Intracellular trafficking of the human oxytocin receptor: evidence of receptor recycling via a Rab4/Rab5 “short cycle”

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Conti F, Sertic S, Reversi A, Chini B. Intracellular trafficking of the human oxytocin receptor: evidence of receptor recycling via a Rab4/Rab5 “short cycle.” Am J Physiol Endocrinol Metab 296: E532–E542, 2009. First published January 6, 2009; doi:10.1152/ajpendo.90590.2008.—As in the case of most G protein-coupled receptors, agonist stimulation of human oxytocin receptors (OTRs) leads to desensitization and internalization; however, little is known about the subsequent intracellular OTR trafficking, which is crucial for reestablishing agonist responsiveness. We examined receptor resensitization by first using HEK293T cells stably expressing human OTRs. Upon agonist activation, the receptors were almost completely sequestered inside intracellular compartments that were not labeled by lysosomal markers, thus indicating that the internalized receptors were not sorted to these degrading organelles. Binding and fluorescence assays showed that almost 85% of the receptors had returned to the cell surface after 4 h, binding and fluorescence assays showed that almost 85% of the receptors had returned to the cell surface after 4 h, by which time cell responsiveness to the agonist was also completely restored, as shown by measuring phospholipase C activation. Similar results were also obtained in the presence of cycloheximide, thus indicating that receptor recycling and not de novo receptor synthesis was responsible for the resensitization. Notably, very similar internalization and recycling kinetics were observed in endogenous OTRs expressed on myometrial cells. We also investigated the role of β-arrestin2 in OTR recycling as these receptors have been previously classified as slowly or nonrecycling receptors on the basis of their stable association with this interacting protein. Our data suggest that the stable OTR/β-arrestin2 interaction plays an important role in determining the rate of recycling of human OTRs, but does not determine the fate of endocyotysed receptors. Subsequent investigations of receptor recycling pathways showed that OTRs localize in vesicles containing the Rab5 and Rab4 small GTPases (markers of the “short cycle”), whereas there was no colocalization with Rab11 (a marker of the “long cycle”) or Rab7 (a marker of vesicles directed to endosomal/lysosomal compartments). Taken together, these data indicate that OTRs are capable of very efficient and complete resensitization due to receptor recycling via the short cycle.

UPON AGONIST STIMULATION, the vast majority of G protein-coupled receptors (GPCRs) undergo a multistep process in which they lose their ability to respond for varying lengths of time (14). During the first desensitization step, the activated GPCRs uncouple from the G proteins physically and/or functionally, usually after phosphorylation by specific kinases (GRKs) and then, after binding to β-arrestins, they are removed from the plasma membrane by means of endocytosis. Depending on the receptor subtype and cell background, the internalized GPCRs are targeted to different intracellular compartments, where their subsequent fate is determined (9, 29, 38): they may be recycled to the cell surface following rapid or slow routes (in a process known as resensitization), or transported to lysosomes where they are degraded (in a process known as downregulation). All of these events together contribute to regulating the extent and duration of intracellular receptor responses, and thus play a crucial role in regulating the temporal and spatial aspects of receptor signaling. It has also been recognized that receptor trafficking is critical to the regulation of selective aspects of receptor signaling, such as MAPK activation (27).

The human oxytocin receptor (OTR) is a GPCR that plays a crucial role in parturition and lactation, as well as in the regulation of a number of neuroendocrine, social, cognitive, and emotional functions, including mating, pair bonding, pup care and nursing, learning, memory, aggression, fear and anxiety (13). It has also been recently postulated that the oxytocin/vasopressin system may be involved in severe neuropsychiatric conditions, such as autism, social phobia, and anxiety-related disorders (17, 22).

Despite the physiological and clinical relevance of OTRs, little is known about their desensitization and resensitization processes in native tissues. It is known that, like the vast majority of GPCRs, they desensitize after agonist application: long ago radioligand binding studies of myometrial cells showed a decrease in binding sites after prolonged oxytocin (OT) application (35), and more recent studies reported the desensitization of the OT-induced increase in intracellular calcium concentrations (37). In the case of OTRs expressed in neuronal cells, no desensitization and internalization data are yet available. Identifying and quantifying the OTRs in neuronal cells are particularly difficult because of their highly restricted and sometimes very low level of expression, and the lack of specific OTR antibodies. These two constraints have so far prevented the isolation of OTR-expressing neurons in which to investigate receptor trafficking.

Heterologous expression systems generate more reproducible responses and are therefore a more suitable context in which to obtain initial insights into the mechanisms involved in receptor regulation. HEK293 cells have been extensively used to investigate and compare the internalization and trafficking of a number of GPCRs, including OT/vasopressin receptors. It has been shown in heterologous expression systems, such as HEK293 and Madin-Darby canine kidney cells, that human OTRs fused to enhanced green fluorescent protein (EGFP) are rapidly and almost completely internalized, as no surface
fluorescence can be seen after 30 min of agonist application (15, 36, 42). In line with this, OT treatment leads to a progressive and almost complete (75–80%) loss of receptors in ~15 min, as estimated by ELISA (42) or by measuring the high-affinity binding of [3H]OT to intact cells (15, 36). Prolonged agonist exposure (18 h) also induces a 50% loss in the number of high-affinity receptors at the cell surface (23).

Some of the events involved in OTR desensitization/internalization have been recently investigated in HEK293 cells; it has been found that OTRs are phosphorylated by the GRK2 protein kinase, which phosphorylates agonist-occupied receptors promoting their subsequent binding to β-arrestin, and their endocytosis via clathrin-coated pits (18, 42). It has been also shown that the endocytosed OTRs in HEK293 cells are stably associated with β-arrestin, which has led to their classification as “class B” receptors (31). Unlike “class A” receptors, whose transient association with β-arrestin allows rapid recycling to the cell surface, class B receptors are sorted to intracellular compartments from where they may very slowly recycle back to the cell surface or be transported to lysosomes for degradation (30, 34). As a retention motif, Oakley et al. (31) identified some serine clusters in the intracellular domain of class B receptors which, once phosphorylated, would account for their stable interaction with β-arrestin. Human OTRs have three serine clusters in their COOH-terminal tail, and it has been shown that mutations in two of them do in fact destroy their stable interaction with β-arrestin. However, none of the above studies analyzed the intracellular trafficking of endocytosed OTRs and particularly their recycling to the cell surface.

We here describe the intracellular fate of endocytosed OTRs in HEK293 cells and in myometrial cells. Our results indicate that, on agonist-induced internalization, human OTRs are not sorted to the lysosomal compartments for degradation but recycle to the cell surface. We also investigated the recycling properties of OTRs and their intracellular trafficking in compartments characterized by the presence of different Rab proteins, the small GTPases that regulate the intracellular trafficking of a number of endocytosed membrane proteins (47), including various GPCRs (38). Our findings indicate that, in HEK293 cells, the OTRs recycle to the cell surface via a Rab4/Rab5 “short cycle.”

**MATERIALS AND METHODS**

**Peptide and Reagents**

OT was obtained from Bachem; cycloheximide was purchased from Sigma. Monoclonal anti-HA antibody (cat. H9658) and anti-c-myc antibody (cat. M5546) were from Sigma. The polyclonal anti-Rab11 antibody 121 (45) was a kind gift of M. Zerial (Max Planck Institute, Dresden, Germany). The monoclonal anti EE1A antibody (cat. 610456) was from BD Bioscience. The polyclonal anti-Rabip4 antibody was a generous gift of M. Cormount (Institut National de la Santé et de la Recherche Médicale, Nice, France). The monoclonal anti-Lamp1 (HA43) and β-tubulin (E7) antibodies were obtained from Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Science. The polyclonal anti-GFP antibody was from MBL (cat. 598). Alexa 647-conjugated transferrin (cat. T23366), Red DND-99 LysoTracker (cat. L7528), and Alexa 568-conjugated secondary anti-mouse and anti-rabbit antibodies were from Molecular Probes. [3H]OT (48 Ci/mmol) was from Perkin Elmer.

**Cell Culture, cDNA Constructs, and Transfections**

HEK293T cells were maintained in DMEM supplemented with 10% FBS, 200 U/ml penicillin, 200 mg/ml streptomycin, and 2 mM L-glutamine (all purchased from Sigma). Stable clones of HEK293T cells expressing the human OTR cDNA fused to EGFP (OTR-EGFP) were produced as described in Ref. 15 and maintained in the presence of geneticin (Invitrogen) at a final concentration of 500 μg/ml. Myometrial h-TERT (5) were used with permission of Geron; the hTERT-C3 clone (8) was obtained by H. Zingg (McGill University, Montreal, QC, Canada). Cells were maintained in DMEM:F12 with 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, and 4 mM L-glutamine (all purchased from Sigma).

The cDNAs encoding for the WT-OTR and OTR-AA2 mutant, tagged at their NH2-terminus with an HA epitope and subcloned in the pCDNA 3.1 vector, were a generous gift of M. Caron (Duke University, Durham, NC); the cDNA encoding GFP-β-arrestin2 and myc-β-arrestin2 (both NH2 terminally tagged) was obtained from M. Scott (Inst. Cochin, Paris, France); the NH2 terminally tagged GFP-Rab4a, GFP-Rab5a, GFP-Rab7, GFP-Rab11a, myc-Rab5N133I, and HA-Rab11S25N cDNAs were a generous gift of C. Bucci (Dept. of Science, Lecce, Italy) and G. P. Schiavo (Cancer Research UK, London, UK).

For transient transfections, cells were seeded on glass coverslips (2 × 10^5 cells/coverslip), allowed to attach for 24 h (60% confluence), and then transfected with lipofectamine 2000 (Invitrogen). For each transfection sample, 1 μg of cDNA was mixed with 6 μl of lipofectamine reagent in 200 μl of Opti-MEM I Medium (GIBCO) and incubated for 20 min at room temperature. Cells were washed twice with Opti-MEM, and DNA/lipofectamine complexes were diluted with 800 μl of Opti-MEM and gently added to the cells. After 5 h at 37°C, the medium was removed, and the cells were washed and maintained in DMEM supplemented with 10% FBS; experiments were performed 48 h after transfection.

**Fluorescence Microscopy and Immunofluorescence Staining**

Cells were washed twice with 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl [low-salt buffer (LS)], and fixed for 20 min at room temperature with 4% (wt/vol) paraformaldehyde. Fixed cells were then immediately mounted or processed for immunofluorescence. After being washed with LS, cells were incubated with primary antibodies in GDB buffer [20 mM sodium phosphate buffer, pH 7.4, 450 mM NaCl, 0.1% (wt/vol) gelatine, 0.3% (vol/vol) Triton X-100] for 2 h at room temperature. After being washed with 20 mM sodium phosphate, pH 7.4, containing 500 mM NaCl [high-salt buffer (HS)], the samples were incubated for 1 h with secondary antibodies in GDB buffer and sequentially washed with HS and LS buffers. Glass coverslips were mounted on glass slides with MOWIOL or VECTASHIELD (Vector Laboratories) and analyzed under a confocal microscope MRC 1024 (Bio-Rad) or LSM 510 Meta (Zeiss). Immunofluorescence with the anti-Lamp1 monoclonal antibody was performed in PBS buffer with 0.1% saponin and 0.1% BSA.

**LysoTracker Labeling of Lysosomal Compartments**

Lysosomal compartments were labeled by Red DND-99 LysoTracker 99. LysoTracker was added at the same time as the agonist, at a final concentration of 500 nM. Inhibition of the activity of the lysosomal enzymes was obtained by pretreating the cells with chloroquine at the final concentration of 10 μM for 30 min at 37°C.

**Internalization and Recycling Assays**

Cells were plated at a concentration of 100,000 cells/cm². In internalization experiments, cells were preincubated for 30 min in serum-free medium at 37°C and treated with OT at a final concentration of 100 nM; after different periods of time, internalization was blocked by placing the dishes on ice and the cells were immediately processed for binding assay or fluorescence microscopy analysis. In the recycling experiments, the
agonist was removed after a period of 45 min with an acid wash (150 mM NaCl, 5 mM CH₃COOH) and the cells were incubated for a subsequent period of time of 45 min, 2 h, 3 h, or 4 h in serum-free medium.

To determine the intracellular origin (recycling vs. newly synthesized receptors) of the receptors observed at the plasma membrane after internalization, the experiments were performed after pretreating the cells for a period of 4 h with cycloheximide at a final concentration of 10 μg/ml in serum-free medium. Cycloheximide at the same final concentration was then maintained for the duration of the experiment.

For colocalization with transferrin, cells were washed five times in TM (DMEM, 0.1% BSA, 20 mM HEPES/NaOH, pH 7.4), preincubated for 30 min at 4°C with Alexa 647 Transferrin at a final concentration of 60 μg/ml in TM, and stimulated with OT at a final concentration of 100 nM for 5, 15, and 45 min.

Fig. 1. A: oxytocin receptors (OTRs) do not colocalize with lysotracker following oxytocin (OT) stimulation. HEK293T cells stably expressing OTR-green fluorescent protein (GFP) were incubated with the lysotracker lysosome-specific marker (red) at a final concentration of 500 nM, with OT (at a final concentration of 10⁻⁷ M) being added at the same time for 15, 30, 60, and 90 min. The cells were then fixed and imaged by confocal microscopy. Scale bar = 10 μm. A ×3 magnification of the box area is shown in the bottom row. The pictures shown are representative of 2 independent experiments. B: endocyosed OTRs do not colocalize with the lysosomal marker Lamp1. HEK293T cells stably expressing OTR-GFP were incubated with OT (at a final concentration of 10⁻⁷ M) for 15, 30, 60, and 90 min. The cells were then fixed, processed for immunofluorescence with an anti-Lamp1 polyclonal antibody, and imaged by confocal microscopy. Scale bar = 5 μm. A ×3 magnification of the box area is shown in the bottom row. The pictures shown are representative of 2 independent experiments. C: endocyosed OTRs do not colocalize with the small GTPase Rab7. The internalization of human OTRs was assayed in HEK293T cells transiently cotransfected with an HA-tagged OTR and a GFP-tagged Rab7 cDNAs. Forty-eight hours after transfection, the cells were incubated for 15, 30, 60, and 90 min with OT at a final concentration of 10⁻⁷ M, fixed, and analyzed by confocal microscopy. Scale bar = 5 μm. A ×3 magnification of the box area is shown in the bottom row. The pictures shown are representative of 2 independent experiments in which we obtained comparable expression levels of the transiently transfected Rab protein. D: Western blot analysis of internalized OTR. OTR-GFP-expressing cells were treated with OT for the indicated periods of time. The cells were lysed and 30 μg of proteins were resolved by means of 8% SDS-PAGE. The OTR-GFP was detected by Western blotting with a polyclonal anti-GFP antibody; a polyclonal anti-β-tubulin antibody was finally used to verify that equal amounts of proteins were loaded in each line. Unsaturated bands from 2 independent experiments were acquired and analyzed with the ImageJ program. OTR-GFP levels normalized to β-tubulin were found not to be significantly different from the basal condition at any time point (data not shown). E: on agonist stimulation, OTRs are located in transferrin-positive vesicles. OTR-GFP-expressing cells were preincubated for 30 min at 4°C with Alexa 647-conjugated transferrin (at a final concentration of 60 μg/ml) shifted to 37°C and treated with OT (at a final concentration of 10⁻⁷ M) for 5, 15, and 45 min. The cells were then fixed and imaged by confocal microscopy. Scale bar = 5 μm. A ×5 magnification of the boxed area is shown in the bottom row. The pictures shown are representative of 2 independent experiments.
Preparation of Cell Lysates and Western Blot Analysis

Cells were stimulated with OT at the final concentration of $10^{-7}$ M for the indicated periods of time (5 min, 15 min, and 3 h). Cells were then washed once in ice-cold PBS and lysed in 50 mM Tris·HCl, pH 6.8, 2% SDS preheated at 100°C. After four to five cycles of freezing in dry-ice and boiling for 2 min, aliquots of the lysates were assayed for protein content using the BCA protein assay (Pierce). Thirty micrograms of cellular proteins were resolved by Laemmli SDS-PAGE in 8% (wt/vol) acrylamide gels (25) and then blotted onto 0.45-μm-pore size nitrocellulose membranes (Costar). Blots were incubated for 90 min at 37°C in TBS (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.2), containing 5% powdered skim milk. The membranes were then incubated for 2 h with primary antibodies anti-GFP and anti-β-tubulin diluted in TBS/milk and for 1 h with horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG (Pierce). Proteins were detected using the SuperSignal chemiluminescent substrate (Pierce). For quantification, unsaturated bands were acquired by mean of a Perfection 4990 photo scanner (EPSON) and analyzed with the ImageJ program (version 1.37v).

Receptor Internalization Binding Assay

To determine cell surface binding, cells subcultured into 24-well dishes, after stimulation for the specified period of time with agonist, were placed on ice and washed twice with an acid buffer (150 mM NaCl, 5 mM CH₃COOH), and sequentially washed with an ice-cold binding buffer [BB; 146 mM NaCl, 4.2 mM KCl, 0.5 mM MgCl₂, 1.0 mM CaCl₂, 1.0 mM NaN₃, 0.2% (wt/vol) NaN₃ to inhibit ATP production and block any further exo-endocytosis]. Thirty micrograms of cellular proteins were resolved by Laemmli SDS-PAGE in 8% (wt/vol) acrylamide gels (25) and then blotted onto 0.45-μm-pore size nitrocellulose membranes (Costar). Blots were incubated for 90 min at 37°C in TBS (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.2), containing 5% powdered skim milk. The membranes were then incubated for 2 h with primary antibodies anti-GFP and anti-β-tubulin diluted in TBS/milk and for 1 h with horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG (Pierce). Proteins were detected using the SuperSignal chemiluminescent substrate (Pierce). For quantification, unsaturated bands were acquired by mean of a Perfection 4990 photo scanner (EPSON) and analyzed with the ImageJ program (version 1.37v).

Inositol Phosphate Assay

Inositol phosphate (InsP) accumulation was measured as previously described (4, 24). Briefly, cells grown in six-well dishes were labeled for 24 h with myo-[2-3H]inositol at a final concentration of 2 Ci/ml in a serum- and inositol-free medium (Gibco BRL). The cells were washed twice in Krebs buffer [146 mM NaCl, 4.2 mM KCl, 0.5 mM MgCl₂, 1.0 mM CaCl₂, 10 mM HEPES base, 1% (wt/vol) glucose, pH 7.4] and preincubated for 10 min at 37°C in the same buffer supplemented with 10 mM LiCl. After incubation for 15 min in the absence (baseline), or presence of peptide at a final concentration $10^{-7}$ M, the reaction was stopped with 5% perchloric acid and the InsPs were extracted and separated using a strong anionic exchange column (Dowex AG1X8, formate form, 200–400 mesh; Bio-Rad). A fraction containing the inositol mono-, bis-, and trisphosphates was then collected and its radioactivity content was determined by liquid scintillation counting. This fraction is referred to as total InsP and expressed as DPM/well.

RESULTS

Endocytosed OTRs Are Not Sorted to Degrading Compartments

To determine the fate of endocytosed OTRs, we first analyzed their sorting to lysosomal compartments. Before agonist exposure (baseline), the edges of the cells expressing OTR-GFP were homogeneously fluorescent, but punctate fluorescence (presumably associated with endocytic vesicles) could be seen as early as 5 min after agonist application and became clearly visible after 15 min; after 30, 60, and 90 min, the fluorescent patches seemed to be almost exclusively localized in intracellular compartments (Fig. 1A). No colocalization of OTR-GFP and lysotracker (a specific marker of lysosomes) was observed at any of the investigated times, thus suggesting that the receptor is not delivered to these compartments. To exclude the possibility that the absence of lysosomal localization may be due to almost immediate receptor degradation (thus preventing accumulation), the same experiment was per-
formed using cells pretreated with chloroquine, a drug that inactivates proteolytic enzymes by destroying the acidic pH of lysosomes. Again, no lysosomal localization of the receptor was observed, thus excluding the possibility that the OTRs were not detected because they had been immediately degraded (not shown). To further confirm that OTRs are not sorted to lysosomal compartments, we verified the absence of internalized OTR in structures labeled by an anti-Lamp1 antibody, a marker of lysosomes. As shown in Fig. 1B, no colocalization of OTR and Lamp1 was observed after 15, 30, 60, and 90 min of OT application, indicating that internalized OTRs are not found in lysosomal compartments. Finally, we verified the absence of OTR in structures labeled by the small GTPase Rab7, a well-established marker of vesicles directed to late endosomal/lysosomal compartments. To this aim, we transiently cotransfected HEK293T cells with a GFP-Rab7 construct and HA-tagged OTRs that internalize and recycle as well as the stably expressed OTR-GFP (not shown). As shown in Fig. 1C, no significant colocalization of OTR and Rab7 was observed after 15, 30, 60, and 90 min of OT application, indicating that internalized OTRs are not sorted to endosomal/lysosomal compartments.

To verify the absence of OTR degradation during OT internalization, we also performed a Western blot analysis on HEK293 cells stably expressing the OTR-GFP and stimulated for 5 and 15 min and 3 h with OT. As shown in Fig. 1D, no significant reduction in the OTR protein was observed within this time frame. For quantification, two different experiments were analyzed; unsaturated bands were acquired and the OTR level in each condition was normalized to β-tubulin; OTR-GFP levels normalized to β-tubulin were not significantly different from the basal condition at any time point (data not shown).

All together, these data indicate that the internalized OTRs are not sorted to the lysosomal compartment after OT stimulation.

**OTRs Recycle to the Plasma Membrane After Agonist Removal**

**Confocal microscopy analysis.** As the endocytosed OTRs are not sorted to lysosomes, we investigated their possible recycling to the cell surface. We first then analyzed OTRs colocalization with transferrin, a classical marker of the recycling pathway(s). After endocytosis, transferrin is very rapidly diverged from lysosome-targeted proteins (t1/2 2–3 min) (10, 44) and recycles to the plasma membrane with a t1/2 of 10–15 min (28). As shown in Fig. 1E, intense colocalization of OTRs and transferrin was observed at 5, 15, and 45 min of OT application, indicating that internalized OTRs are indeed sorted to recycling compartments labeled by transferrin.

Fig. 3. OTRs recycle to the plasma membrane after agonist removal. Binding analysis and desensitization assay. **A:** time course of receptor internalization. After OT stimulation, at a final concentration of 10–7 M, for different periods of time (5, 15, 60, and 120 min), the dishes were placed on ice and processed for the determination of specific binding at the cell surface, as described in MATERIALS AND METHODS. **B:** recycling binding assay. The internalization binding assays were performed as described in MATERIALS AND METHODS. Briefly, the cells were incubated for 45 min in the presence of OT, washed in acidic buffer, and kept in the absence of agonist for 45 min or 4 h. One hundred percent represents specific binding under baseline conditions (Bas); the other conditions are shown as ratios between the specific binding determined at each time point and specific baseline binding. The mean values ± SD of 3 independent experiments each performed in triplicate were as follows: 16 ± 3% (45’ OT), 53 ± 7% (45’ OT + 45’ wash), and 86 ± 3% (45’ OT + 4 h wash). **C:** recycling binding assay under protein synthesis inhibition. The experiment was performed in cells preincubated for 4 h in the presence of 10 μg/ml cycloheximide to block protein synthesis. The experiment is representative of 2 independent experiments whose results did not diverge by more than 90%. Each point was done in triplicate. **D:** desensitization assay. Total inositol phosphate (InsP) production was determined under Bas, and after 15 min of OT stimulation at a final concentration of 10–7 M (45’ OT + 4 h wash + 15’ OT). The experiment is representative of 2 independent experiments whose results did not diverge by more than 90%. Each point was done in triplicate.
We then analyzed the possible receptor recycling to the plasma membrane after removing the agonist by acid washing. As shown in Fig. 2A, after 45 min of agonist application, the receptors had completely lost their baseline membrane localization and were located in intracellular vesicular compartments. When the agonist was removed by acid washing, and the receptor was allowed to traffic for another 45 min, fluorescence reappeared at the plasma membrane and progressively increased over time until it was as intense as under baseline conditions after 4 h, and there was a parallel progressive decrease in intracellular fluorescence. To verify that the appearance of receptors at the cell surface was not due to new protein synthesis, we performed the same experiments in the presence of cycloheximide, an inhibitor of protein synthesis. Figure 2B shows that this treatment had no effect on receptor internalization or their reappearance at the cell surface, thus indicating that the membrane staining was mainly due to internalized receptors that had recycled to the plasma membrane.

Binding and coupling analysis. To quantify the receptor recycling to the cell surface, we performed binding assays on intact cells using [3H]OT as a radiotracer. Figure 3A shows the high-affinity binding sites measured under baseline conditions and after various times of agonist application. It can be seen that there was a rapid decrease in the total number of receptors at the cell surface: 51% of the number originally present on the cell surface after 5 min, 21% after 15 min, 9% after 60 min, and 14% after 120 min, with an estimated t_{1/2} of 4.42 min. These data confirmed the rapid and almost complete OTR internalization previously reported in transiently transfected HEK293T cells (42). When the agonist was washed out and the binding measured after 45 min or 4 h, there was a progressive increase in the number of high-affinity membrane receptors that, after 4 h, had reached 86% of the number originally present on the cell surface, thus indicating that more than 80% of the internalized receptors were recycled to the cell surface (Fig. 3B). The same results were obtained when the experiment was performed in the presence of cycloheximide, thus confirming that de novo receptor synthesis does not play a major role in the reappearance of the receptors at the cell surface (Fig. 3C).

Finally, we checked the coupling properties of the recycled receptors by comparing the production of InsP induced by 15 min of OT stimulation before and after 4 h of receptor recycling. As shown in Fig. 3D, the total amount of InsP produced after 4 h of receptor recycling was identical to that measured before any agonist-induced internalization, indicating that the recycled receptors are fully functional and that a complete resensitization of the receptor response was observed.

OTR Recycling in Myometrial Cells

As receptor internalization and recycling may vary in different cellular systems, and in particular in cells expressing endogenous vs. stably or transiently transfected cells, we checked the internalization and recycling properties of human OTRs endogenously expressed in myometrial cells. To this purpose, we used myometrial cells immortalized with human telomerase reverse transcriptase (hTERT), a procedure that endows the cells with an unlimited life span in culture (5). In particular, we employed the hTERT-C3 subclone, which was shown to express high-affinity OTRs (8). As shown in Fig. 4A, a rapid decrease in the total number of receptors on the cell surface could be detected in hTERT-C3 cells after OT application, indicating that in myometrial cells the endogenous OTR is rapidly and almost completely internalized with an estimated t_{1/2} of 2.36 min. When receptor recycling was measured after 45 min and 4 h, we found a progressive increase in the number of high-affinity membrane receptors that, after 4 h, had reached 113% of the number originally present on the cell surface, indicating that all of the internalized receptors were recycled to the cell surface (Fig. 4B). These data indicate that in myometrial cells the human OTRs completely recycle to the cell surface after OT-induced internalization.

Role of β-Arrestin2 in Regulating the Rate of OTR Recycling

OTRs have been classified as class B receptors on the basis of their persistent interaction with β-arrestin (31), a postendocytic event that allows the retention of class B receptors inside intracellular compartments and directs their sorting preferentially to degradative or slow recycling organelles (30, 32). As we did not observe any lysosomal targeting of OTRs after endocytosis, we checked whether persistent OTR/β-arrestin2 colocalization was maintained under our experimental conditions. As shown in Fig. 5A, OTR and β-arrestin2 colocalized in intracellular compartments for up to 45 min, thus suggesting
their long-lasting association after agonist-induced receptor internalization. We also investigated the persistence of OTR/β-arrestin2 colocalization after agonist removal. In this experiment, OT was washed out after 5 min of OT application and colocalization was monitored after a further 5, 15, and 30 min; Fig. 5B shows that OTR/β-arrestin2 colocalization was lost somewhere between 15 and 30 min after agonist removal.

To check whether stable β-arrestin2 interaction with the phosphorylated COOH-terminal tail of OTRs may slow OTR recycling, we analyzed the recycling properties of an OTR mutant (OTR-AAA2) lacking one serine cluster responsible for stable receptor interaction with β-arrestin2 (31). As shown in Fig. 5C, colocalization of β-arrestin2 with OTR-AAA2 mutants was observed after 5, 10, 15, and 30 min of OT application. However, unlike those containing the WT OTR, we found that several vesicles containing the OTR-AAA2 mutant were devoid of β-arrestin2 after 30 min of agonist application. Furthermore, OTR-AAA2 staining of the cell surface was apparent throughout the time of OT stimulation. Binding experiments quantifying the number of OTR-AAA2 receptors at the cell surface during OT application (Fig. 6A) showed a peak reduction of only 60% of the initial OTR-AAA2 binding sites after 45-min treatment, when steady state was achieved. One possible explanation is that OTR-AAA2 receptors recycle so rapidly that they are never completely lost from the cell surface in the continuous presence of agonists because of several cycles of internalization and recycling: Fig. 6B shows that all of the internalized receptors were recycled to the cell surface after only 10 min of agonist washout. In line with this hypothesis, the coexistence of OTR-AAA2-positive vesicles with and without β-arrestin2 after 30 min indicates that the receptors are located in various positions along the internalization/recycling pathways. Taken together, these data suggest that destroying the long-lasting interaction of OTRs with β-arrestin2 accelerates their recycling rate.

Evidence of Receptor Recycling Via the Short Cycle: Endocytosed OTRs Colocalize with Rab4 and Rab5 but not with Rab11

The small GTPases of the Rab family regulate the intracellular trafficking of endocytosed membrane proteins (47), in-

Fig. 5. A: colocalization of OTRs and β-arrestin2 after agonist-induced receptor internalization in presence of agonist. OTR-GFP-expressing cells were transiently transfected with a myc-tagged β-arrestin2 cDNA. After stimulation with OT at a final concentration of $10^{-7}$ M for the indicated periods of time, cells were fixed, processed for immunofluorescence, and imaged by confocal microscopy. The pictures shown are representative of 2 independent experiments, in which comparable expression levels of β-arrestin2 were obtained. Scale bar = 5 µm. A ×5 magnification of the box area is shown in the bottom row. B: colocalization of OTRs and β-arrestin2 after agonist-induced receptor internalization in absence of agonist. OTR-GFP-expressing cells were transiently transfected with a myc-tagged β-arrestin2 cDNA. After stimulation with OT at a final concentration of $10^{-7}$ M for 5 min, the agonist was washed, and the cells were incubated in complete medium for 5, 15, and 30 min, processed for immunofluorescence, and imaged by confocal microscopy. The pictures shown are representative of 2 independent experiments. Scale bar = 5 µm. A ×5 magnification of the box area is shown in the bottom row. C: colocalization of OTR-AAA2 and β-arrestin2 after agonist-induced receptor internalization. HEK293 cells were transiently cotransfected with HA-tagged OTR-AAA2 mutant and GFP-β-arrestin2. After stimulation with OT at a final concentration of $10^{-7}$ M for the indicated periods of time, cells were fixed, processed for immunofluorescence, and imaged by confocal microscopy. The pictures shown are representative of 2 independent experiments. Scale bar = 5 µm. A ×5 magnification of the box area is shown in the bottom row.
The internalization binding assays were performed as described in MATERIALS AND METHODS. Briefly, the cells were incubated for 45 min in the presence of receptor internalization. After OT stimulation at a final concentration of 10^{-7} M for different periods of time (5, 15, 45, and 90 min), the dishes were placed on ice and processed for the determination of specific binding at the cell surface, as described in MATERIALS AND METHODS. The internalization binding assays were performed as described in MATERIALS AND METHODS. Briefly, the cells were incubated for 45 min in the presence of OT, washed in acidic buffer, and kept in the absence of agonist for 5 and 10 min. One hundred percent represents specific binding under Bas. The other conditions are shown as ratios between the specific binding determined at each time point and specific baseline binding. The mean values ± SD of 2 independent experiments each performed in triplicate were as follows: 36 ± 8% (45’ OT), 76 ± 7% (45’ OT + 5’ wash), and 99 ± 13% (45’ OT + 10’ wash).

Our findings provide evidence that, after agonist-induced internalization, human OTRs efficiently recycle to the cell surface without trafficking through Rab11-positive compartments. Rab5-positive organelles are actively involved in intracellular OTR trafficking.

We finally investigated the colocalization of HA-tagged WT-OTR and GFP-Rab4a, the small GTPase that identifies vesicle recycling from both EEs and REEs to the plasma membrane (43). As shown in Fig. 7A, extensive colocalization was observed after 45 min of agonist application and 45 min and 4 h postwashing, thus indicating that OTRs traffic in Rab4-positive compartments.

We then investigated the colocalization of the HA-tagged WT-OTR and GFP-Rab11a (43). As shown in Fig. 7A, almost no colocalization of Rab11 and OTRs was observed after 45 min of agonist-induced internalization. Similarly, no significant colocalization was observed 45 min or 4 h postwashing, thus indicating that OTRs do not traffic in Rab11-positive vesicles. To confirm that Rab11 compartments are not involved in OTR trafficking, we performed an immunofluorescence study using an anti-Rab11 antibody on cells treated with OT for 15, 60, and 120 min, without finding any evidence of the colocalization of the endogenous Rab11 protein and the internalized OTRs (Supplementary Fig. 1; the online version of this article contains supplemental data).

The presence of internalized OTR in Rab5/Rab4 compartments was also investigated by labeling specific effectors of Rab5 and Rab4. As shown in Fig. 7B, OTR colocalized with EEA1, a Rab5 effector (40), at 45 min after OT application as well as 45 min postwashing. Similarly, we found colocalization of OTR with Rabip4, an effector of Rab4 (6), at 45 min after OT application as well as 45 min postwashing.

Finally, to further check the role of Rab5 and Rab11 in the intracellular trafficking of the OTR, we transfected HEK293T cells stably expressing the OTR-GFP with the dominant-negative Rab mutants Rab5N133I and Rab11S25N (Supplementary Fig. 2). Even if preliminary, our data indicate that in Rab5N133I-transfected cells, the OTR was expressed at the cell surface and was completely internalized after 45 min of OT application; after 45 min of agonist washing, internalized receptors were almost completely retained in intracellular compartments marked by the Rab5N133I protein and extensive intracellular colocalization of the receptor with Rab5N133I was also observed after 4 h of washing, when only a fraction of the receptor was recycled to the cell surface. On the contrary, no effects on the basal localization, internalization, and recycling were observed in cells transfected with the Rab11S25N mutant.

Taken together, these data indicate that human OTRs recycle to the cell surface via Rab4/Rab5-positive organelles, without trafficking through Rab11-positive compartments.

**DISCUSSION**

Our findings provide evidence that, after agonist-induced internalization, human OTRs efficiently recycle to the cell surface. This is particularly relevant to understanding the role played by OTR internalization and recycling in regulating uterine contractility. The human uterus is in a state of relative quiescence during most of pregnancy, but switches to a state of phasic contractions at the time of parturition. OT is one of the most potent hormones inducing uterine contractility, and drugs acting on OTRs are widely used to block (1) or sustain uterine
contractions (41). However, although OT is one of the most frequently used drugs in modern obstetric practice, there is considerable controversy concerning its administration, and so a wide range of doses and intervals between increasing doses are routinely used. The intensity and duration of the response to OT are mainly determined by the number of OTRs expressed in myometrial cells, which may be influenced by pre- and posttranscriptional mechanisms. The dramatic upregulation of OTRs before parturition has been extensively investigated at the transcriptional level and found to be due to a more than 300-fold increase in receptor mRNA levels compared with basal levels (31).

In vitro, human OTRs are effectively recycled to the cell surface on agonist-induced internalization (35). We obtained comparable expression levels of the transiently transfected Rab proteins. The internalization and recycling of human OTRs were assayed in OTR-GFP-expressing HEK293T cells. The cells were incubated for 45 min in the presence of OT, washed in acidic buffer to remove the agonist, and kept in the absence of agonist for 45 min or 4 h. At the end of the recycling period, the cells were fixed, processed for immunofluorescence, and analyzed by confocal microscopy. Scale bar = 5 μm. A ×5 magnification of the box area is shown in the bottom row. The pictures shown are representative of 2 independent experiments.

It has been shown that a number of signals and interacting proteins play crucial roles in GPCR trafficking (9). The best-characterized signals for receptor internalization include intracellular serine and threonine residues, which, once phosphorylated by specific GRKs, mediate receptor interactions with β-arrestins. GPCRs are classified as class A or class B receptors depending on whether these interactions are transient or stable, and, unlike class A receptors (whose transient association with β-arrestin allows rapid recycling to the cell surface), class B receptors are sorted to intracellular compartments from where they may very slowly recycle back to the cell surface or be transported to lysosomes for degradation (30, 34). As a retention motif, Oakley et al. (31) identified some serine clusters in the intracellular domain of class B receptors which, once phosphorylated, would account for their stable interaction with β-arrestin: human OTRs have three serine clusters in their COOH-terminal tail, and it has been shown that mutations in two of them destroy their stable interaction with β-arrestin, which led to the hypothesis that OTRs recycle very poorly to the cell surface or are targeted to lysosomes for degradation. However, our findings indicate that the OTRs in HEK293T and myometrial cells efficiently recycle to the cell surface on agonist removal, thus allowing the full resensitization of receptor response. It has been shown that the desensitization of OTR responses in myometrial cells takes place over a long time; a recent study found that 6 h were necessary to obtain full inhibition (37), and an earlier study found that it took 48 h to obtain a 50% reduction in the OT-induced increase in intracellular Ca2+ (33), which is consistent with the efficient receptor recycling necessary to sustain a functional response for so long. It will be interesting to investigate whether longer treatment with OT (4–6 h) eventually shifts OTRs to other pathways, finally leading to receptor desensitization and downregulation as originally suggested. However, the toxic effects of cycloheximide do not allow receptor recycling to be measured over such long periods, and the lack of specific antibodies to human OTRs and very poor transfection efficiency do not allow receptor traffic to be followed in myometrial cells.

Fig. 7. OTRs recycle to the cell surface via Rab5/Rab4-positive vesicles. A: analysis of OTR colocalization with GFP-tagged Rab5, Rab4, and Rab11. The internalization and recycling of human OTRs were assayed in HEK293T cells transiently cotransfected with an HA-tagged OTR cDNA and GFP-tagged Rab5a, Rab4a, or Rab11a cDNA constructs. Forty-eight hours after transfection, the cells were incubated for 45 min in the presence of OT, washed in acidic buffer to remove the agonist, and kept in the absence of agonist for 45 min or 4 h. At the end of the recycling period, the cells were fixed, processed for immunofluorescence, and analyzed by confocal microscopy. Scale bar = 5 μm. The pictures shown are representative of 2 independent experiments in which we obtained comparable expression levels of the transiently transfected Rab proteins. B: analysis of OTR colocalization with Rab4 and Rab5 effectors. The internalization and recycling of human OTRs were assayed in OTR-GFP-expressing cells HEK293T. The cells were incubated for 45 min in the presence of OT, washed in acidic buffer to remove the agonist, and kept in the absence of agonist for 45 min or 4 h. At the end of the recycling period, the cells were fixed, processed for immunofluorescence with anti-EEA1 and anti-Rabip4 antibodies, and analyzed by confocal microscopy. Scale bar = 5 μm. A ×5 magnification of the box area is shown in the bottom row. The pictures shown are representative of 2 independent experiments.
signal blocking receptor recycling, because mutations in these serines allow recycling but do not determine the final destination of the internalized receptor (21). It has very recently been found that a protein interacting with the COOH-terminal 23 amino acid of the V2 receptor greatly increases receptor degradation (46), thus further supporting the idea that the intracellular sorting mechanisms rely on proteins other than β-arrestins, whose main role may actually be to regulate the rate of receptor recycling.

As OTRs have slower kinetics than other recycling GPCRs expressed in HEK293T cells, such as the β2-adrenergic receptors which are >80% recycled back to the cell surface in less than 1 h (2), we tested whether the stable interaction between OTRs and β-arrestin could slow down the OTR recycling rate. To this end, we measured the recycling of one OTR mutant (OTR-AAA2) that lacks one of the serine clusters accounting for the stable receptor/β-arrestin interaction and found that OTR-AAA2 receptors are completely recycled to the cell surface after 10 min of agonist application, thus supporting the idea that OTRs/β-arrestin2 interactions control the rate of receptor recycling but not the receptor’s intracellular fate (recycling vs. degradation).

To investigate intracellular OTR traffic, we used HEK293T cells in which it has been previously shown that human OTRs internalize via clathrin-coated pits. Upon endocytosis, the internalized membrane receptors entering via clathrin-coated pits may immediately recycle to the cell surface via the short cycle, or be targeted to the perinuclear endosomal compartment, from where they may recycle via the long cycle or be sorted to the lysosomal compartments for degradation (9, 29, 38). Rab GTPases coordinate vesicle transport between a numbers of intracellular compartments and are used to identify the pathways followed by specific membrane proteins, including a number of GPCRs (38, 47). However, individual Rab proteins label more than one intracellular compartment: Rab5 is found on both endocytosed and recycling vesicles of the short cycle, Rab4 on recycling vesicles of the short and long cycles, and Rab11 on recycling vesicles of the long cycle and vesicles directed to lysosomes (43). To investigate the fate of an endocytosed membrane protein, it is therefore necessary to analyze its colocalization with multiple Rab proteins. The absence of OTRs in Rab11-labeled structures allowed us to exclude recycling via the long cycle and/or lysosomal degradation, and their presence in Rab4-positive vesicles indicated that they recycle via the short cycle, as has been shown in the case of other GPCRs, such as the cannabinoid CB1 receptor (26), the prostanoid DP receptor (12), and the corticotropin-releasing factor 1α receptor (19). It is worth mentioning the recent finding that Rab4 enhances β2AR recycling and activation in cardiac myocytes, an event that may contribute to the cardiac hypertrophy observed in transgenic mice overexpressing Rab4, and also suggests alternative strategies for regulating myocardial diseases (11). Whether variations in Rab4 levels in myometrial cells may be responsible for altered OTR responses is an open question that deserves further investigation.

Although Rab GTPases can modulate GPCR traffic without receptor binding (as in the case of the β2AR) (16), it has been reported that direct interactions between Rab5 and the AT1R are very important for preventing receptor sorting to lysosomes (7, 39). As the traffic of human OTRs also depends on Rab5, it would be very interesting to see whether a direct OTR-Rab5 interaction prevents sorting to the degradation compartments and allows receptor recycling. Moreover, as the expression of a Rab5 dominant-negative mutant does not affect the internalization of AT1R (39) but completely blocks that of β2AR (16), our finding that OTR internalization in unaltered in Rab5 dominant-negative-expressing cells highlights a further resemblance between OTRs and the AT1Rs that may be worth investigating.

In conclusion, as the preservation of sensitivity to agonist exposure has a great impact in a number of physiological and pathological conditions, we believe that identifying the intracellular pathways involved in OTR trafficking and recycling is fundamental to understanding the regulatory mechanisms that influence the outcome of receptor stimulation and may contribute toward the development of novel therapeutic approaches.

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