiescent unless their activity is stimulated by specific stimuli and effectors. To
drive phosphorylation by active endogenous PKC, we incubated non-denatured recombinant His-tagged P2X1, P2X2 and P2X3 receptors isolated by Ni²⁺ NTA agarose affinity chromatography from dodecylmaltoside extracts of X. laevis oocytes with PMA-stimulated lysates of X. laevis oocytes (Fig. 5a) or HEK293 cells (Fig. 5b) in the presence of [γ-32P]ATP. As a positive control, a specific peptide PKC substrate RRGRTGRGRRGIYR was incubated with the lysates. This peptide was equally well phosphorylated by addition of either cell lysate or a commercial preparation of the rat brain PKC subunit (Fig. 5a, b). In contrast, however, no 32P incorporation could be detected in the SDS-PAGE migration position of the P2X1, P2X3 or P2X2 receptor subunit at 56 kDa, 54 kDa or 66 kDa, respectively. The presence of purified P2X1, P2X2 and P2X3 receptor subunit protein in this assay was verified by immunoblotting using P2X1-, P2X2- or P2X3-specific antibodies (Fig. 5c). The phosphorylation of the synthetic peptide clearly demonstrates that both X. laevis oocytes and HEK293 cells contain sufficient endogenous PKC to drive at least the phosphorylation of a synthetic peptide substrate.

Discernible phosphorylation of P2X2 and P2X3 receptors might be obscured by a low level of endogenous PKC. Therefore, an analogous experiment was performed, in which the purified P2X2 and P2X3 receptors were incubated with purified rat brain PKC catalytic subunit rather than cell lysate in the presence of [γ-32P]ATP. Again, the specific peptide PKC substrate was efficiently phosphorylated as demonstrated both by direct phosphorimaging (Fig. 6a) and immunoblotting with the phosphotheorine monoclonal antibody (Fig. 6b). Phosphorylated P2X2 or P2X3 subunits could not be detected by either method (Fig. 6a, b) despite the presence of abundant amounts of the respective P2X subunits in the assays (Fig. 6c).
P2X<sub>3</sub> receptors in intact HEK cells

In a final set of experiments, we attempted to directly demonstrate phosphorylation in vivo by using metabolic [<sup>32</sup>P]orthophosphate labelling combined with immunoprecipitation analysis as a read out. Mock-transfected and P2X<sub>3</sub> receptor-transfected HEK293 cells were incubated with [<sup>32</sup>P]orthophosphate in phosphate-free DMEM, followed by incubation with the PKC activator PMA and/or the phosphoserine/phosphothreonine phosphatase inhibitor calyculin A. The PhosphorImager scan revealed an identical pattern of phosphorylated bands almost irrespective of the experimental conditions, with no evidence for a significant [<sup>32</sup>P]-labelling of the P2X<sub>3</sub> subunit (Fig. 7, top panel). P2X<sub>3</sub> receptor expression was verified by immunoblotting (Fig. 7, bottom panel).

Discussion

Both P2X<sub>3</sub> and P2X<sub>2,3</sub> receptors are present on sensory neurons, where they play an important role in nociception. In addition to mediating transient nociceptive responses, these receptors contribute critically to the hyperexcitability that underlies abnormal pain states elicited by nerve injuries. One mechanism for the increased responsiveness to ATP is sensitization of P2X<sub>3</sub> and P2X<sub>2,3</sub> receptors arising from a significant enhancement in the trafficking of P2X<sub>3</sub> subunit-containing receptors and/or an increase in function of pre-existing receptors [17]. An obvious candidate mechanism for functional modulation is phosphorylation, which is believed to occur universally in all ligand-gated ion channels as reviewed by Levitan [33].

Evidence for a control of P2X receptor channel function by PKC-mediated phosphorylation

Site-directed elimination of the putative N-terminal PKC site has already previously been shown to severely affect the electrophysiological phenotype of several P2X receptor subtypes, including P2X<sub>1</sub>, P2X<sub>2</sub>, and P2X<sub>3</sub> receptors from rat and/or human [16, 19, 20, 34] and P2X<sub>5</sub> receptors from frog [35]. Replacing the putative phospho-acceptor residue<sup>18</sup> for the basic residue in the P+2 position<sup>29</sup> by A or a non-basic residue, respectively, imposed rapidly desensitizing properties onto the non-desensitizing wt P2X<sub>2</sub> receptor without grossly affecting the current amplitude. In contrast, when analogous mutations were introduced into the rapidly desensitizing receptor subtypes P2X<sub>1</sub> or P2X<sub>3</sub>, peak currents declined to low or undetectable levels, respectively [16, 20, 34]; the same observation was also made with the slower desensitizing frog P2X<sub>5</sub> receptor [35]. Overall, the results obtained by site-directed elimination of the N-terminal TXR/K motif can be plausibly reconciled with the hypothesis that the rate of P2X receptor channel desensitization is controlled by a phosphorylation-dephosphorylation mechanism, with phosphorylation at this site being associated with a decreased rate of desensitization.

Additional evidence for a possible involvement of PKC-dependent phosphorylation comes from experiments with the PKC activator PMA, which was consistently found to elicit a severalfold increase in currents mediated by the P2X<sub>1</sub> receptor [36] or the P2X<sub>3</sub> receptor [16, 18, 21] without changing the rate of desensitization. A PMA-induced change of the current desensitization was noted for C-terminally truncated P2X<sub>2</sub> receptors, which are known to exhibit a desensitizing phenotype in contrast to the parent full-length P2X<sub>2</sub> receptors [19, 37]. PMA treatment converted the rapidly desensitizing phenotype of truncated P2X<sub>2</sub> receptors to slowly desensitizing, but did not affect the kinetic properties or current amplitudes of the full-length P2X<sub>2</sub> receptors. The same lack of effect of PMA has been observed with wild-type P2X<sub>3</sub> receptors stably expressed in HEK293 [38]. It has been inferred from these data that P2X<sub>2</sub> receptors are constitutively phosphorylated and that this phosphorylation is responsible for their non-desensitizing phenotype. Consistent with this view, immunoblot analysis with a phosphothreonine-proline-specific monoclonal antibody efficiently detected wild-type P2X<sub>2</sub> receptors with an intact PKC site, but not P2X<sub>2</sub> receptor mutants with a disrupted PKC site [19]. Constitutive phosphorylation has been also demonstrated for the P2X<sub>1</sub> receptor expressed in HEK293 cells [34]. Further studies demonstrated that exogenously added uridine triphosphate (UTP) potentiated the current mediated by endogenous and expressed P2X<sub>3</sub> receptors in response to αβ-meATP as agonist. Substitution of phospho-acceptor residues of PKC consensus sites located in the ectodomain of the P2X<sub>3</sub> receptor abolished this UTP-induced current potentiation, suggesting a role of ecto-protein kinase C in P2X<sub>3</sub> receptor regulation [39, 40].

Evidence against a control of P2X receptor channel function by PKC-mediated phosphorylation

In direct contrast to the results of Boue-Grabot et al. [19], we were unable to demonstrate a direct phosphorylation of wild-type P2X<sub>2</sub> receptors expressed in X. laevis oocytes and HEK293 cells. The various experimental approaches for phosphorylation detection that we applied included immunoblot analysis of purified P2X<sub>2</sub> receptors. In the experiments shown, we used a phosphothreonine-specific monoclonal antibody instead of a phosphothreonine-proline-specific antibody, because the TXK motif of the P2X<sub>3</sub> receptor contains a threonine in the -X-position instead of
the proline found in the amino acid sequence of the P2X1 and P2X2 subunit (cf. Fig. 1b). However, even when we used the same phosphothreonine-proline-specific antibody as Boue-Grabot et al. [19], we could not demonstrate phosphorylation of P2X2 receptors in our experiments (data not shown).

Recently, it has been shown that a proline in the P+1 position strongly disfavours substrate recognition by PKC [41], suggesting that our negative results with P2X2 receptors may not result from technical limitations, but reflect the inherent inability of P2X2 receptors to serve as substrates for PKC. Notably, P2X3 receptors, despite harbouring a T in the P+1 position, could also not be demonstrated to become phosphorylated by PKC in our experiments, as well as in a recently published paper [21]. Further evidence against a direct PKC-mediated P2X receptor phosphorylation comes from co-expression studies of P2X2 and G protein-coupled receptors. Co-expression of the metabotropic glutamate receptor mGluR1α with P2X1 receptors produced a 2.5-fold increase of the P2X1 receptor-mediated current that was blocked by the PKC inhibitor staurosporine and could be mimicked by PMA treatment of singly expressed P2X1 receptors. Although the pharmacological data are basically consistent with PKC-mediated receptor phosphorylation being involved, mutant P2X1 receptors with a disrupted N-terminal PKC site (T<sup>18V</sup>) still showed PMA-mediated and mGluR1α-mediated potentiation. Also, PMA treatment did not lead to an increase in the (rather weak) basal phosphorylation of the P2X1 receptor [36].

Functional data supporting a role of PKC in P2X receptor regulation have been detailed above. There are, however, also functional data that are inconsistent with the view that the TXR/K motif represents a true PKC site: (1) replacement of the phospho-acceptor threonine by an acidic residue to mimic incorporation of a negatively charged phosphate of the phospho-acceptor threonine by an acidic residue to the TXR/K motif represents a true PKC site: (1) replacement also functional data that are inconsistent with the view that regulation have been detailed above. There are, however, is unlikely to involve direct PKC-mediated phosphorylation of P2X receptors. The existence of a proline in the P+1 position in several P2X subunit isoforms (P2X<sub>1</sub>, P2X<sub>2</sub>, P2X<sub>4</sub>) might be one causative factor preventing direct phosphorylation by PKC. Additional steric constraints may explain why P2X<sub>1</sub> receptors also do not serve as PKC substrates, despite lacking the proline in P+1. The exact role of the TXR/K motif awaits further clarification.

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