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Structural Basis of Agonist-induced Desensitization and Sequestration of the $P2Y_2$ Nucleotide Receptor

CONSEQUENCES OF TRUNCATION OF THE C TERMINUS*

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Molecular determinants of $P2Y_2$ receptor desensitization and sequestration have been investigated. Wild-type $P2Y_2$ receptors and a series of five C-terminal truncation mutants of the receptor were epitope-tagged and stably expressed in 1321N1 cells. These constructs were used to assess the importance of the intracellular C terminus on 1) UTP-stimulated increases in intracellular calcium concentration, 2) homologous desensitization of the receptor, and 3) agonist-induced decreases in cell-surface density (receptor sequestration) of epitope-tagged receptors using fluorescence-activated cell sorting. The potency and efficacy of UTP were similar for the wild-type and all mutant $P2Y_2$ receptors. Truncation of 18 or more amino acids from the C terminus increased by ~30-fold the concentration of UTP necessary to desensitize the receptor. Both the rate and magnitude of UTP-induced receptor sequestration were decreased with progressively larger truncations of the C terminus. Furthermore, the recovery from sequestration was slower for the most extensively truncated receptor. Complete desensitization was obtained with >50% of the original receptor complement remaining on the cell surface. Protein kinase C activation, which desensitizes the $P2Y_2$ receptor, had no effect on sequestration, consistent with the ideas that desensitization and sequestration are discrete events and that agonist occupancy is required for receptor sequestration.

Responses to extracellular nucleotides are mediated by P2 receptors that belong to two receptor superfamilies: P2Y G protein-coupled receptors (GPCRs)[1] and P2X ligand-gated ion channels. Multiple P2Y subtypes have been classified pharmacologically and molecularly and are predominantly linked to activation of phospholipase C and increased levels of inositol 1,4,5-trisphosphate and diacylglycerol, leading to elevations in the intracellular free calcium concentration ($[Ca^{2+}]_i$) and the activation of protein kinase C (PKC) (1–4). The $P2Y_2$ nucleotide receptor subtype (formerly the P_{2U} receptor) is distinguished pharmacologically from the other known mammalian P2Y receptor subtypes by the equal potency and efficacy of the naturally occurring agonists ATP and UTP.

Activation of $P2Y_2$ receptors present in airway epithelium increases Cl^- secretion through Ca^{2+}-dependent and outwardly rectifying Cl^- channels (5). It has been shown that $P2Y_2$ receptor activation by UTP in airway epithelia of cystic fibrosis patients can increase Cl^- secretion, thereby effectively bypassing the defective cAMP-dependent Cl^- transport (6). Like other members of the GPCR superfamily, $P2Y_2$ receptors undergo agonist-induced desensitization (7), but little is known about the mechanisms involved in desensitization of the $P2Y_2$ receptor. It seems likely that a fuller understanding of desensitization and the signaling pathways that affect the $P2Y_2$ receptor may lead to improved therapies targeted to this receptor.

Desensitization of GPCRs is a complex process involving phosphorylation of the receptors by multiple protein kinases, including G protein-coupled receptor kinases, cAMP-dependent protein kinase, and PKC. The mechanisms of GPCR desensitization have been studied most extensively with the β_2-adrenergic receptor (reviewed in Ref. 8). The activation of β_2-adrenergic receptors by agonist results in G protein-coupled receptor kinase-mediated phosphorylation of serine and threonine residues in the carboxyl-terminal tail (9). The subsequent agonist-induced increase of cyclic AMP can result in the phosphorylation of additional residues within the β_2-adrenergic receptor by protein kinase A. Phosphorylation of β_2-adrenergic receptors leads to desensitization and sequestration of the receptors from the cell surface, where they can no longer bind hydrophilic ligands and apparently undergo dephosphorylation prior to recycling to the cell membrane (10–13). Prolonged exposure to agonist results in down-regulation of the receptor, which is a decrease in receptor number that requires new protein expression to reestablish receptor levels.

We investigated the role of the serine- and threonine-rich C terminus of the $P2Y_2$ receptor in agonist-induced receptor desensitization and sequestration. The C terminus of the $P2Y_2$ receptor contains two consensus phosphorylation sites for PKC (another site is present in the third intracellular loop) and possible sites for G protein-coupled receptor kinase-mediated phosphorylation. To determine the role of the C terminus in receptor desensitization and sequestration, truncation mutants of a $P2Y_2$ receptor cDNA encoding an epitope-tagged

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[1] The abbreviations used are: GPCRs, G protein-coupled receptors; $[Ca^{2+}]_i$, intracellular free calcium concentration; PKC, protein kinase C; PCR, polymerase chain reaction; HA, hemagglutinin; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.

protein were constructed by polymerase chain reaction (PCR). The truncated receptor cDNAs were expressed in a clonal human 1321N1 astrocytoma cell line devoid of P2Y nucleotide receptors. UTP-induced desensitization was compared in cells expressing mutant or wild-type $P2Y_2$ receptors. Cell-surface receptor density and agonist-induced sequestration in 1321N1 cells were assessed by immunofluorescence detection of the epitope-tagged receptors.

EXPERIMENTAL PROCEDURES

Mutagenesis, Epitope Tagging, and Subcloning—Wild-type murine $P2Y_2$ receptor cDNA was subcloned into the retroviral vector pLXSN at the *Eco*RI/*Bam*HI sites of the multiple cloning site. The open reading frame of the wild-type $P2Y_2$ receptor cDNA was modified to incorporate, at the amino terminus of the expressed protein, the hemagglutinin (HA) epitope (YPYDVPDYA) from influenza virus using PCR. The forward and reverse HA primers were 5′-gatcgtgaattctgatgtatccatatgatgttccagattatgctgcagcagacctggaaccctgg-3′ and 5′-gatcgtggatcccctgactgaggtgctatagccg-3′, respectively. The PCR solution contained primers (0.7 μM each), 10 μl of 10× Vent polymerase buffer (New England Biolabs Inc., Beverly, MA), 100 ng of template DNA, 2.5 units of Vent (exo^-) polymerase, 0.25 units of Vent (exo^+) polymerase, and 20 μl of dNTP mixture (0.2 mM dATP, dCTP, dTTP, and dGTP) in a final volume of 100 μl. The PCR parameters were as follows: 96 °C for 1 min, 62 °C for 1 min, and 72 °C for 2.5 min for 25 cycles. The PCR conditions were identical for creation of the five truncation mutants. In each case, the HA epitope was inserted using the forward HA primer and the following reverse primers: 5′-tgctagggatcccgctactatgtccgtcttgagtcgtcact-3′ (truncation mutant 1), 5′-tgctagggatcccgtcatcagtcgtcactactgactgacaaatc-3′ (truncation mutant 2), 5′-tgctagggatcccgctactaacttccagctggtgtggactc-3′ (truncation mutant 3), 5′-tgctagggatcccgtcatcacaaatctttcctcacagttctgttag-3′ (truncation mutant 4), and 5′-gcatcggatcctcatcagttaggcctgtgcaggccc-3′ (truncation mutant 5). After verification of the PCR amplification by agarose gel electrophoresis, the products were purified using a PCR Wizard kit (Promega, Madison, WI). The purified PCR products and pLXSN DNA were digested overnight with *Eco*RI and *Bam*HI and ligated together, followed by transformation of competent *Escherichia coli* and identification of positive clones (14). All mutant DNAs were sequenced on both strands to ensure that mutagenesis had occurred as predicted, using an ABI Prism automated sequencing apparatus (Perkin-Elmer) and fluorescence dideoxynucleotide technology.

Cell Culture—Human 1321N1 cells were transfected with HA-tagged murine $P2Y_2$ receptor cDNA ($P2Y_2$-1321N1 cells), using the pLXSN retroviral vector, as described previously (15). The $P2Y_2$-1321N1 cells were grown to a density of $\sim 5 \times 10^6$ cells/75-cm^2 flask in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml Geneticin (G418, Life Technologies, Inc.) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The day prior to use of the cells, the growth medium was replaced with G418-free medium.

Calcium Measurements—Changes in $[Ca^{2+}]_i$ were detected by dual-excitation spectrofluorometric analysis of $P2Y_2$-1321N1 cell suspensions loaded with fura-2 as described previously (16, 17). Cells were assayed in 10 mM Hepes-buffered saline (pH 7.4) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ unless indicated otherwise. Desensitization experiments were performed by incubating $P2Y_2$-1321N1 cell suspensions with varying concentrations of UTP for 5 min at 37 °C. The cells were pelleted in a microcentrifuge and resuspended in 2 ml of buffer. The EC_{50} value of UTP, determined during the concentration-response experiments (see Fig. 3), was used to re-challenge the cells. Concentration-response data were analyzed with the Prism curve fitting program (GraphPAD Software for Science, San Diego, CA).

Sequestration Assays—$P2Y_2$-1321N1 cells, grown to 80% confluency in 35-mm^2 dishes, were incubated with UTP (Amersham Pharmacia Biotech) or phorbol 12-myristate 13-acetate (PMA; Sigma) for the times and at the concentrations indicated in the figure legends. Control cells were incubated without UTP (to allow an estimation of the total cell-surface complement of $P2Y_2$ receptors) for 180 min.

The $P2Y_2$-1321N1 cells were washed with 2 ml of ice-cold Hepes-buffered saline (pH 7.4) and incubated at 4 °C for 1 h with gentle rocking in 1 ml of Hepes-buffered saline containing 10 μg of anti-HA antibody 12CA5 (Boehringer Mannheim) that was affinity-purified as follows. The antibody was added to a HiTrap protein G column (Amersham Pharmacia Biotech) equilibrated with 20 mM phosphate buffer (pH 7.5) and eluted with 1-ml fractions of 100 mM glycine (pH 2.75). The eluted fractions were immediately neutralized with 1 M Tris buffer (pH 8.5), dialyzed against phosphate-buffered saline (PBS), and stored for use at a protein concentration of 1.5 mg/ml as estimated by a modification (18) of the method of Lowry *et al.* (19).

Cells incubated with antibody 12CA5 were washed with 2 ml of Dulbecco's PBS and incubated in 1 ml of Dulbecco's PBS containing 10 μl of Fc-specific fluorescein isothiocyanate-labeled goat anti-mouse antibody (Sigma) in the dark at 4 °C with gentle rocking for 1 h. Control cells were incubated with primary or secondary antibody alone to detect nonspecific fluorescence. The $P2Y_2$-1321N1 cells were washed with 2 ml of Dulbecco's PBS and detached in 2 ml of Hepes-buffered saline (pH 7.4) containing 2 mM EDTA. The cells were centrifuged and resuspended in 1 ml of 1% (v/v) formaldehyde in PBS and incubated in the dark at 4 °C for 10 min.

Cells were centrifuged and resuspended in 0.5 ml of PBS and analyzed on an EPICS 753 flow cytometer (Coulter Corp., Hialeah, FL) with a 5-watt argon laser tuned at 488 nm using 150-milliwatt output. The instrument was optically aligned using DNA-Check fluorospheres (Coulter Corp.) with the coefficients of variation ≤2%. For each analysis, 10,000 cells were collected using a dual-parameter histogram of forward angle light scatter *versus* log 90° light scatter to gate out debris. Integral green fluorescence emission (525 band path filter) of fluorescein isothiocyanate-labeled cells was displayed as a single-parameter histogram on a three-decade log scale. Data analysis was performed using EASY2 software (Coulter Corp.). Fluorescence intensity was used as a measure of differences in receptor density on the cell surface for the various receptor constructs. The computer cursor was set at a point representing half the peak width at half the peak height, and the channel number of this position was noted as the fluorescence intensity of the histogram. Fluorescence intensity was measured after logarithmic amplification with the use of a three-decade log scale, but illustrated on a linear scale of 253 channels or 85.33 units/decade. Fluorescence intensity (FI) was converted to linear arbitrary units (LAU) using the following formula: $LAU = 10^{(FI/85.33)}$. LAU was corrected for fluorescence due to secondary antibody alone. Cells expressing wild-type or mutant $P2Y_2$ receptors that were not incubated with UTP but were incubated with antibody 12CA5 and secondary antibody were considered to have the maximum number of receptor molecules on the cell surface.

Calculation of Relative Receptor Densities—Cells expressing $P2Y_2$ receptors were diluted from 1:2 to 1:8 with nontransfected 1321N1 cells, and the amount of fluorescein isothiocyanate fluorescence was measured in a Spex dual-excitation/emission spectrofluorometer using 488 and 525 nm for the excitation and emission wavelengths, respectively. Determinations of total protein/ml of cells were made using a modification (18) of the method of Lowry *et al.* (19). A standard curve of fluorescence was constructed with HEK-293 cells heterologously expressing N-terminally HA-tagged β_2-adrenergic receptors (B_{max} = 500 fmol/mg of total cell protein (20)) diluted with nontransfected HEK-293 cells.

RESULTS

Five cDNAs encoding $P2Y_2$ receptors with different length C-terminal truncations (Fig. 1) were constructed by PCR and expressed in 1321N1 cells to determine the role of the C terminus in agonist-induced desensitization of receptor-mediated calcium mobilization and receptor sequestration. The most extensively truncated receptor (truncation mutant 5) lacks two of three protein kinase C consensus phosphorylation sites and potential sites of phosphorylation by G protein-coupled receptor kinase present in the intracellular domains of the wild-type $P2Y_2$ receptor.

Recombinant $P2Y_2$ Receptor Expression Levels in 1321N1 Cells—In the absence of an established method to detect $P2Y_2$ receptors on the cell surface, all receptor constructs were N-terminally tagged with an epitope of the HA antigen from influenza virus. The fluorescence intensities of cells expressing the epitope-tagged $P2Y_2$ receptor constructs were determined by flow cytometry, and the resulting scans were superimposed (Fig. 2). These data indicate similar fluorescence intensities for each receptor, with truncation mutant 3 and wild-type receptors representing the high and low values, respectively, of the range of receptor expression. Thus, the expression of the six receptor constructs was approximately the same, with a maximal 2-fold difference in receptor density (Table I). The number

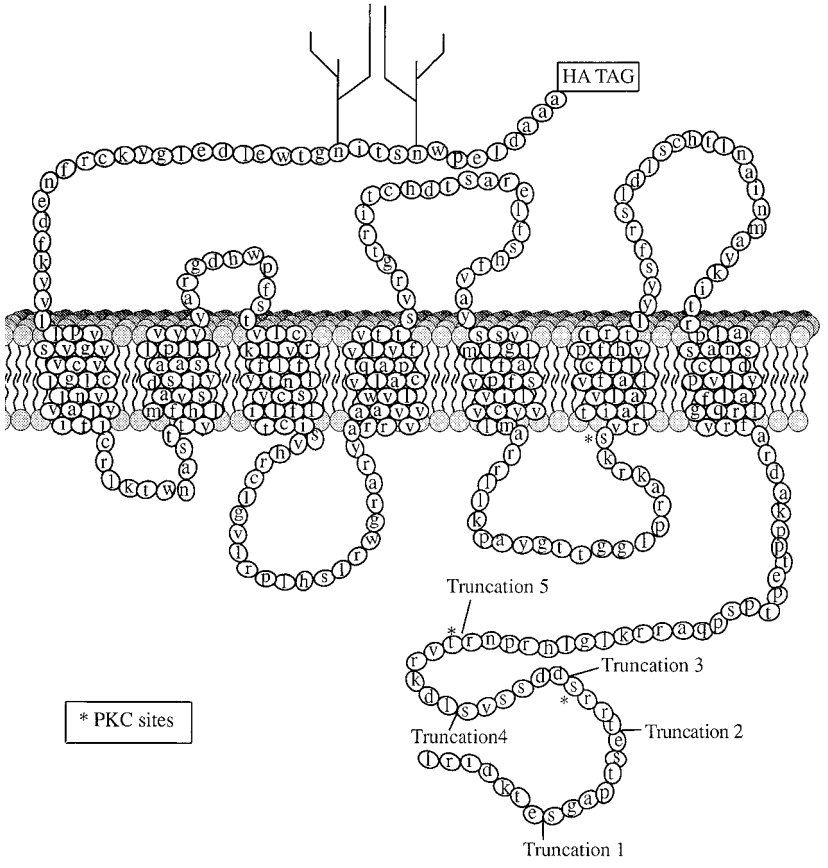

FIG. 1. **Predicted secondary structure of the wild-type P2Y$_2$ receptor and positions of truncation mutant receptors.** All recombinant receptors used in this study were N-terminally tagged with the HA epitope. The positions of the truncations made and the PKC consensus phosphorylation sites (*) are shown.

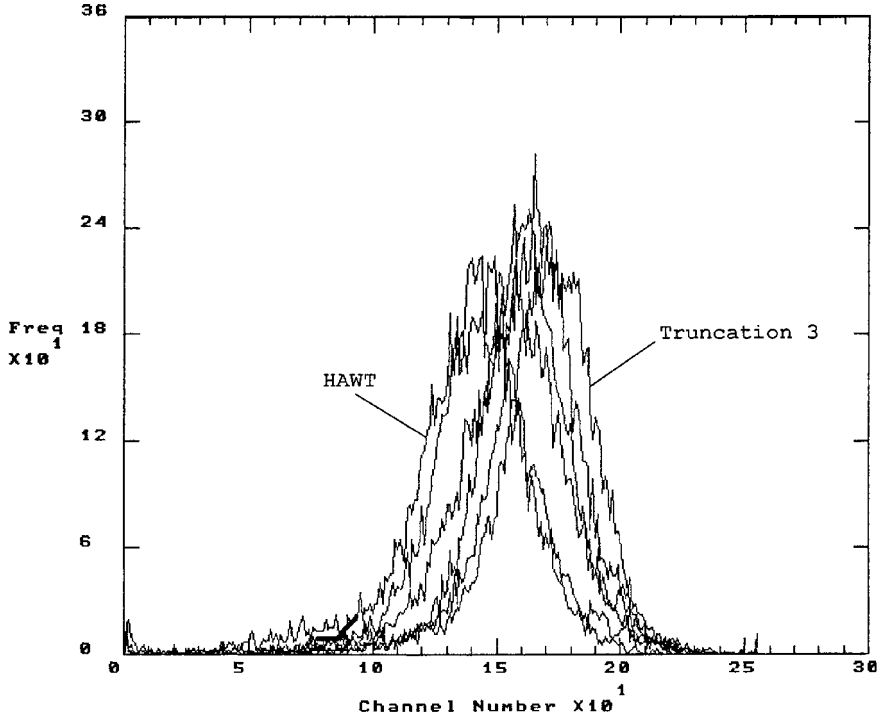

FIG. 2. **Expression levels of recombinant receptors.** Flow cytometry scans of HA-tagged wild-type (*HAWT*) and truncation mutant P2Y$_2$ receptors expressed in 1321N1 astrocytoma cells were superimposed. The wild-type and truncation mutant 3 receptors represent the low and high range, respectively, of expression levels for recombinant receptors in 1321N1 cells.

of HA-tagged wild-type P2Y$_2$ receptors in 1321N1 cells was estimated by comparison with the fluorescence intensity due to HA-tagged β_2-adrenergic receptors (500 fmol/mg of total protein) in HEK-293 cells (20). The β_2-adrenergic receptor density was determined to be 32,667 ± 6692 receptors/cell compared with 22,767 ± 9753 receptors/cell for the wild-type P2Y$_2$ receptor.

Progressive Truncations of the C Terminus Result in Altered Agonist Concentration-effect Curves for Receptor Desensitization—As shown in Fig. 3 (*A–F*), wild-type and truncated P2Y$_2$ receptors had similar concentration-effect curves for UTP-induced increases in $[Ca^{2+}]_i$. The similarity of the EC_{50} and maximal response values with UTP for the six P2Y$_2$ receptor constructs (Fig. 3 and Table I) indicates that the truncations had no marked effect on receptor activation and signaling when the various constructs were expressed at approximately the

TABLE I

EC_{50}/IC_{50} values of agonist-induced activation and desensitization of $[Ca^{2+}]_i$, together with receptor expression and estimated $t_{1/2}$ values and maximum levels of receptor sequestration

Concentration responses (EC_{50} and IC_{50}) were determined from the data collected in Fig. 3 (*A–F*). Normalized receptor expression levels were calculated by determining the ALI ($n = 3$) for each receptor construct and comparing each value to the wild-type receptor, which was regarded as 1. Estimated $t_{1/2}$ values of sequestration were from Fig. 4 (*A–F*). Maximal receptor sequestration (the percentage of receptor complement removed from the cell surface) was determined from the data used to construct Fig. 4 (*A–F*).

Recombinant $P2Y_2$ receptors	Concentration-response EC_{50} of UTP (activation)	Concentration-response IC_{50} of UTP (desensitization)	Normalized receptor expression (wild-type = 1)	Estimated $t_{1/2}$ values of sequestration	Maximal receptor sequestration after 180 min
	μM			*min*	*%*
Wild-type	0.25 ± 0.03	0.43 ± 0.1	1.00 ± 0.10	<5	90 ± 1
Truncation 1	0.47 ± 0.07	1.23 ± 0.8	1.07 ± 0.15	5–10	90 ± 1
Truncation 2	0.61 ± 0.23	3.37 ± 1.8	1.95 ± 0.18	45	80 ± 1
Truncation 3	0.05 ± 0.01	7.78 ± 4.8	2.00 ± 0.15	60	80 ± 2
Truncation 4	0.10 ± 0.02	12.3 ± 3.3	1.09 ± 0.17	150	50 ± 2
Truncation 5	0.18 ± 0.04	9.75 ± 5.1	1.72 ± 0.36	120	67 ± 14

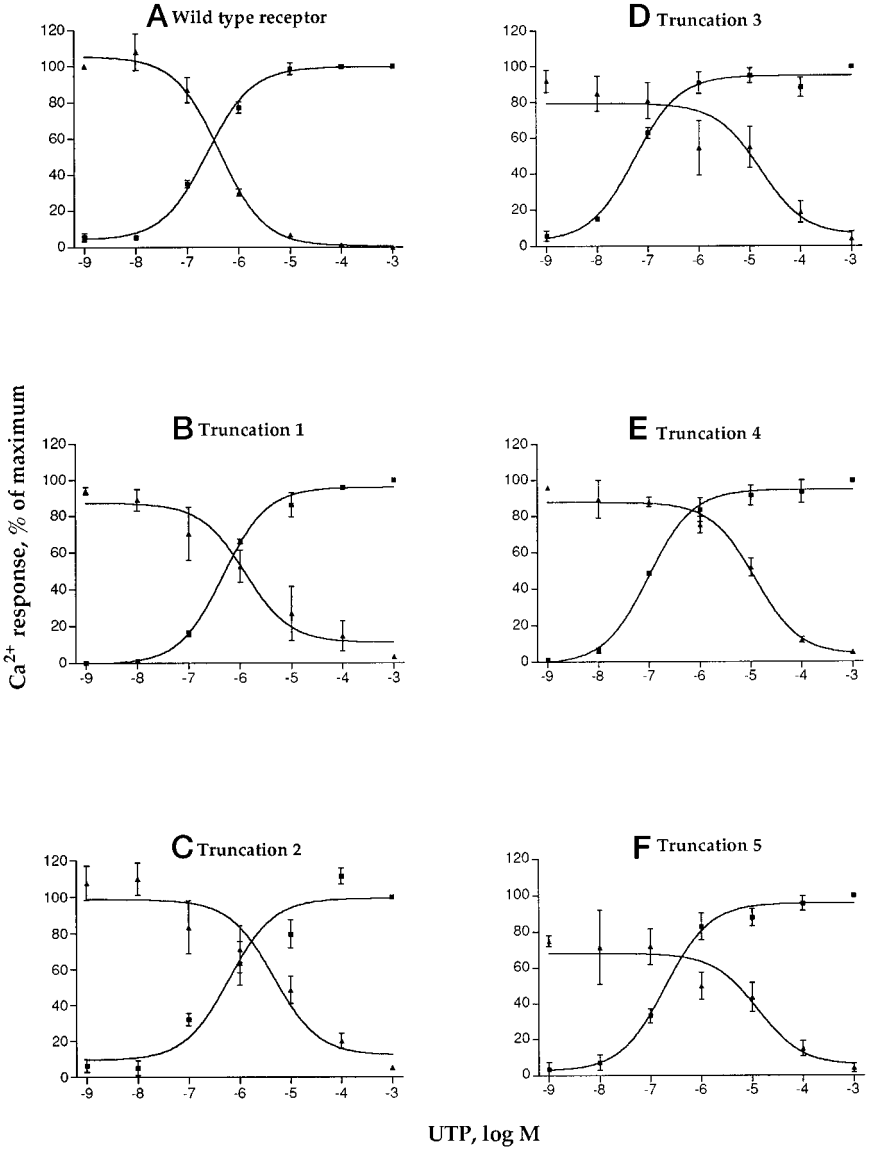

FIG. 3. **UTP concentration-effect curves for activation and agonist-induced desensitization of wild-type and truncation mutant $P2Y_2$ receptors.** Cells expressing recombinant HA-tagged $P2Y_2$ receptors were prepared, and $[Ca^{2+}]_i$ was measured in response to the indicated concentrations of UTP (■) as described under "Experimental Procedures." $[Ca^{2+}]_i$ is expressed as a percentage of the maximal response to allow comparisons among different receptor constructs. The maximal increases in $[Ca^{2+}]_i$ in response to UTP obtained for each receptor construct were as follows: wild-type, 282 ± 82 nM (*A*); truncation mutant 1, 733 ± 13 nM (*B*); truncation mutant 2, 341 ± 132 nM (*C*); truncation mutant 3, 341 ± 130 nM (*D*); truncation mutant 4, 276 ± 33 nM (*E*); and truncation mutant 5, 318 ± 80 nM (*F*). Desensitization (▲) was assessed by incubating 1321N1 cells expressing the different receptor constructs for 5 min with the indicated concentrations of UTP, followed by re-challenging with the EC_{50} value of UTP, determined in the receptor activation experiments (see Table I). The data presented are expressed as a percentage of the peak calcium response to the EC_{50} value of UTP in cells that were not preincubated with the agonist. The peak values were as follows: wild-type, 176 ± 43 nM (*A*); truncation mutant 1, 247 ± 43 nM (*B*); truncation mutant 2, 252 ± 97 nM (*C*); truncation mutant 3, 240 ± 84 nM (*D*); truncation mutant 4, 231 ± 47 nM (*E*); and truncation mutant 5, 420 ± 188 nM (*F*). The values shown are the mean ± S.E. of three experiments.

same density. The truncated receptors exhibited rapid and transient increases in $[Ca^{2+}]_i$ in response to UTP, followed by a return to base-line levels within 3 min (data not shown), similar to the wild-type receptor. The desensitization of the calcium response to UTP was determined for each receptor following a 5-min preincubation with the indicated concentration of UTP (Fig. 3). Progressive truncations of the C terminus increased the IC_{50} values for UTP-induced desensitization to a maximum of 25-fold with truncation mutant 4 (Table I).

Truncations of the C Terminus Decrease Receptor Sequestration—Fig. 4 shows that >80% of the wild-type receptors were sequestered within 10 min. The $t_{1/2}$, the time at which 50% of the cell-surface receptor complement is sequestered, was <5 min. Progressive truncations of the C terminus of the $P2Y_2$

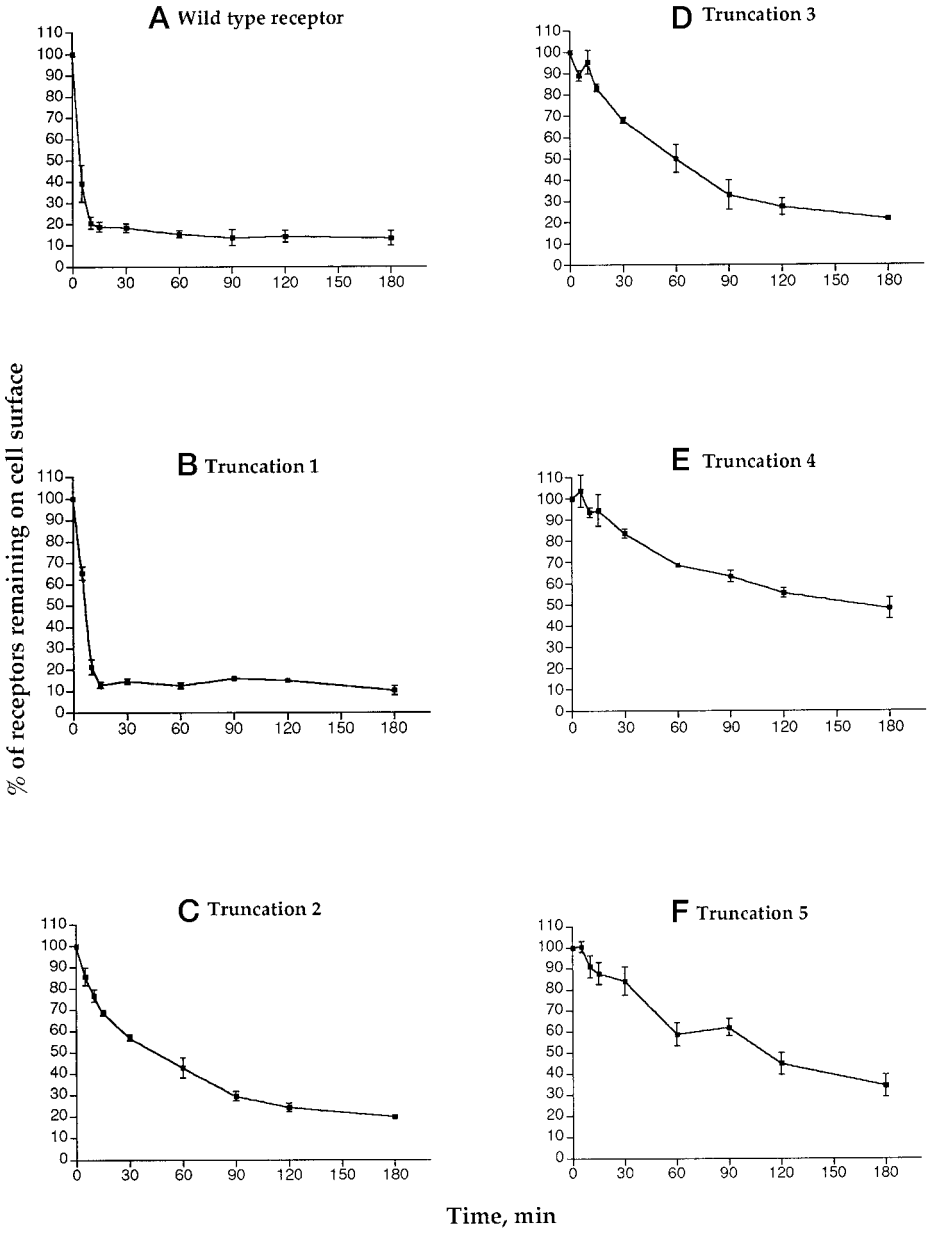

FIG. 4. **Time course for sequestration of recombinant P2Y$_2$ receptors expressed in 1321N1 cells.** Cells expressing recombinant P2Y$_2$ receptors were incubated with 1 mM UTP for the indicated times, and receptor density on the cell surface was determined as described under "Experimental Procedures." Cells expressing recombinant P2Y$_2$ receptors and incubated in the absence of UTP were regarded as having the full complement of cell-surface receptors, from which the percentage of receptors present on the cell surface following UTP incubation was calculated. The values shown are the mean ± S.E. of 10 experiments for the wild-type P2Y$_2$ receptor, seven experiments for truncation mutant 5, and three experiments for truncation mutants 1–4.

receptor increased the $t_{1/2}$ for sequestration. For the most truncated forms of the receptor (truncation mutants 4 and 5), the $t_{1/2}$ values were ~25-fold greater, and at least 35% of the receptors remained on the cell surface after 180 min. UTP concentration-effect curves show that 50% of the wild-type receptors were sequestered after a 10-min incubation with ~6 μM UTP, whereas sequestration of 50% of the truncation mutant 5 receptors required a 120-min incubation with 1 mM UTP (Fig. 5). Hydrolysis of UTP by 1321N1 cells was determined by HPLC analysis to be negligible, with 96% of the UTP remaining after 3-h incubation (data not shown). The original UTP stock solution contained a similar percentage of UTP, with 4% contamination with UDP. We were thus unable to detect any significant hydrolysis of UTP under these experimental conditions, unlike Lazarowski *et al.* (21), who demonstrated significant ecto-apyrase activity in 1321N1 cells. It is possible that, during our experiments, too much UTP and too few 1321N1 cells were present to demonstrate significant hydrolysis of nucleotide.

P2Y$_2$ Receptor Sequestration Is Reversible—The reversibility of sequestration of the wild-type P2Y$_2$ receptor was determined after preincubating cells for 10 min with 1 mM UTP. Following incubation of the cells for various times in the absence of agonist, cell-surface receptor expression was determined. As shown in Fig. 6, recovery was time-dependent and nearly complete (~90%) after 240 min. Half of the receptor complement returned to the cell surface in ~60 min.

UTP-induced Receptor Desensitization and Sequestration Are Distinct Events—Fig. 7 illustrates the relationship between agonist-induced desensitization and receptor sequestration for the wild-type and truncation mutant 5 receptors. It is apparent for both receptors that 50% of the cell-surface receptor complement was still present when the UTP-induced increase in $[Ca^{2+}]_i$ was desensitized. Furthermore, the wild-type and truncation mutant 5 P2Y$_2$ receptors had nearly all of the receptor complement on the cell surface when 50% of the UTP-induced calcium response was desensitized.

The effects of the protein kinase C activator PMA on desensitization of UTP-stimulated increases in $[Ca^{2+}]_i$ were examined in cells expressing the wild-type and truncation mutant 5 P2Y$_2$ receptors. Fig. 8 shows that the wild-type and truncation mutant 5 receptors have similar IC_{50} values (35 ± 17 and 62 ± 35 nM, respectively) for PMA-induced desensitization. How-

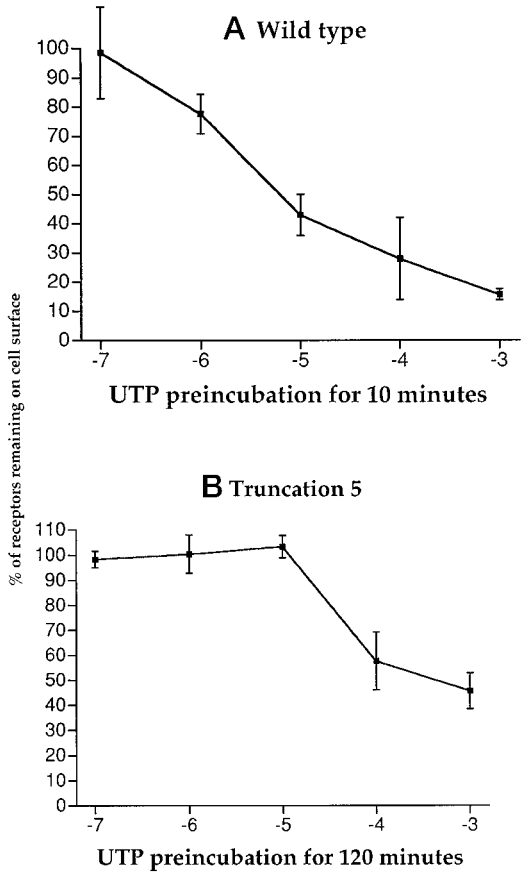

FIG. 5. **Concentration-dependent effect of UTP on sequestration of recombinant $P2Y_2$ receptors.** *A*, 1321N1 cells expressing the wild-type $P2Y_2$ receptor were incubated with the indicated concentrations of UTP for 10 min, and receptor density on the cell surface was determined as described under "Experimental Procedures." *B*, 1321N1 cells expressing truncation mutant 5 were incubated with the indicated concentrations of UTP for 120 min, and receptor sequestration was determined as described for *A*. The values shown are the mean ± S.E. of data from three experiments.

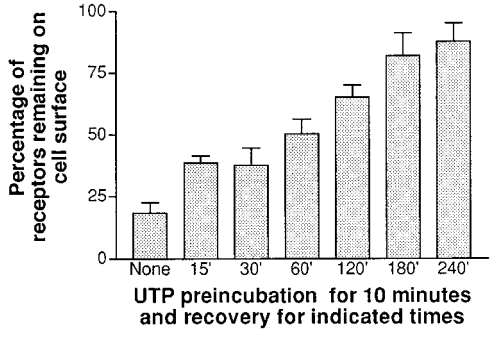

FIG. 6. **Time course of recovery of the cell-surface $P2Y_2$ receptor complement.** 1321N1 cells expressing the wild-type $P2Y_2$ receptor were incubated with 1 mM UTP for 10 min. UTP was removed; the medium was replaced; and the cells were incubated at 37 °C in 5% CO_2 and allowed to recover for the indicated times before the percentage of receptors remaining on the cell surface was determined as described under "Experimental Procedures." The receptor complement is expressed as a percentage of the level of receptors present in 1321N1 cells incubated in the absence of UTP. The values shown are the mean ± S.E. from three experiments.

ever, truncation mutant 5 required greater concentrations of PMA to induce desensitization. For example, truncation mutant 5 was ~65% desensitized at 1 μM PMA, a concentration that fully desensitized the wild-type receptor. These data indicate a strong desensitizing effect of PMA on UTP-stimulated Ca^{2+} mobilization.

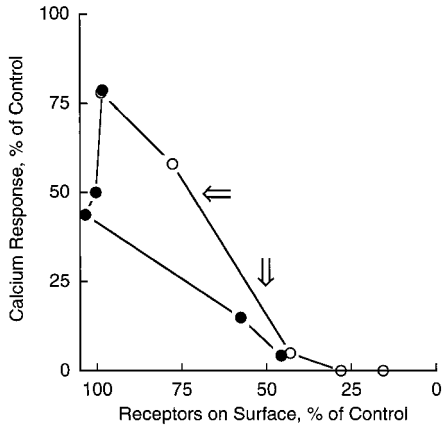

FIG. 7. **Relationship between agonist-induced desensitization of recombinant $P2Y_2$ receptors and the percentage of receptors remaining on the surface of 1321N1 astrocytoma cells.** Values for UTP-induced desensitization (after a 10-min preincubation with agonist) of calcium mobilization by wild-type (○) and truncation mutant 5 (●) $P2Y_2$ receptors expressed in 1321N1 cells were plotted against the percentage of receptors remaining on the cell surface after incubation with the concentrations of UTP indicated in Fig. 5(*A* and *B*).

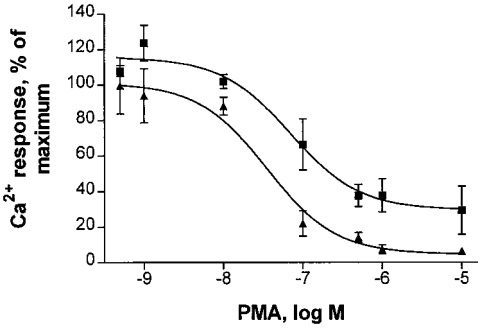

FIG. 8. **Concentration-effect curves for PMA-induced desensitization of wild-type and truncation mutant 5 $P2Y_2$ receptors.** Cells expressing recombinant HA-tagged $P2Y_2$ receptors were prepared, and $[Ca^{2+}]_i$ was measured and desensitization was assessed as described under "Experimental Procedures" by incubating 1321N1 cells expressing the different receptor constructs (wild-type (▲) and truncation mutant 5 (■) $P2Y_2$ receptors) for 5 min with the indicated concentrations of PMA, followed by re-challenging with the EC_{50} value of UTP, determined in receptor activation experiments (Fig. 3). $[Ca^{2+}]_i$ is expressed as a percentage of the maximal response to allow comparisons of the receptor constructs. The maximal increases in $[Ca^{2+}]_i$ in response to UTP obtained for each receptor construct were as follows: wild-type, 189 ± 43 nM; and truncation mutant 5, 196 ± 55 nM. The values shown are the mean ± S.E. from three experiments.

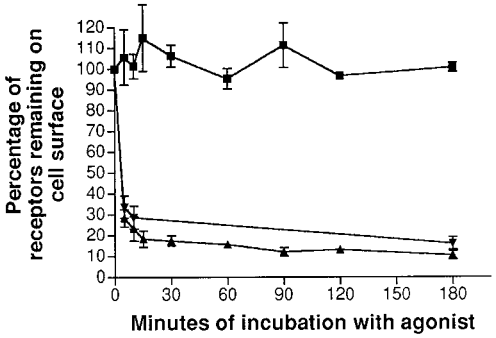

FIG. 9. **Effect of PKC activation on wild-type $P2Y_2$ receptor sequestration.** 1321N1 cells expressing the wild-type $P2Y_2$ receptor were incubated for periods of up to 180 min with 1 mM UTP (▲), 1 mM UTP and 1 μM PMA (▼), or 1 μM PMA (■), and the percentage of receptors remaining on the cell surface was determined as described under "Experimental Procedures." The receptor complement is expressed as a percentage of the level of receptors present in 1321N1 cells incubated in the absence of UTP. The values shown are the mean ± S.E. from three experiments.

However, exposure of cells to 1 μM PMA for times ranging from 5 to 180 min failed to induce sequestration of wild-type $P2Y_2$ receptors (Fig. 9). Furthermore, co-incubation with 1 μM PMA and 1 mM UTP produced a pattern of sequestration similar to that obtained with UTP alone. These findings suggest no role for PKC in the sequestration of the $P2Y_2$ receptor and thus dissociate receptor desensitization from sequestration.

DISCUSSION

Desensitization of the G protein-coupled $P2Y_2$ receptor can attenuate responses to nucleotides, which may impair the therapeutic effects of nucleotides in treatment of diseases such as cystic fibrosis. G protein-coupled receptor desensitization can involve several protein kinases, including protein kinase A, PKC, and G protein-coupled receptor kinases (reviewed in Ref. 8). However, the pathway involved in $P2Y_2$ receptor desensitization is not well understood. $P2Y_2$ receptor activation is coupled to phospholipase C, leading to the production of diacylglycerol, an activator of PKC. The presence of three consensus sites for phosphorylation by PKC and possible sites for G protein-coupled receptor kinase phosphorylation in the intracellular domain of the $P2Y_2$ receptor prompted us to investigate the role of the serine/threonine-rich C terminus in agonist-induced $P2Y_2$ receptor desensitization.

It has been established in this study that the recombinant $P2Y_2$ receptor expressed in 1321N1 cells desensitizes like the endogenous receptor in bovine aortic endothelial cells (7). Desensitization of the $P2Y_2$ receptor occurs in response to UTP or activation of PKC with PMA, although in some cells, PKC may not mediate $P2Y_2$ receptor desensitization (22).

The lack of a reliable radioligand binding assay and antibodies to the endogenous $P2Y_2$ receptor necessitated the incorporation of the HA epitope of influenza virus at the N terminus of the receptor to monitor sequestration with the 12CA5 monoclonal antibody. Truncation of 30 amino acids from the C terminus of the $P2Y_2$ receptor (truncation mutant 5) increased the concentration of UTP required for receptor desensitization and decreased the ability of these receptors to sequester as efficiently as the wild-type $P2Y_2$ receptor. This trend was also exhibited with truncation mutants 3 and 4. However, truncation mutant 2 had a similar UTP concentration response for desensitization, but different sequestration properties compared with the wild-type $P2Y_2$ receptor, whereas truncation mutant 1 desensitized and sequestered like the wild-type receptor. The cause for the change in sequestration properties that occurred between truncation mutants 1 and 2 requires further investigation, but may involve the loss of one or more essential amino acids from truncation mutant 2 that impact on sequestration, but not desensitization. Clearly, these changes do not affect receptor activation since UTP-induced increases in $[Ca^{2+}]_i$ for all the recombinant $P2Y_2$ receptor constructs had similar concentration-response curves. The wild-type $P2Y_2$ receptor lacks any cysteine residues in its C terminus that are apparently required for palmitoylation of the β_2-adrenergic receptor (23, 24) and the formation of tertiary structure required for agonist-induced receptor desensitization (25).

The wild-type $P2Y_2$ receptor expressed in 1321N1 cells desensitizes and sequesters in a time-dependent manner similar to other GPCRs, and although the $P2Y_2$ receptor recovers from desensitization like other GPCRs, its recovery from sequestration is slower (11, 26). It may be that the presence of the HA epitope affects the sequestration kinetics of the $P2Y_2$ receptor, but until reliable radioligands are developed for this receptor, we are unable to address this question. Although *de novo* protein synthesis may be responsible for recovery of some receptor activity, resensitization is more likely due to receptor recycling given the short incubation period with UTP. However, recovery from receptor sequestration was difficult to evaluate in cells expressing truncation mutant 5 because of the necessity to incubate these cells for 180 min (a period that may involve receptor down-regulation) to get adequate sequestration. Experiments on transcriptional regulation of the endogenous $P2Y_2$ receptor will be necessary to determine the mechanisms of recovery from receptor down-regulation.

Truncations of the carboxyl-terminal tail of G protein-coupled receptors can have a wide range of effects on receptor desensitization and sequestration. Truncation of the terminal 45 amino acids of the angiotensin II type 1A receptor inhibited receptor internalization with no effect on radioligand binding, downstream signaling, or agonist-induced desensitization (27). Removal of 72 of 86 amino acids from the C terminus of the serotonin type 2 receptor had no effect on the ability of the receptor to stimulate phospholipase C or to be internalized even though three of five PKC consensus phosphorylation sites and potential G protein-coupled receptor kinase phosphorylation sites were removed (28). However, the authors noted the presence in the truncated and wild-type $5HT_2$ receptors of the consensus NP*XX*Y sequence, in which the tyrosine residue has been reported to be essential for β_2-adrenergic receptor sequestration. In contrast, Thomas *et al.* (27) have reported the presence of an NP*XX*Y sequence in an internalization-resistant mutant of the angiotensin II type 1A receptor. The $P2Y_2$ receptor has a DP*XX*Y sequence that remains after the most radical truncation (truncation mutant 5), and it will be of interest to see if this tyrosine residue has any role in receptor sequestration.

Another study of the phospholipase C-linked G protein-coupled α_{1B}-adrenergic receptor indicated the importance of the C terminus in agonist-induced receptor phosphorylation and desensitization (29). A 147-amino acid truncation of the C terminus of the α_{1B}-adrenergic receptor did not decrease agonist-induced inositol 1,4,5-triphosphate accumulation compared with the wild-type α_{1B}-adrenergic receptor. However, the truncated receptor was resistant to desensitization and was not phosphorylated in response to activation by its ligand, epinephrine. The truncated α_{1B}-adrenergic receptor was able to sequester, but more slowly than the wild-type receptor.

Removal of 43 amino acids from the C terminus of the lutropin/choriogonadotropin receptor (30) or 48 amino acids from the C terminus of the β_2-adrenergic receptor (31) led to a decrease in agonist-induced uncoupling of the receptors from adenylyl cyclase. Compared with the wild-type β_2-adrenergic receptor, the truncation mutant required longer periods of agonist exposure to desensitize, presumably indicating the importance of β-adrenergic receptor kinase phosphorylation sites that were lost upon receptor truncation.

It will be of interest to investigate the specific amino acid residues of the $P2Y_2$ receptor that are involved in both desensitization and sequestration of this receptor. Fredericks *et al.* (9) identified G protein-coupled receptor kinase phosphorylation sites in the β_2-adrenergic receptor and found similarities between these sites and those found in rhodopsin. The most amino-terminal phosphorylation site in both rhodopsin and the β_2-adrenergic receptor has pairs of acidic residues at its amino-terminal side. Truncation mutant 3 of the $P2Y_2$ receptor truncates the receptor directly after two acidic residues and directly before a serine residue. It is tempting to speculate the importance of these amino acids in $P2Y_2$ receptor function since truncation mutant 3 is the least truncated form of the $P2Y_2$ receptor used in this study that required an increased concentration of UTP to induce desensitization compared with the wild-type receptor.

Very few studies have directly investigated the role of func-

tionally important domains of P2Y receptors. Specific amino acids in the transmembrane-spanning domains of the $P2Y_2$ (32) and $P2Y_1$ (33) receptors have been shown to affect agonist potency and specificity. The present study has defined the importance of the C terminus of the $P2Y_2$ receptor in desensitization and sequestration. Considering the widely varying roles of the C terminus in G protein-coupled receptor functions, future studies should better delineate the relevance of this domain to $P2Y_2$ receptor regulation. Studies are also needed to fully evaluate the role of PKC and other protein kinases in the desensitization and sequestration of the $P2Y_2$ receptor. A complete understanding of the signaling pathways involved may help minimize receptor desensitization and sequestration to optimize nucleotide therapies, such as those proposed for cystic fibrosis, that are directed at the $P2Y_2$ receptor.

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