

# Protein kinase C regulation of P2X<sub>3</sub> receptors is unlikely to involve direct receptor phosphorylation

David A. Brown, David I. Yule\*

Department of Pharmacology and Physiology, School of Medicine and Dentistry, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, USA

Received 3 April 2006; received in revised form 25 August 2006; accepted 13 September 2006  
Available online 19 September 2006

## Abstract

P2X receptors (P2XR) act as ligand-gated, cation-selective ion channels. A common characteristic of all seven P2X family members is a conserved consensus sequence for protein kinase C (PKC)-mediated phosphorylation in the intracellular N-terminus of the receptor. Activation of PKC has been shown to enhance currents through P2X<sub>3</sub>R, however the molecular mechanism of this potentiation has not been elucidated. In the present study we show that activation of PKC can enhance adenosine triphosphate (ATP)-mediated Ca<sup>2+</sup> signals ~2.5-fold in a DT-40 3KO cell culture system (P2 receptor null) transiently overexpressing P2X<sub>3</sub>R. ATP-activated cation currents were also directly studied using whole cell patch clamp techniques in HEK-293 cells, a null background for ionotropic P2XR. PKC activation resulted in a ~8.5-fold enhancement of ATP-activated current in HEK-293 cells transfected with P2X<sub>3</sub>R cDNA, but had no effect on currents through either P2X<sub>4</sub>R- or P2X<sub>7</sub>R-transfected cells. P2X<sub>3</sub>R-transfected HEK-293 cells were metabolically labeled with <sup>32</sup>PO<sub>4</sub><sup>-</sup> and following treatment with phorbol-12-myristate-13-acetate (PMA) and subsequent immunoprecipitation, there was no incorporation of <sup>32</sup>PO<sub>4</sub><sup>-</sup> in bands corresponding to P2X<sub>3</sub>R. Similarly, in vitro phosphorylation experiments, utilizing purified PKC catalytic subunits failed to establish phosphorylation of either P2X<sub>3</sub>R or P2X<sub>3</sub>R-EGFP. These data indicate that PKC activation can enhance both the Ca<sup>2+</sup> signal as well as the cation current through P2X<sub>3</sub>R, however it appears that the regulation is unlikely to be a result of direct phosphorylation of the receptor.

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**Keywords:** Protein kinase C; Phosphorylation; Calcium signaling; P2X; Purinergic receptor; P2X<sub>3</sub>R

## 1. Introduction

Extracellular ATP can initiate signal transduction by activating purinergic receptors of the P2X and P2Y subtypes. P2XR are ligand-gated, cation selective ion channels; whereas P2Y receptors (P2YR) are G-protein coupled receptors (GPCR). Seven genes have been identified as coding for P2XR (P2X<sub>1</sub>–P2X<sub>7</sub>R) [1]. The functional channel is formed by three P2X subunits [2]. Each subunit contains two transmembrane spanning domains, with both the N- and C-termini facing the cytoplasm, and the large extracellular loop containing the ATP binding site [1].

The N- and C-termini of P2XR provide putative unique areas of regulation/modulation by intracellular factors. One conserved feature of all members of the P2XR family is a consensus

sequence for PKC mediated phosphorylation in the N-terminal domain. This region consists of the amino acids Thr-X-Arg/Lys, or T-X-R/K (X=amino acid). The importance of this site in the N-terminus of P2X<sub>2</sub>R was first implicated by studies showing that the fast-desensitizing kinetics of a C-terminal truncated receptor could be transformed into one with slow-desensitizing kinetics following treatment with PKC activators [3]. This study also showed that mutations in the PKC consensus sequence exhibited rapid desensitization kinetics and that residue Thr<sup>18</sup> was phosphorylated in wild-type P2X<sub>2</sub>R [3].

Expression of P2X<sub>3</sub>R were first described in sensory neurons [4,5]. Inflammatory mediators, including the phospholipase C coupled ligands, substance P and bradykinin together with phorbol ester treatment to directly activate PKC have also been shown to augment P2X<sub>3</sub>R currents [6]. However, this particular study was unable to determine whether PKC-mediated phosphorylation occurred directly, presumably at the N-terminal PKC site, or alternatively that the effect was mediated

\* Corresponding author. Tel.: +1 585 275 6128; fax: +1 585 273 2652.

E-mail address: [David.Yule@urmc.rochester.edu](mailto:David.Yule@urmc.rochester.edu) (D.I. Yule).

via phosphorylation of an unknown accessory protein [6]. In fact, recent studies have even suggested the possibility of an external PKC site on the receptor [7,8]. Therefore the goal of this study was to determine specifically whether P2X<sub>3</sub>R are directly phosphorylated by PKC.

## 2. Materials and methods

### 2.1. Digital imaging of intracellular Ca<sup>2+</sup>

DT-40 3KO cells were used as a null background for P2X<sub>3</sub>R [9]. These cells lack all three (3KO) inositol 1,4,5-trisphosphate receptors (InsP<sub>3</sub>R) thus avoiding any possible Ca<sup>2+</sup> response as a consequence of P2Y<sub>R</sub> activation. DT-40 3KO cells were kindly provided by Dr. Kurosaki (Kansai Medical University, Japan) [10] and maintained as previously described [11–14]. DT-40 3KO cells provided a convenient system for digital Ca<sup>2+</sup> imaging studies since multiple cells from a single experimental run could be averaged. HEK-293 cells could not be used for Ca<sup>2+</sup> imaging studies since they express endogenous P2Y<sub>R</sub> that are activated by extracellular ATP [15]. DT-40 3KO cells were loaded with the Ca<sup>2+</sup> sensitive dye Fura-2 AM (2 μM, TEFLABS, Austin, TX) by incubation for 15 min at room temperature (RT). Subsequently, cells were removed from the Fura-2 AM containing solution, and resuspended in a physiological saline solution used for imaging experiments that contained (mM): 137 NaCl, 0.56 MgCl<sub>2</sub>, 4.7 KCl, 1 Na<sub>2</sub>HPO<sub>4</sub>, 10 HEPES, 5.5 glucose, 1.26 CaCl<sub>2</sub>, pH 7.4. Rapid solution changes were performed utilizing an electronic solenoid controlled perfusion system and gravity fed reservoirs (Warner Instruments, Hamden, CT). Imaging was performed using an inverted epifluorescence Nikon microscope with a 40× oil immersion objective lens (numerical aperture, 1.3). Fura-2 loaded cells were excited alternately with light at 340 and 380 nm using a monochromator-based illumination system and the emission at 510 nm captured using a high speed, digital CCD camera (TILL Photonics, Pleasanton, CA). The fluorescent ratio of 340 nm/380 nm was calculated and all data is presented as the change in ratio units. Images were acquired at a rate of 1 Hz with an exposure of 20 ms. All imaging experiments were performed at RT, essentially as previously described [16,17]. Traces are from a single cell, representative of multiple individual cells in a particular experimental run and *n* represents the number of experimental runs, with at least 3 cells per experimental run.

### 2.2. Transfection of DT-40 3KO cells

Human P2X<sub>3</sub>R cDNA, kindly provided by R.A. North (University of Manchester, UK), was transiently transfected into DT-40 3KO cells using a Nucleofector System (Amaxa, Gaithersburg, MD) following the instructions provided. Specifically, 5 × 10<sup>6</sup> cells were resuspended in 100 μL of Cell-line Nucleofector Kit T solution and were co-transfected with 5 μg of the P2X<sub>3</sub>R cDNA and 1 μg of pHcRed 1-N1 cDNA (red fluorescent protein for visualization of positively transfected cells) using Nucleofector program B-23. Immediately after transfection, 500 μL of media was added to the Nucleofector cuvette, followed by transfer of the cells to a single well of a 6 well culture plate which contained 1.5 mL of media. Experiments were performed 24 h after transfection.

### 2.3. Creation of P2X<sub>3</sub>R-EGFP construct

Human P2X<sub>3</sub>R cDNA was amplified by PCR. *Hind*III and *Sall* restriction sites were incorporated into the oligonucleotides used for PCR amplification. The PCR products were restriction enzyme-digested and ligated into pEGFP-N3 at the *Hind*III and *Sall* sites (BD Biosciences Clontech, San Jose, CA). This construct was verified by sequencing and creates a fusion protein with EGFP at the C-terminus of the human P2X<sub>3</sub>R.

### 2.4. Transfection of HEK-293 cells

Human P2X<sub>3</sub>R, human P2X<sub>3</sub>R-EGFP, rat P2X<sub>4</sub>R, rat P2X<sub>7</sub>R (rat P2X<sub>3</sub>R cDNA also kindly provided by R.A. North), human 3HA-M3R (obtained from the UMR cDNA resource center, available on the World Wide Web at [www.cdna.org](http://www.cdna.org)), or rat S1<sup>-</sup>/S2<sup>+</sup> InsP<sub>3</sub>R type I cDNA (kindly provided by Dr. S. Joseph,

Thomas Jefferson University) was transiently transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the instructions provided. Specifically, 5 × 10<sup>4</sup> cells were grown on 25 mm cover slips in 6 well culture plates and were co-transfected with 1 μg of the P2X<sub>3</sub>R cDNA and 100 ng of pHcRed 1-N1 cDNA as described previously [17].

### 2.5. Whole cell patch clamp recordings

ATP-activated cation currents were recorded at a sampling rate of 1 kHz using an Axopatch 200A patch clamp amplifier (Axon Instruments, Union City, CA), Axon digital interface, and pCLAMP version 9.0 software under whole cell patch clamp conditions. To measure ATP-activated currents in HEK-293 cells, cells were perfused with an extracellular solution containing (mM): 140 NaCl, 5 CsCl, 1.2 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 10 HEPES–CsOH, 10 D-glucose, pH 7.4. Internal patch solution contained (mM): 140 Cs-acetate, 1.22 MgCl<sub>2</sub>, 10 HEPES–CsOH, 0.1 EGTA, 10 NaCl, 0.0365 CaCl<sub>2</sub>, pH 7.2. Intervals of 2–3 min were allowed between patch rupture and stimuli to allow for equilibration with the patch pipette solution. HEK-293 cells were held at a holding potential of –30 mV. Experiments were performed at RT.

### 2.6. Immunoprecipitation, electrophoresis and immunoblotting

Immunoprecipitated samples were prepared from either mock-, InsP<sub>3</sub>R type I-, 3HA-M3R-, P2X<sub>3</sub>R-, or P2X<sub>3</sub>R-EGFP-transfected HEK-293 cells. The media was removed and cells were washed in ice-cold PBS, followed by resuspension in 400 μL of ice-cold RIPA (RadioImmunoPrecipitation Assay) lysis buffer that contained (mM): 50 NaF, 2 EDTA, 150 NaCl, 0.01 Na-Phosphate, 1% nonidet P-40 (NP-40), 1% Na-deoxycholate, 0.1% SDS, and 1 Complete EDTA-free protease inhibitor mixture tablet (Roche, Mannheim, Germany). Samples were left on ice with gentle agitation for 30 min to solubilize. Immunoprecipitating primary antibodies, polyclonal α-P2X<sub>3</sub>R from Alomone Labs (Jerusalem, Israel), polyclonal α-InsP<sub>3</sub>R type I from Calbiochem (San Diego, CA), monoclonal α-GFP from Roche (Mannheim, Germany), and monoclonal α-HA.11 from Covance (Princeton, NJ) were used at a 1:100 dilution and incubated for 2 h at 4 °C with rotation. Protein A or G sepharose (70 μL or 50 μL) was added to each sample and rotated at 4 °C for 1 h, then washed and centrifuged seven times, before final resuspension in 1× SDS loading buffer. Samples were resolved on 7.5% SDS-PAGE and transferred as described previously [17]. Polyclonal α-P2X<sub>3</sub>R (1:200 dilution) and α-InsP<sub>3</sub>R type I (1:750 dilution) primary antibodies (see above) were used following the manufacturer's instructions for immunoblotting. Monoclonal α-GFP (1:3000 dilution) and α-HA.11 (1:1000 dilution) primary antibodies (see above) were used following the manufacturer's instructions for immunoblotting. Proteins were visualized as previously described [17].

### 2.7. Metabolic labeling of HEK-293 cells

Mock-, InsP<sub>3</sub>R type I-, or P2X<sub>3</sub>R-transfected HEK-293 cells were metabolically labeled by incubating for 3 h with 150 μCi/mL <sup>32</sup>PO<sub>4</sub> (PerkinElmer, Boston, MA) in a phosphate-free DMEM (Invitrogen, Carlsbad, CA). Following incubation, cells were treated with or without 100 nM PMA for 10 min at RT. Cells were washed once in an ice cold TBS solution that contained (mM): 20 Tris, 138 NaCl, pH 7.6. Cells were then resuspended in 400 μL of ice-cold RIPA lysis buffer and immunoprecipitations were performed as mentioned above. Samples were resolved on 7.5% SDS-PAGE and then the gel was placed on a gel dryer for 1 h at 80 °C (Bio-Rad, Hercules, CA). The dried gel was then placed in a 20 × 25 cm phosphor screen (Amersham Biosciences, Piscataway, NJ) for 22–72 h before visualizing using a Molecular Dynamics Phosphor-Imager. Dried gels were then rehydrated for 30 min in SDS-PAGE running buffer that contained (mM): 25 mM Tris, 192 Glycine, 0.1% SDS, pH 8.3. Rehydrated gels were transferred to nitrocellulose and proteins were visualized as previously described in the immunoblotting section.

### 2.8. In vitro PKC phosphorylation assay

P2X<sub>3</sub>R, P2X<sub>3</sub>R-EGFP, 3HA-M3R, or InsP<sub>3</sub>R type I were immunoprecipitated (see above protocol) from mock-, P2X<sub>3</sub>R-, P2X<sub>3</sub>R-EGFP-, 3HA-M3R-, or

InsP<sub>3</sub>R type I transiently transfected HEK-293 cells and transferred from ice cold RIPA lysis buffer after three washes to PKC phosphorylation buffer containing 20 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, and Complete, EDTA-free protease inhibitor cocktail tablets (Roche, Mannheim, Germany), pH 7.5. Samples were briefly centrifuged and washed three times at 4 °C in PKC phosphorylation buffer. After the final wash, all remaining buffer was removed and samples were then resuspended in PKC phosphorylation buffer containing 20 μM ATP, 8 μL of [ $\gamma$ -<sup>32</sup>P]-ATP (40 μCi) (PerkinElmer, Boston, MA), and either 10 ng of a catalytically active PKC fragment from rat brain (Calbiochem, San Diego, CA), which does not require Ca<sup>2+</sup> or phosphatidylserine for its activity [18], were added or omitted as indicated (+ or - PKC). Final volume was 400 μL. Samples were mixed gently and incubated at 30 °C for 15 min. Reactions were quenched by adding 1.3 mL of ice-cold PKC phosphorylation buffer containing 1 mM ATP. Samples were briefly centrifuged at 4 °C and washed twice with 1.5 mL of phosphorylation buffer containing 1 mM ATP, finally resuspended in 1X SDS loading buffer. Samples were resolved on 7.5% SDS-PAGE and then the gel was dried. The dried gel was then placed in a 20×25 cm phosphor screen (Amersham Biosciences, Piscataway, NJ) for 60 h before visualizing using a Molecular Dynamics PhosphorImager. Dried gels were then rehydrated and proteins were visualized as described above.

### 2.9. Statistical analysis

Statistical significance was determined using either a paired or unpaired *t* test as indicated. Data from several cells in a particular experimental run were averaged, and experimental averages were used to calculate the mean±S.E. Two-tailed *p* values of less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Effects of PKC activation on ATP-evoked Ca<sup>2+</sup> signals in DT-40 3KO cells

DT-40 3KO cells are a useful screening tool for P2XR studies since activity from multiple cells can be recorded in a single experimental run and unlike most cells lines, including HEK-293 cells, they have no endogenous P2YR [9]. To verify that DT-40 3KO cells represent a null background for P2R, Ca<sup>2+</sup> signaling events were first evaluated in mock-transfected DT-40 3KO cells following ATP stimulation [9]. No Ca<sup>2+</sup> signals were evoked by 200–500 μM ATP or 100 nM PMA in mock-transfected cells (Fig. 1A). However, cyclo-piazonic acid (CPA) treatment, to inhibit the sarco-endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) and promote Ca<sup>2+</sup> leak from intracellular stores, confirmed the viability of the measurement (Fig. 1A). In P2X<sub>3</sub>R-transfected DT-40 3KO cells, stimulation with 200 μM ATP caused a small reproducible Ca<sup>2+</sup> transient (Fig. 1B, P2X<sub>3</sub>R control; second response 85±16% of initial stimulation, *n*=6). Importantly, after treatment of P2X<sub>3</sub>R-expressing cells with 100 nM PMA for 4–5 min, subsequent reapplication of 200 μM ATP caused a significant ~2.5-fold enhancement of the Ca<sup>2+</sup> signal over control values (Fig. 1C, P2X<sub>3</sub>R PMA treatment; second response 268±42% of initial stimulation, *n*=12, *p*=0.008). Note treatment with CPA resulted in a similar Ca<sup>2+</sup> response in either mock- or P2X<sub>3</sub>R-transfected DT-40 3KO cells. When desensitization of the second ATP-induced Ca<sup>2+</sup> signal in the absence of PMA was considered, this potentiation reached ~3.2-fold (Control; second response 85±16% of initial stimulation *versus* PMA treatment; second response 268±42% of initial stimulation). These data are summarized in Fig. 1D.

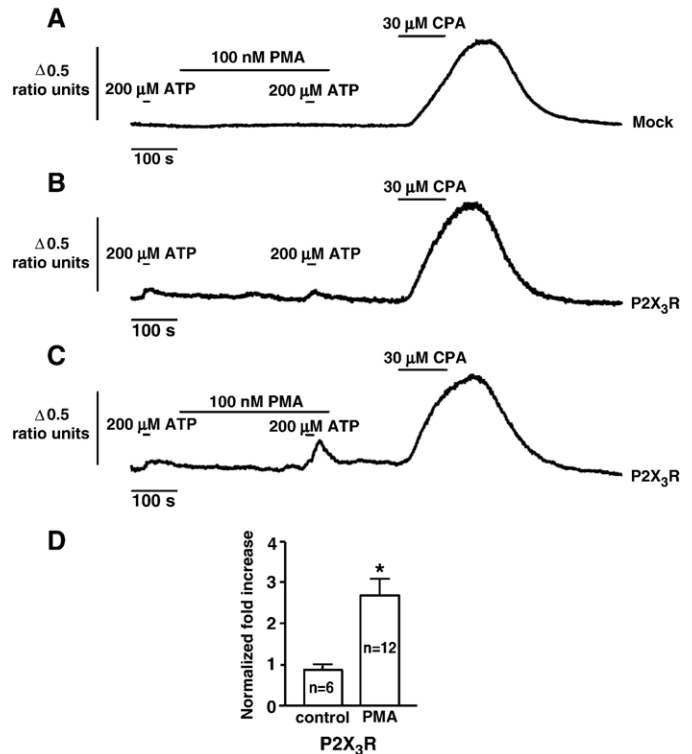


Fig. 1. PMA-induced potentiation of ATP-activated P2X<sub>3</sub>R-mediated Ca<sup>2+</sup> signals in DT-40 3KO cells. (A) Mock-transfected DT-40 3KO cells do not respond to 200 μM ATP in the absence or following 100 nM PMA treatment. Cells were confirmed to be responsive since 30 μM CPA caused Ca<sup>2+</sup> release from intracellular stores. (B) P2X<sub>3</sub>R-transfected DT-40 3KO cells elicit a small, reproducible Ca<sup>2+</sup> response upon 200 μM ATP stimulation. (C) Treatment with 100 nM PMA of P2X<sub>3</sub>R-transfected DT-40 3KO cells resulted in a ~2.5-fold enhanced Ca<sup>2+</sup> signal. Note CPA treatment resulted in a Ca<sup>2+</sup> response in P2X<sub>3</sub>R-transfected DT-40 3KO cells similar to mock-transfected cells. (D) Data from paired experiments, where the maximum Ca<sup>2+</sup> response over baseline of the second application of ATP was normalized relative to the first application of ATP either in the presence or absence of PMA. The data are presented as the mean±S.E. (\**p*<0.05). Each trace is representative of three or more experiments.

### 3.2. PMA significantly enhances P2X<sub>3</sub>R current

Quantifying the magnitude of the PMA-induced enhancement on P2X<sub>3</sub>R-mediated Ca<sup>2+</sup> signaling was complicated by the initial small responses observed in DT-40 3KO cells. One possible reason for the small Ca<sup>2+</sup> response is that these indirect measurements of channel activity rely on a spatially averaged global signal from the Ca<sup>2+</sup> indicator, which might underestimate the signal if locally defined. In addition, the fact that the receptor desensitizes very rapidly may compound this problem. As a direct measurement of channel activity, we therefore used an electrophysiological approach to study the regulation of P2X<sub>3</sub>R in isolation following expression in P2XR-null HEK-293 cells [1,19–24].

Fig. 2 shows membrane currents recorded in the whole cell configuration of the patch clamp technique in response to extracellular ATP stimulated at a holding potential of -30 mV. The pipette solution and holding potential were chosen to isolate inwardly directed cation currents, predominately carried by Na<sup>+</sup>, as we have reported previously [17]. No inward currents were

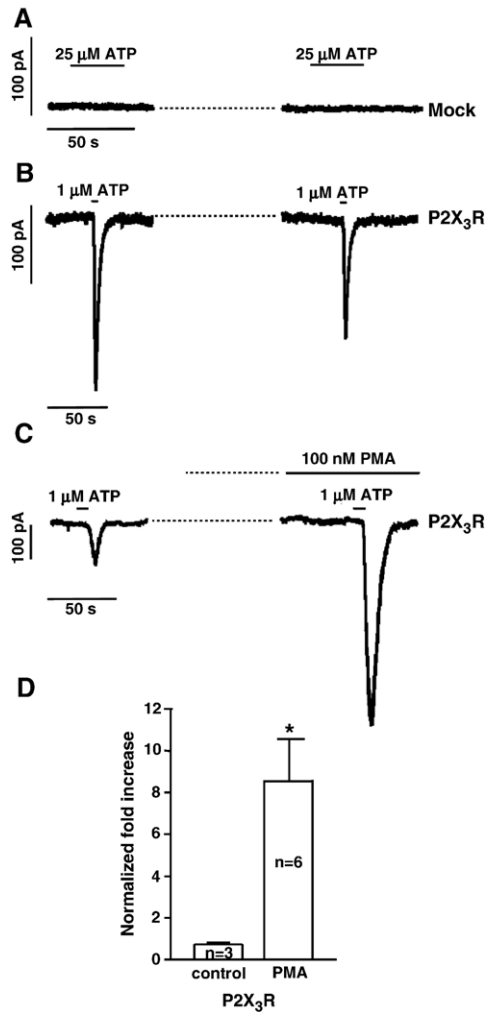


Fig. 2. PKC activation enhances ATP-activated current through P2X<sub>3</sub>R in HEK-293 cells. Mock- or P2X<sub>3</sub>R-transfected HEK-293 cells were whole cell patch clamped at a holding potential of  $-30$  mV. (A) Mock-transfected HEK-293 cells did not elicit any current upon ATP stimulation. (B) P2X<sub>3</sub>R-transfected HEK-293 cells elicit current when treated with  $1 \mu\text{M}$  ATP. (C) PMA treatment of P2X<sub>3</sub>R-transfected HEK-293 cells resulted in a  $\sim 8.5$ -fold enhanced inward current when cells were re-exposed to ATP (control  $72 \pm 7\%$  of initial response versus PMA  $857 \pm 199\%$  of initial response,  $n=3$  and  $n=6$ , respectively,  $p=0.03$ ). (D) Whole cell patch clamp data from paired experiments, where the baseline subtracted maximum inward current of the second application of ATP was normalized relative to the first application of ATP either in the presence or absence of PMA. The data are presented as the mean  $\pm$  S.E. ( $*p < 0.05$ ). Each trace is representative of three or more experiments.

evoked by  $25 \mu\text{M}$  ATP in mock-transfected cells (Fig. 2A). Stimulation of P2X<sub>3</sub>R-transfected cells with  $1 \mu\text{M}$  ATP resulted in an inward current, repeated stimulation after 4–5 min revealed a measurable desensitization of the P2X<sub>3</sub>R activity (Fig. 2B, P2X<sub>3</sub>R control; second response  $72 \pm 7\%$  of initial stimulation,  $n=3$ ). In spite of this desensitization, treatment with PMA (4 min) in P2X<sub>3</sub>R expressing cells significantly enhanced the maximum amplitude of the inward current  $\sim 8.5$ -fold (Fig. 2C, P2X<sub>3</sub>R PMA treatment; second response  $857 \pm 199\%$  of initial stimulation,  $n=6$ ,  $p=0.03$ ). When desensitization of the second ATP response in the absence of PMA was considered, this

potentiation reached almost 12-fold (Control; second response  $72 \pm 7\%$  of initial stimulation versus PMA treatment; second response  $857 \pm 199\%$  of initial stimulation). These data are summarized in Fig. 2D.

### 3.3. PMA has no effect on P2X<sub>4</sub>R or P2X<sub>7</sub>R current

One possible mechanism underlying this observation is a phosphorylation event occurring on the PKC consensus site in the N-terminal domain, conserved among all P2XR family members. Table 1 shows the amino acid alignment of the N-terminal regions of all seven rat P2XR [1]. It should be noted that human P2X<sub>3</sub>R is identical to rat P2X<sub>3</sub>R in this region. The underlined residues represent the PKC consensus site, where the highlighted Thr (T) is presumably the putative phosphorylation site [1]. Given the similarities in this region among P2XR, we next determined if treatment with PMA would also enhance signaling through both P2X<sub>4</sub>R and P2X<sub>7</sub>R.

Stimulation of P2X<sub>4</sub>R-transfected HEK-293 cells with  $25 \mu\text{M}$  ATP elicited a robust inward current, which was unaffected by PMA treatment (Fig. 3A, P2X<sub>4</sub>R PMA treatment; second response  $74 \pm 2\%$  of initial stimulation,  $n=4$ ). Stimulation of P2X<sub>7</sub>R-transfected HEK-293 cells with  $100 \mu\text{M}$  ATP evoked inward currents of similar magnitude to those stimulated by  $25 \mu\text{M}$  ATP in P2X<sub>4</sub>R-transfected HEK-293, consistent with reported EC<sub>50</sub> values for each receptor (Fig. 3B) [25]. Similarly, PMA treatment did not significantly enhance the current (Fig. 3B, P2X<sub>7</sub>R PMA treatment; second response  $72 \pm 7\%$  of initial stimulation,  $n=10$ ). Currents through both P2X<sub>4</sub>R and P2X<sub>7</sub>R in the presence of PMA were similar to control values that we have previously reported under identical conditions [17]. These data are summarized in Fig. 3C.

### 3.4. Mechanism of PMA enhancing P2X<sub>3</sub>R channel activity

Our results have shown that following PKC activation, P2X<sub>3</sub>R channel activity is significantly augmented in two different cell lines following transient expression of the receptor. The simplest explanation of the underlying mechanism for this enhancement would be a PKC-mediated phosphorylation event occurring at the N-terminal region of the receptor. Contrary to this idea, it should be noted that PMA treatment can enhance P2X<sub>3</sub>R, but not P2X<sub>4</sub>R or P2X<sub>7</sub>R inward currents, yet all three receptors share a similar N-terminal PKC phosphorylation site. These findings might suggest that the conserved N-terminal PKC site might not be a universal substrate for phosphorylation/regulation by PKC. To determine if receptor

Table 1  
N-terminus alignment of P2XR amino acid sequences

P2X <sub>1</sub>	MARRLQDELSA-FFFEYD <u>TPR</u>
P2X <sub>2</sub>	MVRRRLARGCWS-AFWDY <u>TPK</u>
P2X <sub>3</sub>	-----MNCIS-DFFTY <u>ETK</u>
P2X <sub>4</sub>	MAGCCSVLG-S-FLFEYD <u>TPR</u>
P2X <sub>5</sub>	MGQAAWKGFV-LSLFDYK <u>TKA</u>
P2X <sub>6</sub>	MASAVAAALVSWGFLDYK <u>TEK</u>
P2X <sub>7</sub>	MPACCSWN----DVFOY <u>ETNK</u>

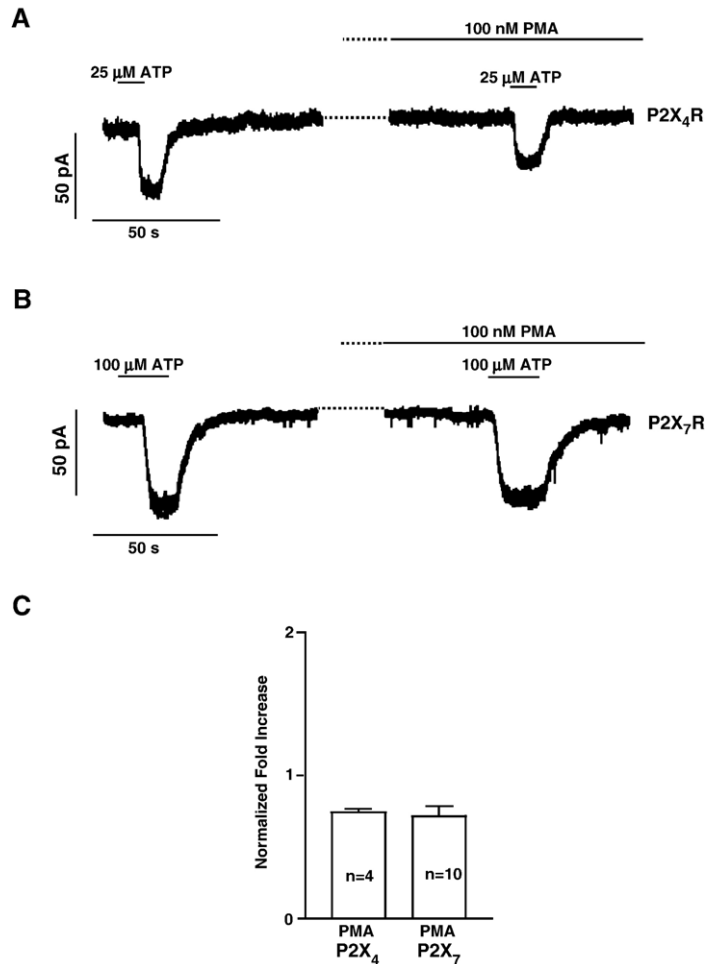


Fig. 3. PKC activation does not enhance ATP-activated current through either P2X<sub>4</sub>R or P2X<sub>7</sub>R in HEK-293 cells. P2X<sub>4</sub>R- or P2X<sub>7</sub>R-transfected HEK-293 cells were whole cell patch clamped at a holding potential of  $-30$  mV. (A) Treatment of P2X<sub>4</sub>R-transfected HEK-293 cells with  $25$   $\mu$ M ATP resulted in an inward current, which was not enhanced by PMA treatment. (B) Treatment of P2X<sub>7</sub>R-transfected HEK-293 with  $100$   $\mu$ M ATP also resulted in an inward current, however again PMA treatment did not enhance the inward current. (C) Whole cell patch clamp data from paired experiments, where the baseline subtracted maximum inward current of the second application of ATP in the presence of PMA was normalized relative to the first application of ATP in the absence of PMA. PMA did not enhance the inward current in HEK-293 cells transiently expressing either P2X<sub>4</sub>R or P2X<sub>7</sub>R (P2X<sub>4</sub>R, PMA  $74 \pm 2\%$  of initial response; P2X<sub>7</sub>R, PMA  $72 \pm 7\%$  of initial response,  $n=4$  and  $n=10$ , respectively). The data are presented as the mean  $\pm$  S.E. ( $*p < 0.05$ ). Each trace is representative of three or more experiments.

phosphorylation was occurring on the P2X<sub>3</sub>R directly, we performed both intact cell and in vitro phosphorylation experiments after immunoprecipitation with a P2X<sub>3</sub>R antibody.

Immunoprecipitation followed by immunoblotting with a P2X<sub>3</sub>R antibody was performed to verify purification of the receptor. In lysates from P2X<sub>3</sub>R-transfected HEK-293 cells, a single major band was identified by a P2X<sub>3</sub>R-specific antibody, which was absent in mock-transfected cells (Fig. 4A). The size of the immunoreactive band was larger than the predicted size ( $44$  kDa in human), as reported previously [26,27]. Where indicated, the lower band marked (\*) near the  $50$  kDa marker in samples depicts the band corresponding to the heavy chain of the antibody used for immunoprecipitation.

As a positive control for PKC-mediated phosphorylation, immunoprecipitation followed by immunoblotting with a InsP<sub>3</sub>R type I antibody was also performed. This control was chosen because this ligand-gated ion channel is also involved in  $\text{Ca}^{2+}$  signaling and has been shown to be a

substrate for PKC [28,29]. Lysates from InsP<sub>3</sub>R type I-transfected HEK-293 cells revealed a single distinct band of the predicted size ( $313$  kDa), which was not evident in mock-transfected cells (Fig. 4B).

To determine if activation of endogenous PKC results in P2X<sub>3</sub>R phosphorylation, we first metabolically labeled mock-, InsP<sub>3</sub>R type I-, or P2X<sub>3</sub>R-transfected cells with  $^{32}\text{PO}_4^-$ . Subsequently, protein that had incorporated  $^{32}\text{PO}_4^-$  was detected by autoradiography. Following treatment with PMA and immunoprecipitation with either InsP<sub>3</sub>R type I or P2X<sub>3</sub>R antibodies, there was an enhanced labeling of a single band in InsP<sub>3</sub>R type I-transfected samples compared to control (Fig. 5A). This demonstrates that InsP<sub>3</sub>R (type I) are directly phosphorylated, presumably by a PKC-mediated process. In contrast, under identical conditions, no detectable signal was observed at the appropriate molecular weight for P2X<sub>3</sub>R (Fig. 5C). All mock-, InsP<sub>3</sub>R type I-, or P2X<sub>3</sub>R-transfected samples shown were run on the same gel, thus we next

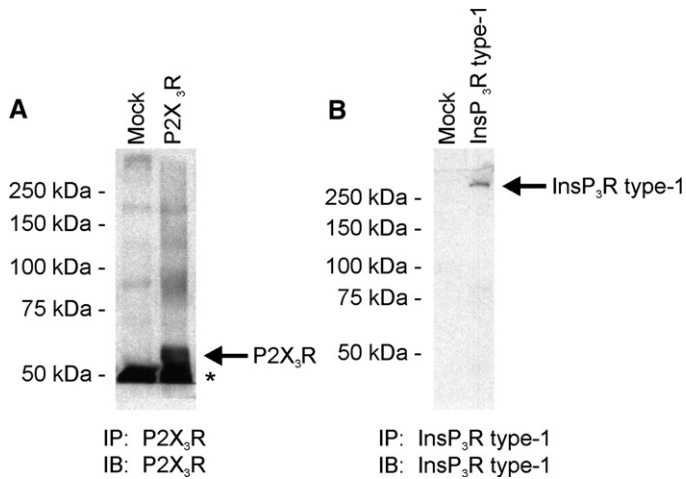


Fig. 4. Immunoprecipitation and immunoblotting using receptor specific antibodies for P2X<sub>3</sub>R and InsP<sub>3</sub>R type I. Mock-, InsP<sub>3</sub>R type 1-, or P2X<sub>3</sub>R-transfected HEK-293 cells were utilized for immunoprecipitation experiments. (A) A commercially available P2X<sub>3</sub>R-specific antibody was used for both immunoprecipitation and immunoblotting, which recognizes a single distinct band in P2X<sub>3</sub>R-transfected HEK-293 cells, however, no band was detected in mock-transfected cells. The lower band located near the 50 kDa marker in both samples is the heavy chain from the antibody used for immunoprecipitation (note \*), which was not present in control experiments where the antibody used for immunoprecipitation was absent (data not shown). (B) A commercially available InsP<sub>3</sub>R type I antibody was used for both immunoprecipitation and immunoblotting, which recognizes a single distinct band in InsP<sub>3</sub>R type I-transfected HEK-293 cells, however, no band was detected in mock-transfected cells.

confirmed expression of transfected protein by rehydrating the gel, transferring to nitrocellulose, and immunoblotting with either InsP<sub>3</sub>R type I or P2X<sub>3</sub>R antibodies. Immunoblotting with a InsP<sub>3</sub>R type I antibody showed that both InsP<sub>3</sub>R type I-transfected samples contained similar amounts of protein and confirmed the identity based on the size of the phosphorylated protein detected in Fig. 5A (Fig. 5B). Immunoblotting with a P2X<sub>3</sub>R antibody demonstrated similar expression of P2X<sub>3</sub>R in P2X<sub>3</sub>R-transfected samples, that was absent in mock-transfected samples (Fig. 5D). These data confirm that the P2X<sub>3</sub>R was present in Fig. 5C. It is formally possible, however, that low level phosphorylation of the P2X<sub>3</sub>R might be below the sensitivity of this detection system. In addition, it is possible that 100 nM PMA stimulation is not sufficient to promote phosphorylation of the receptor, even though functional effects are readily evident. To address these issues, we next attempted to directly phosphorylate the P2X<sub>3</sub>R directly using active catalytic PKC subunits in vitro.

To determine whether the P2X<sub>3</sub>R can be directly phosphorylated by PKC, the receptor was purified by immunoprecipitation, followed by treatment with a catalytically active PKC fragment from rat brain and [ $\gamma$ -<sup>32</sup>P]-ATP in vitro. This PKC fragment has been shown previously to phosphorylate PKC substrates such as the  $\delta$ 2 glutamate receptor [30]. Again, as a positive control, InsP<sub>3</sub>R type I-transfected HEK-293 cells were included. After a 15 min incubation there was no detectable incorporation of <sup>32</sup>P in either mock or P2X<sub>3</sub>R

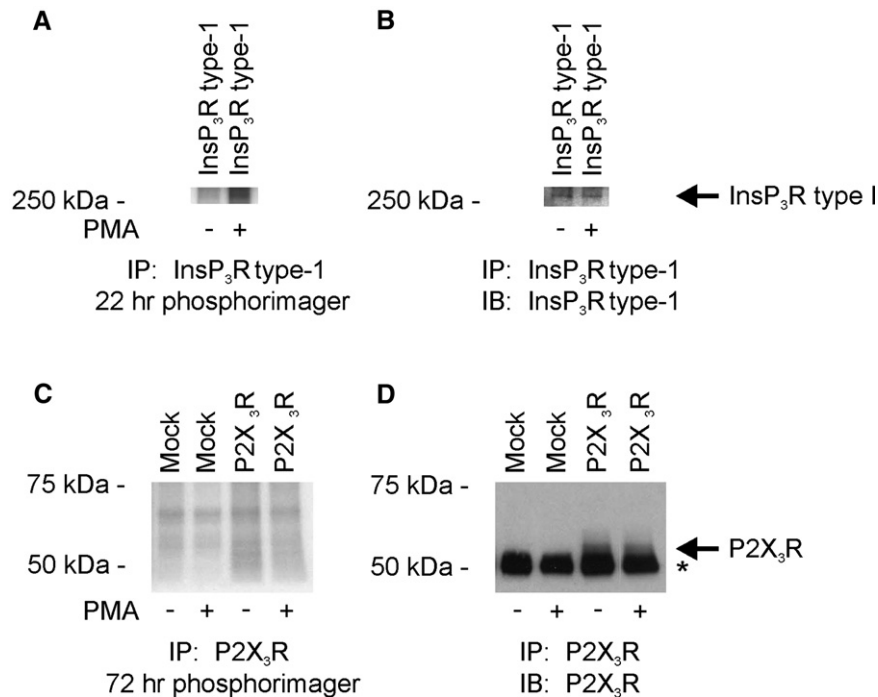


Fig. 5. PMA treatment phosphorylates InsP<sub>3</sub>R type I, but does not phosphorylate P2X<sub>3</sub>R in intact cells. Mock-, InsP<sub>3</sub>R type 1-, or P2X<sub>3</sub>R-transfected HEK-293 cells were metabolically labeled with <sup>32</sup>PO<sub>4</sub><sup>-</sup> and incubated with (+) or without (-) 100 nM PMA for 10 min and then utilized for immunoprecipitation experiments. (A) An increase in <sup>32</sup>P incorporation was observed in InsP<sub>3</sub>R type 1-expressing cells after PMA treatment (Lane 2, +) compared to control treatment (Lane 1, -). (B) Immunoblot of rehydrated gel shown in Panel A, using a InsP<sub>3</sub>R type I antibody, confirms similar expression levels in both samples and confirms protein size (see arrow). (C) There was no <sup>32</sup>P incorporation observed in either mock- or P2X<sub>3</sub>R-transfected lanes treated with PMA compared to control. (D) Immunoblot of rehydrated gel shown in Panel C, using a P2X<sub>3</sub>R antibody, confirming immunoprecipitation of the P2X<sub>3</sub>R (see arrow) from P2X<sub>3</sub>R transfected HEK-293 cells, that was not present in mock-transfected cells (\*Indicates heavy chain). All blots were repeated with similar results.

immunoprecipitated samples between the 50 and 75 kDa markers, even after substantial overexposure (Fig. 6A). Following rehydration and transferring this gel to nitrocellulose, immunoblotting with a P2X<sub>3</sub>R antibody confirmed the presence of the P2X<sub>3</sub>R protein in P2X<sub>3</sub>R- but not mock-transfected cells (Fig. 6B, bottom). InsP<sub>3</sub>R type I-transfected cells showed an increase in <sup>32</sup>P incorporation after 15 min of PKC and [γ-<sup>32</sup>P]-ATP treatment at the correct molecular weight of the receptor. The presence of the InsP<sub>3</sub>R type I was confirmed after rehydrating the gel, transferring to nitrocellulose, and immunoblotting these samples with a InsP<sub>3</sub>R type I antibody (Fig. 6B, top). This increase in <sup>32</sup>P incorporation was not present in the lane lacking PKC addition. These results demonstrate that P2X<sub>3</sub>R do not appear to be a substrate for PKC-mediated phosphorylation.

A possibility exists that the P2X<sub>3</sub>R was not being efficiently immunoprecipitated from our samples, since immunoblotting for P2X<sub>3</sub>R only resulted in a modest signal. To address this issue we generated a C-terminal EGFP tagged human P2X<sub>3</sub>R. This construct was confirmed to be functional by electrophysiological recordings and retained enhancement by PMA treatment (data not shown). Epitope tagging this receptor with EGFP has two distinct advantages; it allows for better resolution from the heavy chain of the antibody used for immunoprecipitation. Secondly, it also allows us to utilize an α-GFP antibody for a more efficient immunoprecipitation. To determine whether the P2X<sub>3</sub>R-EGFP can be directly phosphorylated by PKC, the receptor was purified by immunoprecipitation, followed by treatment with a catalytically active PKC fragment from rat brain and [γ-<sup>32</sup>P]-ATP *in vitro*. In addition, InsP<sub>3</sub>R type I- and 3X HA-tagged human M3R- (3HA-M3R) transfected HEK-293 cells were included as positive controls. Human M3R has been utilized previously as a positive control for P2X<sub>1</sub>R phosphorylation [31], and it is similar in size to P2X<sub>3</sub>R-EGFP.

After a 15 min incubation there was no detectable incorporation of <sup>32</sup>P in either mock or P2X<sub>3</sub>R-EGFP immunoprecipitated samples between the 75 and 100 kDa markers, even after substantial overexposure (Figs. 7A and B). Following rehydration and transferring this gel to nitrocellulose, immunoblotting with an α-GFP antibody confirmed the presence of the P2X<sub>3</sub>R-EGFP protein in P2X<sub>3</sub>R-EGFP- but not mock-transfected cells (Fig. 7C, right). 3HA-M3R- and InsP<sub>3</sub>R type I-transfected cells showed an increase in <sup>32</sup>P incorporation after 15 min of PKC and [γ-<sup>32</sup>P]-ATP treatment at the correct molecular weight of each receptor (Figs. 7A and B). The human M3 receptor has been shown previously to migrate as a diffuse band running at approximately 97–110 kDa due to post-translational modification of the receptor [32]. The presence of both receptors was confirmed after rehydrating the gel, transferring to nitrocellulose, and immunoblotting these samples with either an α-HA or α-InsP<sub>3</sub>R type I antibody (Fig. 7C, left and center). This increase in <sup>32</sup>P incorporation was not present in the lanes lacking PKC treatment. These results demonstrate that P2X<sub>3</sub>R-EGFP do not appear to be a substrate for PKC-mediated phosphorylation. This additional information reinforces the notion that the regulation of P2X<sub>3</sub>R by PKC is likely indirect and thus presumably involves the phosphorylation of an unknown accessory protein.

#### 4. Discussion

The present study demonstrates that PKC activation can significantly enhance both the Ca<sup>2+</sup> signal as well as the cation current through P2X<sub>3</sub>R in different cell lines. More importantly, this is the first study that has specifically addressed whether the P2X<sub>3</sub>R is directly phosphorylated after PKC activation. Utilizing two different techniques, activation of PKC failed to increase the phosphorylation of the P2X<sub>3</sub>R.

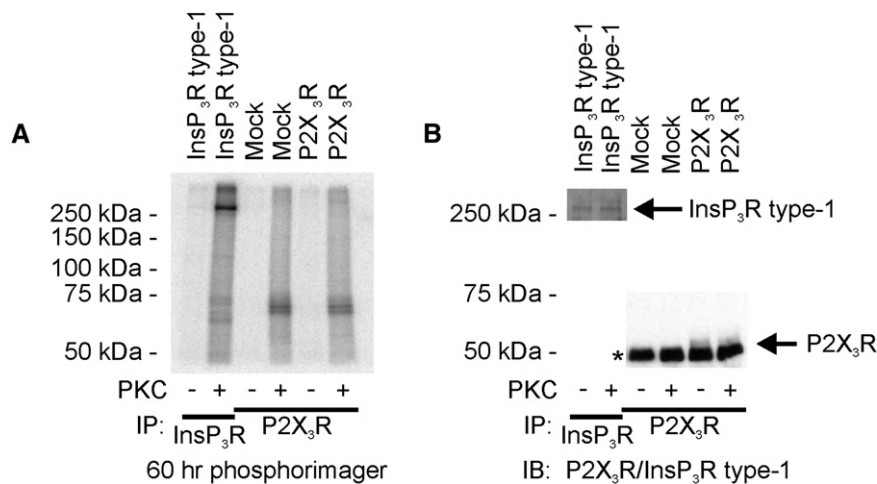


Fig. 6. PKC activation phosphorylates InsP<sub>3</sub>R type I *in vitro*, but does not phosphorylate P2X<sub>3</sub>R. Mock-, InsP<sub>3</sub>R type I-, or P2X<sub>3</sub>R-transfected HEK-293 cells were utilized for immunoprecipitation experiments. (A) Immunoprecipitated samples were incubated with (+) or without (-) purified PKC catalytic subunits for 15 min in the presence of [γ-<sup>32</sup>P]-ATP *in vitro*. <sup>32</sup>P incorporation was observed in InsP<sub>3</sub>R type I-expressing cells when PKC was present (Lane 2), however, there was no detectable <sup>32</sup>P incorporation in the region of the P2X<sub>3</sub>R for either mock- or P2X<sub>3</sub>R-transfected HEK-293 cells when PKC was present (Lane 4 and Lane 6). (B) Immunoblots of the rehydrated gel shown in Panel A, using a InsP<sub>3</sub>R type I antibody (top), or a P2X<sub>3</sub>R antibody (bottom), confirms similar expression levels in both InsP<sub>3</sub>R type I- and P2X<sub>3</sub>R-transfected samples and confirms protein size (see arrows, \*Indicates heavy chain). All blots were repeated with similar results.

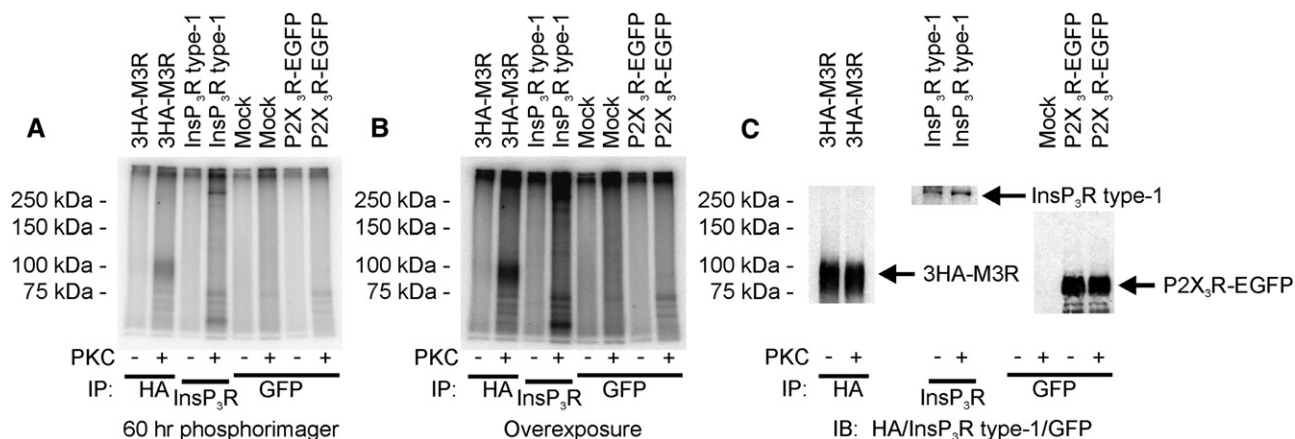


Fig. 7. PKC activation phosphorylates InsP<sub>3</sub>R type I and 3HA-M3R *in vitro*, but does not phosphorylate P2X<sub>3</sub>R-EGFP. Mock-, InsP<sub>3</sub>R type 1-, 3HA-M3R-, P2X<sub>3</sub>R-EGFP-transfected HEK-293 cells were utilized for immunoprecipitation experiments. (A) Immunoprecipitated samples were incubated with (+) or without (–) purified PKC catalytic subunits for 15 min in the presence of [ $\gamma$ -<sup>32</sup>P]-ATP *in vitro*. <sup>32</sup>P incorporation was observed in 3HA-M3R- and InsP<sub>3</sub>R type 1-expressing cells when PKC was present (Lanes 2 and 4), however, there was no detectable <sup>32</sup>P incorporation in the region of the P2X<sub>3</sub>R-EGFP for either mock- or P2X<sub>3</sub>R-EGFP-transfected HEK-293 cells when PKC was present (Lane 6 and Lane 8) even after a longer exposure (B). (C) Immunoblots of the rehydrated gel shown in (A and B), using a HA antibody (left), a InsP<sub>3</sub>R type I antibody (center), or a GFP antibody (right), confirms similar expression levels in 3HA-M3R-, InsP<sub>3</sub>R type 1-, and P2X<sub>3</sub>R-EGFP-transfected samples and confirms protein size (see arrows). All blots were repeated with similar results.

There is no consensus in the literature regarding the regulation of P2XR by PKC. It was first reported that phorbol ester treatment caused a mutant P2X<sub>2</sub>R with truncated C-terminus to convert from one exhibiting rapid desensitizing currents to slow desensitizing currents [3]. In addition, P2X<sub>2</sub>R were shown to be phosphorylated at Thr<sup>18</sup> using an antibody that recognizes a phosphothreonine–proline motif, whereas the P2X<sub>2</sub>R mutant K20T was not recognized [3]. These authors showed that P2X<sub>2</sub>R are constitutively phosphorylated, thus giving these receptors their characteristic slow rate of desensitization; however they did not demonstrate whether the truncated C-terminus mutant P2X<sub>2</sub>R was phosphorylated at this N-terminal site following phorbol ester treatment. It should be noted that the particular antibody used to detect PKC-mediated phosphorylation would not be of use in the present study because it specifically recognizes a motif absent in P2X<sub>3</sub>R.

Ennion and Evans demonstrated that disruption of the N-terminal PKC site in P2X<sub>1</sub>R (T18A) caused reduced peak current amplitude as well as rapid desensitization kinetics [33]. The authors speculated that PKC-mediated phosphorylation/dephosphorylation of P2X<sub>1</sub>R was a mechanism for regulating channel function [33]. In a further study, the P2X<sub>1</sub>R mutants T18A, T18N, P19V, and R20T were made and the authors found that all mutations except P19V significantly reduced the current [34]. They also indicated that the wild-type P2X<sub>1</sub>R was phosphorylated, whereas the mutant R20T was not, after immunoblotting with an antibody that recognizes a phosphothreonine–proline motif [34]. However, Evans and colleagues later showed that activation of PKC, mediated either by GPCR stimulation (metabotropic glutamate receptor 1 $\alpha$ , P2Y<sub>1</sub>R, or P2Y<sub>2</sub>R) or PMA, still enhanced current through point mutants with the N-terminal PKC site disrupted (R20I, R20A, and T18V). These data suggested PKC activation does not involve P2X<sub>1</sub>R phosphorylation in this region [31]. This prediction was confirmed after radiolabeling phosphorylated proteins in the presence or absence of PMA, where P2X<sub>1</sub>R were basally

phosphorylated and no enhanced phosphorylation was observed after PMA treatment. These data in total suggest that the mechanism of PKC-mediated P2X<sub>1</sub>R regulation likely involves the phosphorylation of an accessory protein [31].

P2X<sub>3</sub>R had been shown to be positively enhanced by G<sub>q</sub>-coupled inflammatory mediators such as substance P and bradykinin. These effects could be mimicked by phorbol ester treatment and blocked by inhibitors of protein kinases [6]. The most plausible explanation for this enhancement was either an N-terminal PKC-mediated phosphorylation or an unidentified protein that was phosphorylated and controls activity of P2X receptors [6]. However, there have been recent reports that P2X<sub>3</sub>R are regulated by ecto-PKC activity [7]. P2X<sub>3</sub>R have seven PKC consensus sequences, including the conserved N-terminal intracellular PKC site as well as a C-terminal intracellular PKC site. Mutation of the C-terminal intracellular PKC site did not inhibit PKC-mediated P2X<sub>3</sub>R potentiation [6]. However, P2X<sub>3</sub>R also have five external PKC consensus sequences. The authors argued that since PKC activators such as PMA and DAG-lactone can transverse the cell membrane, that it gives no specificity as to the intracellular or extracellular location of the phosphorylation site. External PKC site mutants T134A and S178A both abolished the UTP-induced potentiation of the current through P2X<sub>3</sub>R, yet mutants T196A and S269A had no effect [7]. Interestingly, activity of the internal PKC site mutant T13A, was not enhanced by UTP in this study, although a previous report has shown that this same mutation, P2X<sub>3</sub>T13A was still enhanced by PKC activation [6]. The substitution of the same four external PKC sites to the negatively charged Asp residue (T134D, S178D, T196D, and S269D), to mimic phosphorylation, all prevented the potentiation by UTP [8]. To summarize, adding a negative charge to T196 and S269, blocked potentiation by UTP, but the alanine mutants (T196A and S269A) were still enhanced by UTP, which renders the



importance of these PKC sites unclear [8]. Regardless, the substrate of the putative ecto-protein kinase is not resolved; in particular, it remains to be determined whether phosphorylation occurs at extracellular receptor sites or an accessory membrane protein(s) [8].

The present study reports a previously unreported finding; namely that PKC-mediated enhancement of P2X<sub>3</sub>R channel activity does not seem to involve direct channel phosphorylation. These results suggest that the PMA-induced potentiation of P2X<sub>3</sub>R signaling involves an unknown accessory protein. In support of this contention, we also have shown that two other P2XR family members, which also possess N-terminal PKC sites, are not regulated by PKC activation, suggesting these N-terminal PKC sites are not important for PKC-mediated regulation. While it is possible that PKC-mediated phosphorylation of P2X<sub>3</sub>R or P2X<sub>3</sub>R-EGFP was below the level of detection in both our intact cell and in vitro systems, this seems unlikely as the InsP<sub>3</sub>R type I and 3HA-M3R, included as a positive controls were phosphorylated after phorbol ester treatment or after incubation with active PKC catalytic subunits as previously reported [28,29,31]. Furthermore, P2X<sub>1</sub>R are positively modulated by PKC activation, however, it does not involve direct phosphorylation of the receptor [31]. It is interesting to note that these two P2X receptors belong to the same sub-group of P2XR based on their rapid desensitization properties and high sensitivity to ATP and selective agonist  $\alpha$ ,  $\beta$ -methylene ATP [3,35]. In support of our findings, Vial and Evans were unable to successfully measure phosphorylation of the P2X<sub>3</sub>R. However, in this case they attributed this finding to insufficient expression levels of P2X<sub>3</sub>R achieved in HEK-293 cells in their experiments [7]. In our transiently overexpressing HEK-293 cell culture system the expression of the human P2X<sub>3</sub>R or P2X<sub>3</sub>R-EGFP was unlikely to be an issue, since the receptor was readily detected by immunoblotting using either a P2X<sub>3</sub>R- or GFP-specific antibody (Figs. 4A and 7C).

One other possibility explaining why PKC-mediated phosphorylation was not detected could be that the number of PKC sites was significantly different in the InsP<sub>3</sub>R type I and 3HA-M3R when compared to the P2X<sub>3</sub>R-EGFP. After PKC-mediated phosphorylation followed by thermolysin digestion and two-dimensional phosphopeptide analysis, the InsP<sub>3</sub>R type I has been shown to produce one major phosphopeptide and two minor phosphopeptides [28], suggesting that the InsP<sub>3</sub>R type I has potentially 3 different PKC sites. The number and location of the active PKC phosphorylation sites in the human M3R has not been well defined, however, the number of active PKC sites in human M1R has been determined to be 2–3 [36]. This number of sites seem reasonable for comparison to potential P2X<sub>3</sub>R-EGFP PKC-mediated phosphorylation. In addition both proteins are similar in size (Fig. 7C). Furthermore, a search of the human 3HA-M3R protein shows it contains 17 PKC consensus sequences compared to 9 PKC consensus sequences in the human P2X<sub>3</sub>R-EGFP using the search criteria of [ST]X[RK] and including all regions of the receptor. If the assumption is made that the stoichiometry of phosphorylation is similar, one would expect to measure approximately half the amount of phosphorylation in the P2X<sub>3</sub>R-EGFP compared to the

3HA-M3R. Thus, it is reasonable to suggest, that since we can robustly detect 3HA-M3R phosphorylation (Fig. 7A and B), that P2X<sub>3</sub>R-EGFP phosphorylation should also be evident.

There is an emerging notion that P2XR activities are modulated by a range of protein kinases [17,37]. Furthermore, it appears that protein kinases can selectively modulate signaling through different P2XR. Recent studies from our laboratory have revealed that mechanisms that increase cellular adenosine 3',5'-cyclic monophosphate (cAMP), activating PKA, can significantly enhance both the Ca<sup>2+</sup> signal and the cation current through P2X<sub>4</sub>R, however, raising cAMP has no effect on P2X<sub>7</sub>R signaling [17]. It remains to be determined if this regulation involves direct receptor phosphorylation or not. Additionally, P2X<sub>2</sub>R have also been shown to be regulated by PKA [37].

Given the preponderance of evidence, our results pose a novel and interesting question, what is the identity of the unknown accessory protein that modulates P2X<sub>3</sub>R? It is intriguing that this protein is conserved in at least two different cell lines of chicken and human origin (DT-40 and HEK-293) and future studies should be directed to ascertain the identity of this seemingly ubiquitous accessory protein and also to address whether it can associate and regulate other P2XR. The current study helps support the notion that accessory proteins play a significant role in both the regulation and modulation of P2XR. In support of this contention, a heat shock protein, HSP90, was recently shown to interact with the P2X<sub>7</sub>R and modulate the receptor when phosphorylated [38].

In summary, PKC modulation of P2X<sub>3</sub>R represents a mechanism resulting in augmented intracellular Ca<sup>2+</sup> signaling. These results support the emerging consensus that protein kinases can regulate P2XR signaling and therefore represent a point of convergence between individual signaling systems. However, importantly, it does not appear that the P2X<sub>3</sub>R is subject to a direct PKC-mediated phosphorylation event. This is nevertheless likely an important mechanism for selectively modulating P2XR and has broad implications for the fidelity of downstream P2XR signaling.

## Acknowledgments

This work was supported in part by Grants R01-DK-054568 and R01-DE014756 from the National Institutes of Health (to D.I.Y.) and the NIDCR, National Institutes of Health Training Grant T32-DE07202 (to D.A.B.). We would like to thank Drs. R.A. North and J.A. Sim for providing the P2XR constructs and Drs. Jim Melvin, Matthew Betzenhauer, Larry Wagner, and Trevor Shuttleworth for helpful discussion during the course of this study.

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