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β-Arrestin-2 Interaction and Internalization of the Human $P2Y_1$ Receptor Are Dependent on C-Terminal Phosphorylation Sites

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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 that 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 human embryonic kidney (HEK)-293 cells and stimulated with 100 μM ADP. Rapid receptor internalization was observed for the wild-type receptor and from 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 enzyme-linked immunosorbent assay. $P2Y_1$-receptor stimulated β-arrestin-2-yellow fluorescent protein (YFP) translocation followed the same pattern as receptor internalization. Hence, no β-arrestin-2-YFP translocation was observed when the distal C-terminal phosphorylation sites were mutated. Individual mutations indicate that residues Ser352 and Thr358 are essential for receptor internalization and β-arrestin-2-YFP translocation. In contrast, protein kinase C (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 calmodulin-dependent protein kinase. Thus, we conclude that $P2Y_1$-receptor desensitization and internalization are mediated by different phosphorylation sites and kinases.

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 G_q-proteins, whereas the second group consisting of the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptor mainly couples to G_i-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 (Abbracchio et al., 2006; Gachet, 2006). Besides expression in platelets, the $P2Y_1$ receptor is expressed in epithelial and endothelial cells and in immune cells and osteoclasts; thus, the receptor offers a diverse therapeutic potential (Jacobson et al., 2002; Abbracchio et al., 2006; Burnstock, 2006). Significant mutagenesis efforts have led to a detailed characterization of the ligand binding site of the $P2Y_1$ receptor (Jiang et al., 1997;

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; 2-MeSADP, 2-methylthio-adenosine 5′-diphosphate; CaM, calmodulin-dependent; DMEM, Dulbecco's modified Eagle's medium; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; eCFP, enhanced cyan 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}; GRK, G protein-coupled receptor kinase; 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; PBS, phosphate-buffered saline; ANOVA, analysis of variance; HEK, human embryonic kidney; HA, hemagglutinin; ELISA, enzyme-linked immunosorbent assay.

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 a complex series of reactions that 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 (Ferguson, 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 and Shenoy, 2005; Gurevich and Gurevich, 2006). In platelet aggregation, ADP has been shown to be an important mediator. However, once stimulated with ADP, platelets become nonresponsive to a second stimulus with the same ligand. This process is transient because platelets fully recover after 15 to 30 min. This effect was shown to be mediated 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; Mundell et al., 2006a,b; Tulapurkar et al., 2006; 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 (Ferguson, 2001; Tobin, 2008), alternative routes of GPCR internalization, which are phosphorylation-independent, have been described by several groups (Ferguson, 2007). The $P2Y_1$ receptor has been shown to incorporate radioactive phosphate upon agonist stimulation (Mundell 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. Although 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 that 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.

Materials and Methods

Materials. ADP, 2-MeSADP, and poly(D-lysine) were purchased form Sigma (Steinheim, Germany). All kinase inhibitors were purchased from Calbiochem (Schwalbach, Germany). 2,2′-Azino-di-[3-ethylbenzthiazoline sulfonate(6)] was from Roche Diagnostics GmbH (Mannheim, Germany). Cell culture reagents were supplied by PAN-Biotech GmbH (Aidenbach, Germany). Effectene was purchased from QIAGEN (Hilden, Germany). cDNA for the human $P2Y_1$ receptor has been described previously (Hoffmann et al., 1999). All polymerase chain reaction 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, Germany), the horseradish peroxidase-conjugated polyclonal goat anti-rabbit antibody was purchased from Dianova (Hamburg, Germany). 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. P2Y receptor constructs were fused to the enhanced variants of cyan (CFP), green (GFP), or yellow (YFP) fluorescent protein (Clontech, Heidelberg, Germany) by standard polymerase chain reaction 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 were confirmed by sequencing.

The constructs are shown in Fig. 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), whereas for 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 article, we used bovine β-arrestin-2 fused C-terminally to enhanced YFP as described previously (Krasel et al., 2005).

Cell Culture. HEK-293 cells were maintained in DMEM with 4.5 g/l glucose, 10% fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37°C, 7% CO_2. All cells were routinely passaged every 2 to 3 days. Culture medium for cells stably expressing the individual HA-$P2Y_1$-CFP receptor constructs was additionally supplemented with 200 μg/ml G418.

Transfection of HEK-293 Cells for Microscopic Analysis. Individual 24-mm glass coverslips were placed in six-well plates and coated for 30 to 60 min using 300 μl of poly-(D-lysine) (1 mg/ml). Poly-(D-lysine) was aspirated, and the glass coverslips were washed once with sterile PBS without Ca^{2+}. HEK-293 cells were seeded onto these coverslips to result in approximately 50% confluence. After attachment of the cells (4–6 h), 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, and 200 ng for β-arrestins. All constructs were in pcDNA3; the amount of DNA was adjusted using empty pcDNA3 vector. Medium was exchanged 12 to 16 h later, and cells were analyzed 48 h after transfection.

Confocal Microscopy. All confocal microscopy experiments were performed on a Leica TCS SP2 system (Leica, Wetzlar, Germany). Coverslips with transfected HEK-293 cells were mounted using an "Attofluor" holder (Invitrogen, Leiden, The Netherlands). Images were taken with a 63× objective lens as described previously (Hoffmann et al., 2008). In brief, CFP was excited with a 430-nm diode laser using a DCLP455 dichroic mirror. GFP was excited using the 488-nm line of an argon laser and a DCLP500 dichroic mirror. YFP was excited with the 514-nm line of the argon laser and a dual beam-splitter 458/514 nm. Settings for recording images were kept constant: 512 × 512-pixel format, line average 4, 400 Hz, resulting in an image acquisition time of 7 s. Time series were recorded using the standard Leica software package (version 2.61). Pictures were taken at 1-min 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 ROIs 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 published previously (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 24 h to approximately 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 4°C for 2 h. 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 at 37°C. 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 four times with PBS, and were blocked in a solution of PBS containing 1 mM $CaCl_2$ and 3% bovine serum albumin for 1 h at room temperature. The cells were incubated with secondary horseradish peroxidase-conjugated polyclonal goat anti-rabbit antibody (1:2500) diluted in PBS supplemented with 1 mM $CaCl_2$ and 1% bovine serum albumin at room temperature. After four additional washes with PBS containing 1 mM $CaCl_2$, antibody binding was detected by addition of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate(6)]. After 25-min incubation at room temperature, 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 Experiments Using a Probe for PKC Activity. Fluorescence imaging of FlAsH-labeled CFP-KCP-2-Flash probe was performed as described previously (Jost et al., 2008) on a Zeiss Axiovert 135 inverted microscope equipped with a Zeiss PlanNeofluar 100x/1.3 oil objective at room temperature (Carl Zeiss GmbH, Jena, Germany). FlAsH labeling 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, Gräfelfing, Germany). 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) and beam-splitter dclp 505 nm. Signals detected by avalanche photodiodes were digitized using an analog-to-digital converter (Digidata1322A; Molecular Devices, Sunnyvale, CA) and stored on a personal computer using Clampex 8.1 software (Molecular Devices).

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

Determination of Intracellular Ca^{2+} Increase. 1321N1 cells were transfected with either HA-$P2Y_1$-wild-type, HA-$P2Y_1$-Gr2, or HA-$P2Y_1$-ST/AA mutant receptor cDNA using the Amaxa Nucleofactor Kit V according to the manufacturer's instructions (Amaxa Biosystems, Gaithersburg, MD). Transfected cells were seeded onto poly(D-lysine)-coated coverslips in six-well plates and grown to approximately 70% confluence in DMEM. To load cells with the calcium indicator, cells were incubated for 35 min at 37°C in loading buffer (10 mM HEPES, 140 mM NaCl, 5,4 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$) supplemented with Fura-2 acetoxymethyl ester (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 Attofluor holder and placed on an inverted fluorescence imaging

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	

Fig. 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 boldface letters as potential phosphorylation sites. In the subsequent mutant constructs, changes to alanine that were made compared with the wild-type receptor, are highlighted in boldface type. Missing sequences represent truncation of the receptor.

microscope Nikon TE-2000U (dichroic mirror: 455DCLP; filter set: 340 and 380 nm excitation filters and 520/20 emission filter; Nikon, Tokyo, Japan). Cells were perfused with measuring buffer (10 mM HEPES, 140 mM $NaCl_2$, 5,4 mM KCl, 1 mM $MgCl_2$, and 5 mM EGTA) or stimulated with 10 nM 2-MeSADP diluted in measuring buffer. Cells were measured with or without preincubation with 500 nM PMA for 5 min. To detect the elicited Ca^{2+} response in single cells, the Fura-2 fluorescence in defined ROIs was recorded using MetaMorph/MetaFluor imaging acquisition and analysis software (Molecular Devices). Data are expressed as the ratio of Fura-2 emission when excited at 340 and 380 nm (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 six-well plates. Forty-eight hours after transfection, cells were labeled with 400 μCi/well of [^{32}P]orthophosphate in phosphate-free DMEM for 2 h at 30°C. Labeled cells were stimulated or not with 10 nM 2-MeSADP for 7 min. Cells were solubilized on ice for 30 min as described previously (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-polyacrylamide gel electrophoresis and quantified by PhosphorImager analysis (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

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 (Fig. 1). Sequence comparison of $P2Y_1$ receptors from six different species (available at http://www.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 Fig. 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, whereas 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

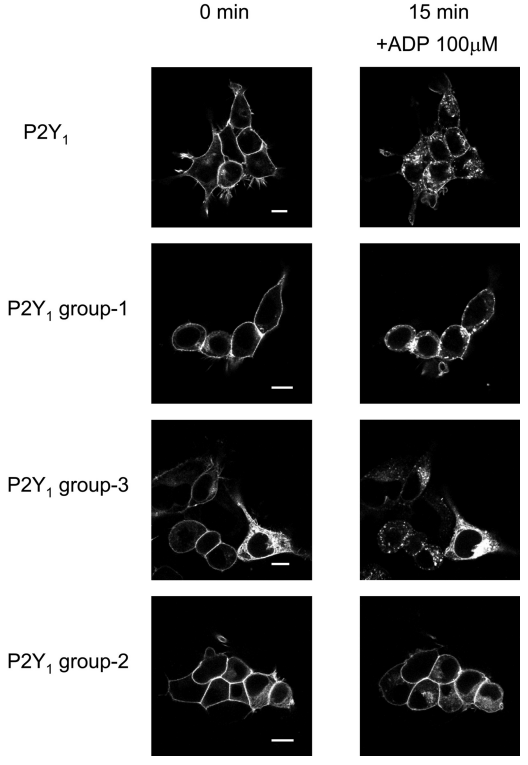

Fig. 2. Agonist-induced internalization of GFP-tagged $P2Y_1$ receptor constructs in HEK-293 cells. Cells were transfected with GFP-tagged $P2Y_1$ receptor constructs and studied for receptor internalization. The left column shows cells before stimulation with ADP. The right column represents the same cells 15 min after ADP exposure (100 μM final concentration). Data are representative examples of at least five individual experiments. Scale bars, 10 μm.

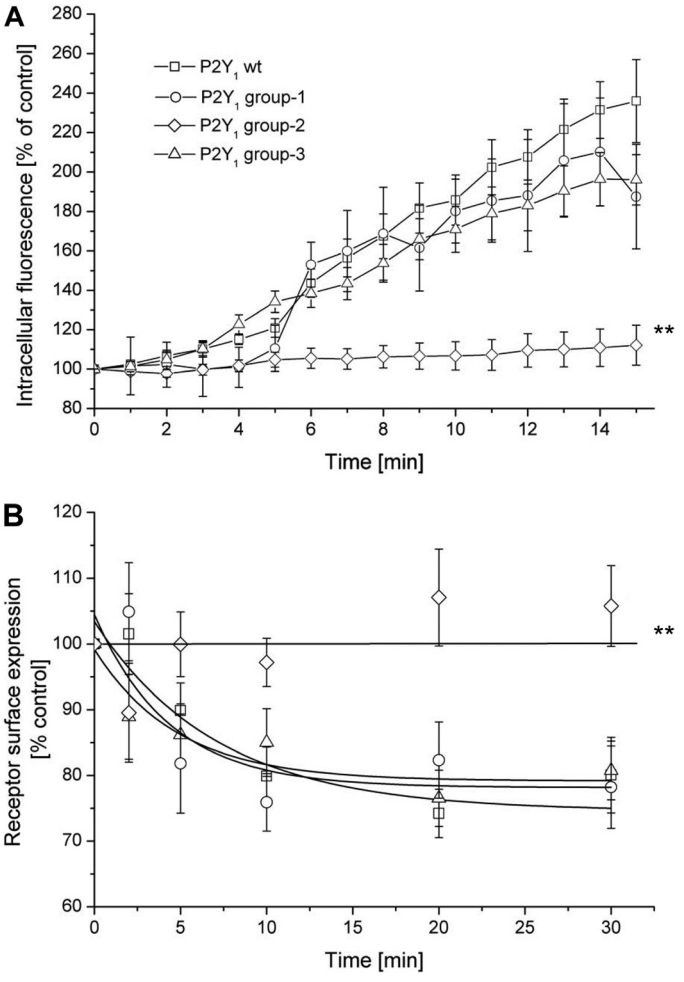

Fig. 3. Quantification of receptor internalization. A, experiments as shown in Fig. 2 were quantified as described under *Materials and Methods*. Data from at least five different experiments were analyzed for each construct. The increase of intracellular fluorescence was calculated as the percentage 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. □, wild-type $P2Y_1$ receptor; ○, $P2Y_1$ group 1; △, $P2Y_1$ group 3; ◇, $P2Y_1$ group 2 mutant. Statistical analysis was performed using a one-way ANOVA, **, $p < 0.01$. 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 more than six different experiments performed in triplicate were analyzed for each construct. The decrease in surface-expressed HA-tagged $P2Y_1$ receptor constructs was calculated as the percentage 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. □, wild-type $P2Y_1$ receptor; ○, $P2Y_1$ group 1; △, $P2Y_1$ group 3 mutant; ◇, $P2Y_1$ group 2 mutant. Statistical analysis was performed using a one-way ANOVA, **, $p < 0.01$.

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 (Fig. 2). Upon stimulation with 100 μM ADP, the wild-type receptor rapidly internalized, as depicted by the appearance of an intracellular punctate pattern. $P2Y_1$ group-1 and $P2Y_1$ group-3 mutant receptors also exhibited rapid internalization upon agonist stimulation (Fig. 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 Fig. 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 that 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, and remaining surface expression of the HA-tagged receptors was quantified as described under *Materials and Methods*. The data are shown in Fig. 3B. A loss of surface expression of wild-type, $P2Y_1$ group 1, and $P2Y_1$ group 3 receptors was readily detectable, whereas 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, because essentially no internalization could be observed for the $P2Y_1$ receptor lacking potential phosphorylation sites at the distal C terminus. Therefore, this group of phosphorylation sites seems 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 11 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 Fig. 4. Again, mutation of all five residues at the same time ($P2Y_1$ group 2) blocked receptor internalization (Fig. 4, A and B), whereas the data for individual mutations of residues S354A, T371A, and S372A indicate that receptor internalization still occurred upon agonist stimulation (Fig. 4, A or B). However, quantification of intracellular fluorescence showed a significant

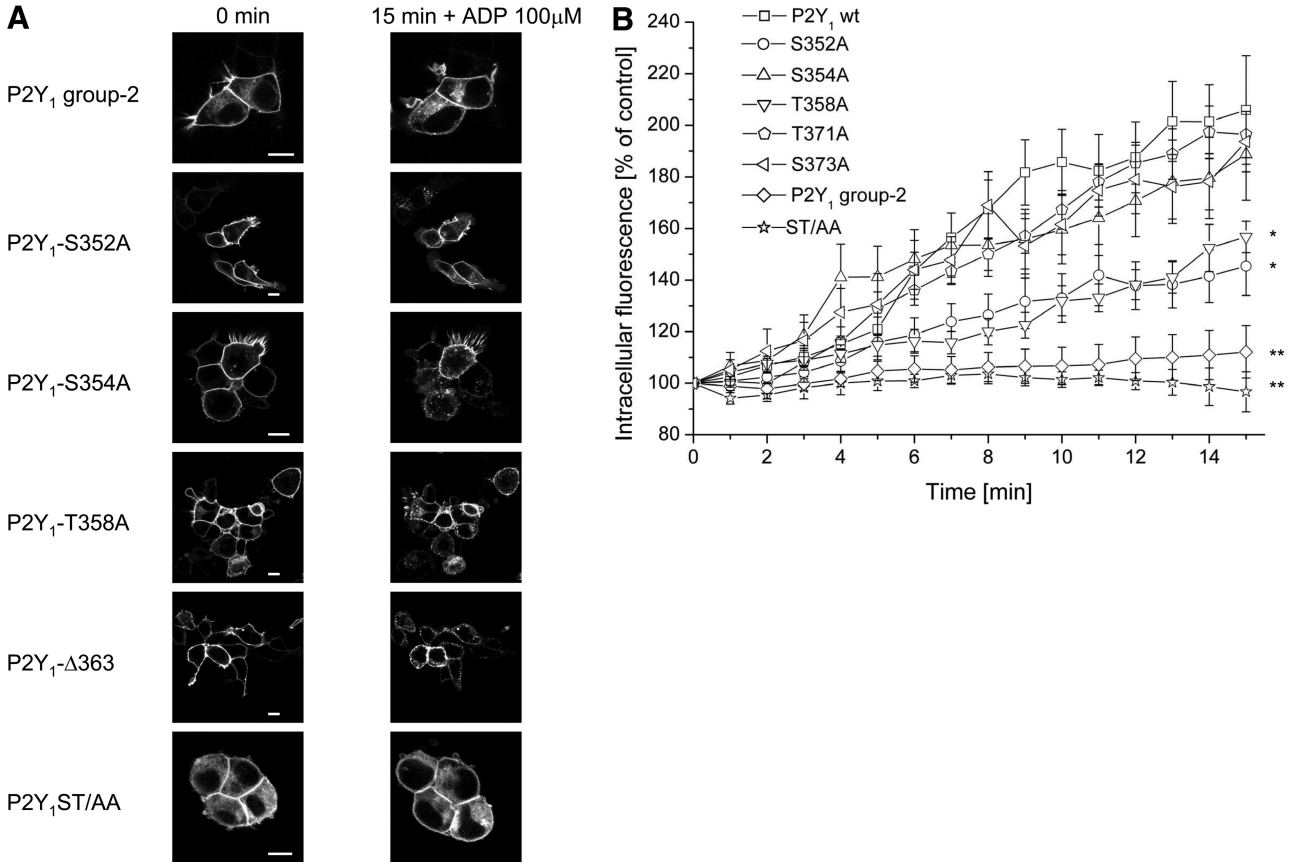

Fig. 4. Mutational analyses of the $P2Y_1$ distal C terminus in HEK-293 cells. A, cells were transfected with GFP-tagged $P2Y_1$ receptor constructs as depicted in Fig. 1. Cells were stimulated with ADP (100 μM final concentration) and studied for receptor internalization. The left column represents cells before agonist stimulation. The right column shows the same cells 15 min after agonist exposure. Data are representative examples of at least four individual experiments. Scale bars, 10 μm. B, quantification of receptor internalization of individual point mutants. Experiments were quantified as described previously (see Fig. 3A). Data from at least four different experiments were analyzed for each construct. The increase of intracellular fluorescence was calculated as the percentage increase above control and plotted against time. Individual traces represent the mean ± S.E.M. of more than 12 different cells from 4 independent experiments. Open squares, wild-type $P2Y_1$ receptor; open triangles, the S354A mutant; open pentagon, the T371A; open triangles left, the S372A; open circles, the S352A; open inverted triangles, the S358A mutant; open stars, the double mutant ST/AA; open diamonds, the group 2 mutant. Statistical analysis was performed using a one way ANOVA test; *, $p < 0.05$; **, $p < 0.01$.

reduction in receptor internalization for the mutant receptors S352A and T358A, but internalization was not fully blocked (Fig. 4, A and B). 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 Thr371, Ser372, and the C-terminal PDZ domain (Fam et al., 2005), had very little influence on receptor internalization (Fig. 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 (Fig. 4, A and B). Thus, potential phosphorylation of serine 352 and threonine 358 sites seem to play a role in the internalization of the $P2Y_1$ receptor.

We have reported recently that the $P2Y_1$ receptor translocates β-arrestin-2 to the plasma membrane upon stimulation with ADP (Hoffmann et al., 2008). Because 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 cotransfected with CFP-tagged group mutant receptors and β-arrestin-2-YFP. Pictures of the CFP-tagged receptors were taken before agonist stimulation to verify receptor expression. Upon stimulation with ADP, a rapid translocation of β-arrestin-2-YFP was observed in cells co-

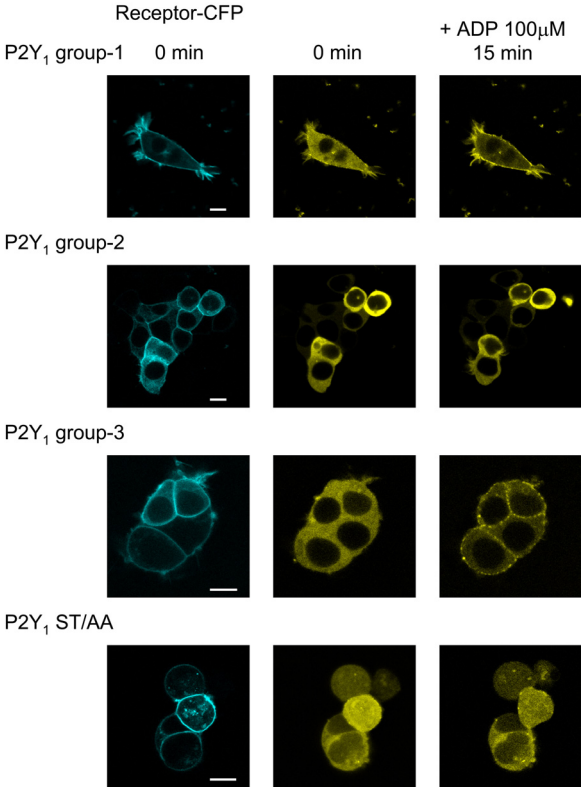

Fig. 5. β-Arrestin-2 translocation induced by stimulation of $P2Y_1$ receptor constructs. HEK-293 cells were cotransfected with the indicated $P2Y_1$ receptor-CFP and the β-arrestin-2-YFP construct. The left column shows localization of the CFP-tagged receptor construct before stimulation to demonstrate that the cells were cotransfected with both β-arrestin-2 and receptor. The middle column represents cells before ADP stimulation, whereas the right column shows the same cells 15 min after exposure to ADP (100 μM final concentration). A clear β-arrestin-2 translocation was observed for the $P2Y_1$ group 1 and $P2Y_1$ group 3 receptor constructs, whereas 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 four individual experiments. Scale bars, 10 μm.

transfected with $P2Y_1$ group 1 or $P2Y_1$ group 3 mutant receptors (Fig. 5). In contrast, although receptor expression was verified, no β-arrestin-2-YFP translocation was observed in cells that were either cotransfected with the $P2Y_1$ group 2 mutant receptor or the double-mutant receptor $P2Y_1$ ST/AA (Fig. 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 with that of the wild-type $P2Y_1$ receptor. The right portion of Fig. 6 shows that all three receptor constructs exhibit little phosphorylation under basal conditions. Upon agonist stimulation with 2-MeSADP for 7 min, only the wild-type $P2Y_1$ receptor showed a significant increase in receptor phosphorylation. Quantitative analysis shows that agonist-stimulated phosphorylation of the mutant receptors ($P2Y_1$ group 2 and $P2Y_1$ ST/AA) was impaired compared with wild-type $P2Y_1$ receptor (Fig. 6, left).

Because 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+} response. Because 1321N1 astrocytoma cells do not express endogenous P2Y receptors, they were chosen for the study of Ca^{2+} responses (Lazarowski et al., 1997). 1321N1 cells transfected with the appropriate receptor constructs were stimulated with 2-MeSADP, and as shown in Fig. 7 (top row), all constructs exhibited similar Ca^{2+} response. However, when cells were preincubated with the PKC activator PMA (5 min) 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 (Fig. 7, bottom row).

Based on studies using different kinase inhibitors (Mundell et al., 2006a; Tulapurkar et al., 2006), 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 $P2Y_1$-

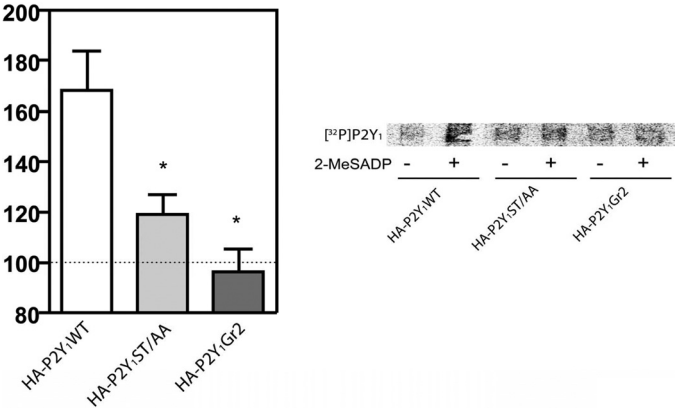

Fig. 6. Phosphorylation of $P2Y_1$ receptor constructs. HEK-293 cells transfected with the indicated $P2Y_1$ receptor-CFP constructs were labeled with [^{32}P]orthophosphate in phosphate-free DMEM and stimulated with 2-MeSADP as described. The agonist-induced phosphorylation was quantified for each construct (left). The results are the mean ± S.E.M. of four independent experiments compared with basal (*, $p < 0.05$; one-way ANOVA). A representative experiment is shown at right.

receptor internalization. Cells were transfected with wild-type $P2Y_1$ receptor fused to GFP. Before stimulation with ADP, cells were incubated for 10 min with different kinase inhibitors at 1 to 10 μM concentrations. Subsequent stimulation with ADP led to visible receptor internalization for each inhibitor tested (Fig. 8). Consistent with this notion, we observed that the addition of PMA did not lead to receptor internalization (Fig. 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 been described previously 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 the addition of 200 nM PMA led to a rapid change in the FRET ratio, whereas subsequent addition of 500 nM Gö-6983 fully reversed the effect (Fig. 9). This indicates that the used concentrations of inhibitor and 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.

Discussion

In this article, 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 focused our interest on β-arrestin-2, because we have shown previously 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; Mundell et al., 2006a,b; Tulapurkar et al., 2006; Hoffmann et al., 2008), and this has been shown to be relevant for the nonresponsiveness of platelets toward 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 Fig. 1. All receptor constructs were expressed at the cell surface in HEK-293 cells (Fig. 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 and a cell surface ELISA. Both techniques showed a total lack of internalization for the

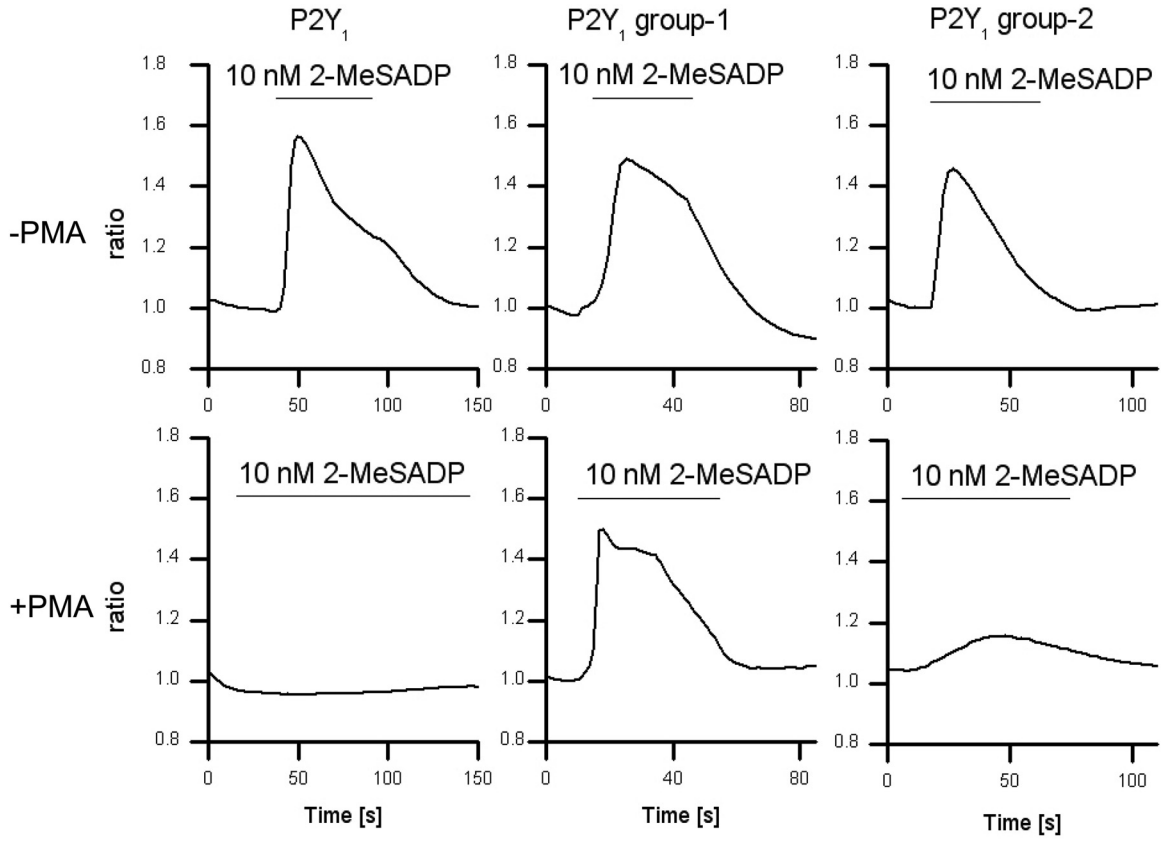

Fig. 7. Ca^{2+} response of $P2Y_1$ receptor constructs. 1321N1 astrocytoma cells were transfected with the indicated $P2Y_1$ receptor-CFP constructs and loaded with the fluorescent Ca^{2+}-sensitive dye Fura-2 acetoxymethyl ester as described. The top row shows Ca^{2+} traces recorded for individual cells when cells were stimulated with 10 nM 2-MeSADP. The bottom row shows Ca^{2+} traces recorded for individual cells when cells were preincubated for 5 min with 500 nM PMA and stimulated with 10 nM 2-MeSADP. Traces are representative for at least nine cells of three independent transfections.

$P2Y_1$ group 2 construct, whereas the other constructs internalize similarly to wild-type receptor (see Figs. 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 an agonist-stimulated increase in intracellular Ca^{2+} (Fig. 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 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 Ser354, Thr371, and Ser372 (Fig. 4).

Because agonist-stimulated receptor phosphorylation was significantly reduced in the $P2Y_1$ group 2 and $P2Y_1$ ST/AA construct compared with the wild-type P2Y1-receptor (Fig. 6), we conclude that the lack of phosphorylation sites Ser352 and Thr358 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 (Ferguson, 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), whereas 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). Because 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, in which several residues need to be phosphorylated to recruit arrestin or β-arrestin to the receptor (Vishnivetskiy et al., 2007, Krasel et al., 2008). It is interesting that different GPCRs seem to use different domains or mechanisms to interact with β-arrestin-2. For the thyrotropin-releasing hormone receptor, it has been described recently that phosphorylation 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 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, because our data demonstrate that the mutation of potential phosphorylation sites in the distal C terminus fully prevented β-arrestin-2 translocation, whereas truncation of the C terminus adjacent to the important sites ($P2Y_1$-Δ363) had no influence on internalization, although

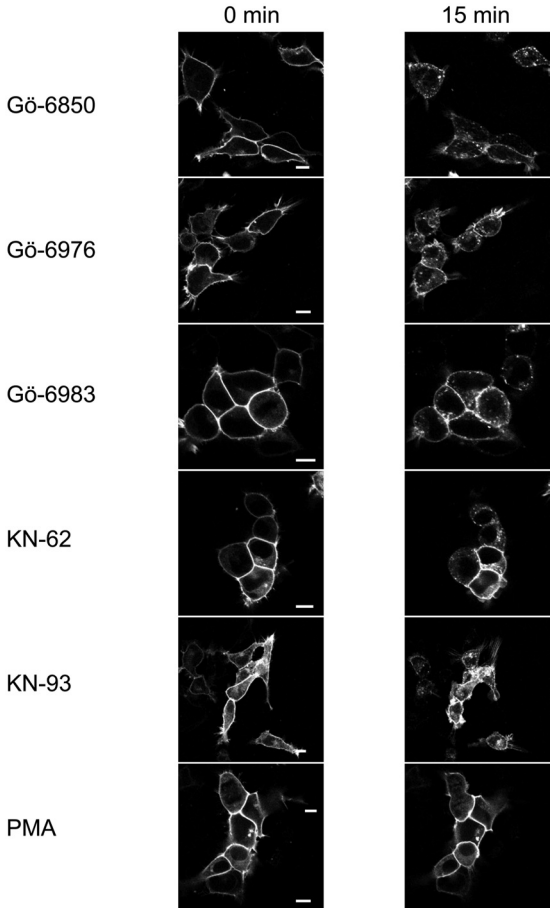

Fig. 8. Effect of different kinase inhibitors on the internalization of GFP-tagged $P2Y_1$ receptor in HEK-293 cells. Cells were transfected with GFP-tagged wild-type $P2Y_1$ receptor and studied for receptor internalization. Cells were treated with the PKC inhibitors Gö-6850, Gö-6976, and Gö-6983 (1 μM final); the CaM-kinase II inhibitors KN-62 and KN-93 (10 μM final); or PMA (500 nM final). After 10-min preincubation, cells were stimulated with ADP (100 μM final). The left column represents cells 10 min after preincubation 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 four individual experiments. Scale bars, 10 μm.

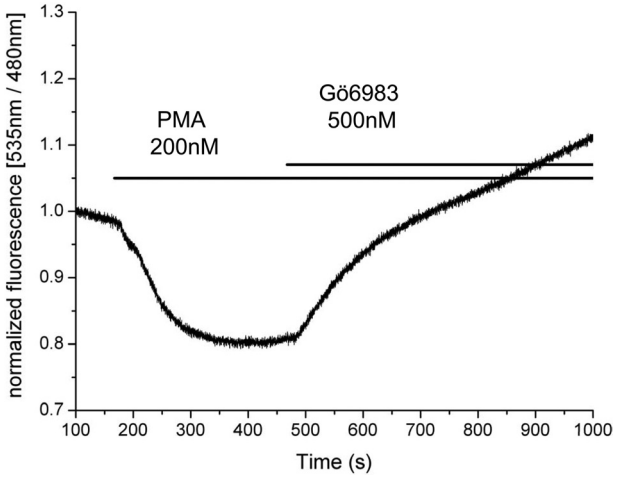

Fig. 9. Monitoring PKC activity in intact HEK-293 cells by FRET. HEK-293 cells were transfected with a FRET-probe named CFP-KCP-2-Flash and monitored for PKC activity by changes in FRET. Cells were excited at 436 nm, and fluorescence emission was measured at 480 and 535 nm wavelength. The recorded emission was corrected for bleed-through and photobleaching as described and calculated as FRET ratio (535/480 nm). Cells were incubated with 200 nM PMA to stimulate PKC activity, and after 5-min incubation, cells were additionally exposed to 500 nM Gö-6983 to block PKC activity.

this domain has been shown recently to be important for receptor dimerization (Choi et al., 2008).

Different kinases have been investigated for their involvement in the phosphorylation process of the $P2Y_1$ receptor. A reduction of GRK-2 or GRK-6 expression levels by small interfering RNA was shown to have little effect on $P2Y_1$ receptor internalization (Hardy et al., 2005; Mundell et al., 2006b), whereas 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 Figs. 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 with data published previously could be explained by the use of different cell lines, a phenomenon that 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 Fig. 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 behavior. Consistent with the finding published by Fam and colleagues (2003), the wild-type $P2Y_1$ receptor desensitized upon pretreatment with PMA. Interestingly, the $P2Y_1$ group 1 did not desensitize (but internalized), whereas the $P2Y_1$ group 2 construct showed desensitization comparable with wild-type (but did not internalize). Thus, $P2Y_1$ receptor desensitization and internalization seem to be differentially controlled by different phosphorylation sites and possibly different kinases. Similar observations have been reported recently 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, because 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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