Molecular Pharmacology Fast Forward. Published on September 9, 2009 as doi:10.1124/mol.109.060467
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MOL #60467

β-ARRESTIN-2 INTERACTION AND INTERNALIZATION OF THE HUMAN $P2Y_1$-RECEPTOR ARE DEPENDENT ON C-TERMINAL PHOSPHORLAYTION SITES

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Molecular Pharmacology Fast Forward. Published on September 9, 2009 as DOI: 10.1124/mol.109.060467
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MOL #60467

RUNNING TITLE PAGE

a) Running Title: Internalisation of P2Y1 receptors

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c) Text pages: 19
Tables: none
Figures: 9
References: 38
Abstract: 240 words
Introduction: 568 words
Discussion: 1108 words

d) List of non-standard abbreviations:

ABTS, 2,2`-azino-di-[3-ethylbenzthiazoline sulfonate(6)],
ADP, adenosine 5´-diphosphate;
2-MeSADP, 2-Methylthio- adenosine 5´-diphosphate;
CaMKII, Calmodulin-dependent Kinase II;
DMEM, Dulbecco´s modified Eagle´s medium;
FCS, fetal calf serum;
FRET, fluorescence resonance energy transfer;
GFP, green fluorescent protein;
Gö-6850, {2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide};
Gö-6976, [12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)
(3,4-c)-carbazole];
Gö-6983, {2-[1-(3-Dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)
Maleimide};
GPCR, G protein-coupled receptor;
GRK, G protein-coupled receptor kinase;
HBSS, Hank´s balanced salt solution;
KN-62, {1-[N,O-*bis*-(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine};
KN-93, {2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-cholocinnamyl)
-N-methylbenzylamine)};
PKC, protein kinase C;
PMA, phorbol 12-myristate 13-acetate;
ROI, region of interest;
XFP, color variant of GFP;

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ABSTRACT

The nucleotide receptor $P2Y_1$ regulates a variety of physiological processes and is involved in platelet aggregation. Using human $P2Y_1$-receptors C-terminally fused with a fluorescent protein we studied the role of potential receptor phosphorylation sites in receptor internalization and β-arrestin-2 translocation by means of confocal microscopy. Three receptor constructs were generated which lacked potential phosphorylation sites in the third intracellular loop, the proximal C-terminus, or the distal C-terminus. The corresponding receptor constructs were expressed in HEK-293 cells and stimulated with 100 μM ADP. Rapid receptor internalization was observed for the wild-type receptor, as well as those constructs mutated in the third intracellular loop and the proximal C-terminus. However, the construct lacking phosphorylation sites at the distal C-terminus did not show receptor internalization upon stimulation. The microscopic data were validated by HA-tagged receptor constructs using a cell surface ELISA. $P2Y_1$-receptor stimulated β-arrestin-2-YFP translocation followed the same pattern as receptor internalization. Hence, no β-arrestin-2-YFP translocation was observed when the distal C-terminal phosphoylation sites were mutated. Individual mutations indicate that residues Ser352 and Thr358 are essential for receptor internalization and β-arrestin-2-YFP translocation. In contrast, PKC mediated receptor desensitization was not affected by mutation of potential phosphorylation sites in the distal C-terminus, but was prevented by mutation of potential phosphorylation sites in the proximal C-terminus. $P2Y_1$-receptor internalization in HEK-293 cells was not blocked by inhibitors of PKC and CaM-Kinase. Thus we conclude that $P2Y_1$-receptor desensitization and internalization are mediated by different phosphorylation sites and kinases.

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INTRODUCTION

The P2Y-receptor family comprises eight different subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) which can further be subdivided into two subgroups based on their G-protein coupling specificity (Von Kügelgen, 2006; Abbracchio, et al. 2006). One group consist of the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptor and couples mainly to Gq-proteins, while the second group consisting of the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptor, mainly couples to Gi-proteins. These receptors mediate the action of extracellular nucleotides on cellular signaling. The $P2Y_1$-receptor is activated by ADP and has been shown to play a major role in the initiation of platelet activation and aggregation (Gachet, 2006; Abbracchio et al., 2006). Besides expression in platelets, the $P2Y_1$-receptor is expressed in epithelial and endothelial cells as well as immune cells and osteoclasts, thus the receptor offers a diverse therapeutic potential (Jacobson et al., 2002; Burnstock, 2006; Abbracchio et al., 2006). Significant mutagenesis efforts have led to a detailed characterization of the ligand binding site of the $P2Y_1$-receptor (Jiang et al., 1997; Moro et al., 1998; Hoffmann et al., 1999) and allowed the design of selective agonists and antagonists for this receptor (Jacobson et al., 2002; Von Kügelgen, 2006). After agonist binding, G protein-coupled receptors (GPCRs) trigger activation of G-proteins but then also undergo complex series of reactions which turn off signal transduction via G-proteins and can start the process of receptor internalization (Lohse, 1993). Receptor phosphorylation of GPCRs has been shown to be an important regulatory process in receptor signaling and internalization (Fergusson, 2001). Phosphorylation of the activated receptors by specialized kinases allows β-arrestins to bind and to switch off receptor signaling through G-proteins, while often opening alternative signaling pathways (Lefkowitz, 2005, Gurevich and Gurevich, 2006). In platelet aggregation, ADP has been shown to be an important mediator. However, once stimulated with ADP platelets become non-responsive to a second stimulus with the same ligand. This process is, transient since platelets fully recover after 15 to 30 min. This effect was shown to be mediated

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by the $P2Y_1$ receptor (Baurand et al., 2000). Therefore, it is important to understand the regulation of the $P2Y_1$ receptor in more detail. Several groups have reported that the $P2Y_1$-receptor internalizes upon agonist stimulation (Baurand et al., 2005; Hardy et al., 2005; Tulapurkar et al., 2006; Mundell et al., 2006a; Mundell et al., 2006b; Hoffmann et al., 2008). However, little information is available on the processes that control this mechanism at the $P2Y_1$-receptor. Although phosphorylation is an important regulatory control in receptor internalization (Fergusson, 2001; Tobin, 2008), alternative routes of GPCR internalization, which are phosphorylation-independent, have been described by several groups (Fergusson, 2007). The $P2Y_1$-receptor has been shown to incorporate radioactive phosphate upon agonist stimulation (Mundel et al., 2006a) and Ser339 was found to play a role in receptor desensitization (Fam et al., 2003). Different kinases have been studied for their involvement in the phosphorylation process. While GRK-2 and -6 were found to have little effect (Hardy et al., 2005; Mundell et al., 2006b), inhibition of protein kinase C isoforms (Mundell et al., 2006a) or calmodulin-dependent protein kinase II (CaM-Kinase II) (Tulapurkar et al., 2006) blocked the agonist-promoted internalization of the $P2Y_1$-receptor. Unfortunately, no information is currently available about the molecular domains which are involved in the regulation of $P2Y_1$-receptor internalization. Therefore, we decided to study the effects of potential phosphorylation sites in the intracellular domains of the human $P2Y_1$-receptor on receptor internalization and β-arrestin translocation.

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MATERIALS AND METHODS

Materials ADP, 2-SMeADP, and poly-D-lysine were purchased form Sigma (Steinheim, Germany). All kinase-inhibitors were purchased from Calbiochem (Schwalbach, Germany). ABTS was from Roche Diagnostics GmbH (Mannheim, Germany). Cell culture reagents were supplied by PAN-Biotech GmbH (Aidenbach, Germany). Effectene was purchased from Quiagen (Hilden, Germany). cDNA for the human $P2Y_1$-receptor has been described previously (Hoffmann et al., 1999). All PCR primers were synthesized by MWG-Biotech GmbH (Ebersberg, Germany). Sequencing reactions were done by Eurofins Medigenomix GmbH (Martinsried, Germany). Primary HA-antibody was a gift from Dr. Stefan Schultz (Jena), the HRP-conjugated polyclonal goat anti-rabbit antibody was purchased from Dianova. OPTI MEM-1 was purchased from Invitrogen (Karlsruhe, Germany). All other chemicals were purchased from commercial suppliers at the highest purity grade available.

Construction of $P2Y_1$ receptors tagged with fluorescent proteins (XFP) P2Y receptor constructs were fused to the enhanced variants of cyan (eCFP), green (eGFP), or yellow (eYFP) fluorescent protein (BD Bioscience Clontech, Heidelberg, Germany) by standard PCR extension overlap technique (Ho et al., 1989). In each case, the C-terminal stop-codon of the receptor and the initial codon for methionine of the fluorescent protein were deleted. Hence, no linker sequence exists between the receptor and the fluorescent protein. All resulting constructs were cloned into pcDNA3 (Invitrogen) and confirmed by sequencing.

The constructs are shown in Figure 1. Mutant $P2Y_1$ receptors were constructed by replacing one or several serine, threonine or tyrosine residues with an alanine residue. The receptor mutant termed $P2Y_1$ group-1 was depleted of all potential phosphorylation sites in the proximal C-terminus (positions 329-346), the receptor mutant termed $P2Y_1$ group-2 mutant was lacking the phosphorylation sites of the distal C-terminus (positions 352-373), while for

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the receptor mutant termed $P2Y_1$ group-3 the potential sites of the intracellular loop three were deleted (positions 241-258).

β-arrestin constructs Throughout all confocal microscopic experiments described in this paper we used bovine β-arrestin-2 fused C-terminally to enhanced YFP as previously described (Krasel et al., 2005).

Cell culture HEK-293 cells were maintained in DMEM with 4.5 g/l glucose, 10% FCS, 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate at 37°C, 7% CO_2. All cells were routinely passaged every two to three days. Culture medium for cells stably expressing the individual HA-$P2Y_1$-CFP receptor constructs was additionally supplemented with 200 µg/ml G-418.

Transfection of HEK-293 cells for microscopic analysis Individual 24 mm glass cover slips were placed in 6-well plates and coated for 30-60 min using 300 µl of poly-D-lysine (1mg/ml). Poly-D-lysine was aspirated and the glass cover slips were washed once with sterile PBS w/o Ca^{2+}. HEK-293 cells were seeded onto these cover slips to result in approximately 50% confluence. After attachment of the cells (4 to 6 hours), the cells were transfected using Effectene according to the manufacturer´s instructions. The following amounts of DNA were used per well: 300 ng for receptors, 200 ng for β-arrestins. All constructs were in pcDNA3; the amount of DNA was adjusted using empty pcDNA3 vector. Medium was exchanged 12-16 hours later, and cells were analyzed 48 hours after transfection.

Confocal Microscopy All confocal microscopy experiments were performed on a Leica TCS SP2 system. Cover slips with transfected HEK-293 cells were mounted using an “Attofluor”

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holder (Molecular Probes, Leiden, The Netherlands). Images were taken with a 63× objective as previously described (Hoffmann et al., 2008). In brief, CFP was excited with a 430nm diode laser using a DCLP455 dichroic mirror. GFP was excited using the 488nm line of an argon laser and a DCLP500 dichroic mirror. YFP was excited with the 514nm line of the argon laser and a dual beamsplitter 458/514nm. Settings for recording images were kept constant: 512*512 pixel format, line average 4, 400Hz, resulting in an image acquisition time of 7sec. Time series were recorded using the standard Leica software package (version 2.61). Pictures were taken at one minute intervals.

Quantification of receptor internalization was done with the Leica software package (version 2.61). Regions of interest (ROI) were defined in the cytosol and quantified over the time recorded. Care was taken that slight movements of the cells did not result in misplacement of the defined ROI`s either onto the membrane or into the nuclear region. To correct for possible photobleaching, control regions were defined that included whole cells and were used to correct the images in the cytosolic regions of interest. To quantify receptor internalization, the resulting fluorescence intensity values were normalized to the initial value and plotted against time.

Receptor surface expression determined by ELISA The experiments followed the procedure as previously published (Desai et al., 2000) with some slight modifications. HEK-293 cells stably expressing the HA-tagged-$P2Y_1$-CFP receptor or the corresponding mutant receptors were grown in poly-D-lysine coated 24-well plates. Equal numbers of cells were seeded per well and grown for 24h to about 85 % confluence. To quantify receptors present at the cell surface, cells were incubated with rabbit anti-HA antibody (1:2000) diluted in OPTI MEM-1 in a humid atmosphere at 4C° for 2h. To detect background binding of the secondary antibody, OPTI MEM-1 without antibody was added and the cells were treated as control cells. After incubation the cells were washed once carefully with OPTI MEM-1 and incubated

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at 37C°. At the indicated time points ADP was added (100 µM final concentration), and cells were further incubated until all samples were incubated for 30 min total time. To stop internalization, cells were washed three times with ice-cold PBS. After this, cells were fixed with Zamboni solution at room temperature for 40 min, washed 4 times with PBS and were blocked in a solution of PBS containing 1 mM $CaCl_2$ and 3% BSA for 1h at room temperature. The cells were incubated with secondary HRP-conjugated polyclonal goat anti-rabbit antibody (1:2500) diluted in PBS supplemented with 1 mM $CaCl_2$ and 1% BSA at room temperature. After 4 additional washes with PBS containing 1 mM $CaCl_2$, antibody binding was detected by addition of ABTS. After 25 min incubation at RT quantification was done by photometric determination of the absorption at 405 nm. Remaining surface receptors were calculated as absorption of treated cells divided by the absorption of untreated cells, both corrected for background. Each experiment was repeated six times in triplicate.

Fluorescence resonance energy transfer (FRET) experiments using a probe for PKC activity Fluorescence imaging of FlAsH labeled CFP-KCP-2-Flash probe was performed as previously described (Jost et al., 2008) on a Zeiss Axiovert 135 inverted microscope equipped with a Zeiss PlanNeofluar 100x/1.3 Oil objective at room temperature. FlAsH labelling was performed as described previously (Hoffmann et al., 2005). Samples were excited at 436 nm (dichroic 450 nm) with light from a polychrome IV (Till Photonics). The light source settings were controlled by Till pmc Communications software version 1.0.5. The emission ratio (FlAsH over eCFP) was measured with emission filters 480/40 nm (eCFP) and 535/30 nm (FlAsH), beam splitter dclp 505 nm. Signals detected by avalanche photodiodes were digitized using an AD converter (Digidata1322A, Axon Instruments) and stored on PC using Clampex 8.1 software (Axon Instruments).

The emission ratio was corrected for bleed-through of eCFP into the FlAsH channel to give a corrected emission ratio (bleed-through of FlAsH into the eCFP channel is negligible). FlAsH

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emission after excitation with light at 490 nm was determined in order to subtract direct excitation of FlAsH.

Determination of intracellular Ca^{2+}-increase 1321N1 cells were transfected with either HA-$P2Y_1$-wt, HA-$P2Y_1$-Gr2 or HA-$P2Y_1$-ST/AA mutant receptor cDNA using the Amaxa Nucleofactor Kit V according to the manufacturers instructions. Transfected cells were seeded onto poly-D-lysine coated coverslips in 6-well plates and grown to about 70% confluence in DMEM. To load cells with the calcium indicator, cells were incubated for 35 min at 37 °C in loading-buffer (10mM HEPES, 140 mM NaCl, 5,4 mMKCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$) supplemented with Fura-2 AM (1 µM). Cells were washed three times with loading-buffer and incubated at room temperature in the dark for 40 min. Coverslips were mounted onto an Attoflour holder and placed on an inverted fluorescence imaging microscope Nikon TE-2000U (Dichroic mirror: 455DCLP; Filter set: 340nm and 380nm excitation filters and 520/20 emission filter). Cells were perfused with measuring buffer (10 mM HEPES, 140 mM $NaCl_2$, 5,4 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA) or stimulated with 10nM 2-MeSADP diluted in measuring-buffer. Cells were measured with or without preincubation with 500 nM PMA for 5min. To detect the elicited Ca^{2+} response in single cells, the Fura-2 fluorescence in defined regions of interest (ROI) was recorded using MetaMorph/MetaFluor imaging acquisition and analysis software. Data were expressed as the ratio of Fura-2 emission when excited at 340 and 380nm. (Harbeck *et al.*, 2006)

Receptor Phosphorylation To determine phosphorylation of the $P2Y_1$ receptor, transiently transfected HEK-293 cells expressing comparable amounts of the HA-tagged $P2Y_1$ receptor or the respective mutant receptor, were seeded in 6-well plates. 48 hours after transfection, cells were labeled with 400 µCi/well of [^{32}P]orthophosphate in phosphate-free DMEM for 2h at 30°C. Labeled cells were (or were not) stimulated with 10 nM 2-MeSADP for 7 minutes.

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Cells were solubilized on ice for 30 min as previously described (Lorenz et al., 2003). The HA-tagged receptors were immunoprecipitated with anti-HA (12CA5) antibodies loaded sepharose beads. The phosphorylated receptor was subjected to SDS-polyacrylamid gel electrophoresis and quantified by phosphorimager analysis.

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RESULTS

The human $P2Y_1$ receptor was analyzed with respect to potential phosphorylation sites in the third intracellular loop and the C-terminus. Thirteen serine, threonine, and tyrosine residues are present in these regions (Figure 1). Sequence comparison of $P2Y_1$ receptors from six different species, listed at GPCR.org, showed that all of these residues are conserved (data not shown). Therefore we decided to construct three mutant receptors each containing three to five mutated residues. The constructs are shown in Figure 1. The receptor mutant termed $P2Y_1$ group-1 was depleted of all potential phosphorylation sites in the proximal C-terminus, the receptor mutant termed $P2Y_1$ group-2 was lacking the phosphorylation sites of the distal C-terminus, while in the receptor mutant termed $P2Y_1$ group-3 the potential sites of the third intracellular loop were deleted. The constructs were functionally not distinct from wild-type $P2Y_1$ receptors with respect to increase in intracellular Ca^{2+} concentration upon receptor stimulation in COS-7 cells (data not shown). All receptor mutants were expressed at the cell surface in HEK-293 cells (Figure 2). Upon stimulation with 100 μM ADP, the wild-type receptor rapidly internalized, as depicted by the appearance of an intracellular punctuate pattern. $P2Y_1$ group-1 and $P2Y_1$ group-3 mutant receptors also exhibited rapid internalization upon agonist stimulation (Figure 2). However, $P2Y_1$ group-2 mutant receptor was virtually devoid of internalization upon exposure to 100 μM ADP. Data were quantified by measuring the increase of intracellular fluorescence over time as described above and shown in Figure 3A. To verify the observed data by an independent method, we generated receptor constructs that, in addition to the mutations, contained an N-terminal HA-tag. HEK-293 cells were stably transfected with these constructs and cells lines were selected which expressed comparable amounts of receptors as judged by fluorescence intensity of the cells. These cell lines were used for further analyses by cell surface ELISA. Cells expressing either wild-type, $P2Y_1$ group-1, $P2Y_1$ group-2 or $P2Y_1$ group-3 receptors were stimulated for 5 to 30 min with ADP

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and remaining surface expression of the HA-tagged receptors was quantified as described in the Materials and Methods section. The data are shown in Figure 3B. A loss of surface expression of wild-type, $P2Y_1$ group-1 and $P2Y_1$ group-3 receptors was readily detectable, while no reduction of surface expressed receptors was observed for the $P2Y_1$ group-2 receptor construct. Thus, the data from confocal microscopy and surface ELISA exhibit the same pattern, since essentially no internalization could be observed for $P2Y_1$-receptor lacking potential phosphorylation sites at the distal C-terminus. Therefore, this group of phosphorylation sites appears to play a critical role in receptor internalization.

To further analyze the role of the individual serine or threonine residues present in the distal C-terminus, we generated receptor constructs each of which carried one individual serine or threonine point mutation in the distal C-terminus. In addition, we generated a construct in which the last eleven amino acids of the C-terminus were deleted. Each construct was expressed in HEK-293 cells and analyzed by confocal microscopy for agonist stimulated receptor internalization. The results are presented in Figure 4. Again, mutation of all five residues at the same time ($P2Y_1$ group-2) blocked receptor internalization (Figure 4A and 4B), while the data for individual mutations of residues S354A, T371A and S372A indicate that receptor internalization still occurred upon agonist stimulation (Figure 4A or 4B). However, quantification of intracellular fluorescence showed a significant reduction in receptor internalization for the mutant receptors S352A and T358A, but internalization was not fully blocked (Figure 4A and 4B). Deletion of the very C-terminus ($P2Y_1$-Δ363), which has been described to be important for receptor dimerization (Choi et al., 2008) and contains T371, S372 as well as the C-terminal PDZ-domain (Fam et al., 2005), had very little influence on receptor internalization (Figure 4A). The combination of S352A and T358A in a double mutant receptor termed $P2Y_1$ ST/AA fully blocked receptor internalization and was indistinguishable from $P2Y_1$ group-2 mutant (Figure 4A and 4B). Thus, potential

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phosphorylation of serine 352 and threonine 358 sites seem to play a role in the internalization of the $P2Y_1$-receptor.

We have recently reported that the $P2Y_1$-receptor translocates β-arrestin-2 to the plasma membrane upon stimulation with ADP (Hoffmann et al., 2008). Since β-arrestin translocation is known to be a phosphorylation-dependent process, we decided to study the influence of the receptor´s potential phosphorylation sites on β-arrestin-2 translocation. HEK-293 cells were co-transfected with CFP-tagged group mutant receptors and β-arrestin-2-YFP. Pictures of the CFP-tagged receptors were taken prior to agonist stimulation to verify receptor expression. Upon stimulation with ADP, a rapid translocation of β-arrestin-2-YFP was observed in cells co-transfected with $P2Y_1$ group-1 or $P2Y_1$ group-3 mutant receptors (Figure 5). In contrast, although receptor expression was verified, no β-arrestin-2-YFP translocation was observed in cells that were either co-transfected with the $P2Y_1$ group-2 mutant receptor or the double mutant receptor $P2Y_1$ ST/AA (Figure 5).

To explore whether the $P2Y_1$ group-2 or the double mutant receptor $P2Y_1$ ST/AA showed altered receptor phosphorylation, we compared the agonist-induced phosphorylation of the mutants to that of the wild-type $P2Y_1$-receptor. The right panel in figure 6 shows that all three receptor constructs exhibit little phosphorylation under basal conditions. Upon agonist-stimulation with 2-MeSADP for 7min, only the wild-type $P2Y_1$-receptor showed a significant increase in receptor phosphorylation. Quantitative analysis show that agonist-stimulated phosphorylation of the mutant receptors ($P2Y_1$ group-2 and $P2Y_1$ ST/AA) was impaired compared to wild-type $P2Y_1$-receptor (Figure 6, left panel).

Since PKC has been described to play a role in $P2Y_1$-receptor desensitization (Fam et a., 2003) we investigated whether the identified phosphorylation sites are also involved in receptor desensitization. Therefore, we tested the wild-type $P2Y_1$-, as well as the $P2Y_1$ group-1- and $P2Y_1$ group-2 receptors for their PKC mediated depression of receptor evoked Ca^{2+}

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response. Since 1321N1 astrocytoma cells do not express endogenous P2Y-receptors they were chosen to study Ca^{2+} responses (Lazarowski et al., 1997). 1321N1 cells transfected with the appropriate receptor constructs were stimulated with 2-MeSADP and as shown in Figure 7 (top row) all constructs exhibited similar Ca^{2+} response. However, when cells were pre-incubated with the PKC-activator PMA (5min) to induce desensitization, the agonist-stimulated Ca^{2+} response was blocked for the wild-type $P2Y_1$-receptor as well as group-2 mutant receptor, whereas the Ca^{2+} response did not desensitize for the mutant receptor $P2Y_1$ group-1 (Figure 7, bottom row).

Based on studies using different kinase inhibitors (Tulapurkar et al., 2006; Mundell et al., 2006a), CaM-Kinase II and PKC isozymes have been described to play a role in $P2Y_1$ receptor internalization. Therefore, we decided to test the influence of PKC and CaM-Kinase II inhibitors on $P2Y_1$-receptor internalization. Cells were transfected with wild-type $P2Y_1$-receptor fused to GFP. Prior to stimulation with ADP, cells were incubated for 10 min with different kinase inhibitors at 1 to 10 μM concentrations. Subsequent stimulation with ADP lead to visible receptor internalization for each inhibitor tested (Figure 8). Consistent with this notion we observed that addition of PMA did not lead to receptor internalization (Figure 8)

To demonstrate that the kinase inhibitors did diffuse into the cells in high enough concentrations to block kinase activity, we tested the inhibition of Gö-6983, which had previously been described to block receptor internalization (Mundell et al., 2006a), on a fluorescence resonance energy transfer (FRET)-based sensor for PKC activity. This FRET-sensor consists of a PKC-phosphorylation sequence which is flanked by two fluorescent groups. Upon phosphorylation the sensor changes its conformation, which leads to a change in FRET dependent on the degree of phosphorylation and thus is able to monitor PKC activity in living cells (Jost et al., 2008). Stimulation of HEK-293 cells by addition of 200 nM PMA lead to a rapid change in the FRET-ratio, whereas subsequent addition of 500 nM Gö-6983 fully reversed the effect (Figure 9). This indicates that the used concentrations of inhibitor and

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incubation times were high and long enough to block kinase activity in intact cells. Thus, CaM-Kinase II or PKC do not seem to play a major role in the internalization of $P2Y_1$ receptors in HEK-293 cells.

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DISCUSSION

In this paper, we analyzed the role of potential phosphorylation sites in the third intracellular loop and the C-terminus of the human $P2Y_1$-receptor for β-arrestin binding and receptor internalization. We focussed our interest on β-arrestin-2, since we have previously shown that the $P2Y_1$-receptor binds β-arrestin-2 with higher affinity than β-arrestin-1 (Hoffmann et al., 2008). Several groups have reported that the $P2Y_1$-receptor internalizes upon agonist stimulation (Baurand et al., 2005; Hardy et al., 2005; Tulapurkar et al., 2006; Mundell et al., 2006a; Mundell et al., 2006b; Hoffmann et al., 2008) and this has been shown to be relevant for the non-responsiveness of platelets towards a second stimulus with ADP (Baurand et al, 2000). However, no information was available on the receptor domains that are involved in the process. Therefore, we created three receptor constructs with deletions of potential phosphorylation sites within the third intracellular loop or the C-terminal domain as shown in Figure 1. All receptor constructs were expressed at the cell surface in HEK-293 cells (Figure. 2). Substitution of serine and threonine residues in the third intracellular loop ($P2Y_1$ group-3) or the proximal C-terminus ($P2Y_1$ group-1) had no negative effect on receptor internalization. In contrast, substitution of distal potential phosphorylation sites ($P2Y_1$ group-2) clearly blocked receptor internalization. We confirmed and quantified our observations using confocal microscopy as well as a cell surface ELISA. Both techniques showed a total lack of internalization for the $P2Y_1$ group-2 construct, while the other constructs internalize similarly to wild-type receptor (see Figure 2 and 3). Consistent with previous findings for C-terminally truncated $P2Y_1$-receptor constructs (Choi et al., 2008), elimination of serine and threonine residues did not prevent receptor mediated signaling to G-proteins as judged by agonist stimulated rise in intracellular Ca^{2+} (compare Figure 7). The detailed analyses of individual mutations revealed that in particular, two mutations, S352A and T358A, reduced receptor internalization upon stimulation. Quantification analysis demonstrated that both mutants

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exhibit reduced internalization without fully blocking it. However, the combined mutations of S352A and T358A fully prevented receptor internalization. No effect on receptor internalization was observed for residues S354, T371 and S372 (figure 4).

Since agonist stimulated receptor phosphorylation was significantly reduced in the $P2Y_1$ group-2 and $P2Y_1$ ST/AA construct compared the wild-type P2Y1-receptor (Figure 6), we conclude that the lack of phosphorylation sites S352 and T358 in the distal C-terminus is specifically involved in the receptor internalization process.

Recruitment of β-arrestins to activated GPCRs is known to be involved in receptor internalization (Fergusson, 2001). Accordingly, β-arrestin-2 translocates to the plasma membrane upon stimulation of $P2Y_1$ receptors, which carry mutations in the third intracellular loop ($P2Y_1$ group-3) or the proximal C-terminus ($P2Y_1$ group-1), while no translocation was observed upon stimulation of $P2Y_1$ receptors with mutations in the distal C-terminus ($P2Y_1$ group-2 construct and $P2Y_1$ ST/AA construct). Since β-arrestin translocation parallels receptor internalization for the human $P2Y_1$-receptor, we propose that the serine and threonine residues in the distal C-terminus are important for β-arrestin-receptor interaction. In particular, two mutations, S352A and T358A, reduced internalization upon receptor stimulation and β-arrestin-receptor interaction.

A contribution of several different phosphorylation sites for effective internalization is consistent with reports for other GPCRs like the rhodopsin- or β_2-adrenergic receptor where several residues need to be phosphorylated to recruit arrestin or β-arrestin to the receptor (Vishnivetskiy et al., 2007, Krasel et al., 2008). Interestingly, different GPCRs seem to utilize different domains or mechanisms to interact with β-arrestin-2. For the TRH-receptor is has recently been described that phosporylation sites in the distal C-terminus were required for β-arrestin-mediated internalization, whereas interaction of β-arrestin-2 with the receptor still occurred when these sites were removed (Jones and Hinkle, 2008). Deletion of the C-terminal

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part directly adjacent to the sites important for internalization blocked β-arrestin-2 interaction. This is different in the case of the $P2Y_1$-receptor, since our data demonstrate that the mutation of potential phosphorylation sites in the distal C-terminus fully prevented β-arrestin-2 translocation while truncation of the C-terminus adjacent to the important sites ($P2Y_1$-Δ363) had no influence on internalization, although this domain has recently been shown to be important for receptor dimerization (Choi et al., 2008).

Different kinases have been investigated for their involvment in the phosphorylation process of the $P2Y_1$ receptor. A reduction of GKR-2 or GKR-6 expression levels by siRNA was shown to have little effect on $P2Y_1$ receptor internalization (Hardy et al., 2005; Mundell et al., 2006b) while inhibition of protein kinase C isoforms (Mundell et al., 2006a) or CaM-kinase II (Tulapurkar et al., 2006) blocked agonist-promoted receptor internalization. In this study, however, inhibition of receptor internalization by the tested inhibitors was not evident, even though pronounced PKC inhibition was demonstrated (compare Figure 8 and 9). Thus, the currently available data on the contributions of PKC or CaM-kinase II activity for the internalization of the $P2Y_1$-receptor are inconclusive. The discrepancy to previously published data could be explained by the use of different cell lines, a phenomenon which has already been reported for other GPCRs (Clark and Rich, 2003).

Previous reports on mutagenesis at the C-terminus of the $P2Y_1$-receptor have indicated that threonine 339 was responsible for receptor desensitization with respect to Ca^{2+}-signaling (Fam et al., 2003) and arginine 333 and 334 were needed for G-protein coupling (Ding et al., 2005). As depicted in Figure 1, residue 339 was mutated to alanine in construct $P2Y_1$ group-1. Thus we tested the wild-type $P2Y_1$-receptor, and the construct $P2Y_1$ group-1 and $P2Y_1$ group-2 for their desensitization behaviour. Consistent with the finding published by Fam and colleagues (Fam et al., 2003) the wild-type $P2Y_1$-receptor desensitized upon pre-treatment with PMA. Interestingly, the $P2Y_1$ group-1 did not desensitize (but internalized), whereas the $P2Y_1$ group-2 construct showed desensitization comparable to wild-type (but did not

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internalize). Thus, $P2Y_1$-receptor desensitization and internalization seem to be differentially controlled by different phophorylation sites and possibly different kinases. Similar observations have recently been reported for the somatostatin sst2A receptor (Liu et al., 2008).

Taking together our data from this study and from previous reports of other groups on the regulation of the $P2Y_1$-receptor, we propose the following scenario: Binding of ADP activates the receptor and allows coupling of the receptor to the G-protein via important interactions with arginine residues 333 and 334 (Ding et al., 2005). Phosphorylation at threonine 339 would add negative charge to the C-terminus and probably weaken the interaction with the G-protein, since threonine 339 has been shown to be responsible for desensitization of Ca^{2+}-signaling (Fam et al., 2003). In addition, serine 352 and threonine 358 might become phosphorylated and mediate interaction with β-arrestin-2, which would now be recruited to the C-terminus and thus would compete with the G-protein and allow additional interactions via β-arrestin-2 with the internalization machinery of the cell.

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Molecular Pharmacology Fast Forward. Published on September 9, 2009 as DOI: 10.1124/mol.109.060467
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FOOTNOTE

This work was supported by the Deutsche Forschungsgemeinschaft -Sonderforschungsbereich "Regulatory Membrane Proteins" [SFB-487 TP-A1]. We thank Dr. Ulrike Zabel for comments and criticism to the manuscript.

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LEGENDS FOR FIGURES

Figure 1:

Schematic representation of the amino acid sequence of the third intracellular loop and C-terminal part of the human wild-type and mutant $P2Y_1$-receptors. Serine, threonine and tyrosine residues in the wild-type receptor are highlighted in bold letters as potential phosphorylation sites. In the subsequent mutant constructs changes to alanine, that were made compared to the wild-type receptor, are highlighted in bold. Missing sequences represent truncation of the receptor.

Figure 2:

Agonist-induced internalization of GFP-tagged $P2Y_1$ receptor constructs in HEK-293 cells. Cells were transfected with GFP-tagged $P2Y_1$-receptor constructs and studied for receptor internalization. The left column shows cells prior to stimulation with ADP. The right column represents the same cells 15 min after ADP exposure (100 μM final). Data are representative examples of at least 5 individual experiments. White scale bars represent 10μm.

Figure 3:

Quantification of receptor internalization. (A) Experiments as shown in Figure 2 were quantified as described under Methods. Data from at least five different experiments were analyzed for each construct. The increase of intracellular fluorescence was calculated as percent increase above control and plotted against time. Individual traces represent the mean ± s.e.m. of at least 15 different cells from 5 independent experiments. Open squares represent the wild-type $P2Y_1$ receptor, open circles the $P2Y_1$ group-1, open triangles the $P2Y_1$ group-3- and open diamonds the $P2Y_1$ group-2 mutant. Statistical analysis was performed using a one way ANOVA-test, ** $p < 0.01$

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(B) Receptor surface expression was determined by ELISA. HEK-293 cells stably expressing the indicated P2Y receptor constructs were stimulated with ADP and analyzed for receptor internalization. Data from >6 different experiments done in triplicate were analyzed for each construct. The decrease in surface expressed HA-tagged $P2Y_1$-receptor constructs was calculated as percent decrease of control at t = 0 min and plotted against time. Individual traces represent the mean ± s.e.m. of at least six independent experiments. Open squares represent the wild-type $P2Y_1$ receptor, open circles the $P2Y_1$ group-1-, and open triangles the $P2Y_1$ group-3 mutant, open diamonds represent the $P2Y_1$ group-2 mutant. Statistical analysis was performed using a one way ANOVA-test, ** $p < 0.01$

Figure 4:

Mutational analyses of the $P2Y_1$ distal C-terminus in HEK-293 cells. (A) Cells were transfected with GFP-tagged $P2Y_1$-receptor constructs as depicted in Figure 1. Cells were stimulated with ADP (100μM final) and studied for receptor internalization. The left column represents cells prior to agonist stimulation. The right column shows the same cells 15 min after agonist exposure. Data are representative examples of at least 4 individual experiments. White scale bars represent 10μm.

(B) Quantification of receptor internalization of individual point mutants. Experiments were quantified as described (see Figure 3A). Data from at least 4 different experiments were analyzed for each construct. The increase of intracellular fluorescence was calculated as percent increase above control and plotted against time. Individual traces represent the mean ± s.e.m. of > 12 different cells from 4 independent experiments. Open squares represent the wild-type $P2Y_1$ receptor, open triangles up the S354A mutant-, open pentagon the T371A-, open triangles left the S372A -, open circles the S352A-, open triangles down the S358A mutant, open stars the double mutant ST/AA, and open diamonds the group-2 mutant. Statistical analysis was performed using a one way ANOVA-test, * $p < 0.05$, ** $p < 0.01$

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Figure 5:

β-Arrestin-2 translocation induced by stimulation of $P2Y_1$ receptor constructs. HEK-293 cells were co-transfected with the indicated $P2Y_1$-receptor-CFP and the β-arrestin-2-YFP construct. The left column shows localization of the CFP-tagged receptor construct prior to stimulation to demonstrate that the cells were co-transfected with both β-arrestin-2 and receptor. The middle column represents cells before ADP stimulation while the right column shows the same cells 15 min after exposure to ADP (100 μM final). A clear β-arrestin-2 translocation was observed for the $P2Y_1$ group-1 and $P2Y_1$ group-3 receptor constructs, while no β-arrestin-2 translocation was observed for the $P2Y_1$ group-2 receptor and the ST/AA double mutant. Data represent the mean of at least 4 individual experiments. White scale bars represent 10 μm.

Figure 6:

Phosphorylation of $P2Y_1$ receptor constructs. HEK-293 cells transfected with the indicated $P2Y_1$-receptor-CFP constructs were labeled with [^{32}P]-orthophosphate in phosphate-free DMEM and stimulated with 2-MeSADP as described. The agonist-induced phosphorylation was quantified for each construct (left panel). The results are the mean ± s.e.m. of 4 independent experiments compared to basal (* $p < 0.05$; one way ANOVA). A representative experiment is shown in the right panel.

Figure 7:

Ca^{2+}-response of $P2Y_1$ receptor constructs. 1321N1 astrocytoma cells were transfected with the indicated $P2Y_1$-receptor-CFP constructs and loaded with the fluorescent Ca^{2+}-sensitive dye Fura-2 AM as described. The top row shows Ca^{2+}-traces recorded for individual cells

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when cells were stimulated with 10 nM 2-MeSADP. The bottom row shows Ca^{2+}-traces recorded for individual cells when cells were pre-incubated for 5min with 500 nM PMA and stimulated with 10 nM 2-MeSADP. Traces are representative for at least 9 cells of three independent transfections.

Figure 8:

Effect of different kinase inhibitors on the internalization of GFP tagged $P2Y_1$ receptor in HEK-293 cells. Cells were transfected with GFP-tagged wild-type $P2Y_1$-receptor and studied for receptor internalization. Cells were treated with the PKC-inhibitors Gö-6850, Gö-6976 and Gö-6983 (1μM final), the CaM-kinase II inhibitors KN-62, and KN-93 (10μM final), or PMA (500 nM final). After 10 minutes pre-incubation, cells were stimulated with ADP (100 μM final). The left column represents cells 10 minutes after pre-incubation with the indicated inhibitor. The right column shows the same cells 15 min after agonist exposure. For PMA application the cells represent time points immediately before and 15 min after PMA exposure. Data are representative examples of at least 4 individual experiments. White scale bars represent 10μm.

Figure 9:

Monitoring PKC-activity in intact HEK-293 cells by FRET. HEK-293 cells were transfected with a FRET-probe named CFP-KCP-2-Flash and monitored for PKC activity by changes in FRET. Cells were excited at 436nm and fluorescence emission was measured at 480nm and 535nm wavelength. The recorded emission was corrected for bleed-through and photobleaching as described and calculated as FRET-ratio (535nm/480nm). Cells were incubated with 200 nM PMA to stimulate PKC activity and after 5min incubation cells were additionally exposed to 500 nM Gö-6983 to block PKC activity.

Figure 1

Construct	Intracellular loop 3	C-terminus
		proximal / distal
	241 258	329 350 373
P2Y$_1$ wild-type	VRALI**Y**KDLDN**S**PLRRK**S**	D**T**FRRRL**S**RA**T**RKA**S**RR**S**EANCQ**S**K**S**EDM**T**LNILPEFKQNGD**TS**L
P2Y$_1$ group-1	VRALIYKDLDNSPLRRKS	D**A**FRRRL**A**RA**A**RKA**A**RR**A**EANCQSKSEDMTLNILPEFKQNGDTSL
P2Y$_1$ group-2	VRALIYKDLDNSPLRRKS	DTFRRRLSRATRKASRRSEANCQ**A**K**A**EDM**A**LNILPEFKQNGD**AA**L
P2Y$_1$ group-3	VRALI**A**KDLDN**A**PLRRK**A**	DTFRRRLSRATRKASRRSEANCQSKSEDMTLNILPEFKQNGDTSL
P2Y$_1$ Δ363	VRALIYKDLDNSPLRRKS	DTFRRRLSRATRKASRRSEANCQSKSEDMTLNIL
P2Y$_1$ S352A	VRALIYKDLDNSPLRRKS	DTFRRRLSRATRKASRRSEANCQ**A**KSEDMTLNILPEFKQNGDTSL
P2Y$_1$ S354A	VRALIYKDLDNSPLRRKS	DTFRRRLSRATRKASRRSEANCQSK**A**EDMTLNILPEFKQNGDTSL
P2Y$_1$ T358A	VRALIYKDLDNSPLRRKS	DTFRRRLSRATRKASRRSEANCQSKSEDM**A**LNILPEFKQNGDTSL
P2Y$_1$ T371A	VRALIYKDLDNSPLRRKS	DTFRRRLSRATRKASRRSEANCQSKSEDMTLNILPEFKQNGD**A**SL
P2Y$_1$ S372A	VRALIYKDLDNSPLRRKS	DTFRRRLSRATRKASRRSEANCQSKSEDMTLNILPEFKQNGDT**A**L
P2Y$_1$ ST/AA	VRALIYKDLDNSPLRRKS	DTFRRRLSRATRKASRRSEANCQ**A**KSEDM**A**LNILPEFKQNGDTSL

Figure 2

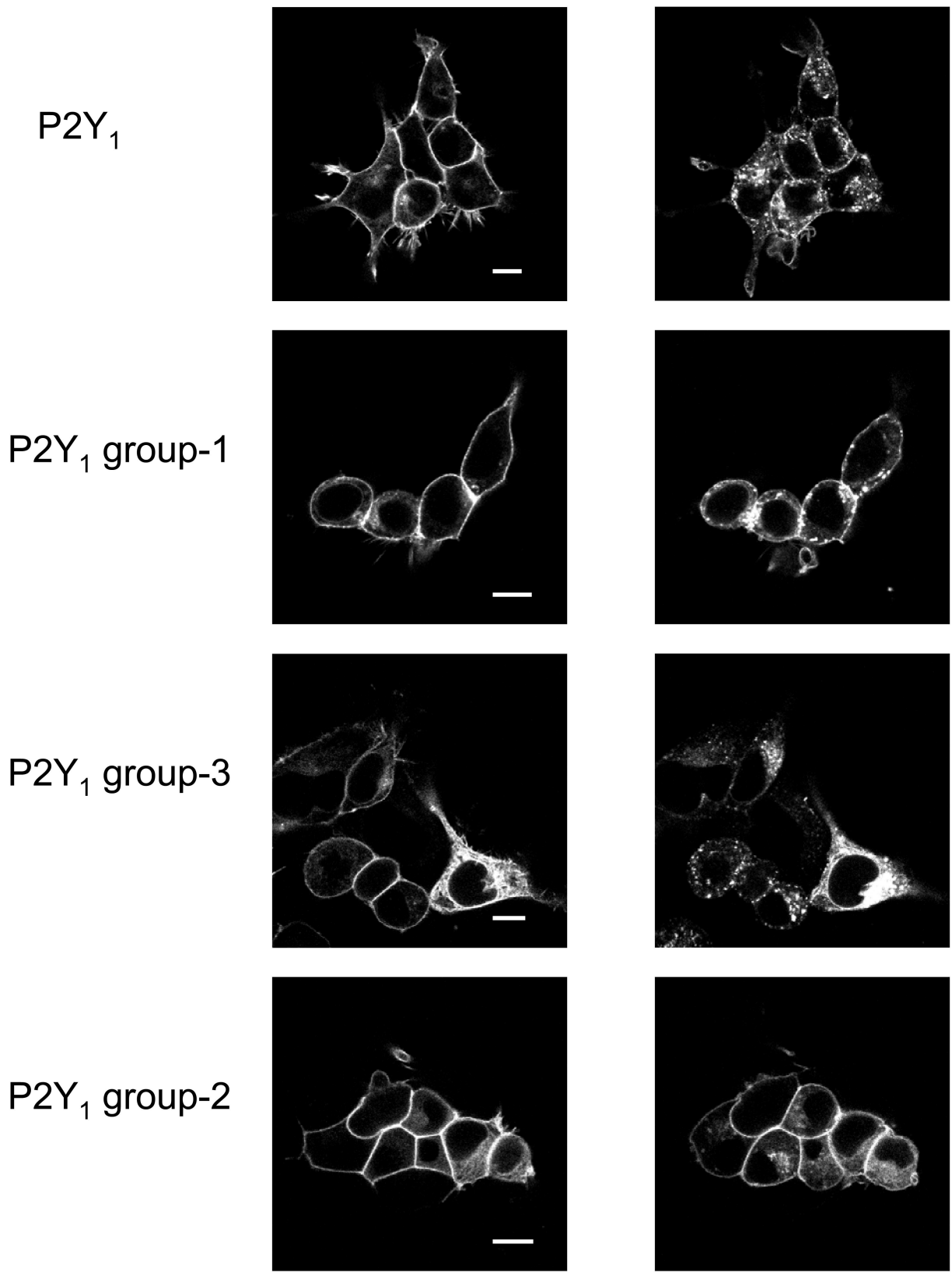

0 min
15 min
+ADP 100μM
P2Y1
P2Y1 group-1
P2Y1 group-3
P2Y1 group-2

Figure 3

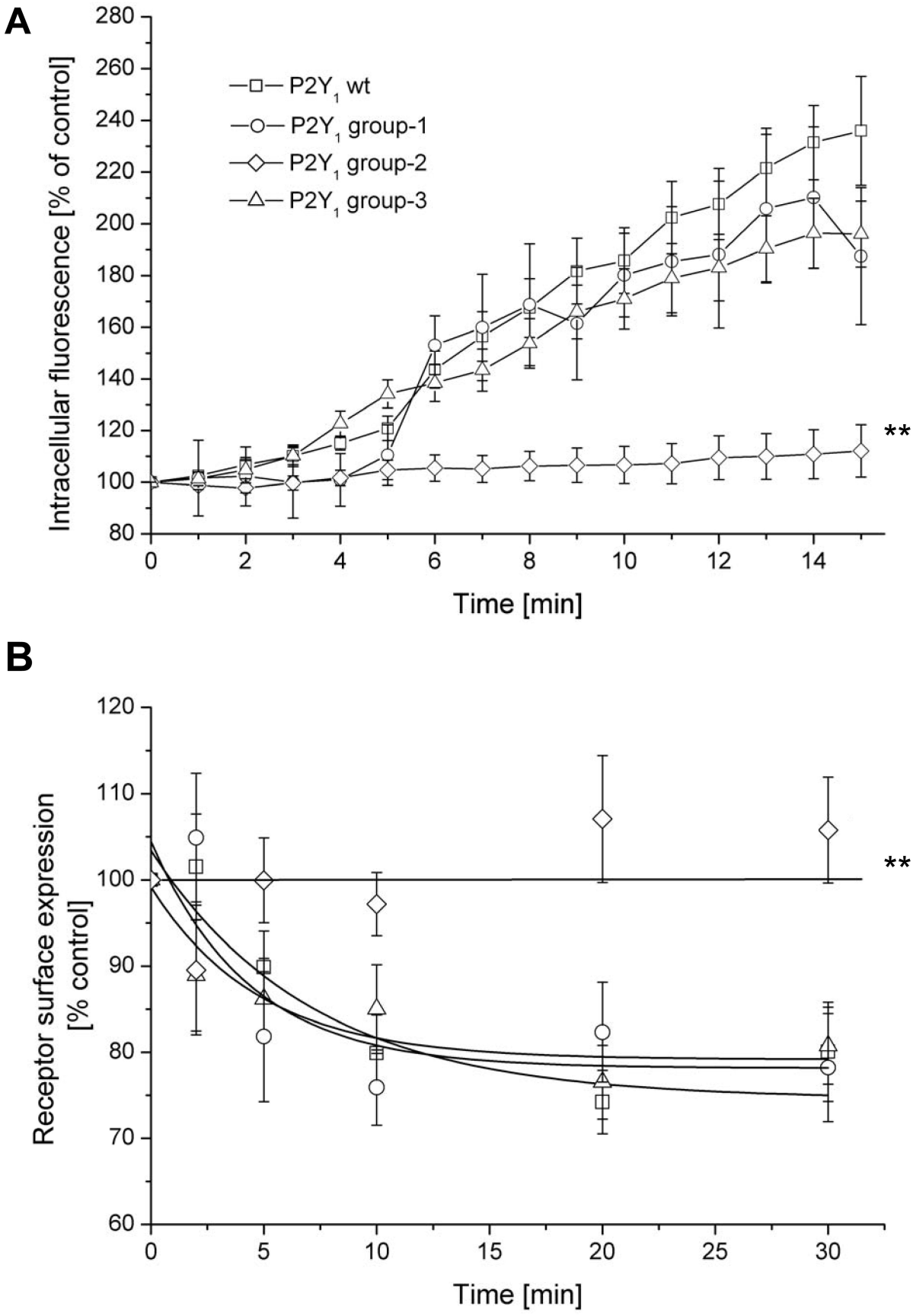

A
Intracellular fluorescence [% of control]
Time [min]
P2Y1 wt
P2Y1 group-1
P2Y1 group-2
P2Y1 group-3
**
B
Receptor surface expression [% control]
Time [min]
**

Figure 4A

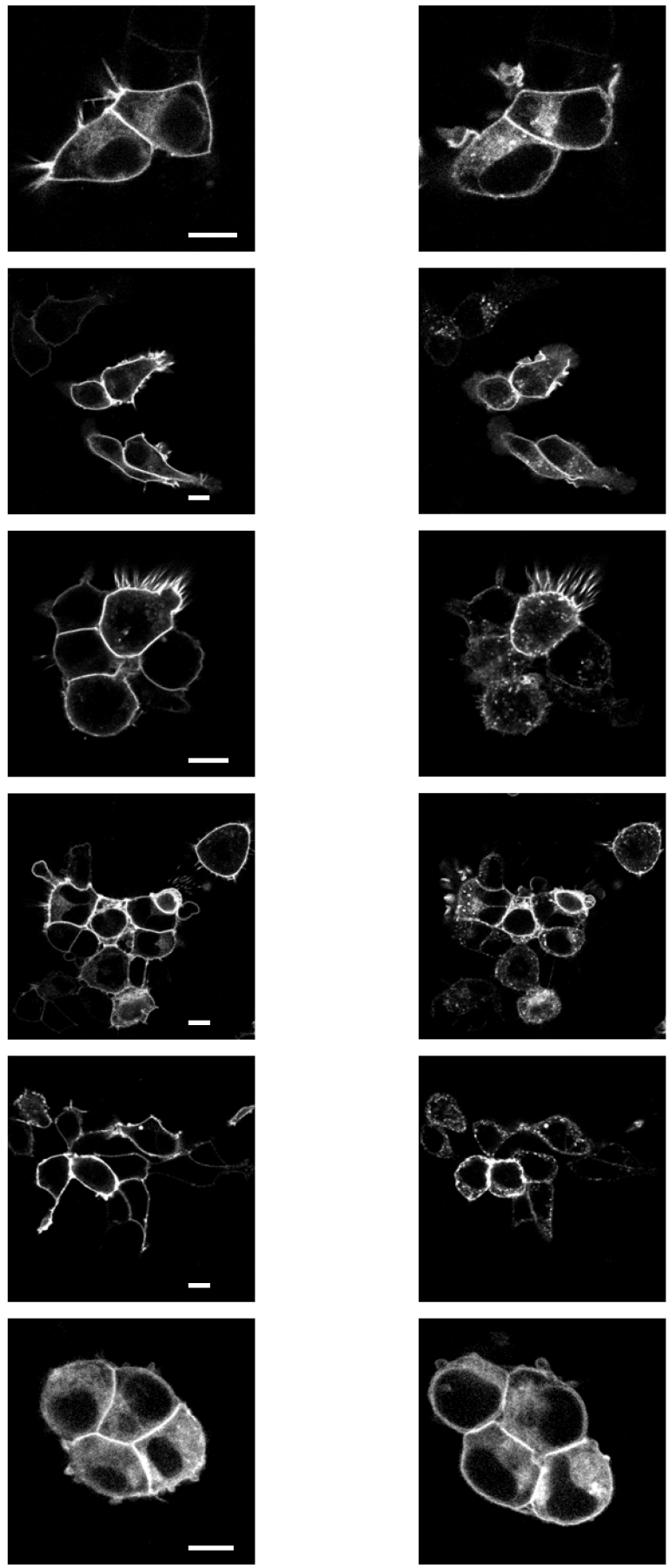

0 min
15 min + ADP 100μM
P2Y1 group-2
P2Y1-S352A
P2Y1-S354A
P2Y1-T358A
P2Y1-Δ363
P2Y1ST/AA

Figure 4B

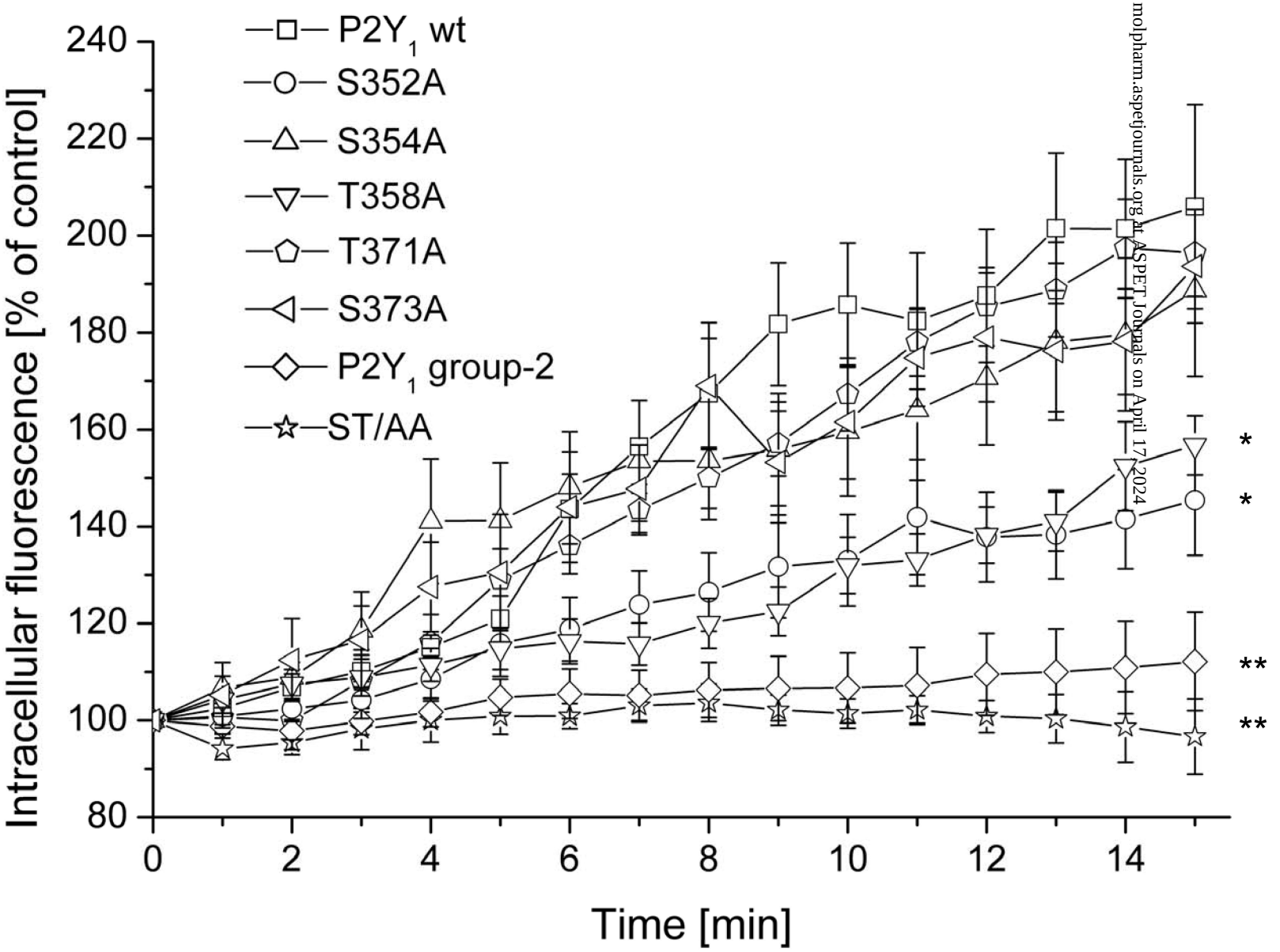


Figure 5 Receptor-CFP

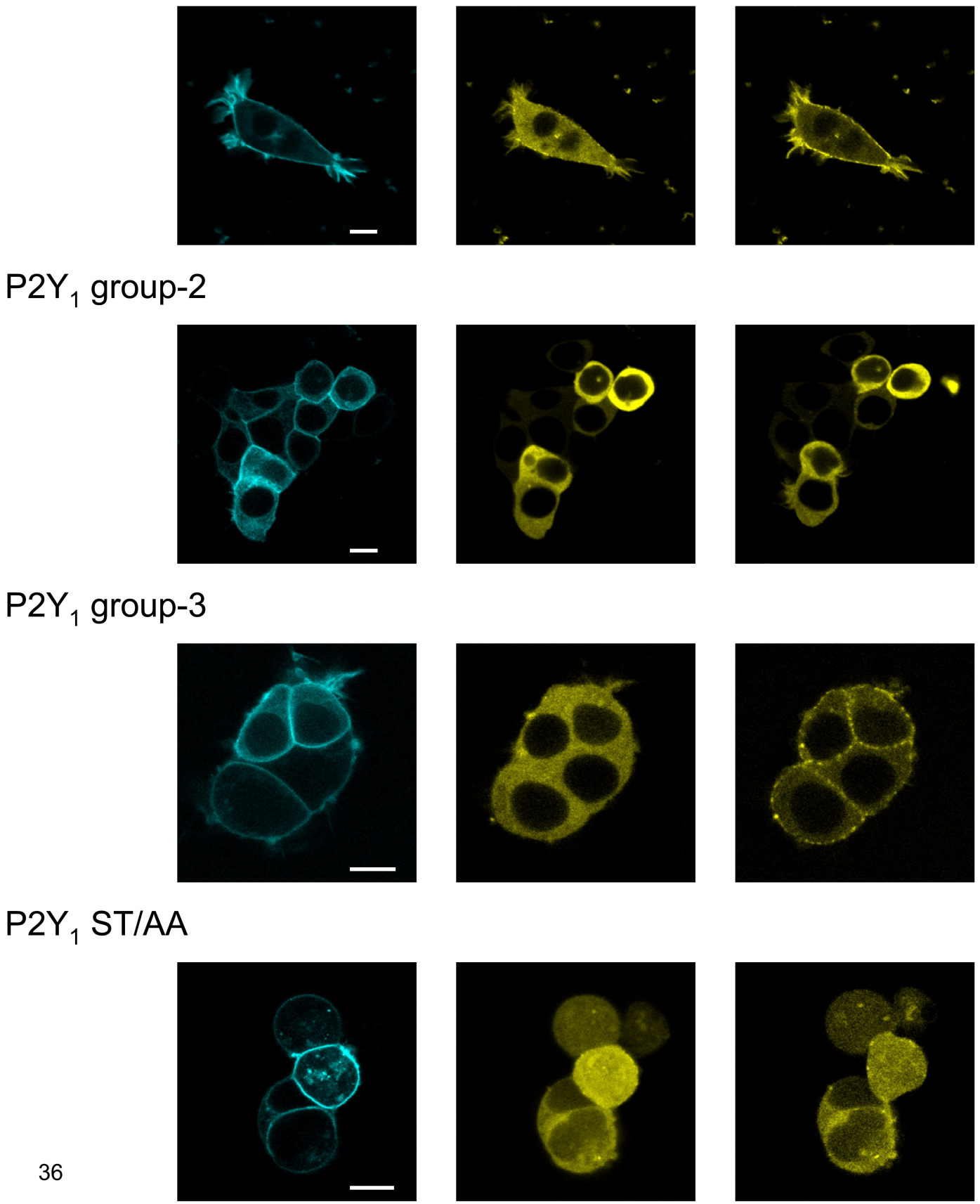
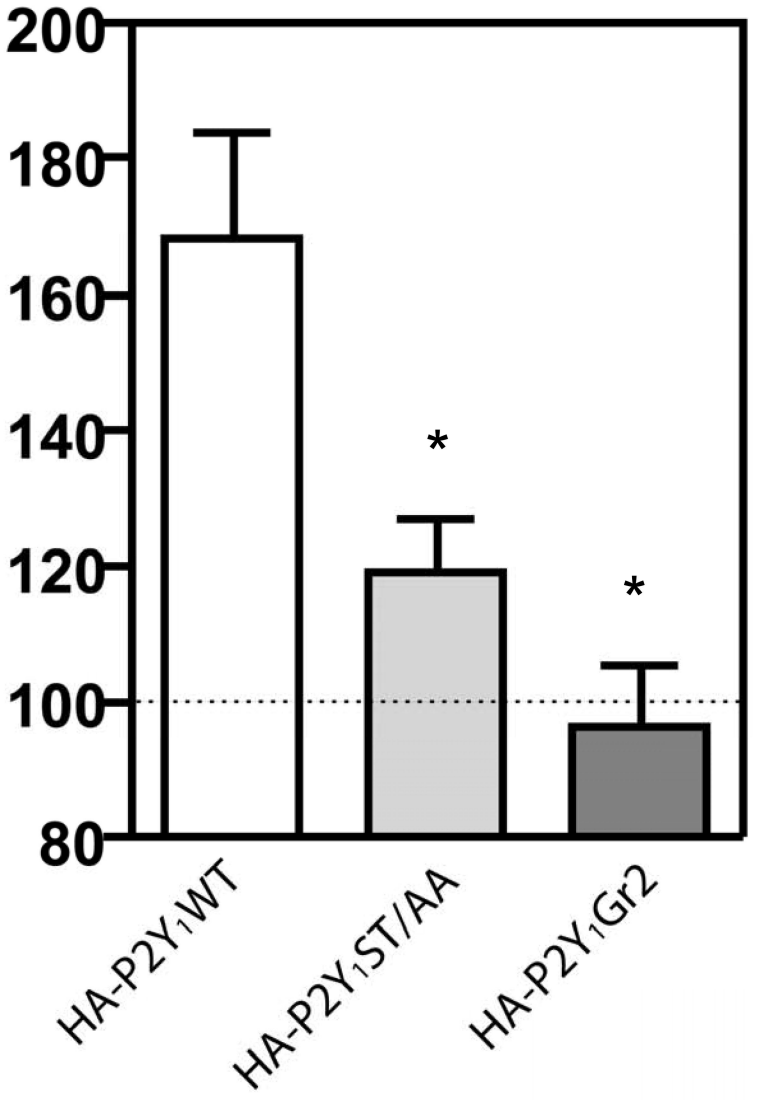


Figure 6



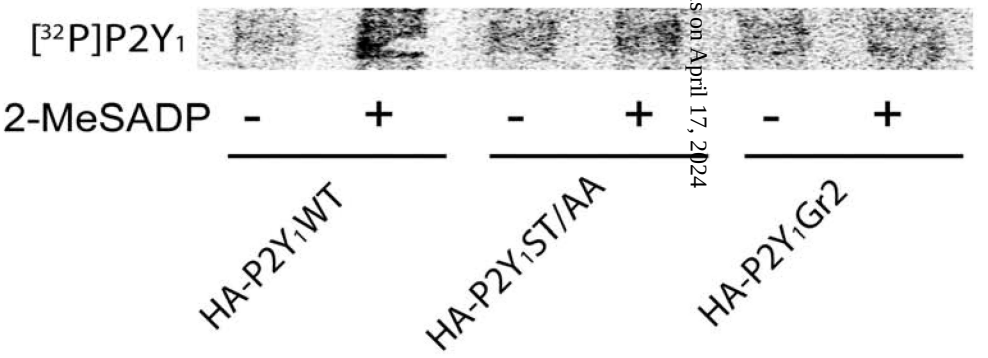


Figure 7

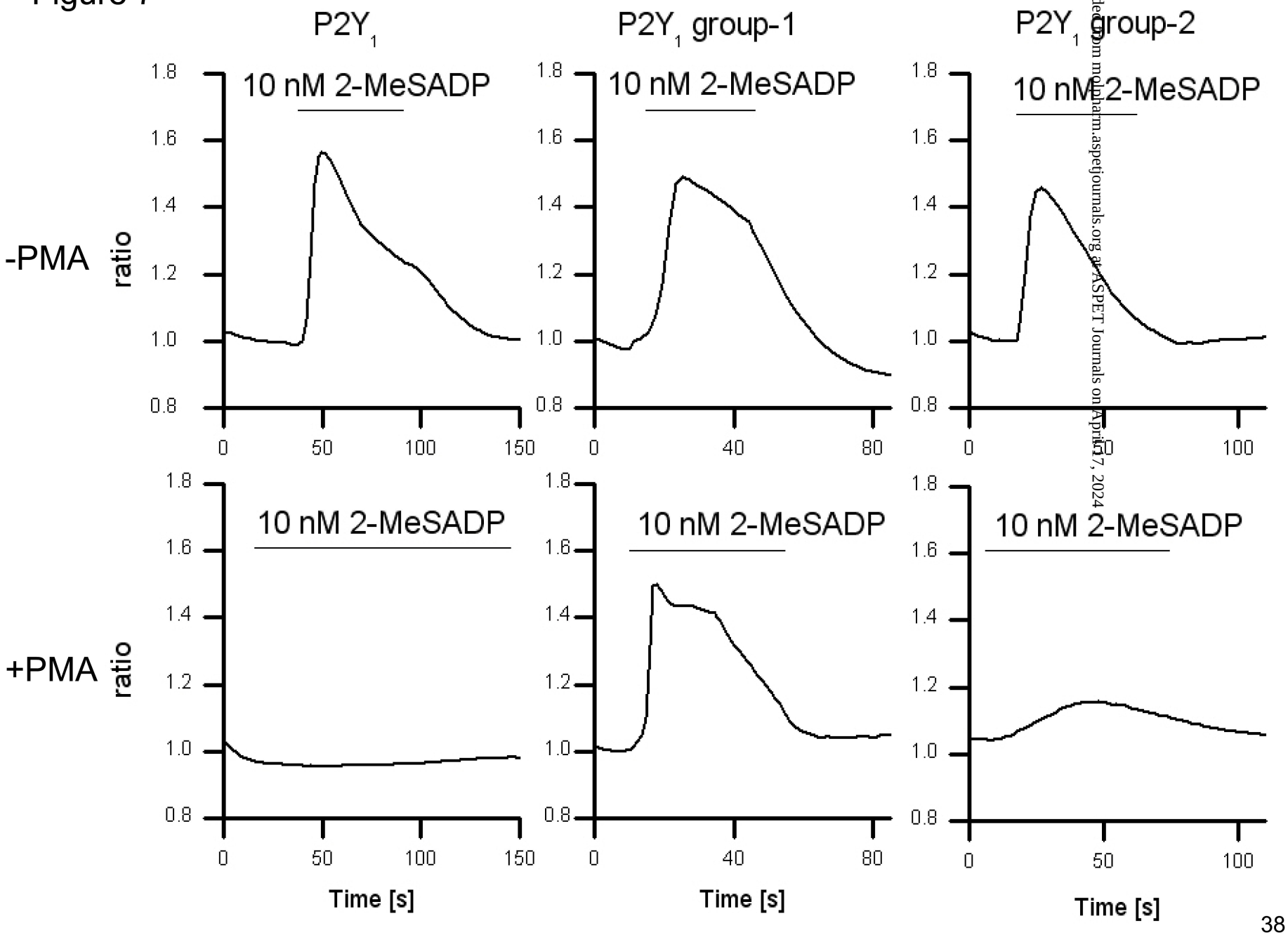


Figure 8

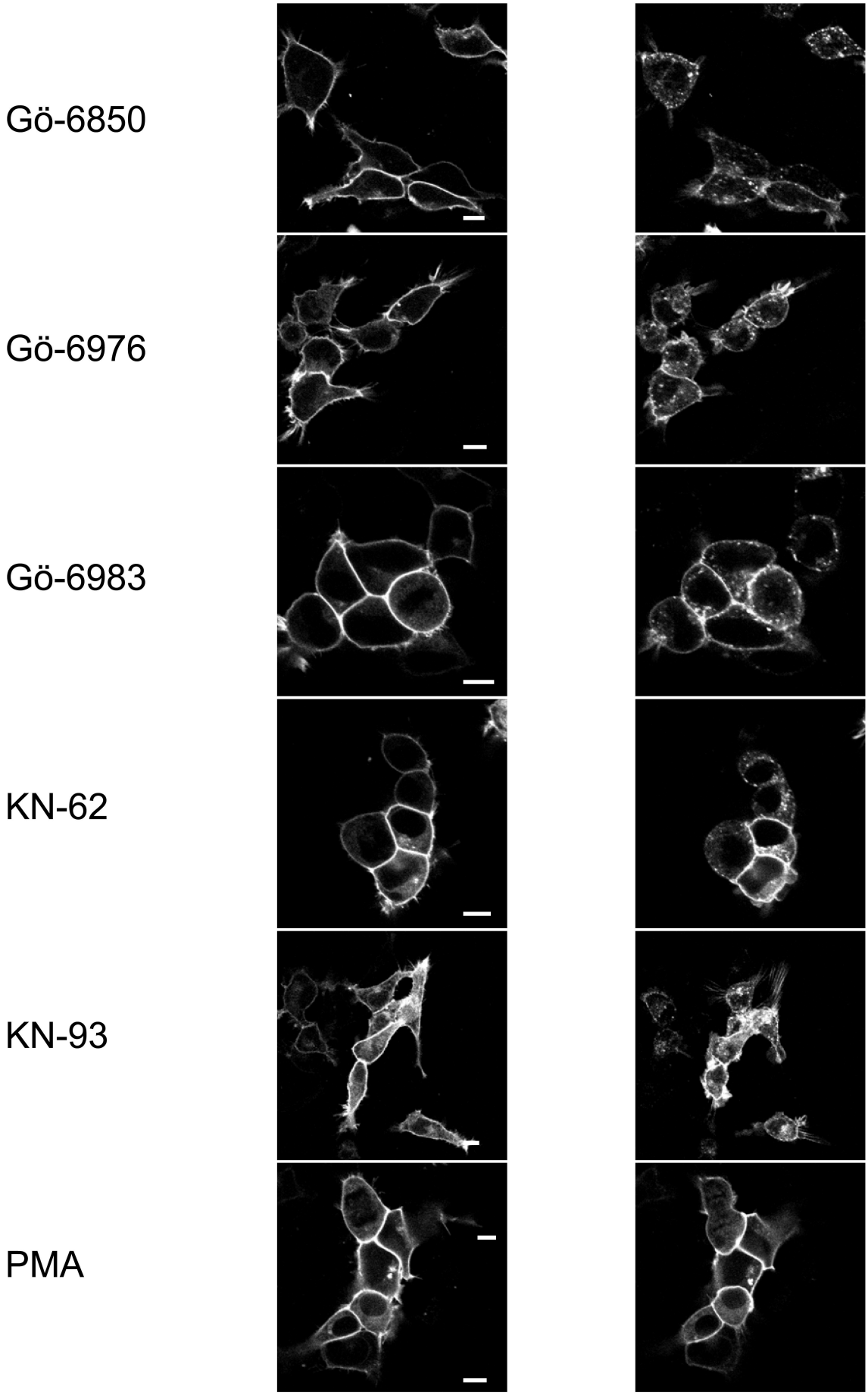


Figure 9