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Differential Regulation of the Uridine Nucleotide-activated P2Y4 and P2Y6 Receptors

SER-333 AND SER-334 IN THE CARBOXYL TERMINUS ARE INVOLVED IN AGONIST-DEPENDENT PHOSPHORYLATION DESENSITIZATION AND INTERNALIZATION OF THE P2Y4 RECEPTOR*

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Agonist-promoted regulation of the uridine nucleotide-activated human P2Y4 receptor (P2Y4-R) and P2Y6 receptor (P2Y6-R) was studied. Incubation of P2Y4-R-expressing 1321N1 human astrocytoma cells with the cognate agonist UTP resulted in rapid desensitization of the inositol phosphate response and a 50% loss of cell surface receptors. In contrast, incubation of P2Y6-R-expressing cells with the cognate agonist UDP caused neither rapid desensitization nor rapid loss of cell surface receptors. Removal of UTP from the medium of UTP-pretreated cells resulted in rapid and complete recovery of surface P2Y4-R even after 12 h of agonist treatment. Although extended incubation with UDP also caused a loss of surface P2Y6-R, rapid recovery of surface P2Y6-R did not occur following removal of agonist. Pharmacological studies indicated that neither protein kinase C nor other Ca^{2+}-activated kinases were involved in agonist-promoted desensitization or loss of surface P2Y4-R or P2Y6-R. Mutational analyses were carried out to identify domains involved in agonist-dependent regulation of P2Y4-R. Sequential truncation of the carboxyl-terminal domain revealed that sequence between amino acids 332 and 343 was necessary for UTP-promoted desensitization and internalization. Further mutational analyses of the three serines in this domain confirmed that Ser-333 and Ser-334 play a major role in these agonist-promoted changes in P2Y4-R. Experiments were carried out with [^{32}P]P_i-labeled cells to ascertain the role of phosphorylation in regulation of P2Y4-R. Incubation with UTP for 2 min caused a marked increase in phosphorylation of both the wild-type P2Y4-R and the P2Y4–343 truncation mutant. In contrast, no UTP-promoted phosphorylation of the P2Y4–332 truncation mutant was observed. Taken together, these results demonstrate differential regulation of uridine nucleotide-activated P2Y4-R and P2Y6-R and indicate that Ser-333 and Ser-334 in the carboxyl terminus of P2Y4-R are important for UTP-dependent phosphorylation, desensitization, and loss of surface receptors.

Adenine nucleotides function as neurotransmitters in the peripheral and central nervous systems and as autocrine or paracrine signaling molecules in most other tissues (1–3). Two large classes of receptors, consisting of the ligand-gated P2X receptors and the G-protein-coupled P2Y receptors (4–6), are broadly distributed and promote a myriad of physiological responses to ATP and ADP, including neurotransmission, muscle contraction, immunological responses, and platelet aggregation (2, 7). A complex family of ecto-enzymes rapidly hydrolyze or interconvert extracellular nucleotides thereby either terminating their signaling action or producing an active metabolite of altered P2 receptor selectivity (8).

The existence of extracellular pyrimidinergic signaling, first suggested over a decade ago (9–11), was brought into focus in studies of UTP-promoted second messenger responses (12–16). UTP was confirmed as an extracellular signaling molecule by demonstration of regulated release of endogenous UTP (17, 18) and by cloning of uridine nucleotide-activated G-protein-coupled receptors (19–22). The P2Y2-R, which is equipotently activated by ATP and UTP, was the first molecularly identified uridine nucleotide-activated receptor (19). However, the demonstration (23), and then cloning (20–22) of uridine nucleotide-specific members of the P2Y receptor family placed the pyrimidinergic signaling hypothesis on firm ground. The human P2Y4-R[1] is specifically activated by UTP (20, 21, 24) and competitively antagonized by ATP (25); nucleoside diphosphates are inactive. In contrast, the human P2Y6-R is specifically activated by UDP and is insensitive to UTP or other triphosphates (22, 24).

The biological significance of P2Y receptors that exhibit strict specificity for either UTP or UDP is not yet understood, and evidence for physiological release of UDP in concentrations that activate P2Y6-R is not available. Thus, the extracellular breakdown of UTP to UDP may provide an important source of activating agonist for P2Y6-R (26). If metabolic formation of UDP is important, then temporal differences might be expected in both the rapidity by which these receptors are activated upon release of nucleotide and in the length of their activation. The relative capacity of P2Y4-R and P2Y6-R to undergo agonist-induced desensitization also would interplay with possible differences in their rapidity and longevity of activation due to differences in the extracellular presence of their cognate agonists.

Little is known about the regulation of P2Y-R in general and of the uridine nucleotide activated P2Y-R in particular. More-

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[1] The abbreviations used are: P2Y-R, P2Y receptor; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; PCR, polymerase chain reaction; HA, hemagglutinin A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBSS, Hanks' buffered saline solution; BSA, bovine serum albumin; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate.

over, P2Y4-R and P2Y6-R differ markedly in potential sites for phosphorylation by second messenger-regulated kinases and GRKs. Thus, we initiated a comparative examination of the agonist-promoted changes in the activities of these receptors and of the mechanisms that underlie these changes. Major differences in the mechanisms of regulation of P2Y4-R *versus* P2Y6-R are described below. We also report identification of two adjacent serines in the carboxyl terminus of the P2Y4-R receptor that play a major role in agonist-dependent phosphorylation, desensitization, and internalization of this receptor.

EXPERIMENTAL PROCEDURES

Construction of Mutant P2Y4-R and P2Y6-R cDNAs—Mutant receptor cDNA constructs were generated by polymerase chain reaction using cloned *Pfu* polymerase (Stratagene) and primers that incorporated an *Eco*RI or MluI restriction site at the amino terminus and a *Xho*I site at the carboxyl terminus. All receptor cDNAs were digested and ligated into a pLXSN retroviral expression vector that incorporated an HA epitope tag (YPYDVPDYAS) after the initial methionine residue. Truncation mutations were made of the P2Y4-R carboxyl terminus using primers that engineered a stop codon directly downstream of Ser-332, Ser-343, and Ser-355. PCR products were generated using the upstream primer 5′-CCGCCTCGATCCTCCCTT-3′ and the following downstream primers: 5′-GAGACTCGAGTCAGTCCTGGGGGGTG-GCC-3′ for P2Y4/355, 5′-GAGACTCGAGTCAATCCTCAGGCAGGGA-CACTA-3′ for P2Y4/343, and 5′-GAGACTCGAGTCAGGCAGCCGT-GCGGGGCT-3′ for P2Y4/332. Point mutations were made in the P2Y4-R carboxyl terminus and P2Y4-R and P2Y6-R using four-primer PCR. The upstream primer employed for all reactions was 5′- CCGC-CTCGATCCTCCCTT-3′, the downstream primer for mutations in the third intracellular loop was 5′-CCTGGGGACTTTCCACAC-3′, and the downstream primer for mutations in the carboxyl terminus was 5′-GACTATGGTTGCTGACTAATTG-3′. The following internal primers were used: 5′-TCTCGCCTCCGCGCTCTCCGCACCA-3′ and 5′-TGGT-GCGGAGAGCGCGGAGGCGAGA-3′ for P2Y4(S243A), 5′-CGTGGCA-AGTCGGCCCGCAT-3′ and 5′-ATGCGGGCCGACTTGCCACG-3′ for P2Y6(A237S), and 5′-CACTAGTGCCAGGGCAGCGGCAGCCGTGCG-GGGCTG-3′ and 5′-GCTGCCCTGGCACTAGTGGCCCTGCCTGAGG-ATAGCAG-3′ for P2Y4(S333A, S334A, S339A). P2Y4 S/T-A, a full-length P2Y4-R construct with 10 serine and threonine residues mutated to alanine, was engineered in a stepwise fashion by first using four-primer PCR with 5′-CCGCCTCGATCCTCCCTT-3′ as the upstream primer, 5′-GACTATGGTTGCTGACTAATTG-3′ as the downstream primer, and 5′-GGCGGCCGCCCACCTGCAGGCGGCAT-CCTCAGGCAGGGACAC-3′ and 5′-TGCAGGTGGGCGGCCGCCCCC-CAGGACAGT-3′ as internal primers. The product from this reaction was amplified with four-primer PCR, the same upstream and downstream primers, and 5′-CACTAGTGCCAGGGCAGCGGCAGCCGTGC-GGGGCTG-3′ and 5′-GCTGCCCTGGCACTAGTGGCCCTGCCTGAG-GATGCCG-3′ as internal primers. The product from this reaction was amplified with the following two primers: 5′-CCGCCTCGATCCTCCC-TT-3′ and 5′-TAGGAGCAGCACAAGCAGCGTCCTGGGGGGCGGC-C-3′ and the final, full-length construct was generated using 5′-CCG-CCTCGATCCTCCCTT-3′ and 5′-GAGACTCGAGTTACAATCTATCTG-CTCTAGGAGCAGCACAAGCAGC-3′. Additional point mutant constructs were made using four-primer PCR and P2Y4/343 as a template. 5′-CCGCCTCGATCCTCCCTT-3′ and 5′-TAGGGGCGGGACTATGGT-TG-3′ were employed as upstream and downstream primers, respectively, and the following internal primers were used: 5′-CACTAGTGC-CAGGGAAGCGGCAGCCGTGC-3′ and 5′-GCTTCCCTGGCACTAGTG-TC-3′ for P2Y4/343(S333A), 5′-CACTAGTGCCAGGGCAGAGGCAGCC-GTGC-3′ and 5′-TCTGCCCTGGCACTAGTGTC-3′ for P2Y4/343(S334A), and 5′-CACTAGTGCCAGGGCAGCGGCAGCCGTGC-3′ and 5′-GCTG-CCCTGGCACTAGTGTC-3′ for P2Y4/343(S333A,S334A). P2Y4/343-(S339A) was generated using full-length P2Y4-R sequence as the template and 5′-CCGCCTCGATCCTCCCTT-3′ and 5′-GAGACTCG-AGTCAATCCTCAGGCAGGGCCACTAGTG-3′ as upstream and downstream primers, respectively. P2Y4/343(S333A,S334A,S339A) was constructed with P2Y4(S333A,S334A,S339A) as the template and 5′-CCG-CCTCGATCCTCCCTT-3′ and 5′-GAGACTCGAGTCAATCCTCAGGC-AGGGCCACTA-3′ as upstream and downstream primers, respectively. The chimera P2Y6/310-Y4 was generated with four-primer PCR using 5′-CCGCCTCGATCCTCCCTT-3′ and 5′-CCTGGGGACTTTCCACA-C-3′ as upstream and downstream primers, respectively, and 5′-CCG-GCGGAACTTCTTCTGG-3′ and 5′-CCAGAAGAAGTTCCGCCGGCAG-CTCCGTCAGCTCTGTG-3′ as internal primers.

Cell Culture and Expression of Receptor Constructs—P2Y-R-expressing 1321N1 human astrocytoma cells were grown in DMEM with 4.5 g/liter glucose (Life Technologies, Inc.) supplemented with 5% FBS (Hyclone) in a 37 °C humidified atmosphere with 5% CO_2 and 95% air. Retrovirus packaging and cell infections were performed as previously described (27). Following infection, receptor-expressing 1321N1 cells were selected in medium containing 1 mg/ml G418 (Life Technologies, Inc.). Stable cell lines were maintained in medium containing 0.6 mg/ml G418.

Radioimmunoassay for Detection of Surface P2Y-R—P2Y-R-expressing 1321N1 cells were seeded at 3×10^5 cells/well in 12-well plates (Corning/Costar) coated with 10 μg/ml fibronectin (Collaborative Bioproducts). Assays were performed on confluent cells 2 days after plating. For most experiments, assays were initiated by replacing the medium with DMEM containing 50 mM HEPES (pH 7.5), and equilibrating for 1 h at 37 °C prior to assay of agonist-promoted responses. Drug incubations of longer than 1-h duration were performed in a humidified, 37 °C/5% CO_2 incubator without a medium change. Drug treatments were terminated by placing plates in an ice bath, aspirating the medium, adding 2% cold paraformaldehyde (Sigma), and incubating at room temperature for 10 min. Following a wash with HBSS containing Ca^{2+} and Mg^{2+}, HEPES/DMEM with 10% heat-inactivated FBS was added to each well for 30 min. Anti-HA.11 raw ascites fluid (BabCo/Covance) was added without changing the medium to a final concentration of 1:1000 for 1 h. Cells were washed two times with HBSS containing Ca^{2+} and Mg^{2+}, followed by addition (typically 100,000 cpm/assay) of ^{125}I-rabbit anti-mouse antibody (PerkinElmer Life Sciences) diluted in HEPES/DMEM with 10% heat-inactivated FBS to a concentration of 1:500. Following a 2-h incubation at room temperature, the cells were washed twice with HBSS containing Ca^{2+} and Mg^{2+}. Cells were solubilized with 1 M NaOH and transferred to glass tubes for quantitation of radioactivity by gamma counting.

In Vivo Labeling and Immunoprecipitation of P2Y4-R—Confluent 100-mm dishes of P2Y4-R-expressing cells were washed with phosphate-free DMEM and incubated in phosphate-free DMEM for 1 h in a 37 °C humidified incubator with 5% CO_2 and 95% air. Cells were labeled with 500 μCi of [^{32}P]orthophosphate for 3 h. Following labeling, cells were treated with UTP for 2 min, transferred to an ice bath, and washed with ice-cold PBS. Ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 200 μM Na_3VO_4, 1% Triton X-100, 200 mM phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine A, 1 mM pepstatin A, 4.3 mg/ml leupeptin, and 10 mg/ml TPCK was added to each dish for 5 min. The cell extracts were transferred to screw cap tubes, rocked for 1.5 h at 4 °C, and centrifuged at 13,000 × *g* for 30 min at 4 °C. The resultant supernatants were transferred to fresh tubes containing 30 μl of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Inc.) that were preblocked with 1% BSA for 30 min. Following rocking for 45 min at 4 °C, the samples were centrifuged for 15 s at 13,000 × *g*, and the supernatants were transferred to tubes containing 2 μl of anti-HA.11 raw ascites fluid (BabCo/Covance) and 50 μl of preblocked protein A/G beads. Tubes were rocked for 1.5 h at 4 °C followed by a quick centrifugation. The pelleted beads were washed twice with the Triton X-100-containing lysis buffer and resuspended in 40 μl of 2× Laemmli sample buffer. Samples were loaded onto a 9% polyacrylamide gel, electrophoresed, and transferred to nitrocellulose. Radioactivity associated with the immunoprecipitated P2Y4-R was measured with a phosphorimaging screen. Immunoblots were generated by blocking with 3% BSA/TBST for 1 h or overnight and incubating with 1:1000 anti-HA-purified monoclonal antibody (Babco/Covance) in 3% BSA/TBST for 2 h. Blots were washed four times with TBST followed by a 2.5-h incubation with 1:10,000 anti-mouse horseradish peroxidase-conjugated antibody in 3% BSA/TBST. Following four washes with TBST, protein bands were visualized with ECL reagent.

Inositol Phosphate Assay—P2Y-R-expressing 1321N1 human astrocytoma cells were seeded in 24-well plates at 1×10^5 cells/well 2 days prior to assay. The evening before each assay, the medium was removed and replaced with 200 μl of inositol-free DMEM containing 2 μCi/ml [^{3}H]inositol. Drug incubations were performed in a 37 °C water bath in the presence of 10 mM LiCl and terminated by addition of 0.9 ml of 50 mM ice-cold formic acid. After a 20-min incubation on ice, 0.3 ml of 150 mM ammonium formate was added to each well. The supernatant from each well was transferred to Dowex AG1-X8 columns, and total [^{3}H]inositol phosphates were quantitated as previously described (23, 24).

Data Analysis—All experiments were repeated at least three times in triplicate assay except where indicated in the figure legends. Results are presented as the mean ± S.E.

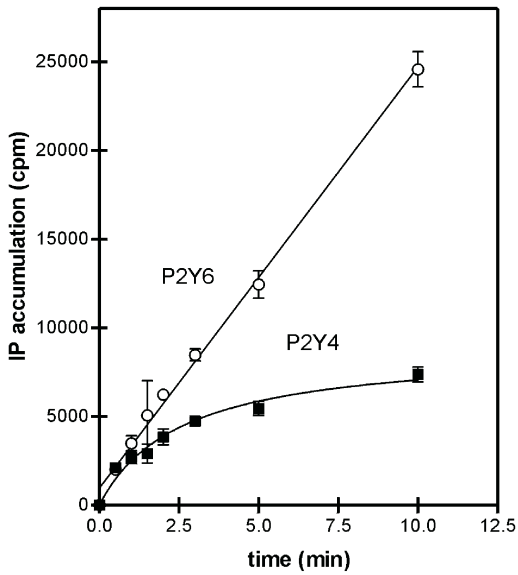

FIG. 1. **Agonist-promoted inositol phosphate accumulation in P2Y4-R- and P2Y6-R-expressing cells.** P2Y4-R- and P2Y6-R-expressing 1321N1 cells were prelabeled with [^{3}H]inositol and then incubated in the presence of 10 mM LiCl and 100 μM UTP (P2Y4-R), or 100 μM UDP (P2Y6-R). Total [^{3}H]inositol phosphate accumulation was quantitated at the indicated times.

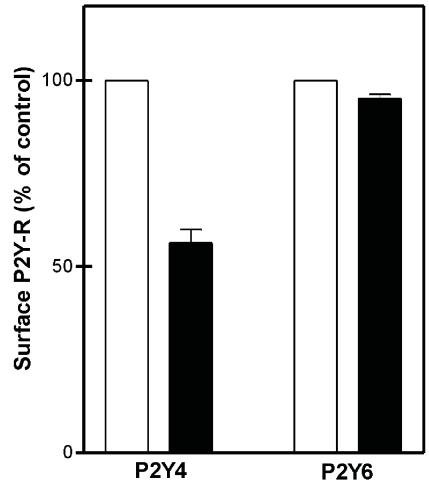

FIG. 2. **Agonist-promoted effects on cell surface P2Y4-R and P2Y6-R.** P2Y4-R and P2Y6-R-expressing 1321N1 cells were incubated in the absence (*open bars*) or presence (*filled bars*) of 100 μM UTP and 100 μM UDP, respectively, for 10 min. Surface P2Y-R were quantitated using anti-HA antibody followed by a ^{125}I-labeled secondary antibody.

RESULTS

Agonist-induced Desensitization and Loss of Cell Surface P2Y4-R and P2Y6-R—Agonist-induced phosphorylation of serine and threonine residues in the third intracellular loop and carboxyl terminus has been implicated in desensitization and subsequent internalization of a broad range of G-protein-coupled receptors (28, 29). Although the P2Y4-R has multiple putative phosphorylation sites in these domains, the P2Y6-R contains only a single threonine residue in the carboxyl terminus, suggesting that these two uridine nucleotide-activated receptors differ significantly in their regulatory mechanisms. To test this hypothesis we examined agonist-induced desensitization by quantitating the rate of accumulation of total inositol phosphates in the presence of LiCl and examined agonist-dependent loss of cell surface P2Y-R utilizing a "surface binding assay" that employs HA-antibody followed by a ^{125}I-labeled secondary antibody (see "Experimental Procedures").

Human 1321N1 astrocytoma cells stably expressing either P2Y4-R or P2Y6-R were labeled with [^{3}H]inositol, and agonist-induced desensitization was assessed. The rate of inositol phosphate accumulation in P2Y4-R-expressing cells rapidly declined in the presence of UTP, reaching a near steady state within 10 min (Fig. 1). In contrast, incubation of P2Y6-R-expressing 1321N1 cells with UDP resulted in a linear accumulation of inositol phosphates for at least 30 min (Fig. 1 and data not shown). Thus, as was reported previously by Robaye *et al.* (30), P2Y4-R undergoes rapid agonist-induced desensitization, whereas P2Y6-R fails to desensitize, even after 30 min of agonist incubation.

The surface binding assay was employed to determine whether the marked differences in capacity of P2Y4-R and P2Y6-R to undergo desensitization were reflected in agonist-dependent changes in surface receptors. Incubation of P2Y4-R-expressing cells with UTP for 20 min resulted in an ~50% decrease in the amount of surface P2Y4-R (Fig. 2). The EC_{50} (886 ± 300 nM) of UTP for inducing loss of surface P2Y4-R during a 20-min incubation (data not shown) was similar to the EC_{50} (541 ± 200 nM) of UTP for promotion of inositol phosphate accumulation and the EC_{50} of UTP (780 ± 60 nM (25)) for stimulation of Ca^{2+} mobilization. In contrast to the changes observed in surface P2Y4-R, a 20-min incubation of P2Y6-R-expressing cells with UDP had essentially no effect on surface receptor levels (Fig. 2). Thus, differences in the capacity of these receptors to undergo rapid agonist-induced desensitization were paralleled by differences in agonist-induced loss of surface receptors.

Extended time courses for agonist-induced loss of surface receptors were generated to further compare P2Y4-R and P2Y6-R. P2Y4-R levels decreased rapidly during agonist incubation reaching a near steady state within 10 min that decreased very slowly if at all over the ensuing 12 h (Fig. 3). Although agonist-promoted changes in surface P2Y6-R occurred more slowly, with extended incubation (>1 h), decreases in the surface levels of this uridine nucleotide-activated receptor also were observed (Fig. 3).

To study the reversibility of agonist-induced loss of surface receptors, we took advantage of the nucleotide-hydrolyzing activity of the enzyme apyrase. This approach was necessary, because antagonists of P2Y4-R or P2Y6-R are not available, and mechanical stimulation caused by medium changes of cultured cells results in release of large amounts of cellular UTP into the medium (17, 18). The reversibility of the loss of surface P2Y4-R or P2Y6-R was examined after various times of incubation with UTP or UDP followed by addition of apyrase at a concentration that completely hydrolyzed nucleotide within 1 min (data not shown). Surprisingly, surface P2Y4-R returned to control levels within 30 min of addition of apyrase, irrespective of the time (up to 12 h) of preincubation with agonist (Fig. 4*A*). The agonist-promoted loss of cell surface P2Y4-R and the complete recovery of P2Y4-R to the surface after hydrolysis of agonist in the medium suggest efficient recycling of internalized receptors. This possibility was supported by the fact that cycloheximide at a concentration (10 μg/ml) that inhibited protein synthesis had no effect on the rapid recovery of P2Y4-R after removal of agonist from the medium (data not shown). Direct evidence for recycling was provided by experiments in which P2Y4-R were prelabeled with primary antibody prior to addition of agonist. Cell surface immunoreactivity moved to an intracellular compartment following addition of agonist (40 ± 15% of control after 20 min in the presence of 100 μM UTP). Antibody-bound receptors then returned to the cell surface after agonist removal by addition of apyrase (95 ± 2% of control levels after removal of agonist for 60 min). The reversibility of

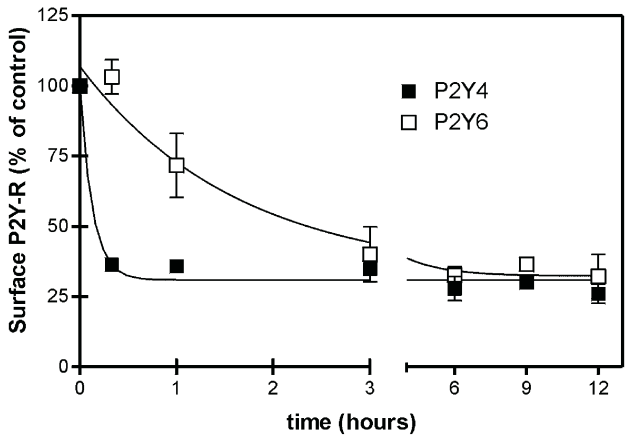

FIG. 3. **Time course of agonist-promoted decrease in cell surface P2Y4-R and P2Y6-R.** P2Y4-R- and P2Y6-R-expressing 1321N1 cells were incubated with 100 μM UTP (■) and 100 μM UDP (□), respectively, for the indicated times. Surface P2Y-R were quantitated using anti-HA antibody followed by a ^{125}I-labeled secondary antibody.

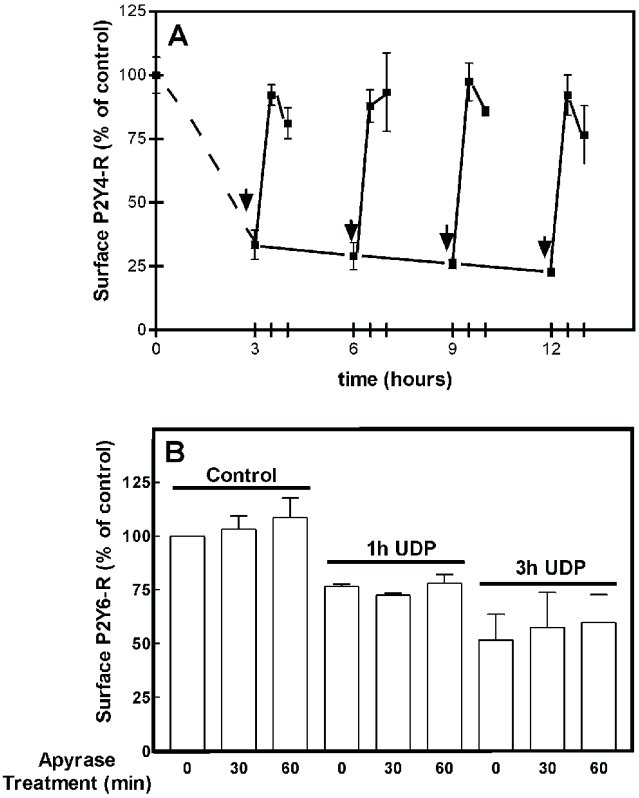

FIG. 4. **Recovery of surface P2Y4-R and P2Y6-R.** *A*, P2Y4-R-expressing 1321N1 cells were incubated for various times with 100 μM UTP. At the indicated times, apyrase (at a concentration that immediately hydrolyzed the UTP) was added to the medium, and cell surface P2Y4-R were measured 30 or 60 min later using anti-HA antibody followed by a ^{125}I-labeled secondary antibody. The data are from triplicate assays in an experiment that was representative of two similar experiments. *B*, P2Y6-R-expressing 1321N1 cells were incubated in the absence (control) or presence of 100 μM UDP for 1 or 3 h. Apyrase (at a concentration that immediately hydrolyzed UDP) then was added to the medium, and cell surface P2Y6-R were measured 30 or 60 min later using anti-HA antibody followed by ^{125}I-labeled secondary antibody. The data are the mean ± S.E. and are representative of results from three experiments.

UDP-induced changes in the cell surface levels of P2Y6-R also was studied after long-term agonist treatment. In marked contrast to P2Y4-R, removal of UDP from the medium with apyrase after 3-h preincubation did not result in rapid recovery of P2Y6-R to the cell surface (Fig. 4*B*). Thus, in the presence of UTP P2Y4-R are internalized and sequestered in an intracellular pool from which receptors rapidly recycle to the cell surface once agonist is removed. In contrast, P2Y6-R are more slowly lost from the cell surface in the presence of agonist and fail to rapidly recycle once agonist is removed from the medium.

Potential Regulation of P2Y4-R and P2Y6-R by Second Messenger-regulated Kinases—P2Y4-R and P2Y6-R are both coupled to Gq and phospholipase C (6), and PKC has been broadly implicated in feed-back regulation of the inositol lipid signaling pathway (31). Moreover, PKC has been shown to phosphorylate a number of Gq/phospholipase C-linked GPCR (32–34). Because the P2Y4-R contains a strong PKC consensus sequence in its third intracellular loop and several weaker sequences in its carboxyl terminus, we investigated the potential involvement of PKC in agonist-promoted loss of surface P2Y4-R and P2Y6-R. Incubation of P2Y4-R-expressing 1321N1 cells with the phorbol ester activator of PKC, PMA, caused only a small loss of surface P2Y4-R compared with that observed during a parallel incubation with UTP (Fig. 5*A*). Although the PKC inhibitor, bisindolemaleimide, blocked the small PMA-induced changes, it had no effect on UTP-induced loss of surface P2Y4-R (Fig. 5*B*). Down-regulation of PKC by overnight incubation with 1 μM PMA also had no effect on the time course of occurrence or extent of UTP-promoted loss of cell surface P2Y4-R (Fig. 5*C*). In contrast, down-regulation of PKC blocked the acute effects of PMA on cell surface P2Y4-R (Fig. 5*C*). As was observed with short-term incubation with UDP, incubation of P2Y6-R-expressing cells with PMA had no effect on cell surface receptors (Fig. 5*A*).

The potential role of the PKC-consensus site (RLRS) in the third intracellular loop of the P2Y4-R also was examined. Mutation of this serine to alanine (P2Y4(S243A); see Fig. 6) failed to inhibit agonist-induced internalization of P2Y4-R (data not shown). Interestingly, although this serine is lacking in the third cytoplasmic loop of the P2Y6-R, the adjoining residues are conserved. Thus, we mutated the corresponding residue of the P2Y6-R to serine (P2Y6/A237S; see Fig. 6) to recapitulate the sequence found in the P2Y4-R. This mutation failed to confer in the P2Y6-R a capacity to undergo rapid agonist-dependent loss of surface receptors (data not shown). Therefore, this potential site for PKC phosphorylation is not involved in agonist-induced changes in surface levels of P2Y4-R, and engineering this site into the P2Y6-R also did not uncover UDP-induced loss of surface receptors. Taken together, these results support the idea that mechanisms largely unrelated to PKC mediate agonist-promoted P2Y4-R sequestration. No effects on agonist-promoted loss of surface P2Y4-R were observed after elevation of Ca^{2+} levels with ionomycin or after increasing cyclic AMP levels with forskolin (data not shown).

Identification of Regulatory Sites in the Carboxyl-terminal Domain of the P2Y4-R by Mutational Analysis—A series of receptor mutants was constructed with the goal of identifying the regions and residues important for regulation of P2Y4-R signaling (Fig. 6). Each mutant P2Y4-R was stably expressed in 1321N1 cells, and its capacity to promote inositol lipid hydrolysis was compared with that of the wild-type P2Y4-R. Concentration effect curves for UTP at wild type and two mutant P2Y4-R are presented in Fig. 7. The EC_{50} values for stimulation (5-min assay) of inositol phosphate accumulation by UTP ranged from 200 to 2000 nM for all of the mutant receptors reported in this study (data not shown). The EC_{50} for UTP (in a 5-min assay) at the wild type P2Y4-R stably expressed in parallel with these various mutants was 440 ± 250 nM. The maximal effect of 100 μM UTP for stimulation of inositol phosphate accumulation in a 5-min assay (0.4–2.5% conversion of

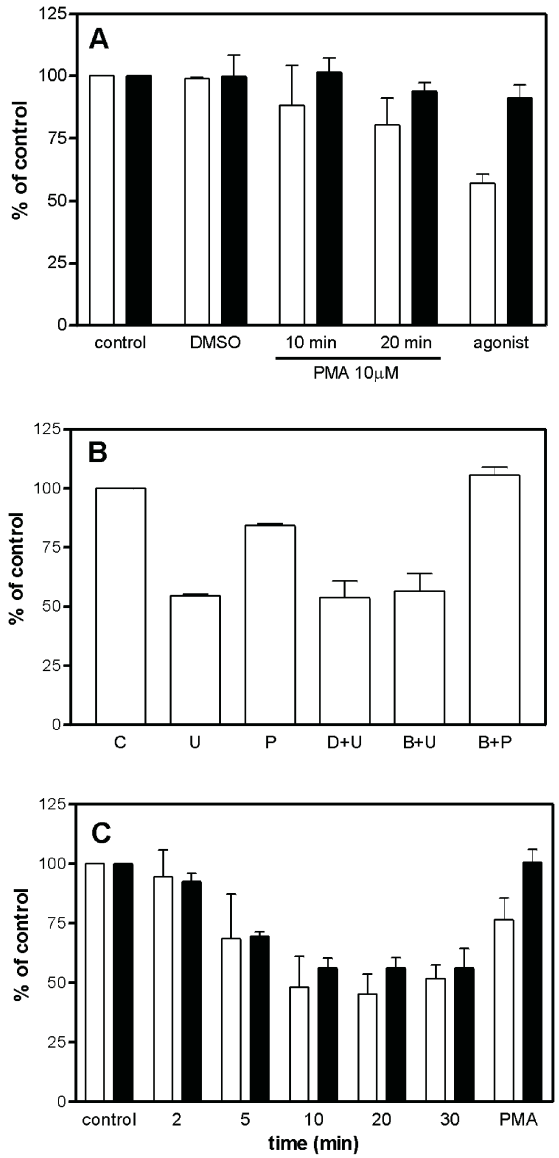

FIG. 5. **Pharmacological analyses of the potential role of PKC in regulation of cell surface P2Y4-R and P2Y6-R.** *A–C*, surface levels of P2Y-R were measured using an anti-HA antibody followed by a ^{125}I-labeled secondary antibody. *A*, P2Y4-R (*light bar*) or P2Y6-R (*dark bar*)-expressing 1321N1 cells were incubated with vehicle (*DMSO*) or 10 μM PMA for 10 or 20 min or with 100 μM UTP (*agonist*) or 100 μM UDP (*agonist*) for 20 min. *B*, P2Y4-R-expressing 1321N1 cells were incubated for 20 min with 100 μM UTP, 10 μM PMA, vehicle + 100 μM UTP (*D+U*), 10 μM bisindolmaleimide + 100 μM UTP (*B+U*), or 10 μM bisindolemaleimide + 10 μM PMA (*B+P*). *C*, P2Y4-R-expressing 1321N1 cells were incubated in the absence (*light bar*) or presence (*dark bar*) of 1 μM PMA overnight to down-regulate PKC. Cells then were incubated with 100 μM UTP for the indicated times or with 10 μM PMA for 30 min.

[^{3}H]inositol lipids into [^{3}H]inositol phosphates) with the mutant receptors also was within the range of maximal effect (0.99 ± 0.42% conversion) observed with 100 μM UTP at the wild type P2Y4-R in a 5-min assay. Thus, each mutant receptor promoted inositol lipid hydrolysis with characteristics similar to that of the wild type receptor. The enhanced maximal response of the P2Y4/332 truncation mutant illustrated in Fig. 7 apparently occurs due to the fact that this receptor does not desensitize (see data and discussion below).

The carboxyl-terminal region of the P2Y4-R is ~55 amino acids in length and contains 11 serines and threonines as potential sites for phosphorylation. To determine whether the carboxyl terminus is important in P2Y4-R desensitization and trafficking, a series of truncations was made after residues 355, 343, and 332 (Fig. 6). Truncations of the carboxyl terminus that removed the final 11 amino acids (and four serines/threonines), *i.e.* P2Y4/355 (data not shown), or final 23 amino acids (and seven serines/threonines), *i.e.* P2Y4/343, had no effect on agonist-induced loss of cell surface P2Y4-R (Fig. 8) or desensitization (Fig. 9). In contrast, removal of an additional 11 residues (and three serines), *i.e.* P2Y4/332, markedly inhibited agonist-induced loss of cell surface P2Y4-R (Fig. 8) and prevented agonist-induced desensitization (Fig. 9; also see Fig. 7). Therefore, residues between 332 and 343 in the carboxyl terminus of P2Y4-R are required for agonist-induced desensitization and loss of surface receptors. Although these data establish the P2Y4-R carboxyl terminus as a critical region for agonist-induced regulation of this receptor, chimeric addition of the P2Y4-R carboxyl terminus to P2Y6-R, *i.e.* P2Y6/310-Y4 (Fig. 6), failed to confer UDP-induced loss of surface receptors to the chimeric P2Y6-R (data not shown).

The data from the carboxyl terminus truncation studies suggest that amino acids within the region that differs between the two mutants, P2Y4/343 and P2Y4/332, are crucial for regulation of P2Y4-R signaling. This region contains three putative phosphorylation sites, *i.e.* Ser-333, Ser-334, and Ser-339. To evaluate their role in regulation of P2Y4-R these serines were mutated to alanines (P2Y4/S333A,S334A,S339A). The capacity of this mutant P2Y4-R to undergo agonist-induced desensitization (Fig. 9) or agonist-promoted loss of surface receptors (Fig. 10) was greatly reduced but not completely inhibited. Because alternative sites in the carboxyl terminus, *i.e.* the seven serines/threonines carboxyl-terminal to Ser-339, could be phosphorylated after mutation of Ser-333, Ser-334, and S339, the truncation mutant P2Y4/343 was used as the "wild type" sequence to assess more directly the role(s) of amino acids between positions 332 and 343. All three of the serines were mutated individually in the truncated receptor, all three serines were mutated simultaneously in a triple mutant, and a double mutant of the serines at positions 333 and 334 also was constructed. Mutation of individual serines did not inhibit agonist-induced loss of surface receptors. In contrast, simultaneous mutation of Ser-333 and Ser-334 or of Ser-333, Ser-334, and S339 reduced the capacity of UTP to induce loss of surface receptors by ~80% (Fig. 11). The inhibition observed with these two mutants was similar to that seen with a full-length construct, designated S/T-A (see Fig. 6), containing mutations to alanine at all potential phosphorylation sites carboxyl-terminal to residue 332 (Fig. 11). Taken together, these data illustrate that serines 333 and 334 are important sites for agonist-induced desensitization and loss of surface P2Y4-R.

Agonist-promoted Phosphorylation of P2Y4-R—Experiments were carried out to demonstrate directly that the P2Y4-R is phosphorylated in an agonist-dependent fashion and to establish whether the regulatory domain identified by mutational analysis also contributes to the residue(s) responsible for this phosphorylation. Cells expressing HA-tagged P2Y4-R were prelabeled with [^{32}P]P_i and then incubated with UTP for 2 min. The extent of [^{32}P]phosphorylation of P2Y4-R was established as described under "Experimental Procedures." A small amount of ^{32}P-phosphorylation of P2Y4-R was detected in the absence of added agonist (Fig. 12). This phosphorylation was not observed in cells not expressing P2Y4-R, and therefore, either represents basal P2Y4-R phosphorylation unrelated to P2Y4-R activation or occurs due to the presence of constitutively released extracellular UTP as we have recently described (26). Addition of UTP for 2 min resulted in a marked increase in P2Y4-R phosphorylation (Fig. 12). This agonist-promoted

C-terminus

P2Y4:	DKYRRQLRQLCGGGKPQPRTAASSLALVSLPEDSSCRWAATPQDSSCSTPRADRL
P2Y4/355:	DKYRRQLRQLCGGGKPQPRTAASSLALVSLPEDSSCRWAATPQD
P2Y4/343:	DKYRRQLRQLCGGGKPQPRTAASSLALVSLPED
P2Y4/332:	DKYRRQLRQLCGGGKPQPRTAA
P2Y4(S333A,S334A,S339A):	DKYRRQLRQLCGGGKPQPRTAA**AA**LALV**A**LPEDSSCRWAATPQDSSCSTPRADRL
P2Y4 S/T-A:	DKYRRQLRQLCGGGKPQPRTAA**AA**LALV**A**LPED**AA**CRWAA**A**PQD**AA**C**AA**PRADRL
P2Y4/343(S333A,S334A,S339A):	DKYRRQLRQLCGGGKPQPRTAA**AA**LALV**A**LPED
P2Y4/343(S339A):	DKYRRQLRQLCGGGKPQPRTAASSLALV**A**LPED
P2Y4/343(S333A):	DKYRRQLRQLCGGGKPQPRTAA**A**SLALVSLPED
P2Y4/343(S334A):	DKYRRQLRQLCGGGKPQPRTAAS**A**LALVSLPED
P2Y4/343(S333A,S334A):	DKYRRQLRQLCGGGKPQPRTAA**AA**LALVSLPED
P2Y6:	KKFRRRPHELLQKLTAKWQRQGR
P2Y6/310-Y4:	KKFRR**QLRQLCGGGKPQPRTAASSLALVSLPEDSSCRWAATPQDSSCSTPRADRL**

Third intracellular loop

P2Y4:	DMARRLYQPLPGSAQSSSRLRSLR
P2Y4(S243A):	DMARRLYQPLPGSAQSSSRLR**A**LR
P2Y6:	LACRLCRQDGPAEPVAQERRGKAAR
P2Y6(A237S):	LACRLCRQDGPAEPVAQERRGK**S**AR

FIG. 6. **Mutations of P2Y4-R and P2Y6-R.** A series of deletions and mutations was made in the carboxyl terminus and third intracellular loop of the human P2Y4-R and human P2Y6-R.

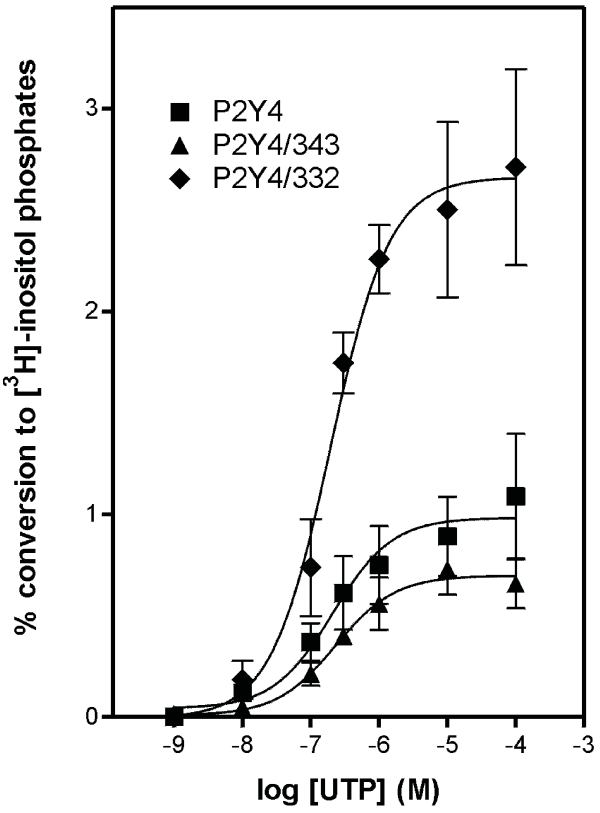

FIG. 7. **Agonist-promoted inositol phosphate accumulation in P2Y4-, P2Y4/343-, and P2Y4/332-R-expressing cells.** P2Y4-, P2Y4/343-, and P2Y4/332-R-expressing 1321N1 cells were prelabeled with [^{3}H]inositol and then incubated in the presence of 10 mM LiCl and the indicated concentrations of UTP for 5 min. Total [^{3}H]inositol phosphate accumulation was measured and divided by the total labeling of [^{3}H]-inositol lipids to determine the percent conversion to [^{3}H]inositol phosphates.

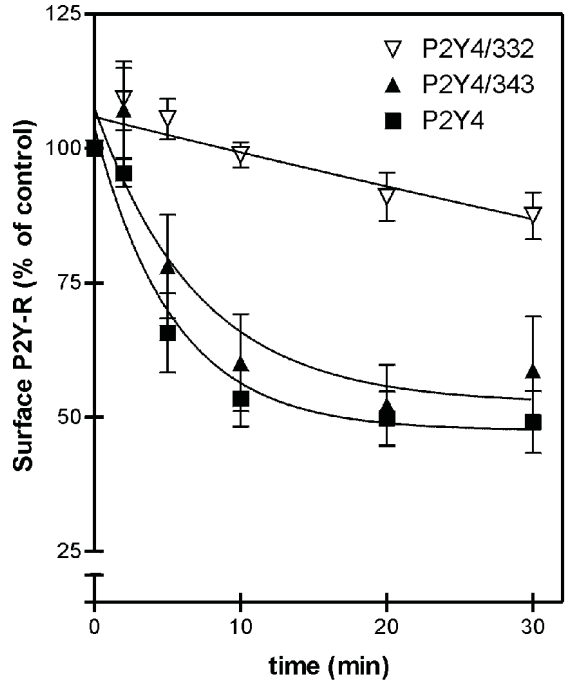

FIG. 8. **Truncation of the carboxyl terminus prevents UTP-promoted loss of cell surface P2Y4-R.** 1321N1 Cells stably expressing wild type P2Y4-R (■), P2Y4-R truncated at amino acid 343 (▲), or P2Y4-R truncated at amino acid 332 (▽) were incubated for the indicated times with 100 μM UTP. Cell surface receptors were detected using anti-HA antibody followed by ^{125}I-labeled secondary antibody.

phosphorylation apparently does not occur in the final seven serines/threonines of the carboxyl terminus, because a similar amount of agonist-dependent phosphorylation was observed with the P2Y4/343-R truncation mutant. In contrast, the capacity of UTP to induce phosphorylation was completely lost with the P2Y4/332 truncation mutant (Fig. 12). These results indicate that phosphorylation occurs on one or more of the three serines present between residues 332 and 343 in the carboxyl terminus. Together with the mutational analyses presented above, these results indicate that Ser-333 and Ser-334 are key regulatory residues in the events of agonist-induced phosphorylation, desensitization, and loss of cell surface P2Y4-R.

DISCUSSION

The observation of a broad range of uridine nucleotide-activated physiological responses (2), the cloning of two receptors that are specifically activated by uridine nucleotides (20–22), and the demonstration of regulated release of cellular UTP (17, 18) establish pyrimidinergic signaling as a physiologically important regulatory pathway. The UTP *versus* UDP specificity of P2Y4-R *versus* P2Y6-R adds potential complexity to pyrimidinergic signaling, and we have demonstrated here that these two P2Y-R exhibit very different regulatory properties. The P2Y4-R undergoes rapid agonist-induced desensitization but does not down-regulate. In contrast, the P2Y6-R does not undergo rapid agonist-induced desensitization but does down-regulate. Delineation of the molecular basis of the very different modes of regulation of these two uridine nucleotide-activated receptors

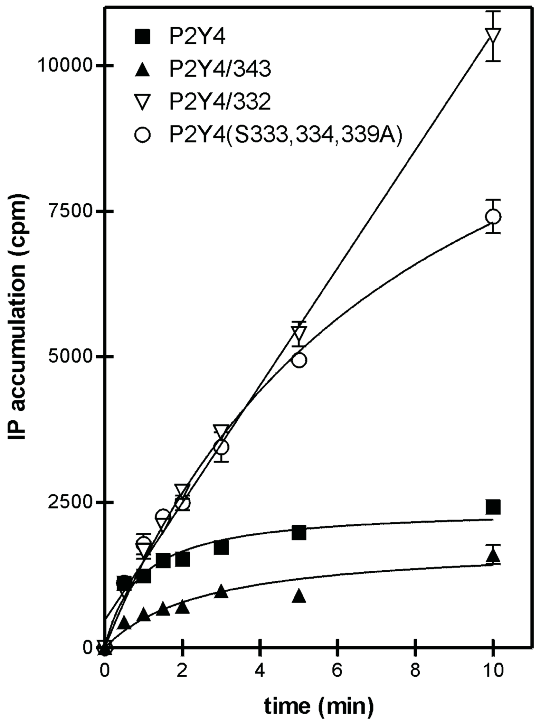

FIG. 9. **Agonist-promoted inositol phosphate accumulation in cells expressing mutant P2Y4-R.** 1321N1 cells stably expressing wild type P2Y4-R (■), P2Y4-R truncated at amino acid 343 (▲), P2Y4-R truncated at amino acid 332 (▽), or P2Y4-R with serine to alanine mutations at residues 333, 334, and 339 (○) were prelabeled with [^{3}H]inositol. The cells were then challenged with 100 μM UTP in the presence of 10 mM LiCl for the indicated times, and total [^{3}H]inositol phosphates were quantitated.

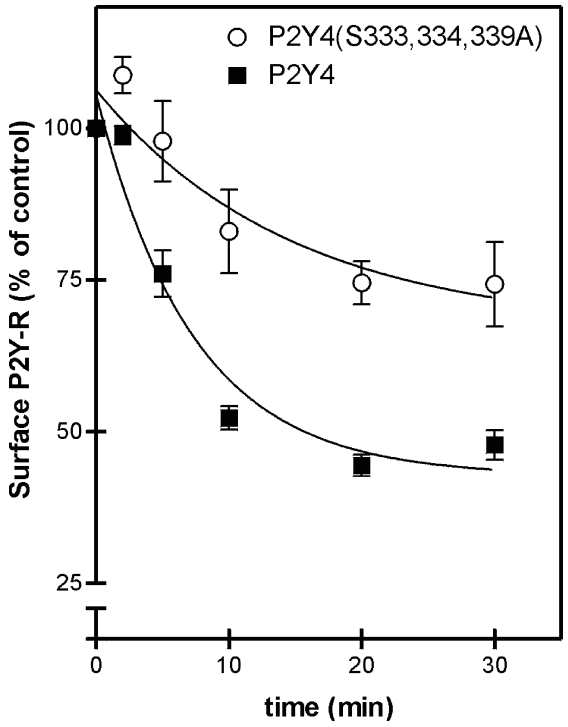

FIG. 10. **Agonist-promoted loss of cell surface P2Y4-R.** 1321N1 cells stably expressing either wild type P2Y4-R (■) or a P2Y4-R with serine to alanine mutations at residues 333, 334, and 339 (○) were incubated with 100 μM UTP for the indicated times. Surface P2Y4-R were quantitated using an anti-HA antibody followed by ^{125}I-labeled secondary antibody.

will be important to establish. In the current study we have shown that agonist-dependent phosphorylation of either of two adjacent serines in the carboxyl terminus of P2Y4-R contributes an initiating step in both desensitization and internalization of this receptor.

The rapidly occurring desensitization of P2Y4-R is a predictable regulatory response of a GPCR and confirms results previously reported by Robaye *et al.* (30) for P2Y4-R. Our kinetic analyses were of insufficient detail to resolve the time course of occurrence of desensitization from that of loss of surface receptors, but desensitization occurred at least as rapidly as did the receptor trafficking response. Agonist-induced desensitization of phospholipase C-linked GPCR has been exceptionally difficult to quantitate (35), because activity cannot be assessed in membranes prepared from agonist-preincubated cells as is the case with studies of adenylyl cyclase-linked receptors. Measurement of intact cell Ins(1,4,5)P_3 levels (35–39) may have certain advantages over quantitation of total inositol phosphates in the presence of LiCl as in the current study. Desensitization of the Ins(1,4,5)P_3 response almost certainly occurs faster than that revealed here in a measurement that includes not only Ins(1,4,5)P_3 but also all of its downstream metabolites. Thus, we only can conclude that agonist-induced desensitization of P2Y4-R is very rapid and likely occurs prior to loss of surface receptors. That agonist-stimulated accumulation of total inositol phosphates is essentially linear during activation of P2Y6-R expressed in the same cells, provides validation of the qualitative, if not quantitative, aspects of our analyses.

The P2Y4/343 carboxyl-terminal truncation mutant retained all of the phenotypical responses of the wild type receptor. In contrast, although UTP activated the P2Y4/332 mutant with properties similar to the wild type receptor, this truncated receptor essentially lost its capacity to undergo UTP-induced desensitization or loss of surface receptors. Although other residues in the 332 to 343 domain could provide these regulatory properties, the serines at positions 333, 334, and 339 are potential targets for kinase-promoted phosphorylation. Our studies showed for the first time that agonist-dependent phosphorylation occurs in a P2Y-R (Fig. 12). Moreover, the occurrence of UTP-stimulated phosphorylation in the P2Y4/343 truncation mutant but not in the P2Y4/332 truncation mutant confirms that the domain between residues 332 and 343 provides the principle sites of agonist-dependent phosphorylation. Mutational analyses of individual or combinations of serines in this domain indicate that Ser-333 and Ser-334 are the important sites of regulation and strongly suggest that phosphorylation of either of these two serines is a key step in agonist-dependent desensitization and loss of surface P2Y4-R. However, our results do not rule out an additional contributing role of phosphorylation at residues elsewhere in P2Y4-R.

Although the P2Y4-R exhibits a commonly described phenotype of rapid desensitization and loss of surface receptors, it is unusual in that agonist-promoted down-regulation of this receptor apparently does not occur. That is, following agonist removal from the medium (after times of preincubation up to 12 h) surface receptors were replenished within 30–60 min of incubation. Such results could be a vagary of a cell line engineered to express a recombinant receptor. However, P2Y6-R and P2Y2-R (40) expressed under the same conditions in 1321N1 cells (and apparently to similar levels as P2Y4-R) both down-regulate. We also have extensively studied down-regulation of the endogenous β2-adrenergic receptor (41, 42) and M3 muscarinic receptor (43, 44) in this same cell line. Thus, the P2Y4-R is a GPCR that both rapidly desensitizes and rapidly recovers from desensitization irrespective of the length of time of activation. It will be important to determine both the physiological significance and structural basis for this unusual property of P2Y4-R.

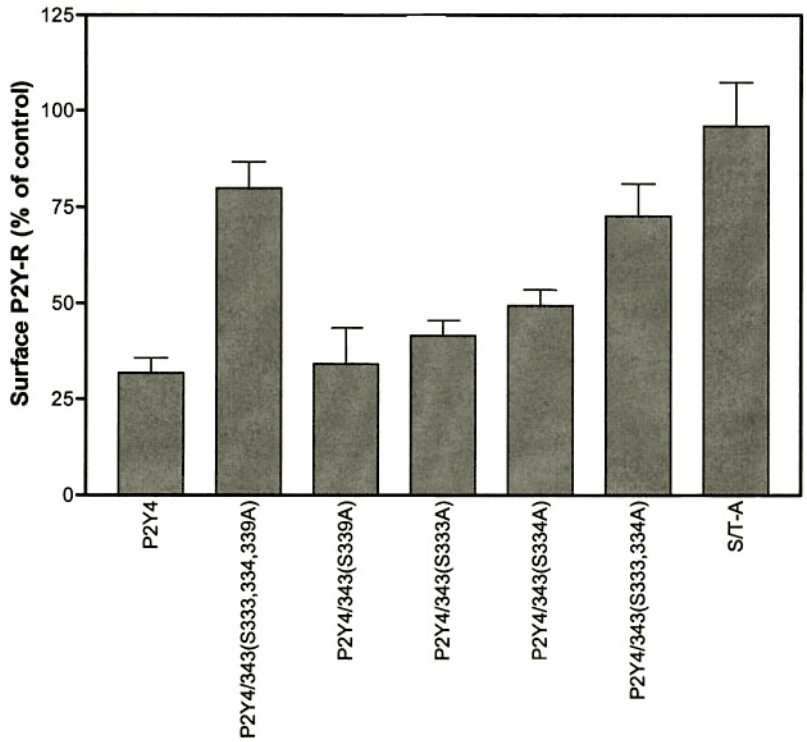

FIG. 11. **Agonist-promoted loss of surface receptors in cells expressing truncated/mutated P2Y4-R.** 1321N1 cells stably expressing mutant P2Y4-R were incubated with 100 μM UTP for 20 min. Surface P2Y4-R were quantitated using an anti-HA antibody followed by ^{125}I-labeled secondary antibody.

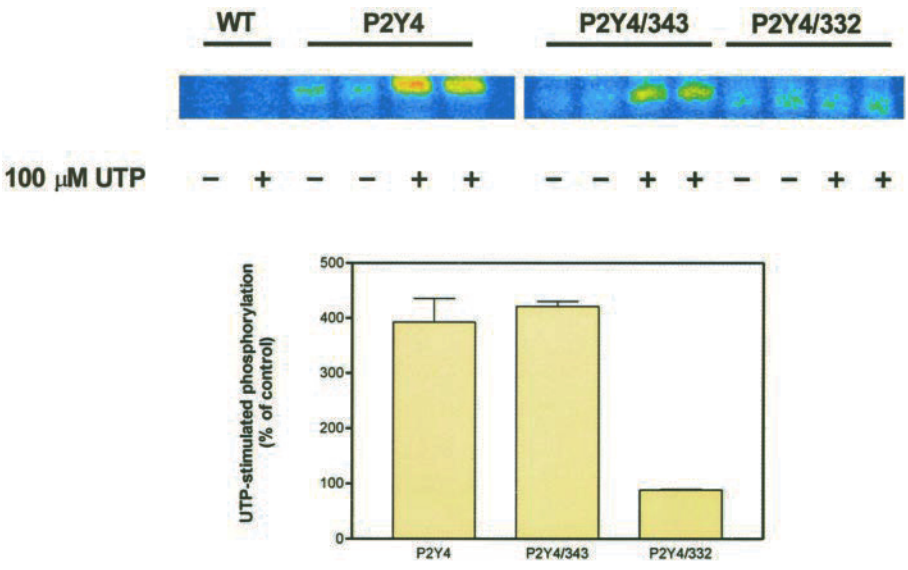

FIG. 12. **Agonist-promoted phosphorylation of wild type P2Y4 and mutant receptors.** Wild type 1321N1 cells (*WT*) or 1321N1 cells stably expressing P2Y4-, P2Y4/343-, and P2Y4/332-R were labeled with 500 μCi of [^{32}P] orthophosphate and incubated with 100 μM UTP for 2 min. Cells were lysed, and receptors were immunoprecipitated, resolved on SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose. A PhosphorImager was used to measure receptor phosphorylation. The data are presented as a densitometric scan of gels from a single experiment and as *bar graphs* of densitometric quantitation averaged from two experiments.

The P2Y6-R falls into a relatively small group of GPCR that do not undergo rapid agonist-induced desensitization. This observation is not entirely surprising, because the P2Y6-R lacks serines and threonines in its third cytoplasmic loop and only a single threonine occurs in the carboxyl-terminal domain. Two serines and two threonines are present in the first cytoplasmic loop, but this domain has not been predictably important in GPCR coupling to heterotrimeric G-proteins or in their agonist-dependent regulation. Simple replacement of the carboxyl-terminal domain of P2Y6-R with that of P2Y4-R did not confer to P2Y6-R a capacity to undergo rapid agonist-induced loss of surface receptors. It is uncertain whether such results reflect a regulation-resistant contribution of noncarboxyl-terminal sequence of P2Y6-R, or whether they simply reflect lack of proper structural context in which the phosphorylation of Ser-333 and Ser-334 apparently produce signals for desensitization/internalization in the P2Y4-R protein.

The overriding view of agonist-induced regulatory changes in GPCR signaling has followed from studies of the adenylyl cyclase-coupled β2-adrenergic receptor (45, 46). The intuitive model of regulation is one of second messenger-regulated kinases immediately downstream of GPCR feeding back to regulate the activities of cohort proteins of the pathway. This model has been substantiated in studies of the β2-adrenergic receptor by demonstrating that cyclic AMP-dependent protein kinase is in part responsible for agonist-induced desensitization (47–49). However, the dominant concept is that members of the GRK family of kinases catalyze receptor phosphorylation to provide the major initiating event in agonist-dependent receptor desensitization and internalization (28, 29).

As with receptor-regulated adenylyl cyclase, much of the early thinking on regulation of receptor-promoted inositol lipid signaling included models involving protein kinase C-mediated feed-back regulation of responsiveness. This view was supported by large inhibitory effects of phorbol ester activators of PKC on receptor-stimulated inositol phosphate accumulation

(50–52), although it could be argued that the extent of PKC activation by PMA greatly exceeds that normally occurring through receptor-promoted activation of phospholipase C. Experiments utilizing pharmacological inhibition or down-regulation of PKC clearly confirmed a role for this second messenger-regulated kinase in agonist-promoted desensitization of inositol lipid signaling (31, 52). However, very few of these studies established that the effects of PKC were at the level of the involved GPCR, and PKC could act at multiple levels in the inositol lipid signaling pathway. For example, phospholipase Cβ is phosphorylated by PKC, and its activity is inhibited (53, 54). Nonetheless, several phospholipase C-linked GPCR are phosphorylated in response to activation of protein kinase C (32–34).

The presence of a potential consensus site (S243) for PKC phosphorylation in the carboxyl-terminal portion of the third cytoplasmic loop of P2Y4-R suggested that PKC might play a role in the marked agonist-induced desensitization and loss of cell surface receptors that occurs upon activation of this receptor. However, incubation of P2Y4-R expressing cells with PMA did not mimic these UTP-promoted effects, and neither down-regulation of PKC nor pharmacological inhibition of its activity altered the time course or extent of agonist promoted loss of surface P2Y4-R. Moreover, serine to alanine mutation of S243 did not modify agonist-dependent regulatory effects. Thus, although UTP induces profound changes in P2Y4-R, little or none of these effects follow from a feed-back involvement from its most proximally activated protein kinase. Because ionomycin also failed to induce changes in UTP responsiveness or modify those occurring during incubation with UTP, Ca^{2+}/calmodulin-regulated kinases also are not involved. GRKs recently have been implicated in regulation of Gq/phospholipase C-coupled GPCR (29, 55–57), and our experiments that exclude PKC in regulation of P2Y4-R indirectly suggest that GRKs also regulate P2Y4-R. However, coexpression of GRK2 with P2Y4-R in Cos-7 cells had no effect on UTP-dependent desensitization or loss of surface receptors whereas in parallel experiments GRK2 expression markedly augmented desensitization and internalization of the P2Y2-R.[2] Perhaps another GRK family kinase, *e.g.* GRK5, is involved in regulation of P2Y4-R. Alternatively, casein kinase 1α catalyzes agonist-dependent phosphorylation and regulation of the Gq/phospholipase C-coupled M3-muscarinic receptor, and potentially other Gq-linked receptors (58, 59).

The P2Y-R family is comprised of a group of five Gq/phospholipase C-linked GPCR that exhibit novel selectivity for extracellular adenine and uridine di- and triphosphates. The ATP- and UTP-activated P2Y2-R was shown previously to undergo agonist-induced internalization (40, 60), and Garrad *et al.* (60) reported that the capacity of UTP to induce P2Y2-R sequestration was reduced by truncation of the carboxyl terminus. The current work reveals that the subfamily of two pyrimidinergic P2Y-R exhibit very different properties of regulation and that each of these receptors exhibits a property of regulation not common among GPCR. Although our work has not established the identity of the kinase involved in regulation of P2Y4-R, it identifies in P2Y-R for the first time sites of phosphorylation important for agonist-dependent regulation of receptor responsiveness and cellular translocation. It will be important to establish whether phosphorylation *per se* is sufficient to functionally uncouple P2Y4-R from Gq and to establish the role this phosphorylation plays, for example, in interaction with arrestin, in internalization, and in a recycling response that rapidly and completely replenishes surface P2Y4-R even during extended activation of the receptor.

[2] S. Delaney and T. K. Harden, unpublished results.

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