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Lack of evidence for direct phosphorylation of recombinantly expressed P2X$_2$ and P2X$_3$ receptors by protein kinase C

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Abstract P2X$_3$ and P2X$_{2+3}$ 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 P2X$_3$ and P2X$_{2+3}$ 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 P2X$_2$ receptors and abolishes P2X$_3$ receptor function. Moreover, the PKC activator phorbol 12-myristate 13-acetate increased P2X$_3$ (but not P2X$_2$) receptor-mediated currents. Biochemically, however, we were unable to demonstrate by various experimental approaches a direct phosphorylation of wild-type P2X$_2$ and P2X$_3$ 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.

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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 Ca^{2+} [1, 2]. A family encompassing seven P2X subunit genes, designated P2X$_{1-7}$, has been identified in rodents and mammals. Six of the seven subunit isoforms (P2X$_1$-P2X$_5$ and P2X$_7$) 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 $\alpha\beta$-meATP sensitive (P2X$_1$ and P2X$_3$) and (2) slowly or non-desensitizing and $\alpha\beta$-meATP insensitive (P2X$_2$, P2X$_4$ and P2X$_7$) 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 P2X$_2$/P2X$_3$ (P2X$_{2+3}$) receptors, which feature $\alpha\beta$-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 P2X$_3$ receptors or heterotrimeric P2X$_{2+3}$ receptors. Conditions and mediators that facilitate P2X$_3$ and P2X$_{2+3}$ 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 intracellularly located N-terminal domain (Fig. 1a, b). Indeed, results obtained with pharmacological activators and inhibitors are consistent with PKC being involved in P2X$_3$ receptor potentiation by substance P and bradykinin [16], as well as the calcitonin gene-related peptide [18]. Direct PKC-mediated phosphorylation has so far been demonstrated only for the P2X$_2$ receptor, with ^{18}T of the ^{18}TPK^{20} sequence as the phospho-acceptor site [19]. Mutational analysis has further shown that the TXR/K sequence of the P2X$_1$, P2X$_2$ and P2X$_3$ receptors 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 P2X$_2$ and P2X$_3$ receptors become directly phosphorylated by PKC in a heterologous system. While this work was in progress, a similar study was published for the P2X$_3$ receptor that arrived at essentially the same conclusion, namely that the P2X$_3$ receptor is unlikely to be a direct PKC substrate [21]. Our results complement and extend this finding by demonstrating that the P2X$_2$ 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 P2X$_1$, P2X$_2$ and P2X$_3$ subunits

Peptides ATSSTLGLQENMRTS (residues 385-399 of the P2X$_1$ subunit), QQDSTSTDPKGLAQL (residues 458–472 of the P2X$_2$ subunit) and VEKQSTDSGAYSIGH (residues 383–397 of the P2X$_3$ 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 P2X$_1$, P2X$_2$ and P2X$_3$, 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 P2X$_1$ subunits (rP2X$_1$, GenBank accession no. X80477), P2X$_2$ subunits (rP2X$_2$, GenBank accession no. U14414) or rat P2X$_3$ subunits (rP2X$_3$, 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-rP2X$_2$-EGFP-pcDNA3.1 with a C-terminal enhanced green fluorescent protein (EGFP) fusion was generated by eliminating the stop codon in His-rP2X$_2$ 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 $CaCl_2$, 1 mM $MgCl_2$ and 10 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), pH 7.4] supplemented with 50 μg/ml of gentamicin.

Heterologous expression in HEK293 cells

cDNAs encoding P2X subunits in pcDNA3.1 vector (Invitrogen, Karlsruhe, Germany) or the splicing factor SF3B1 in EGFP-C1 vector were transiently transfected into HEK293 cells using Lipofectamine LTX (Invitrogen, Karlsruhe, Germany). Cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) (PAA Laboratories, Linz, Austria), 100 U/ml of penicillin G and 100 μg/ml of streptomycin.

Metabolic [^{35}S]methionine labelling of oocytes

For metabolic [^{35}S]methionine labelling of oocyte-expressed recombinant proteins, cRNA-injected oocytes and non-injected controls were incubated overnight with L-[^{35}S] 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 $MgCl_2$), 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 Ni^{2+} 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 ethylenediamine-tetraacetate (EDTA)-free Halt™ protease inhibitor cocktail (Pierce, Rockford, IL, USA), 50 mM NaF and 50 mM $Na_4P_2O_7$ 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 Ni^{2+} NTA agarose beads with non-denaturing elution buffer consisting of 250 mM imidazole/HCl (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 $Na_4P_2O_7$, 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 phenylmethylsulfonyl fluoride (PMSF), 0.5% Triton X-100 v/v and 25 mM iodoacetamide], supplemented 1:1,000 with the desired peptide-specific $P2X_2$ or $P2X_3$ 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 RRGIYR, 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-^{32}P]ATP (10 mCi/ml, Amersham Biosciences GE Healthcare, Freiburg, Germany) in phosphorylation buffer containing 6 mM $CaCl_2$, 10 mM $MgCl_2$, 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 detailed below.

In vivo phosphorylation assay

Transiently transfected HEK293 cells were metabolically labelled by a 3-h incubation with 500 μCi/ml of [^{32}P] 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 [^{14}C]-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 radiolabelled 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 P2X$_2$ and P2X$_3$ 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 Ca^{2+}-free bath medium (designated Mg-ORi) to avoid activation of endogenous Ca^{2+}-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 $\Delta V = 10$ mV were applied from the holding potential to elicit a capacitive transient I_{cap}, the integral of which yields the charge movement Q_{cap} and thus the membrane capacitance $C_m = Q_{cap}/\Delta V$ [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 $P2X_2$ 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 $P2X_2$ and $P2X_3$ 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 $P2X_2$ receptors (Fig. 1c, left current trace). The $P2X_2$ subunit shares with other P2X subunit isoforms a conserved consensus site for PKC phosphorylation (TXR/K, where T is the phosphorylation
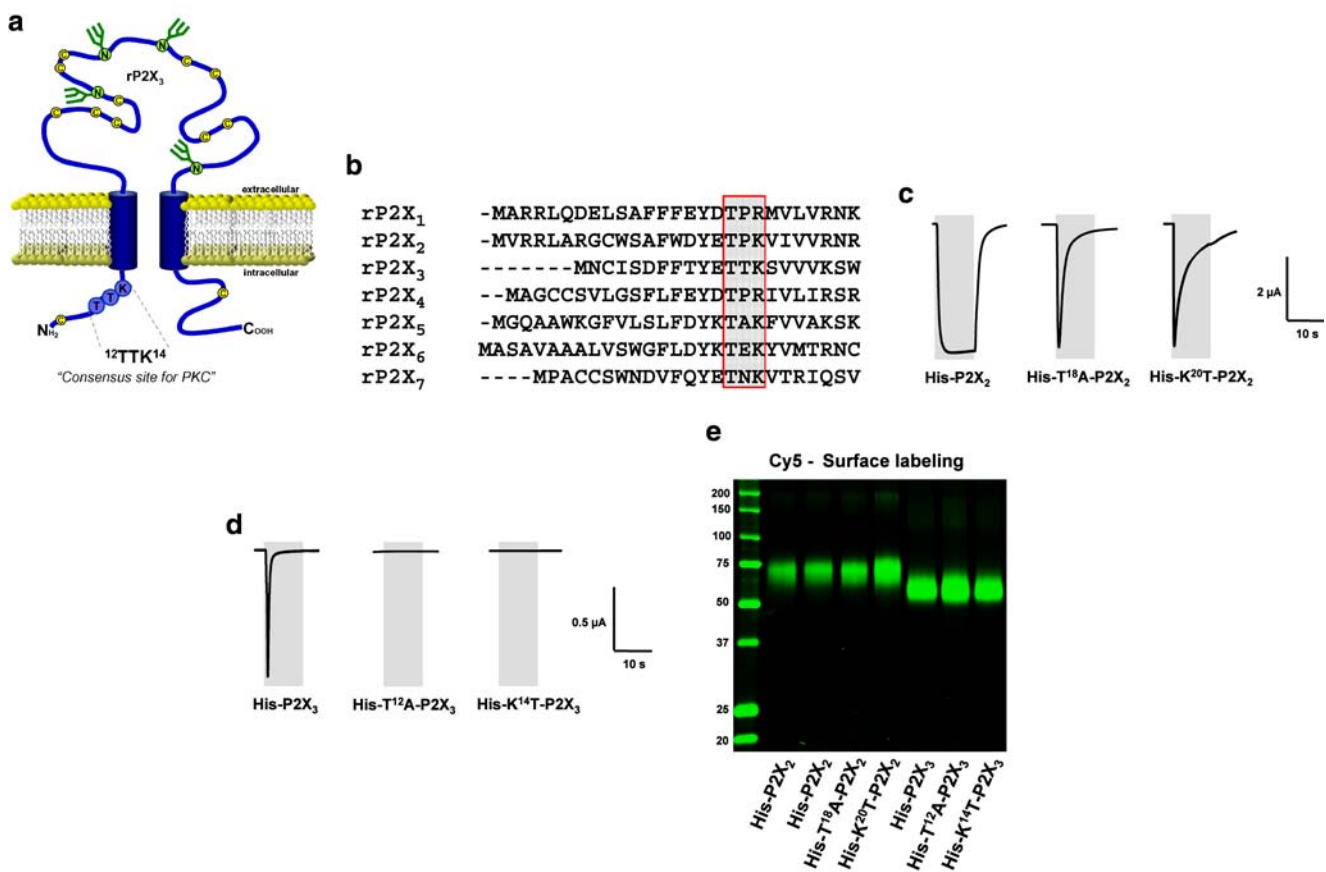
Fig. 1 Effect of site-directed modification of a putative PKC motif on currents mediated by ATP-activated $P2X_2$ and $P2X_3$ receptors. **a** Schematic model of the transmembrane topology of the rat $P2X_3$ subunit illustrating the N-terminal position of the [12]TTK[14] sequence. **b** Alignment of 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 $P2X_2$ receptors. **d** Typical current traces elicited by applying 10-s pulses of 100 μM ATP to oocytes expressing the indicated wild-type or mutant $P2X_3$ receptors. *Gray areas* indicate the duration of ATP application. **e** All the $P2X_2$ and $P2X_3$ 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 Ni^{2+} chelate chromatography and resolved by reducing SDS-PAGE. Shown is a fluorescence scan of an SDS-PAGE gel
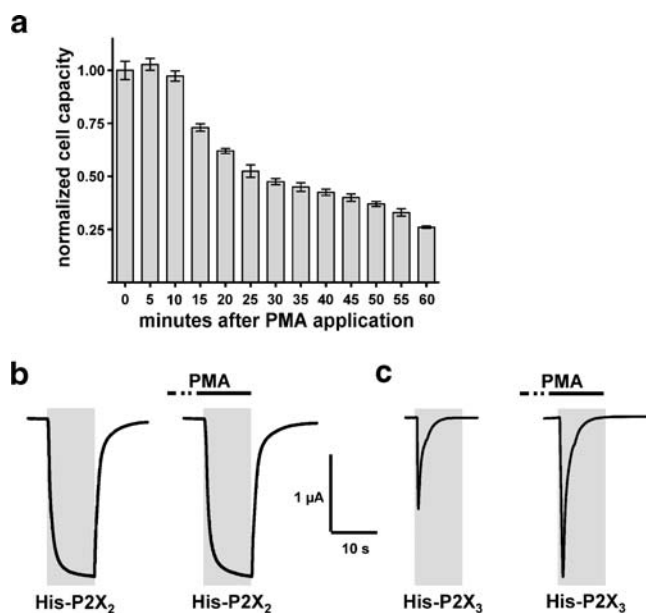
Fig. 2 Effect of PMA on ATP-induced currents mediated by expressed $P2X_2$ and $P2X_3$ receptors in *X. laevis* oocytes. **a** The electrical membrane capacitance was monitored as a measure of the oocyte surface area during sustained stimulation with PMA. Each *bar* represents the mean $C_m \pm SEM$ calculated from the areas under the current transients elicited in 10-s intervals by five consecutive 10-mV depolarizing steps. **b**, **c** Representative current traces elicited by 10-s lasting pulses of 100 μM ATP applied in 10-min intervals to oocytes expressing the indicated P2X receptors or P2X receptor mutants. Where denoted by a *black bar*, oocytes were pre-incubated with 100 nM PMA before ATP was co-applied. PMA was without effect on the $P2X_2$ receptor-mediated current (**b**), but induced a marked increase of the current amplitude mediated by $P2X_3$ receptors (**c**). *Gray areas* indicate the duration of ATP application

site and X denotes any amino acid; see Fig. 1a, b). Disruption of the conserved N-terminal PKC site by mutating the phosphor-acceptor [18]T to A resulted in a rapidly desensitizing channel (Fig. 1c, middle current trace). Also, replacement of [20]K 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 [20]K in the P+2 position by a non-basic residue such as T will destroy the putative PKC site despite preserving the phosphor-acceptor [18]T. Virtually identical findings had previously been reported by Boue-Grabot et al. [19]. The average half-times of desensitization (τ) of the [18]A and [20]T $P2X_2$ mutants were quite similar, with mean τ values ($\pm SEM$) 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 $P2X_3$ 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 $P2X_2$ subunit in the analogous positions of the $P2X_3$ receptor $T^{12}A$ or $K^{14}T$, completely abolished $P2X_3$ 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).
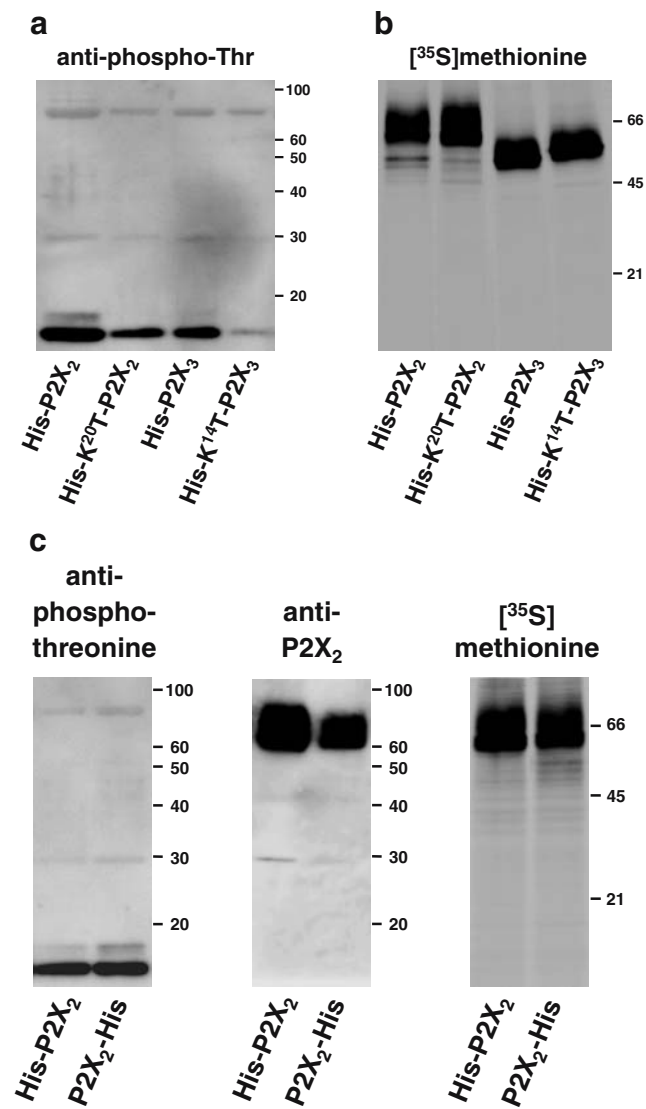
The PKC activator PMA augments $P2X_3$ receptor-mediated currents, yet leaves $P2X_2$-mediated currents unchanged

Since the mutagenesis data for the $P2X_2$ and $P2X_3$ 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 $P2X_2$ or $P2X_3$ 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 $P2X_2$ or $P2X_3$ receptor-expressing oocytes before and after a 10-min exposure to PMA. PMA neither affected the shape nor the amplitude of $P2X_2$ receptor-mediated current (Fig. 2b). However, PMA produced a marked increase of the current amplitude mediated by the $P2X_3$ receptor (Fig. 2c). In one experiment with four oocytes, a statistically significant increase of the $P2X_3$-mediated current amplitude by PMA of $155 \pm 42\%$ (SEM) was determined.

$P2X_2$ and $P2X_3$ receptors exist in a non-phosphorylated state in both *X. laevis* oocytes and HEK293 cells
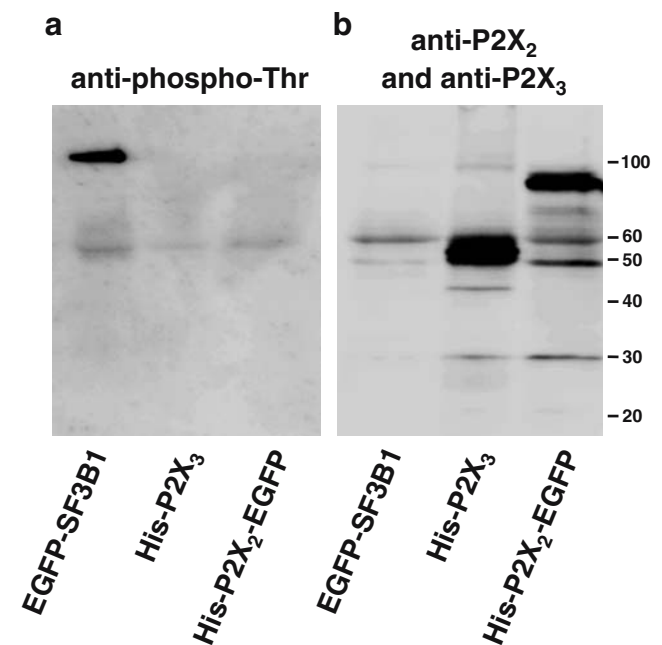
To explain the accelerated desensitization upon site-directed perturbation of the $^{18}TXK^{20}$ motif, it has been suggested that the wild-type $P2X_2$ receptor is constitutively phosphorylated at [18]T and that this phosphorylation is responsible for the slow desensitization rate [19]. To address this issue, we next examined biochemically whether $P2X_3$

receptors also exist in a constitutively phosphorylated state; the $P2X_2$ receptor was also analysed to allow for a direct comparison of the results. Both parent P2X receptors and mutants were purified by Ni^{2+} chelate affinity chromatography from dodecylmaltoside extracts of $[^{35}S]$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 $[^{35}S]$methionine-labelled $P2X_2$ and $P2X_3$ 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 $P2X_3$ or $P2X_2$ receptor subunits, which are separated by only 12 or 20 residues, respectively, we performed analogous experiments with a C-terminally His-tagged $P2X_2$ receptor, $P2X_2$-His. As apparent from Fig. 3c, neither N-terminally nor C-terminally His-tagged $P2X_2$ receptor became phosphorylated, as evidenced by immunoblotting with a

Fig. 3 Immunoblots show no constitutive phosphorylation of oocyte-expressed $P2X_2$ or $P2X_3$ receptors. The indicated wild-type or mutant P2X subunits were purified by Ni^{2+} chelate chromatography from $[^{35}S]$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 $P2X_2$ and $P2X_3$ subunits, which are indicated by the adjacent PhosphorImager scan in panel b. **b** Direct PhosphorImager visualization of metabolically $[^{35}S]$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 $P2X_2$ subunits tagged with either an N-terminal or C-terminal hexahistidine sequence (designated His-$P2X_2$ or $P2X_2$-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 $P2X_2$ subunits, which were visualized by immunoblotting with the $P2X_2$ subunit polyclonal antibody (*middle panel*) or PhosphorImager scanning of incorporated $[^{35}S]$methionine (*right panel*)

Fig. 4 Immunoblotting reveals no constitutive phosphorylation of HEK293 cell-expressed $P2X_2$ and $P2X_3$ receptors. The indicated proteins were isolated by immunoprecipitation using the peptide-specific $P2X_3$ subunit polyclonal antibody or an EGFP-specific polyclonal antibody as appropriate and resolved by reducing SDS-PAGE. **a** Shown is an immunoblot probed by phosphothreonine-specific monoclonal antibody and a peroxidase-conjugated secondary antibody. Note that phosphorylation of the $P2X_3$ receptor or the EGFP-tagged $P2X_2$ receptor is not detectable, whereas a positive control—the EGFP-tagged splicing factor SF3B1 (migration mass 97 kDa [23])—was detected by the antibody. **b** The same samples as in panel a were immunoblotted using peptide-specific $P2X_2$ and $P2X_3$ subunit polyclonal antibodies to verify the expression of the transfected genes
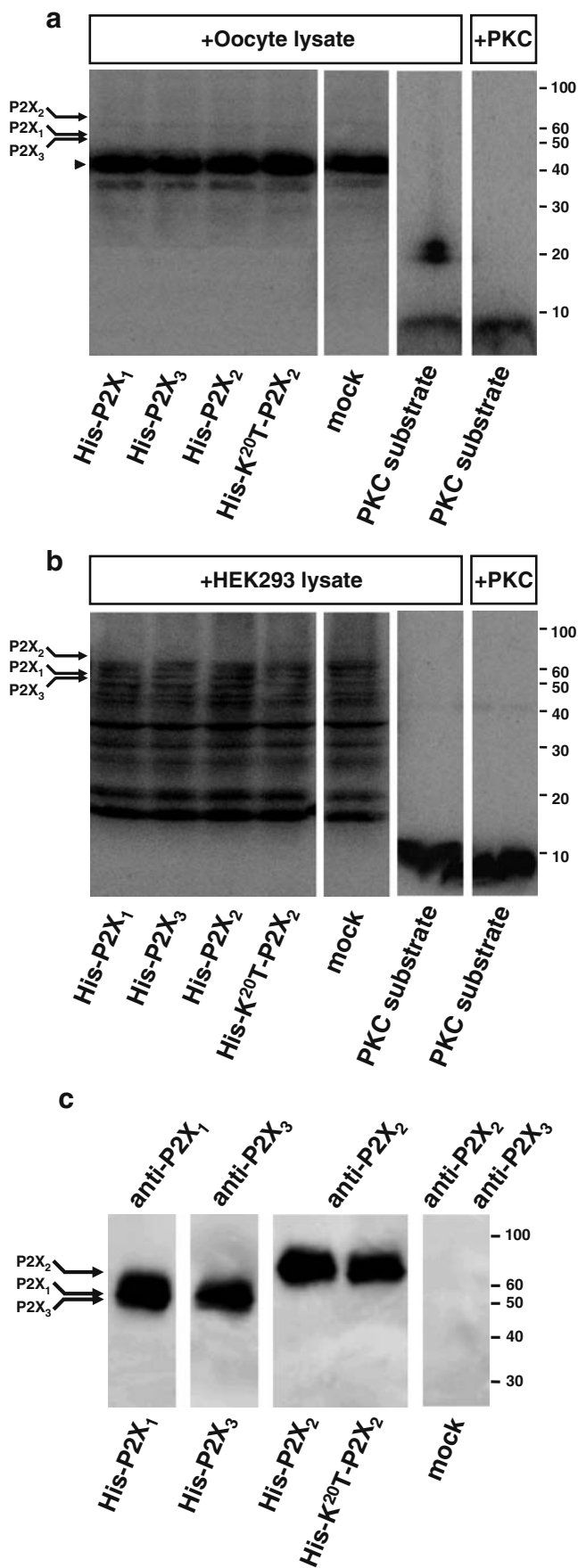
Fig. 5 Lysates of *X. laevis* oocytes or HEK293 cells contain endogenous PKC, but do not support PMA-driven phosphorylation of $P2X_2$ or $P2X_3$ receptors. The indicated wild-type and mutant P2X receptors were purified by non-denaturing Ni^{2+} chelate chromatography from *X. laevis* oocytes, supplemented with $[\gamma\text{-}^{32}P]ATP$, 100 nM PMA and oocyte lysate (**a**) or HEK293 cell lysate (**b**), and incubated for 30 min at ambient temperature. In parallel, a peptide PKC substrate (final concentration 0.5 μg/μl) was incubated with $[\gamma\text{-}^{32}P]$ATP, PMA and either of the two cell lysates or the purified rat brain PKC catalytic subunit as indicated. The proteins were resolved by reducing SDS-PAGE and visualized by phosphorimaging. Note that the band pattern was similar irrespective of whether protein of mock-injected oocytes or P2X receptor-expressing oocytes was used as a substrate, arguing against specific labelling of either P2X receptor. *Arrows* indicate migration positions of $P2X_1$, $P2X_2$ and $P2X_3$ subunits. *Arrowhead* in a, dominant background band that is present in all oocyte samples. The peptide PKC substrate was similarly labelled by lysates or purified rat brain PKC, confirming the presence of endogenous PKC in *X. laevis* oocytes and HEK293 cells. **c** The same samples as in panels a and b were resolved on a separate SDS-PAGE gel. The indicated P2X subunits were detected by immunoblotting using the appropriate P2X subunit-specific polyclonal antibodies. A sample of mock-transfected cells was simultaneously probed with the $P2X_2$ and $P2X_3$ subunit-specific polyclonal antibodies

phosphor-specific anti-threonine antibody (Fig. 3c). The presence of $P2X_2$ receptor protein was verified by immunoblotting and metabolic labelling with $[^{35}S]$methionine (Fig. 3c, middle and right panel).

Boue-Grabot et al. [19] have demonstrated phosphorylation of $P2X_2$ receptors expressed in HEK293 cells. To address the possibility that P2X receptor phosphorylation is host cell specific, we analysed HEK293 cells transiently transfected with $P2X_2$ or $P2X_3$ expression plasmids. P2X receptor proteins were isolated by immunoprecipitation and resolved by reducing SDS-PAGE. The efficient expression of the $P2X_3$ receptor or the C-terminally EGFP-tagged $P2X_2$ receptor in HEK293 cells was verified by immunoblotting with subunit bands migrating at 54 kDa or 83 kDa (27 kDa contributed to the $P2X_2$ subunit by the EGFP tag), respectively (Fig. 4b). However, phosphorylated $P2X_2$ or $P2X_3$ 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 $P2X_2$ and $P2X_3$ 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 $P2X_2$ or $P2X_3$ receptors

A majority of protein kinases are quiescent unless their activity is stimulated by specific stimuli and effectors. To
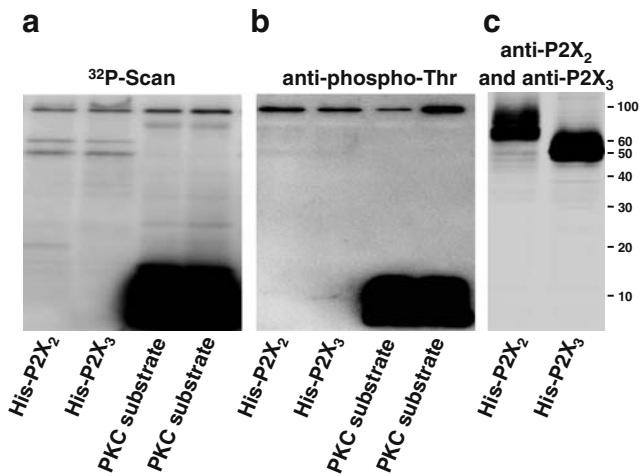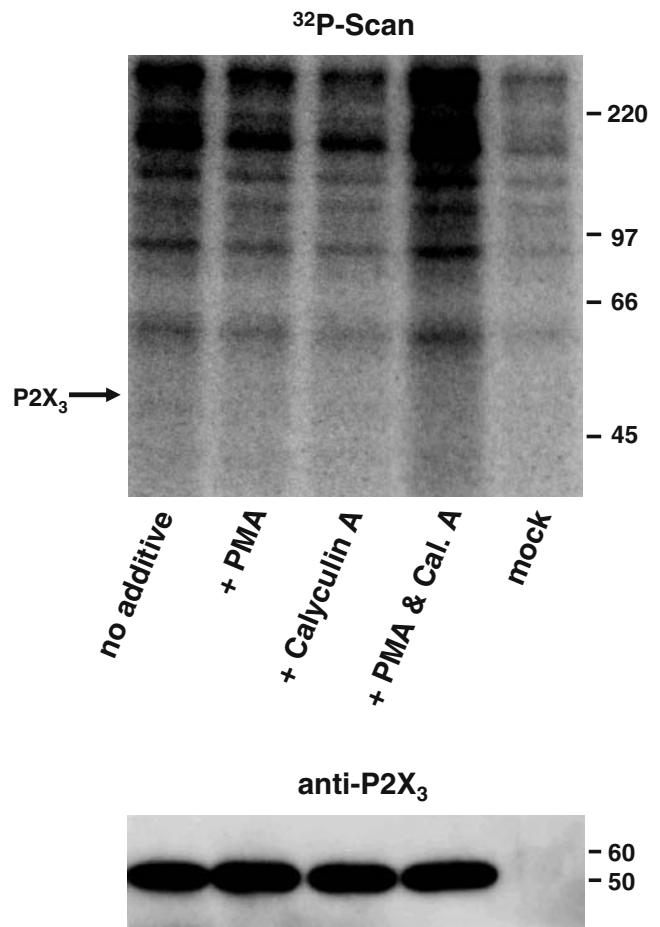
Fig. 6 PKC phosphorylates a specific PKC substrate in vitro, but does not phosphorylate P2X$_2$ or P2X$_3$ receptors. The immunoprecipitated recombinant P2X$_2$ or P2X$_3$ receptor from *X. laevis* oocytes or a synthetic peptide (1 or 10 μg) derived from the non-structural protein 3 of hepatitis C virus, which serves as a specific substrate for PKC, were incubated for 30 min with purified rat brain PKC catalytic subunit (0.02 μg corresponding to 0.02 U/μl) in the presence of 50 μM [γ-^{32}P]ATP (10 mCi/ml), and then subjected to SDS-PAGE and phosphorimaging. The rat brain PKC catalytic subunit does not require Ca^{2+} and phosphatidylserine for activity [42]. ^{32}P was incorporated into the PKC substrate (*left panel*) and was also detected by immunoblotting with an anti-phosphothreonine antibody (*middle panel*). However, there was no detectable ^{32}P incorporation at a migration position of ~66 kDa or ~54 kDa of the P2X$_2$ or P2X$_3$ subunit, respectively, the presence of which could be verified by immunoblotting (*right panel*)

which the purified P2X$_2$ and P2X$_3$ receptors were incubated with purified rat brain PKC catalytic subunit rather than cell lysate in the presence of [γ-^{32}P]ATP. Again, the specific peptide PKC substrate was efficiently phosphorylated as demonstrated both by direct phosphorimaging (Fig. 6a) and immunoblotting with the phosphothreonine monoclonal antibody (Fig. 6b). Phosphorylated P2X$_2$ or P2X$_3$ 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).

drive phosphorylation by active endogenous PKC, we incubated non-denatured recombinant His-tagged P2X$_1$, P2X$_2$ and P2X$_3$ receptors isolated by Ni^{2+} 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-^{32}P]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 ^{32}P incorporation could be detected in the SDS-PAGE migration position of the P2X$_1$, P2X$_3$ or P2X$_2$ receptor subunit at 56 kDa, 54 kDa or 66 kDa, respectively. The presence of purified P2X$_1$, P2X$_2$ and P2X$_3$ receptor subunit protein in this assay was verified by immunoblotting using P2X$_1$-, P2X$_2$- or P2X$_3$-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 P2X$_2$ and P2X$_3$ receptors might be obscured by a low level of endogenous PKC. Therefore, an analogous experiment was performed, in

Fig. 7 PMA and calyculin A do not induce phosphorylation of P2X$_3$ receptors in intact HEK cells. Mock- and P2X$_3$ receptor-transfected HEK293 cells were incubated with [^{32}P]orthophosphate (500 μCi/ml) for 3 h in phosphate-free DMEM, followed by a 10-min incubation with 100 nM of each, the PKC activator PMA and/or the phosphatase inhibitor calyculin A. Triton X-100 extracts were then prepared from the cells and subjected to immunoprecipitation using the peptide-specific P2X$_3$ subunit polyclonal antibody, followed by reducing SDS-PAGE and phosphorimaging. The migration position of the P2X$_3$ subunit is marked by an *arrow*, indicating the absence of ^{32}P incorporation into the P2X$_3$ subunit. P2X$_3$ receptor expression was verified by immunoblotting with the P2X$_3$ subunit polyclonal antibody (*bottom panel*)

PMA or calyculin A do not enhance phosphorylation of P2X$_3$ receptors in intact HEK cells

In a final set of experiments, we attempted to directly demonstrate phosphorylation in vivo by using metabolic [32P] orthophosphate labelling combined with immunoprecipitation analysis as a read out. Mock-transfected and P2X$_3$ receptor-transfected HEK293 cells were incubated with [32P]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 32P-labelling of the P2X$_3$ subunit (Fig. 7, top panel). P2X$_3$ receptor expression was verified by immunoblotting (Fig. 7, bottom panel).

Discussion

Both P2X$_3$ and P2X$_{2+3}$ 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$_3$ and P2X$_{2+3}$ receptors arising from a significant enhancement in the trafficking of P2X$_3$ 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$_1$, P2X$_2$ and P2X$_3$ receptors from rat and/or human [16, 19, 20, 34] and P2X$_5$ receptors from frog [35]. Replacing the putative phospho-acceptor residue [18]T or the basic residue in the P+2 position ([20]K) by A or a non-basic residue, respectively, imposed rapidly desensitizing properties onto the non-desensitizing wt P2X$_2$ receptor without grossly affecting the current amplitude. In contrast, when analogous mutations were introduced into the rapidly desensitizing receptor subtypes P2X$_1$ or P2X$_3$, peak currents declined to low or undetectable levels, respectively [16 ,20, 34]; the same observation was also made with the slower desensitizing frog P2X$_5$ 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$_1$ receptor [36] or the P2X$_3$ 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$_2$ receptors, which are known to exhibit a desensitizing phenotype in contrast to the parent full-length P2X$_2$ receptors [19, 37]. PMA treatment converted the rapidly desensitizing phenotype of truncated P2X$_2$ receptors to slowly desensitizing, but did not affect the kinetic properties or current amplitudes of the full-length P2X$_2$ receptors. The same lack of effect of PMA has been observed with wild-type P2X$_2$ receptors stably expressed in HEK293 [38]. It has been inferred from these data that P2X$_2$ 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$_2$ receptors with an intact PKC site, but not P2X$_2$ receptor mutants with a disrupted PKC site [19]. Constitutive phosphorylation has been also demonstrated for the P2X$_1$ receptor expressed in HEK293 cells [34]. Further studies demonstrated that exogenously added uridine triphosphate (UTP) potentiated the current mediated by endogenous and expressed P2X$_3$ receptors in response to αβ-meATP as agonist. Substitution of phospho-acceptor residues of PKC consensus sites located in the ectodomain of the P2X$_3$ receptor abolished this UTP-induced current potentiation, suggesting a role of ecto-protein kinase C in P2X$_3$ 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$_2$ 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$_2$ 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$_3$ receptor contains a threonine in the -X-position instead of

the proline found in the amino acid sequence of the P2X$_1$ and P2X$_2$ 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 P2X$_2$ 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 P2X$_2$ receptors may not result from technical limitations, but reflect the inherent inability of P2X$_2$ receptors to serve as substrates for PKC. Notably, P2X$_3$ 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 P2X$_1$ and G protein-coupled receptors. Co-expression of the metabotropic glutamate receptor mGluR1α with P2X$_1$ receptors produced a 2.5-fold increase of the P2X$_1$ receptor-mediated current that was blocked by the PKC inhibitor staurosporine and could be mimicked by PMA treatment of singly expressed P2X$_1$ receptors. Although the pharmacological data are basically consistent with PKC-mediated receptor phosphorylation being involved, mutant P2X$_1$ receptors with a disrupted N-terminal PKC site (T^{18}V) 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 P2X$_1$ 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 group by constitutive phosphorylation was unable to restore the function of P2X$_1$ receptors [34] and P2X$_3$ receptors [16]; (2) the PMA-induced potentiation of hP2X$_1$ receptor currents could be blocked by the PKC inhibitor staurosporine, but surprisingly, not by site-directed elimination of the PKC site [36]; thus it is much more likely that PMA exerts its effect on the P2X$_1$ receptor (and the P2X$_3$ receptor) through a yet to be identified accessory protein.

Taken together, there is no doubt that the TXR/K motif plays an important role in the functioning of P2X receptors, and that PMA is capable of modulating P2X$_1$- and P2X$_3$-mediated receptor currents. The underlying mechanism, 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$_1$, P2X$_2$, P2X$_4$) might be one causative factor preventing direct phosphorylation by PKC. Additional

steric constraints may explain why P2X$_3$ 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.

References

1. Burnstock G (2004) Introduction: P2 receptors. Curr Top Med Chem 4:793–803
2. North RA (2002) Molecular physiology of P2X receptors. Physiol Rev 82:1013–1067
3. Aschrafi A, Sadtler S, Niculescu C et al (2004) Trimeric architecture of homomeric P2X$_2$ and heteromeric P2X$_{1+2}$ receptor subtypes. J Mol Biol 342:333–343
4. Barrera NP, Ormond SJ, Henderson RM et al (2005) Atomic force microscopy imaging demonstrates that P2X$_2$ receptors are trimers but that P2X$_6$ receptor subunits do not oligomerize. J Biol Chem 280:10759–10765
5. Jiang LH, Kim M, Spelta V et al (2003) Subunit arrangement in P2X receptors. J Neurosci 23:8903–8910
6. Khakh BS, Burnstock G, Kennedy C et al (2001) International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. Pharmacol Rev 53:107–118
7. Mio K, Kubo Y, Ogura T et al (2005) Visualization of the trimeric P2X$_2$ receptor with a crown-capped extracellular domain. Biochem Biophys Res Commun 337:998–1005
8. Nicke A, Bäumert HG, Rettinger J et al (1998) P2X$_1$ and P2X$_3$ receptors form stable trimers: a novel structural motif of ligand-gated ion channels. EMBO J 17:3016–3028
9. Gever JR, Cockayne DA, Dillon MP et al (2006) Pharmacology of P2X channels. Pflugers Arch 452:513–537
10. Burnstock G (2005) Purinergic P2 receptors as targets for novel analgesics. Pharmacol Ther 110:433–454
11. Chizh BA, Illes P (2001) P2X receptors and nociception. Pharmacol Rev 53:553–568
12. Jarvis MF (2003) Contributions of P2X$_3$ homomeric and heteromeric channels to acute and chronic pain. Expert Opin Ther Targets 7:513–522
13. Kennedy C, Assis TS, Currie AJ et al (2003) Crossing the pain barrier: P2 receptors as targets for novel analgesics. J Physiol 553:683–694
14. North RA (2004) P2X$_3$ receptors and peripheral pain mechanisms. J Physiol 554:301–308
15. North RA (2003) The P2X$_3$ subunit: a molecular target in pain therapeutics. Curr Opin Investig Drugs 4:833–840
16. Paukert M, Osteroth R, Geisler HS et al (2001) Inflammatory mediators potentiate ATP-gated channels through the P2X$_3$ subunit. J Biol Chem 276:21077–21082
17. Chen Y, Li GW, Wang C et al (2005) Mechanisms underlying enhanced P2X receptor-mediated responses in the neuropathic pain state. Pain 119:38–48
18. Fabbretti E, D'Arco M, Fabbro A et al (2006) Delayed upregulation of ATP P2X$_3$ receptors of trigeminal sensory

neurons by calcitonin gene-related peptide. J Neurosci 26:6163–6171

19. Boue-Grabot E, Archambault V, Seguela P (2000) A protein kinase C site highly conserved in P2X subunits controls the desensitization kinetics of P2X$_2$ ATP-gated channels. J Biol Chem 275:10190–10195

20. Ennion SJ, Evans RJ (2002) P2X$_1$ receptor subunit contribution to gating revealed by a dominant negative PKC mutant. Biochem Biophys Res Commun 291:611–616

21. Brown DA, Yule DI (2007) Protein kinase C regulation of P2X$_3$ receptors is unlikely to involve direct receptor phosphorylation. Biochim Biophys Acta 1773:166–175

22. Gloor S, Pongs O, Schmalzing G (1995) A vector for the synthesis of cRNAs encoding Myc epitope-tagged proteins in Xenopus laevis oocytes. Gene 160:213–217

23. de Graaf K, Czajkowska H, Rottmann S et al (2006) The protein kinase DYRK1A phosphorylates the splicing factor SF3b1/SAP155 at Thr434, a novel in vivo phosphorylation site. BMC Biochem 7:7

24. Schmalzing G, Gloor S, Omay H et al (1991) Up-regulation of sodium pump activity in Xenopus laevis oocytes by expression of heterologous β1 subunits of the sodium pump. Biochem J 279:329–336

25. Duckwitz W, Hausmann R, Aschrafi A et al (2006) P2X$_5$ subunit assembly requires scaffolding by the second transmembrane domain and a conserved aspartate. J Biol Chem 281:39561–39572

26. Borowski P, Resch K, Schmitz H et al (2000) A synthetic peptide derived from the non-structural protein 3 of hepatitis C virus serves as a specific substrate for PKC. Biol Chem 381:19–27

27. Rettinger J, Schmalzing G (2003) Activation and desensitization of the recombinant P2X$_1$ receptor at nanomolar ATP concentrations. J Gen Physiol 121:451–461

28. Rettinger J, Schmalzing G (2004) Desensitization masks nanomolar potency of ATP at the P2X$_1$ receptor. J Biol Chem 279:6426–6433

29. Hausmann R, Rettinger J, Gerevich Z et al (2006) The suramin analog 4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzenetriylbis (carbonylimino)))tetra-kis-benzenesulfonic acid (NF110) potently blocks P2X$_3$ receptors: subtype selectivity is determined by location of sulfonic acid groups. Mol Pharmacol 69:2058–2067

30. Adrian RH, Almers W (1974) Membrane capacity measurements on frog skeletal muscle in media of low ion content. J Physiol 237:573–605

31. Vasilets LA, Schmalzing G, Mädefessel K et al (1990) Activation of protein kinase C by phorbol ester induces downregulation of the Na+/K+-ATPase in oocytes of Xenopus laevis. J Membr Biol 118:131–142

32. Schmalzing G, Richter H-P, Hansen A et al (1995) Involvement of the GTP binding protein Rho in constitutive endocytosis in Xenopus laevis oocytes. J Cell Biol 130:1319–1332

33. Levitan IB (1999) Modulation of ion channels by protein phosphorylation. How the brain works. Adv Second Messenger Phosphoprotein Res 33:3–22

34. Liu GJ, Brockhausen J, Bennett MR (2003) P2X$_1$ receptor currents after disruption of the PKC site and its surroundings by dominant negative mutations in HEK293 cells. Auton Neurosci 108:12–16

35. Jensik P, Cox T (2002) ATP-induced internalization of amphibian epithelial P2X receptors is linked to channel opening. Pflugers Arch 444:795–800

36. Vial C, Tobin AB, Evans RJ (2004) G-protein-coupled receptor regulation of P2X$_1$ receptors does not involve direct channel phosphorylation. Biochem J 382:101–110

37. He ML, Zemkova H, Koshimizu TA et al (2003) Intracellular calcium measurements as a method in studies on activity of purinergic P2X receptor channels. Am J Physiol Cell Physiol 285:C467–C479

38. Chow YW, Wang HL (1998) Functional modulation of P2X$_2$ receptors by cyclic AMP-dependent protein kinase. J Neurochem 70:2606–2612

39. Wirkner K, Stanchev D, Koles L et al (2005) Regulation of human recombinant P2X$_3$ receptors by ecto-protein kinase C. J Neurosci 25:7734–7742

40. Stanchev D, Flehmig G, Gerevich Z et al (2006) Decrease of current responses at human recombinant P2X$_3$ receptors after substitution by Asp of Ser/Thr residues in protein kinase C phosphorylation sites of their ecto-domains. Neurosci Lett 393:78–83

41. Zhu G, Fujii K, Belkina N et al (2005) Exceptional disfavor for proline at the P+1 position among AGC and CAMK kinases establishes reciprocal specificity between them and the proline-directed kinases. J Biol Chem 280:10743–10748

42. Huang KP, Huang FL (1986) Conversion of protein kinase C from a Ca2+-dependent to an independent form of phorbol ester-binding protein by digestion with trypsin. Biochem Biophys Res Commun 139:320–326