Constitutive and Agonist-induced Dimerizations of the P2Y<sub>1</sub> Receptor

RELATIONSHIP TO INTERNALIZATION AND SCAFFOLDING

In living cells, P2Y<sub>1</sub> receptor dimerization was quantitated by an improved version of fluorescence resonance energy transfer donor photobleaching analysis. 44% of the P2Y<sub>1</sub> receptors expressed in HEK293 cell membranes exist as dimers in the resting state, inducible by agonist exposure to give 85–100% dimerization. Monomer and constitutive dimers are fully active. Agonist-induced dimerization follows desensitization and is fully reversible upon withdrawal of agonist. Receptor dimers are required for internalization at 37 °C but are not sufficient; at 20 °C dimerization also occurs, but endocytosis is abolished. Removal of the C-terminal 19 amino acids abolished both dimerization and internalization, whereas full activation by agonists was retained up to a loss of 39 amino acids, confirming active monomers. This receptor is known to bind through its last four amino acids (DTSL) to a scaffolding protein, Na/H exchanger regulatory factor-2, which was endogenous here, and DTSI removal blocked constitutive dimerization specifically. Distinction should therefore be made between the following: 1) constitutive dimers tethered to a scaffolding protein, together with effector proteins, within a signaling micro-domain, and 2) free dimers in the cell membrane, which here are inducible by agonist exposure. For the class A G-protein-coupled receptors, we suggest dimers in the cell membrane, which here are inducible by agonist exposure. For the class A G-protein-coupled receptors, we suggest dimers in the cell membrane, which here are inducible by agonist exposure.
EXPERIMENTAL PROCEDURES

Materials and Purity of Nucleotides—Materials not specified here were from Sigma or Invitrogen. MRS2179 was from Tocris (UK). FITC-anti-Myc-Ab, Cy3-anti-Myc-Ab, and their parent anti-Myc-Abs were from Abcam (UK) and Sigma, respectively. The anti-phospho-ERK1/2 and anti-ERK1/2 Abs were from New England Biolabs. The mean content of fluorophore per IgG molecule in the samples used for FRET was determined by direct spectrophotometry on aliquots and found to be three for the FITC-anti-Myc-Ab.4 It was seven for the Cy3-anti-Myc-Ab).

Plasmid Constructs and Transfections—hP2Y1, R cDNA (18) was subcloned into pCMV-Tag3 vector (Stratagene) to produce N-terminal-Myc-hP2Y1, R. The ΔT1, ΔT2, and ΔTSL truncations were made on that plasmid using QuickChange site-directed mutagenesis kit (Stratagene) to terminate at amino acid 354, 334, or 371, respectively, with the reverse primers 5′-AACTCGAGTCAACTCTTGATGAAATTT-3′ (ΔT1), 5′-AACTCGAGCTCACTCTCTTCTGAAAGTATC-3′ (ΔT2), and 5′-AACTCGAGTCATCATTCTGCAAGAATCTAGG-3’ (ΔTSL). (Stop codons are in boldface.) Each construct was transfected into HEK293 cells, using Lipofectamine 2000 (Invitrogen), to express either transiently (only where specified) or with selection (to full dilution cloning) for stable expression, each being cultured, as per Simon et al. (17), in Dulbecco’s modified Eagle’s medium (DMEM) containing G-418 (for transient) as defined there. P2Y1R expression at comparable levels for each clone was confirmed by Ca2+ mobilization assays as below, at maximum response levels.

Ca2+ Mobilization and Desensitization Studies—HEK293 cells transiently (where stated) or stably expressing the full or truncated Myc-P2Y1, R were grown to form confluent monolayers (at similar densities) in clear flat-bottomed, black-walled Costar 96-well microtiter plates. The cells were then loaded with 2 μM Fluo-4AM in Hanks’ balanced salt solution (HBSS; but without CaCl2), 20 mM HEPES, pH 7.5, for 45 min, followed by wash (150 μl) and equilibration in HBSS (containing 2 mM CaCl2) per well. Agonist reactions and washes were performed in the latter medium, as were all the cell incubations in the studies described here. All operations were at 37 °C. Changes in fluorescence were measured (integrating the area under each peak, where quantitated) in each well in a FlexStation II robotic spectrophotometer (Molecular Devices, Wokingham, UK) with excitation at 488 nm and emission at 525 nm.

Detection of NHERF-1 and NHERF-2 mRNAs—The PCR amplifications were performed on first-strand cDNA, made from Myc-hp2Y1, R HEK293 cells using standard protocols, with primer sets specific to the human NHERF-1 or NHERF-2 sequences as follows: NHERF-1, 5′-AACGAAAATGACCCCTGCCGA-3′ (sense) and 5′-GAGGTAGACGAGGCGCTGT-3′ (antisense); NHERF-2, 5′-AAGGGCTGTGGGGGAGGCCAGT-3′ (sense) and 5′-GGTGTGACCCGAAGCCGCTT-3′ (antisense). Typically, a 50-μl reaction mixture contained 1st strand cDNA (3 μl), the appropriate primers (200 nM), 3 mM MgCl2, each dNTP (200 μM), and 2.5 units of Taq polymerase (Roche Applied Science). Cycling conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 94 °C for 60 s, 52 °C for 60 s, 72 °C for 60 s with a final extension step of 72 °C for 10 min. PCR products were resolved by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

ERK Phosphorylations—The Myc-hP2Y1, R cells, unlabelled or antibody-labeled separately with FITC or Cy3, were pre-stained for serum (4 h), incubated with agonist (10 min) as shown, washed with 1 mM Na3VO4medium, solubilized in boiling SDS sample buffer, 5% β-mercaptoethanol, and analyzed on 10% SDS-polyacrylamide gels, with details as given in Refs. 14, 19. The quantitations of the phosphorylated ERK1 and -2 isoforms were performed as in Ref. 14, including use of control lanes with serial dilutions of one of the samples tested, for confirming linearity of the response in the gel and conditions used.

FRET Analysis by Donor Photobleaching—HEK293 cells stably expressing Myc-hP2Y1, R were cultured on 13-mm coverslips. At room temperature the living cells were pre-blocked with 5% normal goat serum in HBSS for 1 h and then doubly labeled (1 h) with FITC-anti-Myc (1:500) and Cy3-anti-Myc (1:50) Abs applied together or alone with either FITC-anti-Myc- or Cy3-anti-Myc-Ab, in HBSS plus apyrase (2 units/ml). (When the anti-Myc antibody was labeled with Cy3, that preparation was more diluted than the FITC-labeled anti-Myc, and the titers of the two led to the difference in the dilutions used, which were shown, see below, to give near-equivalent labeling on the cells.) After washing (2× 200 μl of HBSS), the cells were used at once in a laser-scanning confocal microscope (Zeiss LSM 510), using a chamber thermostatted at 37 °C or (where noted) 20 °C. The doubly labeled cells showed FITC (donor) and Cy3 (acceptor) fluorescent label together on almost all of the cells, and these were selected here. (Control cells transfected with empty vector gave no significant reaction with either labeled Ab; data not shown). The rate of loss of the FITC fluorescence emission peak centered at 515 nm was measured during FITC photobleaching at 488 nm of one cell at a time, in an optically isolated constant region of its cell membrane. Bleaching was for repeated periods of 4 s, with 5-s rest intervals between them, during which time the image was captured, and continuing until the FITC signal was <10% of its original. The control cells labeled with Cy3 alone were also bleached at 488 nm, and in the intervals excited instead at 543 nm to test for bleed-through bleaching by measuring its Cy3 emission at 565 nm. All images were acquired well below saturation of the pixel intensity. The operations were standardized, and emission signals were integrated and processed in Zeiss LSM image browser package and further analyzed with Image J software.

For agonist activation studies, the cultures were incubated with 2-MeSADP at the stated final concentration at 20 or 37 °C. Donor photobleaching runs, as above for each case, were performed immediately prior to the addition of agonist and on cells in samples exposed for the increasing times shown. When a P2Y1, R antagonist was applied, it was present prior to the agonist addition and during the photobleaching. In the agonist

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4 A. Uustare, unpublished data.
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wash-out case in Fig. 6D, the cultures were rapidly washed twice with 200 μl of HBSS before each photobleaching was applied. When BAPTA-AM was used it was present throughout the antibody labeling, washing, agonist incubation, and the photobleaching.

Analysis of Donor Photobleaching Kinetics—From the successive emission readings on a given cell membrane region, the decline of FITC emission amplitude was analyzed using the GraphPad Prism 3 software. The great majority of the doubly labeled cells gave values highly significant for a bi-exponential fit and were used in the calculation below. (The small remainder giving different kinetics was attributable to damaged cells or optical artifact.) These decay curves could be resolved into two mono-exponential components, defined by \( \tau_{fast} \) and \( \tau_{slow} \). To verify that a significant difference existed there, the data set from every cell used was separately subjected to a variance ratio test for a fit to two-component dependence, calculating its \( F \)-value with the appropriate degrees of freedom. When Cy3 was absent, the FITC decay consistently gave a highly significant mono-exponential fit, with time constant \( \tau_{con} \) (with \( F \)-values showing its identity to \( \tau_{fast} \) above). For each set of conditions and agonist exposure time tested, the means ± S.E. for these component time constants were derived from the full time series from each of six cells and using two cultures. From the theory of resonance energy transfer, a two-state model (i.e. for one monomer and one dimer state) gives the following relationship (15, 20):

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\text{dimerization} = \frac{f_{ET}}{f_a} \times 100,
\]

where \( f_a \) is the acceptor-labeled fraction in the total of labeled receptors, and \( f_{ET} = \frac{f_{ET}}{f_a} \) is the fraction of the total donor present, which is in FRET. The relative contributions of the slow component, because of donor-acceptor dimers, and of the non-FRET fast component can be obtained from their pre-exponential coefficients (the zero time values), hence giving the value of \( f_{ET} \). Although D-D dimers will also be present, to a smaller and predictable degree, being FRET-negative (like A-A dimers) will not register in the slow component and do not affect the representation by \( f_{ET} \) of the total of dimers present; when the D-A dimer is maximal, total dimers are maximal. The factor \( f_a \) (in equation above) is then needed for the relative occupancy of the Myc site by the donor- and the acceptor-labeled antibodies. Because the acceptor-labeled antibodies reacted in competition and in great excess to the membrane-bound receptors, the occupancy is determined by the ratio of the affinities of those antibodies for the Myc tag on the P2Y<sub>1</sub>R and the actual concentrations of the two IgGs in the reaction mixture. The value of \( f_a \) was found by reacting at 20 °C (40 min) aliquots of the living HEK293 cells expressing Myc-hP2Y<sub>1</sub>R with the Cy3-labeled anti-Myc Ab, alone or in competition with the unlabeled anti-Myc Ab (parent of the FITC-labeled form used), precisely in the conditions used in the dimerization analyses. The ratio of receptor-bound Cy3 in the two cases was then determined by Cy3 spectrofluorimetry in the FlexStation II system. This method avoids the acceptor spectral change because of donor addition and FRET, and can be used because it was shown (Fig. 1) that the labeled anti-Myc Ab and the same unlabeled Ab have (as expected) the same affinity for the Myc-hP2Y1R. The \( f_{ET} \) for each experimental case, plus the \( f_a \) factor, then yielded, as above, the values of the % dimerization.

Acceptor Photobleaching—Living HEK293 cells on 13-mm coverslips stably expressing Myc-hP2Y<sub>1</sub>R were treated with 2-MeSADP (10 μM, 20 min, 20 °C) and immediately fixed by 4% paraformaldehyde, 10 min, followed by 50 mM NH₄Cl, 25 min, and HBSS washing. Cultures were pre-blocked by 5% goat serum as above and then incubated with both the FITC-conjugated (1:500) and the Cy3-conjugated (1:50) anti-Myc antibodies (4 °C, 16 h, in HBSS) followed by washes (five times for 10 min) and ethanol dehydration. The mounted cultures were analyzed by laser confocal microscopy of a defined membrane region of each cell as above. Cy3 photobleaching at 543 nm was for a series of 10-s periods, with intervals of 10 s between them, during which spectral scanning was made. The size of the initial Cy3 peak (set as 100%) provided an internal standard for each membrane region studied. It was checked that no significant local heating occurred in the bleaching conditions used. Cy3 was preferred over the rhodamines usually used as bleachable acceptors for FITC, despite Cy3 bleaching more slowly, because the spectra of the rhodamines (but not Cy3) change considerably and suffer partial quenching when conjugated to a protein (21), compromising quantitation of FRET.

Receptor Internalization—Living HEK293 cells stably expressing Myc-hP2Y<sub>1</sub>R were labeled with FITC-anti-Myc-Ab (1:500) in HBSS at room temperature for 1 h. Following HBSS washing (two times for 5 min), they were incubated with HBSS or with 10 μM 2-MeSADP in HBSS in the confocal microscope chamber at 37 or 20 °C. Images were captured at the times shown. Phase contrast images were taken simultaneously to confirm focusing. Images were analyzed by the LSM Image Analyzer software, with line scanning across the cell or Z-stacking, but avoiding the nucleus. Where fixation is specified, live cultures were incubated ± agonist similarly at 37 °C, 1 h, followed by 4% paraformaldehyde (without permeabilizing agent), 10 min, and 50 mM NH₄Cl, 25 min. They were pre-blocked by 5% normal goat serum in phosphate-buffered saline (PBS) (20 °C, 1 h) and then incubated with anti-Myc antibody (1:1000) in blocking solution at 4 °C, 16 h. After PBS washes (five times for 10 min), TO-PRO-3 (1:2000, 20 °C, 2 h) was applied to show cell nuclei. Alexa-488-labeled secondary antibody (Invitrogen) was used here for the anti-Myc case to increase the fluorescent label number to detect low levels of internalization. After PBS washes (three times for 10 min) and ethanol dehydrations, the cultures were mounted in anti-fade medium (DAKO, UK). Labeled cells were Z-scanned in confocal microscopy, in 1-μm steps from base to top; excitation 488 nm/emission 520 nm (Alexa-488) and 642 nm/660 nm for the TO-PRO-3 pseudo-color.

Statistics—Significance, determined by \( t \) test, was set at \( p < 0.05 \). The mean ± S.E. is stated, or in the quantitative figures is shown by bars (except where too small to show), based on three or more samples, each measured in triplicate, unless otherwise stated. Dose-response plots were best fit curves to a Hill equation (in GraphPad Prism 3). In all the imaging, dose response, gel analysis, and desensitization figures, the results shown are representative of three or more independent replicates of the experiments illustrated.
RESULTS

P2Y<sub>1</sub> Receptors with Extracellular FRET Donor or Acceptor Probes—Anti-Myc antibodies directly conjugated to fluorescein (FITC; FRET donor, D) or to Cy3 (acceptor, A) were applied together to living HEK293 cells stably expressing N-terminal Myc-tagged hP2Y<sub>1</sub>Rs. The two alternative products, designated as FITC-hP2Y<sub>1</sub>R and Cy3-hP2Y<sub>1</sub>R proteins, each showed in confocal microscopy a distribution over the whole cell surface, indistinguishable for those two (Fig. 1A). Intracellular Ca<sup>2+</sup> mobilization by the surface P2Y<sub>1</sub>R activity was evoked by exposure to the selective agonist 2-MeSADP; the agonist EC<sub>50</sub> value was unchanged after the addition of the Myc epitope (Fig. 1B). There was also no change in EC<sub>50</sub> after the further attachment of the labeled Ab (illustrated in Fig. 1, E and F) for P2Y<sub>1</sub>R downstream signaling through ERK and with the weaker agonist 2-MeSATP.

Dimers of Cell Surface P2Y<sub>1</sub>Rs, Two Photobleaching-based Approaches—Fluorescein is readily photobleached at 488 nm. This process was detected maximally at its emission peak of 515 nm, monitored in cells containing only FITC-anti-Myc-hP2Y<sub>1</sub> (Fig. 2A, top panels). This signal was measured at the cell membrane (as optically isolated), showing an exponential decay with that exposure (Fig. 2B, open circles). In the cells containing both FITC-P2Y<sub>1</sub>R and Cy3-P2Y<sub>1</sub>R, the same 488 nm exposure produced a slower decline of the FITC fluorescence (Fig. 2, A, lower panels, and B, closed circles). This protection is because of FRET between the attached FITC and Cy3 groups. There was negligible photobleaching at 488 nm of Cy3 in the same cells (Fig. 2B).

This retardation of donor photobleaching by acceptor interaction was found to be enhanced by P2Y<sub>1</sub> agonist stimulation, to a maximum after 20 min with 10 μM 2-MeSADP (37°C), as detailed below. Those conditions were therefore applied in an independent confirmation of FRET by spectral analysis. FRET decreases donor emission and gives a donor-sensitized increase in acceptor emission. This was assessed by suppressing the effect of FRET on the donor by progressive photobleaching of the acceptor. The FITC emission then rose in each cell, which can only be due to initial FRET (Fig. 2, C–E). This produced, at 30% loss of Cy3 emission (in 20 agonist-treated bleached cells from three independent experiments), a doubling of the FITC emission, denoting a high FRET level and dimer content.

Quantitation of Dimer Formation and Its Dual Response to Sustained Agonist Reaction—The photobleaching of the donor-labeled receptors, when present alone so that FRET was absent (control), best fitted a single exponential decay curve in all cases, with a time constant designated τ<sub>con</sub>. In the dual-labeled cells, in contrast, the bleaching decay (Fig. 2B) showed complex kinetics, which could be resolved analytically (see “Experimental Procedures”) into two mono-exponential components, defined by time-constants τ<sub>fast</sub> and τ<sub>slow</sub>. In all such dual labeling analyses, τ<sub>fast</sub> was constant and not significantly different from τ<sub>con</sub> of the control FITC-labeled receptors without FRET. The τ<sub>fast</sub> component represents control (non-FRET) donor bleaching in monomers and in the lesser fraction of donor-donor dimer present. The relative contribution of the τ<sub>slow</sub> component to the total decay there was thus readily determined (representing the fraction of P2Y<sub>1</sub>Rs in energy transfer, f<sub>ET</sub>, i.e. in dimers). The observed f<sub>ET</sub> value requires correction for the relative labeling of donor and acceptor in the reaction conditions used. That ratio was determined directly, by competition and fluorimetry, to correct f<sub>ET</sub> (see “Experimental Procedures”) to yield the percentage of dimers in the P2Y<sub>1</sub>R population in each set of conditions tested.

Living cells doubly labeled as before were subjected to donor photobleaching (Fig. 2A) to thus determine the % dimerization


**FIGURE 2. Demonstrations of FRET between labeled P2Y1Rs.**

A, donor photobleaching. Cells stably expressing Myc-hP2Y1R were labeled by FITC-labeled anti-Myc antibody or by that plus Cy3-labeled anti-Myc antibody simultaneously. In the presence of 2-MeSADP (see "Experimental Procedures"), under excitation of living cells at 488 nm, confocal images were captured of FITC or Cy3 emission at their peaks (five intervals shown). The photobleaching of FITC is distinctly slowed in the D + A state because of FRET. Bar, 10 μm. B, fluorescence decay over 32 s of such donor-photobleaching was quantified in single cells expressing Myc-hP2Y1R, labeled with FITC (D) or FITC + Cy3 (D + A). (Cells with FRET increased by agonist induction were used here.) Relative fluorescence intensity (RFI) of each is normalized to its initial level. Cells with Cy3 (only), label shows negligible 488 nm bleaching. C, reference emission spectra of FITC or Cy3, superimposed from cells labeled singly, each normalized to its maximum. D, acceptor photobleaching. Myc-P2Y1R-expressing cells were pre-activated by 2-MeSADP (30 min, 20 °C) and then doubly labeled before cumulative Cy3 bleaching at 543 nm was applied, FITC and Cy3 emissions are shown on the same cell. Below, corresponding spectral scans (488 nm excitation). These consistently showed that the relative emission of FITC is much lowered in the initial state because of FRET, but as Cy3 bleaches (open triangles), this effect on FITC is progressively relieved (closed triangles). Relative fluorescence intensity (RFI) is normalized to initial Cy3 peak as 100%. E, as in D but acceptor is absent; FITC emission is unaffected by 543 nm bleaching.

in several conditions as follows. (i) Because the P2Y1R agonists ATP and ADP accumulate in the medium of those cultured cells, they were removed by their scavenger apyrase up to the start of bleaching, to establish the nucleotide-free constitutive level. This reduced significantly the observed dimer content, from “basal” of 44 ± 3% to 28 ± 2% (Fig. 3A), i.e. the automatic exposure to endogenously released agonists had increased P2Y1R dimer formation. (ii) Using the apyrase-pretreated cells, 2-MeSADP produced a further increase in dimerization, which is concentration-dependent up to a maximum at 10 μM agonist; that increase was preventable with the P2Y1R antagonist (22) MRS 2179 (Fig. 3A). The antagonist-resistant dimer content and also that in the apyrase-treated control confirm a constitutive level of 28% dimers for the hP2Y1R at 37 °C. (iii) Binding of the Ab does not affect the agonist-promoted dimerization (Fig. 3B). This was confirmed independently in the acceptor photobleaching study; there, the activation by 10 μM 2-MeSADP, which was given prior to dual Ab labeling, gave a high degree of FRET (Fig. 2D) similar to that noted above.

Therefore, apyrase-pretreated cells carrying dually labeled P2Y1Rs were incubated with 10 μM 2-MeSADP, and the donor photobleaching kinetic analysis as above was performed on single cells. Dimers in the cell membrane increased, to 85 ± 4% of the receptors after 20 min in agonist at 37 °C or 30 min at 20 °C. Beyond that, the dimer content declined, at 20 °C to a steady level of 45 ± 5%, but after ~30 min at 37 °C the receptor con-
tent of the membranes became too low for dimer analysis (Fig. 3C). At 37 °C this loss was shown to be due to dimer internalization and sequestration in sub-membranal compartments, by following the fate of pre-labeled receptors on the intact cells. Thus, after 20–50 min of subsequent agonist treatment at 37 °C, the initial localization in the cell membrane disappeared, and the receptors became dispersed in a zone below the membrane. When the intensity profile across the cytosol was plotted by line scanning of confocal images, no receptors were detectable on the membrane after 50 min of agonist exposure, but the label had then moved to the interior (Fig. 4A). In confirmation, unlabeled cells were incubated (60 min) in medium (control) or agonist as before, and the receptors were then post-labeled. These were subjected to Z-stack confocal microscopic analysis; images 1 μm apart were collected from the base to the top of the unpermeable cell membrane. In control cells the Ab-labeled P2Y1 receptors were detectable over all of the cell membrane, whereas in the agonist-treated cultures the membrane has lost all of those receptors prior to the labeling, except for a weak signal at the adhering base of the cell where there is some protection from the medium (Fig. 4B). Vertical cross-sections of assembled Z-stacks confirmed that in the cells incubated for 60 min at 37 °C without agonist the receptors remain at the membrane over the whole surface region, whereas in parallel agonist-treated, post-labeled cultures the label was virtually all absent (Fig. 4C). The internalization induced by agonist was not dependent upon Ab binding to the receptor (cf. prior attachment in Fig. 4A with final attachment in Fig. 4, B and C). We conclude that after the maximum of dimerization is reached at 37 °C the P2Y1 receptors internalize.

**P2Y1R Desensitization Is Distinct from Dimer Formation or Internalization**—Exposure to 2-MeSADP readily desensitized the intracellular Ca2+ mobilization response of P2Y1 receptors to a second application of agonist. Desensitization was high at 10 nM and complete at 100 nM within 10 min at 37 °C (Fig. 5C). Likewise, in the P2Y1R-evoked phosphorylation of ERK1/2, there was again complete desensitization at 100 nM and above, even with the weaker P2Y1R agonist, 2-MeSATP (Fig. 6A). This sensitivity contrasts with the P2Y1R dimerization, where the maximum effect required 10 μM 2-MeSADP presence for 20 min (Fig. 3, A and C). At 100 nM agonist the dimer content above the constitutive level is low (Fig. 3A).

Desensitization was evoked with equal sensitivity when the receptors carried the anti-Myc Ab (Cy3-labeled or not; Fig. 5, C, I, and J). Activation of another Gq-linked receptor present on the cells, endogenous endothelin-1 (ET-1) receptor, was unaffected when the P2Y1R showed full desensitization (Fig. 5, D, G, and H); hence, depletion of Ca2+ stores or of signaling intermediates did not interfere. Thus, at the agonist concentrations needed to dimerize, the receptors will be fully desensitized long before that process occurs.

At temperatures well below 37 °C the clathrin-mediated endocytosis of membrane GPCRs (which operates for the P2Y1R, see Ref. 23) is known to become blocked (24). Indeed, this block has been confirmed for other Gq-linked P2YR subtypes at 28 °C or below, where tested (25, 26). Therefore, this effect was applied here to test separation of P2Y1R internalization from the other events studied. At 20 °C, by 30 min the P2Y1R dimer formation is maximally induced by 10 μM 2-MeSADP (Fig. 3C), and it then suffers no internalization (Fig. 4D). As expected, it was found to be totally desensitized, as could then be shown immediately after agonist removal (Fig. 6B, 30-min point). During a further 25 min without agonist, it recovers its full activity and original EC50 value (Fig. 6, B and C). During this recovery the dimer dissociates to the basal level (Fig. 6D). The regain of activity is parallel to this dissociation, but it is important to note that when 100% of the activity is recovered there remains 42% dimerization (Fig. 6, B and D), in agreement with the 44% basal dimerization seen initially for the fully active receptor (Fig. 3A, left). Hence, at 20 °C prolonging the agonist treatment to 50–60 min leads to a final steady-state content of ~50% dimers (Fig. 3C and Fig. 6D, filled circles) with full retention of these desensitized receptors at or close to the membrane (Fig. 4D), and at 37 °C the receptor was by then fully internalized (Fig. 4, A–C). Hence, for the agonist-dependent internalization to also occur, desensitization is not a sufficient condition.

**An Intracellular Ca2+ Rise Is Involved in Agonist-induced Dimer Formation**—In nucleotide-free medium the constitutive level of dimerization was not significantly changed when intracellular Ca2+ was chelated by incubation with BAPTA-AM (Fig. 6E). However, in the agonist treatment the dimerization...
Roles of the C Terminus in P2Y1R Dimerization, Desensitization, and Internalization—We tested truncations of the C-terminal domain, which has been implicated in the desensitization and internalization of P2Y2R and P2Y4R (27, 28). By cDNA deletions the Myc-hP2Y1R protein was terminated at position 354 (construct Myc-hP2Y1−/H9004T1) or at position 334 (Myc-hP2Y1−/H9004T2) to remove almost all of the C-terminal tail (Fig. 7A). Each truncated form was expressed separately in HEK293 cells. Staining with directly labeled anti-Myc Abs showed that these forms are at the cell membrane, as before (Fig. 7, D and E). For activation by 2-MeSADP of Ca2+ mobilization, essentially identical EC50 values were found for hP2Y1R, Myc-hP2Y1R, and Myc-hP2Y1−/H9004T1; for Myc-hP2Y1−/H9004T2, the EC50 was 7-fold weaker (Fig. 1B and Fig. 7B). This was confirmed in a downstream response (through ERK1/2 phosphorylation) and with a less potent agonist, 2-MeSATP; the P2Y1R EC50 was again not significantly changed by the T1 truncation and was weaker with the T2 truncation (Fig. 7C).

For both transductions, the desensitization and the recovery from it on washing were fully retained in both Myc-hP2Y1−/H9004T1 and Myc-hP2Y1−/H9004T2 proteins (Fig. 8, A and B), as in intact hP2Y1R (Fig. 5 and Fig. 6A). In contrast, other properties were lost by the C-terminal truncation. On cells expressing truncated Myc-hP2Y1Rs, dually labeled and exposed to 10 μM 2-MeSADP (20 min, 37 °C), FRET was examined using acceptor photobleaching, as used previously (Fig. 2D) on the full-length protein. In complete contrast to the behavior of the latter, no FRET was thus detected after either the T1 or T2 truncations (Fig. 7D and E); no initial depression of the donor fluorescence was seen, and extensive photobleaching of Cy3 produced no increase in the donor emission peak.
including that this C-terminal tail region of the P2Y1R is not confirmed their maintenance in the membrane (Fig. 7).

Furthermore, in cells carrying only the FITC-labeled even after doubling the previously applied period of irradiation.

Because the final sequence, DTSL, in this tail can interact with a scaffolding protein, NHERF-2 (29), we then deleted

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FIGURE 5. Desensitization behavior of Myc-hP2Y1R at 37 °C. On stably transfected HEK293 cells, intracellular Ca2+ changes were measured using Fluo-4 with 2 mM Ca2+ in the external buffer, as in Fig. 4A. Here, no correction for the small fraction of endogenous of hP2Y1R present was needed, because it behaved like the expressed Myc-hP2Y1R. Open triangles, first application of agonist (sustained, broken line) or of buffer alone; closed triangles and solid line, second application. A–D, first application (12 min) of 2-MeSADP at 100 nM or above was able to desensitize fully the response to 1 μM 2-MeSADP. E and F, reversibility was shown after buffer washes (two times for 30 s and one time for 7 min) before 2-MeSADP (1 μM) was re-applied. G and H, similar first application (sustained) of 2-MeSADP (2-MeS) at 1 μM (in H only) plus a second application instead of ET-1 at 1 μM showed no cross-desensitization. I and J, with bound anti-Myc antibody, either unlabeled or Cy3-labeled, the full homologous desensitization of P2Y1R as seen in C and D was likewise shown.

FIGURE 6. Functional comparisons of the hP2Y1R monomer and homodimer. A, activity in the phosphorylation of ERK proteins also shows full homologous desensitization. This is present after applying 2-MeSADP (down to 100 nM) for 10 min. After the challenge by a second application as indicated above, the phosphorylation of ERK2 was quantitated. B and C, cells were pre-treated for 30 min at 20 °C as in Fig. 3C, either in 10 μM 2-MeSADP (●) to maximize the % dimerization or in buffer alone for the basal level (○). After two washes and recovery in buffer alone over a further 25 min, dose-response curves (B) were obtained for Ca2+ responses (as in Fig. 5) to 2-MeSADP. After 25 min of recovery, these curves are identical for those two cases (likewise found for this when pretreatment was instead at 37 °C, 20 min; data not shown). C, their plateau values are plotted to represent the activity regained during the 25-min recovery (30 min = 0 time). Longer recovery gave no further increase (not shown). A, no recovery offered (●. i.e. 10 μM 2-MeSADP was renewed). D, loss of induced dimers in the absence of agonist. As in C, after the 10 μM 2-MeSADP (30 min, 20 °C) treatment the agonist was either removed (○) or renewed (●. here, the dimer content decreased progressively. However, no significant difference is seen in the two cases. E, chelation of intracellular Ca2+ decreases the agonist-induced dimerization. Cells were treated with 10 μM 2-MeSADP at 20 °C as in Fig. 3C, for the times shown but with BAPTA-AM (50 μM) also pre-loaded or absent (control) throughout. Difference from control is significant at p = 0.036 (*). B–E, plots were reproduced in two replicated experiments.

DTSL alone. This product retained full receptor activity (data not shown), as predicted from that retention in hP2Y1,ΔT1. FRET analysis by acceptor bleaching as in Fig. 2D without agonist showed that there now was no constitutive dimerization (Fig. 7G). This was confirmed for those truncated forms using multiple sets of such spectra to quantify the changes in mean FRET values on a relative basis (Fig. 9). The clear susceptibility of the FRET, wherever it occurs, to a partial photobleaching of the acceptor confirmed the interpretation of the spectra. The
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**A**

Myc-hP2Y<sub>1</sub>

Myc-hP2Y<sub>1</sub>-ΔT1

Myc-hP2Y<sub>1</sub>-ΔT2

**B**

[Graph showing Δ[Ca<sup>2+</sup>] (%)]

**C**

[Graph showing [2-MeSADP] (Log M)]

**D**

Myc-hP2Y<sub>1</sub>-ΔT1

FITC

Cy3

[RFI (%)]

Wavelength (nm x 10<sup>-2</sup>)

**E**

Myc-hP2Y<sub>1</sub>-ΔT2

FITC

Cy3

[RFI (%)]

Wavelength (nm x 10<sup>-2</sup>)

**F**

[Images showing FITC and Cy3 fluorescence changes over time]

**G**

Myc-hP2Y<sub>1</sub>-ΔDTSL

FITC

Cy3

[RFI (%)]

Wavelength (nm x 10<sup>-2</sup>)

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**FIGURE 7. C-terminally truncated receptors.** A, truncated constructs used. B, transiently expressed truncated receptors, assayed as in Fig. 5, retain their activity. For 2-MeSADP, EC<sub>50</sub> (nm) = 3.9 ± 0.3 (Myc-hP2Y<sub>1</sub>, Ri); 5.1 ± 0.6 (Myc-hP2Y<sub>1</sub>, R-ΔT1); 28.9 ± 0.9 (Myc-hP2Y<sub>1</sub>, R-ΔT2). C, this holds similarly for ERK phosphorylations and 2-MeSADP, shown as before after exposure for 10 min (symbols as in B). The ERK2 band was quantitated as for Fig. 1D, expressed as a multiple of the basal level (agonist absent). EC<sub>50</sub> (nm) = 38.2 (Myc-hP2Y<sub>1</sub>, Ri); 46.7 (Myc-hP2Y<sub>1</sub>, R-ΔT1); 109.6 (Myc-hP2Y<sub>1</sub>, R-ΔT2). In both B and C, only the ΔT2 truncation gives a significant decrease. D and E, each form was preincubated with 10 μM 2-MeSADP (20 min, 37°C); FRET analysis was as in Fig. 2D. Cy3 is progressively bleached as expected, but the donor (FITC) signal now remains unchanged for both truncations. Identical results were seen in eight cells for each series. F, expressing cells shown are labeled with FITC-anti-Myc antibody alone. Representative images (as in Fig. 4A) before and after 10 μM 2-MeSADP treatment (50 min, 37°C) are shown. No receptor internalization was seen with either truncation. This was confirmed by line-scanning analyses as shown. The truncated receptors were still localized in the cell membrane region after 50 min of agonist exposure, when the full-length protein was fully internalized. G, as for D and E, but with only the final DTSL sequence removed. The original increase in the FRET peak with Cy3 bleaching is now only partly lost (as analyzed in Fig. 9).

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The reproducible loss of all dimerization in the hP2Y<sub>1</sub>-ΔT1 form is clear here. It can be seen that in the DTSL-less mutant the agonist-dependent dimer fraction is largely retained, most of the FRET decrease there being due to its total loss of the constitutive dimerization.

In view of the changes seen with full-length or truncated receptors during the various agonist incubations of up to 60 min, we tested for possible agonist losses therein. Negligible loss of 2-MeSADP concentration then occurs (Fig. 8C).

**DISCUSSION**

Dimers of P2Y<sub>1</sub>, R in the cell membrane were demonstrated by concurrent results from two modes of FRET analysis. The first, being kinetic, can quantitate the dimer population. The quantitation shows 28 ± 2% constitutive dimers (44 ± 3% at resting nucleotide levels), and dimers are further inducible by a P2Y<sub>1</sub>, R agonist (Fig. 3, A and C). The full rise in dimerization then seen is to ~85%, with that being a minimum estimate, as discussed below. This agonist reaction is extremely slow, requiring for completion (at 37°C) 20 min of exposure to 10 μM 2-MeSADP.

We can therefore exclude, for any state of the P2Y<sub>1</sub>, R observed here, the alternative model of resonance energy transfer arising only upon a conformational change produced by the agonist binding to a pre-existing dimer, as has been suggested for some GPCRs (6, 7, 30). First, one pool of pre-existing P2Y<sub>1</sub>, R dimers was able to exhibit their full FRET output here in the complete absence of agonist. That first pool could be explained by assigning it to the class of GPCRs (6, 7, 30) in which prior dimerization occurs well before they are delivered to the cell surface and exposed to agonist. The second P2Y<sub>1</sub>, R pool measured here, the agonist-induced dimers, may be small when in the native situation, as we shall propose below. For that set, the evidence is also against the FRET signal arising from agonist activation of pre-formed but undetected...
The EC₅₀ value of 2-MeSADP at the P₂Y₁R is distinct from the desensitization, which is complete in 10 min at only 100 nM 2-MeSADP (Fig. 5). At the 10 μM concentration of 2-MeSADP needed for full dimer induction, desensitization is complete in <30 s (not shown), consistent with previous data for full or partial agonists, ADP or its β-thio analog, acting on native hP₂Y₁R in platelets (23, 34). Commonly in GPCRs, endocytosis rapidly follows desensitization, being mediated by β-arrestin and involving clathrin or caveolin pathways (24). However, whereas hP₂Y₁R internalize via clathrin-coated pits when activated (23, 35), this process is slower, without recy-
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cling, and is dependent upon PKC and not on arrestin nor on GPCR kinases (35). A similar rate is seen here for hP2Y<sub>1</sub> R internalization and is shown to be preceded by receptor dimerization. In summary, the stages of P2Y<sub>1</sub> R agonist action lie in the series: Ca<sup>2+</sup> signaling rate > desensitization rate > dimerization rate > internalization rate.

The sequence above is consistent with agonist-induced P2Y<sub>1</sub> R dimerization being a prerequisite for internalization. Supporting this proposal, removal of the last 19 amino acids prevented dimerization and with it the internalization. The desensitization was unaffected, confirming it to be a preceding but independent action. Furthermore, at 20 °C where internalization was suppressed (Fig. 4D), both the desensitization and the maximum of dimerization were maintained. (This also confirms that attachment to clathrin-coated pits is not the cause of the agonist-induced element of P2Y<sub>1</sub> R dimer formation.) Some other rhodopsin-class GPCRs show evidence for agonist-induced internalization as dimers (e.g., Refs. 5, 7, 16), but for others, dimerization without such internalization was proposed, e.g., β<sub>1</sub>-adrenergic (36) or some somatostatin (2) or opioid receptors (8, 37), so that the behavior seen here could not have been predicted.

**Functional Activities of the Forms**—No activity is detectable in the agonist-induced dimer, it being fully desensitized. When agonist was removed, the basal composition of 44% dimer/56% monomer was restored in 20 min, regaining full initial activity (Fig. 6, B–D). The monomer is fully active, because the ΔT1-truncated protein is essentially monomeric and fully active (Fig. 7B and Fig. 9). The constitutive dimer is also fully active, seen from equal activities of the intact P2Y<sub>1</sub> R (with 28% constitutive dimers) and of its ΔT1 truncation with none (Fig. 7, B and C).

**Molecular Requirements for P2Y<sub>1</sub>, R Dimerization and Desensitization**—We found evidence for an essential determinant of the constitutive dimerization (only) in the P2Y<sub>1</sub> R C-terminal DTSL sequence (conserved in all species reported). That terminal tetrapeptide can bind P2Y<sub>1</sub> R (29) to NHERF-2, a scaffolding protein endogenous in our cells (Fig. 10). However, for both the slow agonist-induced dimerization and the subsequent internalization, another determinant resides within the next 15 amino acids of the tail (ΔT1 form). That contains several candidate protein kinase sites. hP2Y<sub>1</sub> R internalization has been reported (35) to require phosphorylations (unidentified) by PKC. For several rhodopsin family GPCR monomers, sites of primary interaction to form dimers have been proposed. These are generally in a transmembrane domain (5), but a required site in the C-terminal tail, as we find, has also been deduced in the somatostatin SSTR5 (2) and adenosine A2A receptors (38). In the P2Y<sub>1</sub> R, at least, this tail site must be assumed to act cooperatively with others elsewhere.

Further removal of the Thr<sup>339</sup> here (ΔT2 change), the homologous desensitization by agonist is nevertheless fully retained at two transduction levels tested (Fig. 8, A and B). In P2Y<sub>1</sub> Rs (both recombinant and native) this homologous desensitization involves specifically PKC (34). Such a difference is already known in the P2Y<sub>2</sub> receptor, where desensitization by agonist was linked to phosphorylation at three potentially PKC-reactive serines and threonines (two of these in the tail) that are phorbol-insensitive (41). For the P2Y<sub>1</sub> R, e.g., in platelets, it has further been found (42) that the novel phorbol-insensitive PKC isoform, PKC-δ, contributes to its desensitization and internalization. We suggest, therefore, that the agonist-induced desensitization process in P2Y<sub>1</sub> R includes phosphorylation at sites (yet unidentified) outside the C-terminal 39 residues, by one or more phorbol-insensitive, Ca<sup>2+</sup>-independent PKC isoforms such as PKC-δ.

**Features of the Dimer Quantitation by Donor Photobleaching**—First, this method provides a check by an internal standard in each micro-region of a cell membrane where the FRET is being measured. This check is given by the observed rate of photobleaching decay of the receptor monomer fraction present, which in theory is independent of the extent of FRET and was found so in practice in essentially all of the intact cells recorded. With that condition met, and because the fluorescence decay time constants are independent of the initial fluorophore concentration, those from different membrane regions or cells can be pooled despite different receptor densities, quenchings, etc. Hence, the absolute percentage of the fluorescent receptors that are in FRET, _i.e._ are in dimers, can be obtained.

Second, here we employ only extracellular fluorophores, avoiding the C-terminal attachment to the receptor in general.
use in the methods where a pair of self-fluorescent proteins introduce FRET. In the latter mode, the intracellular green fluorescent protein (etc.) can decrease dimeric interaction between GPCR monomers through secondary bindings to them (43), and the restricted rotation of that fluorophoric protein can decrease the FRET. Here, the small organic fluorophores are freely rotating. There is no evidence for any such obstruction by attachment of an N-terminal antibody (as here), a conclusion supported by the absence of quantitative change then in the receptor activity (Fig. 1D), and by findings in the very few other studies where FRET was shown with labels carried thus (16, 37). Participation of the tail as we saw here also very few other studies where FRET was shown with labels carried thus (16, 37). Participation of the tail as we saw here also

does not interfere, because this would form species containing either Cy3 alone or FITC alone and thus oppose FRET. It therefore could not give the high content (85% seen when the agonist activation was complete) of FITC-Cy3 co-labeled protein nor explain the slow increase in FRET with agonist exposure. The data also confirm that sterically two antibodies can bind to one P2Y,R attaching a C-terminal green fluorescent protein is indeed known to greatly weaken its agonist potencies (46), presumably through secondary mutual bindings, which is avoided here.

Third, bivalent cross-linking of monomers by antibody alone does not interfere, because this would form species containing either Cy3 alone or FITC alone and thus oppose FRET. It therefore could not give the high content (85% seen when the agonist activation was complete) of FITC-Cy3 co-labeled protein nor explain the slow increase in FRET with agonist exposure. The data also confirm that sterically two antibodies can bind to one P2Y,R dimer. In confirmation of the absence of cross-linking, when conditions known to induce a high degree of FRET were applied, post-labeling with the two antibodies after dimer formation gave the same yield as when the usual pre-labeling with them was made.

On the other hand, there might be some uncertainty because of multiple fluorescent labeling of the antibody probes used, a factor common to antibody probes in other types of FRET study. It is desirable to have an excess of label groups on the acceptor partner, because they do not register in the decay curves but increase the probability of a donor label in a dimer finding an acceptor. That number here was a mean of seven acceptor Cy3 groups per IgG but three for the FITC-hP2Y,R. However, because we found up to 85% dimers, it is not possible for >7.5% of the donor fluorophores to be sterically unable to interact for FRET. Flexibility in the Ab and free rotation of the fluorophore about its linkage presumably allow most of the potential FRET pairings of the three FITC groups to occur. This means that the 85% dimerization found is a minimum estimate of the final level and that the true value may be 100%, with up to 7.5% of donor monomers blocked by this steric factor in the label.

Significance of the P2Y,R Dimers—P2Y receptors on cell surfaces are now known to be constantly in contact with a low but significant local level of the agonists ATP and ADP, because of tonic autocrine and paracrine ATP release from neurons and glia and to co-localized ecto-nucleotidases forming ADP (see Refs. 47, 48). We relate the increase seen of constitutive dimer content (28% to basal 44%) to this resting ATP/ADP concentration. The 2-MeSADP-induced dimerization has EC50~0.9 μM, within the equivalent levels of extracellular ATP during physiological or pathological activity (47–49). This potency is much lower than that (Fig. 1C and Fig. 7B) for P2Y,R transductions, but this difference is frequently found also for agonist mediation of GPCR internalizations, including other P2Y receptors (28), and likewise (where known) of GPCR dimerizations. This lower potency could be explained by multiple equilibria in the further pathway to dimerization or if agonist binding is weakened after a phosphorylation in the desensitized state.

Forming a dimer would decrease the surface exposure of some regions of the protein, which may act to facilitate endocytosis of desensitized P2Y,Rs via (see above) clathrin-coated pits. At 37 °C, internalization is complete by 50 min (Fig. 4, A and B). At 20 °C this internalization is inhibited (Fig. 4D), as reported for other cases to be due to the receptor-binding clathrin lattice being unable to deform for pit formation (24). Then the dimer content slowly declines to ~50% (Fig. 3C, left and Fig. 6D), an effect we repeatedly confirmed and not because of loss of agonist in the prolonged incubation (Fig. 8C). In this phase at 20 °C the P2Y,Rs remain but spread into a zone just under the membrane (Fig. 4D; note the broadened peaks at 50 min). This should represent the initial attachment of the receptor to the clathrin complex (24). A final reversion to the monomer is thus occurring at a temperature-arrested intermediate state of the endocytosis process. Similar spreading also appears at 37 °C after 20–30 min (Fig. 4A, right), prior to full internalization.

Without added agonist, as noted above ~56% of membrane P2Y,Rs were found present as monomers; from our evidence, we suggest that this arises largely from the use of heterologous expression of constructs, which has been required universally for studies of receptor dimers in cells by the presently available FRET or BRET techniques (1). As noted above, this behavior is not specifically due here to overexpression, which need not always occur with those techniques (5, 7, 16, 50), although whenever present it must also contribute to this effect. In the cell membrane, where co-anchorage via scaffold proteins of different receptors plus effectors in micro-domains is now seen as integrating GPCR signaling (45), an exogenous GPCR, deriving from transcription outside its normal chromosomal location, will not necessarily be processed and targeted to its required scaffold (such as NHERF-2 for P2Y,R) in the same manner as its endogenous form. This, as well as any overexpression of it if present, would lead to two populations of that receptor, tethered and free. The dispersal of the P2Y,Rs will differ in those two cases, and our evidence on the effect of the NHERF-2 binding site (DTS) suggests that the P2Y,R clustering on a scaffold favors the presence of constitutive homodimerization. The binding at the scaffold could act thus by concentrating them there as a sink for dimers arriving at the cell membrane. Indeed, the resting level seen of ~44% dimers in normal tonic ATP/ADP conditions may be equivalent to near 100% for P2Y,Rs in the cell membranes of native tissues where the excess non-scaffolded fraction is likely normally to be minimal.

Because the agonist-induced phase of FRET was equally present when tethering at the scaffold was prevented by mutation (Fig. 9), that phase cannot be attributed to increased clustering nor to higher oligomers forming upon the constitutive dimers. It is deduced to arise from dimer formation, but with a different mechanism to the constitutive homodimerization;
there was evidence (see above) that it instead involves phosphorylation, with one of the determinants residing earlier in the C-terminal domain. That phase of dimerization may occur only, or mainly, in heterologous expression of GPCRs and not necessarily for all of those. In a survey of reports on 26 class A GPCRs studied in such expression by FRET or BRET, Pfleger and Eidne (50) found that at least 24 showed evidence for constitutive homodimerization, and where agonist treatment was tested, about half then showed a clear further increase in signal. The actual percentages of the dimerizations could not be obtained by these previous techniques. We therefore suggest that the behavior we find here is typical of most class A GPCRs in heterologous expression.

Clustering at a scaffold could also facilitate heterodimerization of P2Y₉R with certain potential binding partners, e.g. the A₁ adenosine receptor (51) or with another P2Y member, co-anchored at its own scaffold if that is itself complexed (44) with NHERF-2. Furthermore, our results indicate that when the tethered dimeric and the free monomeric P2Y₁Rs present in the membrane encounter higher agonist levels, both readily desensitize and (at 37 °C) both slowly and simultaneously internalize. This suggests that the specific phosphorylations required for these actions also occur on the tethered dimer and lead to its detachment from NHERF-2 and dissociation, as part of a deduced life cycle of such receptors, for which confirmation by independent methods should now be sought.

**Conclusion**—We suggest that constitutive dimers held in membrane microdomains are a form likely to be common in GPCRs like the P2Y₉R (i.e. those in the majority class A) in intact tissues in vivo. Agonist-induced native dimers could in principle also arise there in specific cases, but this is as yet uncertain because they may be favored by the use of heterologous expression.

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