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Effects of cholesterol manipulation on the signaling of the human oxytocin receptor

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Submitted 17 May 2006; accepted in final form 19 May 2006

Reversi, Alessandra, Valeria Rimoldi, Silvia Brambillasca, and Bice Chini. Effects of cholesterol manipulation on the signaling of the human oxytocin receptor. Am J Physiol Regul Integr Comp Physiol 291: R861–R869, 2006. First published June 1, 2006; doi:10.1152/ajpregu.00333.2006.—We have recently shown that oxytocin inhibits cell growth when the vast majority of oxytocin receptors (OTRs) are excluded from detergent-resistant membranes (DRMs; the biochemical counterpart of lipid rafts), but has a strong mitogenic effect when the receptors are targeted to these plasma membrane domains upon fusion with caveolin-2, a resident raft protein. The aim of this study was to investigate whether the manipulation of total cell cholesterol can influence OTR localization and signaling. Our data indicate that cholesterol depletion in HEK-293 cells does not affect the signaling events mediated by the OTRs located outside DRMs. When treated with 2 mM methyl-β-cyclodextrin (MβCD), the receptors remained outside and continued to inhibit cell growth. On the contrary, the MβCD treatment of cells expressing receptors fused to caveolin-2 led to their redistribution outside DRMs, and converted the receptor-mediated proliferative effect into cell growth inhibition. These data indicate that 1) once released from DRMs, the receptors fused to caveolin-2 signal exactly as wild-type OTRs and 2) their DRM location is responsible for the specific OTR signaling leading to cell proliferation. Finally, we evaluated whether cholesterol loading could force the OTRs into lipid rafts and change their signaling, but, after cell treatment with an MβCD/cholesterol complex, receptor stimulation continued to lead to cell growth inhibition, thus indicating that increasing cell cholesterol levels is not sufficient per se to affect OTR signaling.

lipid rafts; proliferation; neurohypophyseal hormones

LIPID RAFTS AND CAVEOLAE are specialized regions of the plasma membrane that are enriched in cholesterol, glycosphingolipids, and lipid-anchored proteins (43). The fact that a number of membrane receptors, signaling molecules, and membrane transporters localize to lipid rafts has led to the formulation of the “rafts/caveolae signaling hypothesis,” which suggests that these domains may act as centers for regulating cell signaling activity (18, 42). It can also be hypothesized that disruption of the plasma membrane environment in which the signaling cascades take place may lead to chaotic signaling and, in the case of growth-control signals, contribute to abnormal cell proliferation and neoplastic transformation. In line with this view, we have previously found that lipid rafts represent a membrane compartment in which the signaling of human oxytocin (OT) receptor [OTR; a Goq11- and Goi-coupled receptor (GPCR)] may be specifically regulated (19, 37).

It was originally thought that OTRs were only present in the uterus, mammary gland, and central nervous system, but they have recently been found in a number of other tissues and organs, as well as in tumor cells of various origin (breast and endometrial carcinomas, neuroblastomas, glioblastomas, Kaposi sarcomas, and small-cell lung carcinomas) (4). Functional OTRs have also been described in osteoblasts (9) and endothelial cells (45), and it has recently been suggested that they may play a specific role in the differentiation of cardiomyocytes (31) and myoblasts (2). Interestingly, although only one OTR encoding gene has been cloned and found to be expressed in various tissues and cell lines, OT may stimulate, inhibit, or have no effect on cell proliferation, depending on the cell system (4). These different effects seem to be mediated by different signaling pathways, including an increase in calcium, the formation of cAMP, and the activation of MAPK (6). However, it is difficult to identify the factors determining the different outcomes of OTR activation because of the extreme variability in signaling intermediates in different cell lines and tissues (e.g., G protein types and levels, adenylate cyclases, PKC isofoms, adhesion molecules).

One possibility is that human OTRs may activate different transduction pathways depending on their localization in different membrane microdomains, such as lipid rafts. Lipid rafts in living cells are too small and/or too dynamic to be detected by means of conventional microscopy, and so a number of biophysical, microscopic, and imaging techniques are being developed in the hope of being able to “see” them (25). However, because of their particular biochemical and biophysical nature, they are resistant to low-temperature solubilization by nonionic detergents, such as Triton X-100, which allows them to be separated by means of differential flotation after density gradient centrifugation in the form of detergent-resistant membranes (DRMs) (26). Although the exact correlation between DRMs and lipid rafts in living cells is still unknown, the partitioning of a protein in DRMs is consistent with its localization in lipid rafts, and the conventional way of analyzing the protein and lipid contents of such microdomains. On this basis, we will use the terms “in lipid rafts” and “in DRMs” interchangeably.

To investigate the role of lipid rafts in OTR signaling, we used stable cells transfected with wild-type (WT) OTRs...
(mainly excluded from lipid rafts) or WT OTRs targeted to lipid rafts as a result of their fusion to the resident raft protein caveolin-2. This targeting system makes it possible to identify the signaling pathways initiated by OTRs inside and outside lipid rafts and how they are responsible for OTR-mediated proliferative or antiproliferative effects (19, 37). In particular, Madin-Darby canine kidney (MDCK) and HEK-293 cells were stably transfected with a cDNA encoding for the full-length OTR fused at its COOH terminus with the enhanced green fluorescence protein (EGFP) to allow direct microscopy visualization and specific antibody recognition. Biochemical experiments based on cold Triton X-100 solubilization followed by separation on a sucrose flotation gradient indicated that the WT-OTR-EGFP is mainly excluded from lipid rafts. We also generated MDCK and HEK-293 clones constitutively expressing a new chimeric receptor in which the full-length coding sequence of caveolin-2 was fused to the COOH terminus of WT-OTR-EGFP (OTR-EGFP-cav2). Biochemical experiments indicated that the addition of caveolin-2, a resident raft protein, was sufficient to target the WT OTR to lipid rafts. Furthermore, our data indicate that OT inhibits cell proliferation when the OTRs are excluded from lipid rafts. However, when OTRs are targeted to lipid rafts, OT has a strong mitogenic effect, thus indicating that the fraction of OTRs compartmentalized in lipid rafts may affect its proliferative and antiproliferative effects (19). We also found that the stimulation of cell growth by OTRs localized in DRMs involves ERK1/2 activation and epidermal growth factor receptor (EGFR) transactivation by means of a Gαi-, cellular-Rous sarcoma virus protein (c-Src)- and phospholipase C (PCL)-independent pathway, whereas the inhibition of cell proliferation that follows the stimulation of receptors excluded from DRMs depends on EGFR phosphorylation and ERK1/2 activation by means of a Gαi-, PLC-, c-Src-, and phosphatidylinositol 3-kinase (PI3-kinase)-dependent pathway. These different signaling mechanisms ultimately lead to different temporal patterns of ERK1/2 and EGFR activation: the stimulation of WT OTRs excluded from lipid rafts leads to sustained ERK1/2 and EGFR phosphorylation, whereas only transient activation is observed after stimulating WT OTRs located inside lipid rafts (37) (Fig. 1).

These findings provide a working hypothesis as to how the stimulation of a single GPCR can mediate very different cell responses within the same cell type and how modulating the type and composition of lipid rafts within a cell can affect GPCR signaling. Because the integrity of lipid rafts primarily depends on plasma-membrane cholesterol, we decided to investigate the effects of altering cell cholesterol content on the signaling of the OTRs expressed in HEK-293 cells. Our data indicate that cholesterol depletion has no effect on the signaling of OTRs outside lipid rafts but reverses the signaling of the OTRs inside them, thus indicating that the integrity of these domains is necessary to preserve the signaling specificity of their OTRs. Furthermore, as cholesterol loading did not affect OTR signaling, it does not seem to be sufficient to target the receptors to lipid rafts.

**MATERIALS AND METHODS**

**Peptides and reagents.** OT, methyl-β-cyclodextrin (MβCD), filipin, and cholesterol were obtained from Sigma. The origins of the primary antibodies were as follows: monoclonal anti-GFP (cat. no. M048–3 clone 1E4) from MBL; monoclonal anti-flotillin-2/ESA from BD Transduction Laboratories (cat. no. 610383); monoclonal anti-human transferrin receptor from Zymed Laboratories (cat. no. 13–6800 clone H68.4); cholera toxin B subunit-peroxidase (cat. no. C4672) from Sigma. The peroxidase-conjugated secondary antibodies came from Pierce.

**Generation of fusion constructs and transfection.** The generation of WT-OTR-EGFP and OTR-EGFP-cav2 cDNA has been previously described (19). For the biochemical, pharmacological and fluorescence studies, the cells were seeded at a density of about 100,000 cells/cm² and routinely used on the second day after seeding.

**Cholesterol determination.** For filipin staining, the cells were grown on glass coverslips precoated with 0.5% gelatin (wt/vol) and then incubated in serum-free medium for 40 min at 37°C, with or without a different concentration of MβCD. After this treatment, the cells were washed twice with PBS and fixed with 4% (wt/vol) paraformaldehyde in phosphate buffer for 20 min at room temperature, before being stained with filipin (100 μg/ml in PBS) under light-protected conditions for 2 h at RT. After two washes, the coverslips were mounted on the glass plates using a drop of phenyldiamine (1 mg/ml) for image processing, and a DAPI filter (excitation 340–380 nm) was used to visualize the filipin.

The samples were viewed and photographed using a Zeiss Axioplan microscope with a ×40 Neofluar objective (numerical aperture, 1.3) equipped with charge-coupled device camera Zeiss AxioCam HRm; the images were then processed using Adobe Photoshop 7. Cholesterol levels were determined using the Amplex Red Cholesterol Assay Kit (Molecular Probes).

**Alteration plasma membrane cholesterol content.** The cells were depleted of cholesterol by treatment with MβCD and then loaded with cholesterol by means of treatment with a cholesterol/MβCD complex, as described by Klein et al., (22). Briefly, 300 mg of cholesterol dissolved in methanol-chloroform (2:1 vol/vol) was added to a solution of MβCD on the bottom (5% wt/vol) while stirring at 80°C. Stirring continued until the initially precipitating steroid was completely dissolved.
For filipin staining, the flotation gradient assays and cholesterol determinations, the cells were treated for 40 min at 37°C with MβCD at a final concentration of 2 mM in a serum-free medium. In growth assays, the cells were pretreated with MβCD 2 mM for 40 min in a serum-free medium, and the drugs were maintained at a final concentration of 1 mM in complete medium for the remaining treatment period.

**Flotation gradients and Western blot analysis.** Confluent monolayers of HEK-293 cells stably transfected with OTR-GFP and OTR-GFP-cav2 were collected from 150-mm dishes in PBS (in mM: 140 NaCl, 2.7 KCl, 8 Na2HPO4, 1.5 KH2PO4, pH 7.4), centrifuged and resuspended in 50 mM Tris·HCl, pH 7.4, and supplemented with 2 mM EDTA and a protease inhibitor mixture (Sigma). After the nuclei were removed by means of low-speed centrifugation (800 g), the cell extract was ultracentrifuged at 100,000 g for 1 h, and the resulting pellet was resuspended in buffer A containing a final concentration of 1% Triton X-100. The detergent insoluble complexes were separated by centrifugation on discontinuous gradients: briefly, 250 µl of buffer A containing 2.4 M sucrose was added to 250 µl of each sample (1 mg of proteins), which was then placed in a centrifuge tube and overlaid with 1 ml of 0.9 M, 0.5 ml of 0.8 M, 1 ml of 0.7 M, and 1 ml of 0.1 M sucrose solutions, all prepared in buffer A. The discontinuous gradients were centrifuged at 335,000 g for 16 h by using a rotor SW 55 Ti (Beckman Instruments), after which 500-µl fractions were taken. The pellets were resuspended in 500 µl of buffer A.

For the receptor analysis, the proteins in 400 µl of each fraction were precipitated overnight at −20°C after the addition of four volumes of ice-cold acetone containing 3 µg of hemoglobin as a carrier. The precipitates were pelleted at 8000 g, dried briefly, resuspended in SDS-sample buffer modified to contain a final concentration of 0.05 M DTT as the reducing element and 0.6 M iodoacetamide, and analyzed by SDS-PAGE, and blotted with monoclonal anti-GFP.

The proteins present in 20 µl of each fraction of the flotation gradient were separated by SDS-PAGE and transferred to 0.45 µm nitrocellulose membranes (Schleicher & Schuell Bioscience), which were blocked overnight at 4°C in TBS (Tris·HCl 20 mM, pH 7.4, NaCl 150 mM) containing 5% powdered nonfat milk. They were then incubated for 2 h with primary antibody (monoclonal anti-flotillin-2 and monoclonal anti-transferrin receptor) diluted in TBS/milk/0.3% (vol/vol) Tween 20, and for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Pierce). The proteins were detected using the SuperSignal chemiluminescent substrate (Pierce).

The specific cholera toxin B ligand conjugated with peroxidase was used to detect cell surface ganglioside GM1 in the gradient fractions. One microliter of each diluted 1:10 fraction was spotted onto a nitrocellulose membrane, blocked overnight at 4°C in TBS/milk, incubated for 3 h with HRP-conjugated cholera toxin (4.2 ng/ml) diluted in TBS/milk/0.3% (vol/vol) Tween 20, and visualized by means of chemiluminescence.

**Cell growth assay.** The experiments were routinely carried out in the log phase of growth after the cells had been seeded in 96-multiwell plates (3/4,000 cells/well) and allowed to adhere for 24–48 h. The cells were cultured in MEM supplemented with 10% FCS, and their growth was determined using an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyme-thoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium, inner salt-based assay (Promega’s CellTiter 96 AQueous One Solution Assay) according to the manufacturer’s instructions. All of the determinations were in sextuplicate. A linear correlation between absorbance and cell counts was established by plating a known number of cells from 0 up to 20,000. The results were statistically analyzed using the Student’s t-test.

**RESULTS**

**Cholesterol depletion.** To investigate the effects of cholesterol manipulation on OTR signaling, we used a targeting system that allows OTRs to be directed inside or outside caveolin-enriched lipid rafts in a single cell type (19), i.e., stable HEK-293 cells transfected with OTRs fused to EGFP (OTR-GFP) or with OTRs targeted to lipid rafts as a result of their fusion to EGFP and the resident caveolar protein caveolin-2 (OTR-GFP-cav2) (37).

The cells were depleted of cholesterol by means of treatment with a relatively low (2 mM) concentration of MβCD, a cholesterol-binding drug that is widely used for this purpose (7, 8, 38). To study the cholesterol content of the membranes, we also used filipin, a UV-fluorescent polynye antibiotic that specifically binds to cholesterol and creates multimolecular filipin/cholesterol complexes. Once applied to living cells, it is incorporated in the cellular membranes in an amount proportional to their cholesterol content, allowing a direct correlation between filipin fluorescence and cholesterol level (23).

As shown in Fig. 2, filipin staining indicates that the 2-mM MβCD dose reduced plasma membrane cholesterol levels without affecting general cell morphology, whereas a 10-mM dose led to much greater cholesterol depletion but was accompanied by a rounding of the cells, suggesting a more relevant change in their physiological properties. Neither condition led to a relevant change in the number of cell surface receptors evaluated by means of receptor-tagged EGFP fluorescence (data not shown).

Total cell cholesterol levels were determined by means of a fluorimetric assay. In the HEK-293 cells transfected with the WT OTR, the MβCD treatment reduced total cholesterol by 62% (from 80 pmol/µg protein to 30.5 pmol/µg protein); in the HEK-293 cells transfected with OTR-GFP-cav2, the reduction was 68% (from 114 to 37 pmol/µg protein). It is worth noting that the cells transfected with OTR-GFP-cav2 had a higher basal cholesterol level, which may be explained by the overexpression of the caveolin-2 tagged to the OTR, because it is well known that caveolins bind cholesterol, and their overexpression may lead to an increase in cell cholesterol and membrane caveolae (41). However, standard electron microscopy did not reveal any increase in the number of caveolae in these cells (data not shown), possibly because of the downregulation of caveolin-1 detected by Western blot analysis (data not shown), because it is known that both caveolin-1 and caveolin-2 are required for the assembly of caveolae (27, 40).

**OTR partitioning in HEK-293 DRMs.** To determine the partitioning of OTR-GFP and OTR-GFP-cav2 proteins in HEK-293 cells under normal and depleted cholesterol conditions, we used flotation sucrose gradients after cell solubilization in cold 1% Triton X-100.

We first analyzed the effect of treatment with MβCD 2 mM on the distribution of plasma membrane proteins. Figure 3A shows that, under basal conditions, the low-density fractions (fractions 2, 3, and 4) corresponding to the insoluble fractions were enriched in ganglioside (GM)-1 and flotillin-2 (two characteristic markers of lipid rafts) (32, 47), whereas the transferrin receptor (a protein that is known to localize outside lipid microdomains) (48) was only present in the bottom Triton X-100 soluble fractions. The same treatment only partially destroyed the lipid rafts, because flotillin-2 levels clearly decreased in fraction 2...
but remained almost unchanged in the others, and there was a small shift in GM-1 among the low-density fractions. The unchanged distribution of the transferrin receptor showed that MβCD specifically acted on the lipid rafts and did not change the distribution of a protein normally located outside them. Figure 3B shows that similar results were obtained with cells stably expressing the OTR-GFP-cav2 receptor.

Figure 4 shows that, under normal culture conditions, WT OTRs were only found in the bottom fractions of the flotation gradient (fractions 6, 7, and 8, which correspond to the bulk of cellular Triton-soluble proteins) (Fig. 4A), whereas the addition of the caveolin-2 moiety was sufficient to target fused receptors to DRMs (fractions 2 and 3) (Fig. 4B). Unlike previous findings in the case of MDCK cells stably expressing the OTR-GFP-cav2 protein (19), we also detected OTR-GFP-cav2 in the bottom fractions of the flotation gradient, possibly because, in our HEK-293 experiments, equal volumes of each gradient fraction (rather than equal amounts of proteins) were loaded onto the gel and transferred to the blotting membranes. The latter approach is appropriate and advantageous, because although equal amounts of protein are initially applied to the flotation gradient, the protein content in the resulting fractions is not equal. Thus loading equal volumes of the flotation gradient fractions for the Western blot analysis reveals the real protein content in each fraction and leads to a more precise evaluation of protein enrichment in the Triton X-100-insoluble vs. the Triton X-100-soluble fractions. Unlike MDCK cells, HEK-293 cells expressing the WT-OTR-GFP and OTR-GFP-cav2 proteins have a pool of intracellular receptors (revealed as intracellular fluorescence) (37), which probably correspond to newly synthesized proteins and may increase the percentage of receptors excluded from DRMs. Furthermore, this intracellular pool is more abundant in OTR-GFP-cav2, which may explain its presence in the pellet of the flotation gradient. Because the pellet contains unsolubilized proteins, such as cytoskeleton proteins (11), it can be postulated that the pool of OTR-GFP-cav2 may be particularly resistant to solubilization.

After cholesterol depletion, the WT receptors did not move from the high-density fractions, although we did observe their redistribution in the Triton X-100 soluble fractions. However, the OTR-GFP-cav2 receptors disappeared from the fractions corresponding to lipid rafts in favor of the bottom region of the sucrose gradient (from fractions 2 and 3 to fraction 7), thus indicating that treatment with even a low concentration of MβCD changes their plasma membrane distribution.

To demonstrate that the shift of the receptor to the bottom fractions of the gradient is due to a variation in plasma membrane cholesterol level rather than a nonspecific effect of MβCD, we performed Triton X-100 extraction and sucrose separation using cells treated first with 2 mM of MβCD and then with a MβCD/cholesterol complex at a final concentration of 2 mM (Fig. 5). In the untreated cells, GM-1 was mainly detected in the low-density fractions (fractions 2, 3, and 4), but, after cholesterol depletion, it was found to be decreased in fractions 3 and 4. Analysis of the gradients obtained from the latter cells revealed a GM-1 similar distribution pattern to that observed under basal conditions, thus suggesting that treatment with the MβCD/cholesterol complex restored the organization...
of the lipid microdomain. Similarly, OTR-GFP-cav2, which disappeared from fractions 3 and 4 after MβCD treatment, shifted back to fraction 4 after cholesterol replenishment. The variability in the gradient fraction-location of OTR from experiment to experiment (e.g., Fig. 4 vs. Fig. 5) reflects the fact that the fractions are manually separated making it difficult to obtain the same exact content of proteins in each fraction in different experiments. This is the reason why in some experiments we observed the receptor in insoluble fractions 2 and 3, such as in Fig. 4, and in others in fractions 3 and 4, as in Fig. 5. Another problem reflected in Figs. 4 and 5 is that the OTR is a heavily glycosylated, high molecular weight protein that can dimerize. As a result, the immunostaining for the OTR consists of several smeared bands that are often difficult to be sized. Finally, to see an enrichment of the OTR in Triton X-100 insoluble fractions against Triton X-100 soluble fractions, we had to load a high volume of each sample so the bottom fractions (they have a higher protein content) are easily saturated and impossible to be quantified.

The proliferative effect of OTR-GFP-cav2 activation is reversed by cholesterol depletion. To explore the role of lipid raft location in determining the proliferative/antiproliferative effects of OTR stimulation, the cells were pretreated for 40 min with 2 mM of MβCD and then kept in the presence of 1 mM for the rest of the experiment. Under these culture conditions, there was a <40% decrease in cell viability (Fig. 6). In these experiments, the inhibition of cell growth was measurable after 48 h of OT treatment, whereas cell growth stimulation was already evident after 24 h. We have used these times of stimulation in previous studies (19, 37), and so maintained the same conditions in the present experiments.

As shown in Fig. 6A, OT stimulation significantly inhibited proliferation in the MβCD-treated cells expressing WT-OTR-GFP, thus indicating that cholesterol depletion has no effect on the signaling of WT-OTR-GFP located outside DRMs. On the contrary, in MβCD-treated cells expressing OTR-GFP-cav2 (Fig. 6B), OT stimulation not only did not produce any proliferative effect, but significantly inhibited cell proliferation. This indicates that OTR-GFP-cav2 receptors relocated...
outside DRMs by MβCD can engage the same signaling program as WT-OTR-GFP (Fig. 6B). These findings also show that the addition of caveolin-2 alone does not alter OTR signaling properties, which only seem to depend on whether the receptor is located inside or outside DRMs.

The antiproliferative effect of OTR-GFP is unaffected by cholesterol loading. Finally, we assayed the effects of increasing plasma membrane cholesterol content on OTR signaling. As shown in Fig. 7A, treating the cells with 2 mM of an MβCD/cholesterol complex (molar ratio 1:1.33) increased the total plasma membrane cholesterol content revealed with filipin staining by 81% (from 80 pmol/μg protein to 145 pmol/μg protein), which was higher than the basal value measured in the cells expressing OTR-GFP-cav2 (114 pmol/μg proteins). Under these conditions, OTR stimulation inhibited cell growth (Fig. 7B), thus indicating that increasing cell cholesterol levels is not sufficient per se to affect OTR signaling.

**DISCUSSION**

Cholesterol is an essential constituent of the plasma membrane, and its concentration is strictly regulated in various cell...
membranes (41). At steady state, as much as 80–90% of total cell cholesterol is in the plasma membrane, and there is very little in the endoplasmic reticulum and mitochondria (35). DRMs have 3–5 times higher cholesterol levels than total membranes, thus suggesting that cholesterol is critical for lipid raft formation and maintenance (3). It is known that alterations in cell cholesterol content can change the properties of these domains, and many studies have shown that cholesterol depletion destabilizes lipid rafts and releases their constituents into the bulk plasma membrane; moreover, their destruction greatly affects the functioning of many of the signaling molecules specifically located in lipid rafts (42).

We investigated the effect of cholesterol manipulation on the human OTR signaling and found that cholesterol depletion does not affect the signaling of the OTRs located mainly outside lipid rafts, whereas the signaling of caveolin-2-fused OTRs targeted to lipid rafts depends on raft integrity.

One widely used method of depleting cell cholesterol is treatment with β-cyclodextrins, cyclic heptasaccharides consisting of β(1–4)-glucopyranose units (8). The most effective cholesterol-depleting drug is MβCD, a water-soluble compound that binds cholesterol with high affinity (29). Although it removes cholesterol from all parts of the plasma membrane, the selective removal of cholesterol from lipid rafts and caveolae may be favored at low MβCD concentrations because of their high cholesterol content.

We found that 2 mM of MβCD altered the distribution of raft-resident components (flotillin and ganglioside GM-1) in the low-density fractions (fractions 2, 3, and 4) of discontinuous sucrose flotation gradients that correspond to DRMs, thus indicating that we only partially destabilized the lipid rafts. In particular, we altered one or more lipid raft subtypes that seem to be particularly sensitive to MβCD. As extensively discussed by Pike (33), there is evidence that lipid rafts might have heterogeneous lipid and protein compositions, some of which may be more sensitive to cholesterol extraction. Interestingly, under our depletion conditions, the OTR-GFP-cav2 receptors were completely redistributed into the soluble fractions, thus indicating that they are located within lipid rafts that are particularly sensitive to MβCD. These properties may be of special interest because these domains may be the first to be influenced by physio/pathological fluctuations in plasma membrane cholesterol levels.

It has been reported that plasma membrane cholesterol modulates the functional activity of many membrane proteins, including such GPCRs as rhodopsin (1), the galanin receptor (30), the µ-opioid receptor (24), the glutamate metabotropic receptor (14), the somatostatin receptor (36), and the OTR (15, 22).

In particular, it has been shown that the pharmacological properties of OTRs are regulated by the level of plasma membrane cholesterol, which controls their agonist and antagonist binding affinity: low cholesterol levels lead to low affinity, and higher levels to high affinity (15, 16, 22). This difference is remarkable because cholesterol-bound OTRs have about 100-fold higher affinity for agonists and antagonists. Finally, low-affinity receptors can be reverted to high-affinity states by the addition of cholesterol and, as cholesterol is enriched in lipid rafts, it can be expected that the OTRs in lipid rafts would have higher affinity. High- and low-affinity OTRs have been reported in various cell systems, such as myometrial (34) and glial cells (12), and so it can be postulated that high-affinity sites correspond to the OTRs preferentially located in lipid rafts.

We have previously reported a difference in OT binding affinity between the WT-OTR-GFP and OTR-GFP-cav2 receptors expressed in MDCK and HEK-293 cells (19, 37), although it is not as marked as that found in other cell systems (22). However, in HEK-293 cells, Gimpl and Fahrenholz (17) did not detect any difference in binding affinity between OTRs in lipid rafts and those located in soluble fractions. This discrepancy might be related to the fact that they used detergent-free methods to purify their lipid rafts (which, as discussed above, may lead to the isolation of a different subset of rafts), and they analyzed untagged receptors, whereas we analyzed receptors fused to caveolin-2, which seem to go to a lipid raft subtype that is very sensitive to MβCD. These factors may lead to receptors in different DRMs and/or the purification of different rafts.

It is still unknown where cholesterol binds to OTR, but the results of electron crystallography experiments using bovine rhodopsin suggest that it may bind in a pocket formed at the interface between the extracellular side of the two adjacent molecules of dymeric rhodopsin (39), and it would be interesting to evaluate whether a similar binding pocket exists in OTRs.

A lipid raft localization and cholesterol not only modulate the binding properties of OTRs but are also very important factors in determining their coupling specificity and signaling properties. We have previously shown that OT inhibits cell growth when the vast majority of OTRs are excluded from DRMs but has a strong mitogenic effect when the receptors are targeted to these plasma membrane domains (19, 37). We have now investigated the effects of cholesterol manipulation on the proliferative and antiproliferative effects elicited by OTRs located inside or outside lipid rafts.

As expected, cholesterol depletion had no effect on the signaling of the OTRs located outside lipid rafts, because the same degree of OT-induced cell growth inhibition was observed in the cells maintained in the absence or presence of MβCD. This indicates that, under our experimental conditions, the perturbation of DRMs does not modify OTR coupling properties and that MβCD treatment alone does not have any nonspecific effect on OTR signaling. On the contrary, cholesterol depletion markedly affected the signaling of OTR-GFP-cav2 receptors; it not only abolished their proliferative effects, but their stimulation actually inhibited cell growth. Once released from lipid rafts into the bulk plasma membrane, OTR-GFP-cav2 receptors signal like the WT-OTR-GFP receptors even when they still bear the caveolin-2 tag, which does not seem to affect their signaling properties by itself. This confirms that a lipid raft location is crucial in determining the signaling specificity of human OTRs.

OTRs have been found in the uterus, mammary gland, and central nervous system, as well as in a number of other tissues and tumor cells of various origins (6), where OT may stimulate, inhibit, or have no effect on cell proliferation (4). On the basis of our previous and current findings, we suggest that these different effects may be due to the of OTRs inside or outside lipid rafts in different cell types. A major problem in testing this hypothesis is determining the localization of endogenous receptors in tissues and cells. In our hands, none of the
currently available antibodies raised against human OTRs was sufficiently specific as they cross-reacted with cells transfected with the human OTR, as well as with parental, untransfected cells (data not shown). The exact localization of human OTRs within lipid rafts will unfortunately remain elusive until a highly specific antibody against endogenous OTRs is available.

It is still unclear what molecular mechanisms specifically address GPCRs to lipid rafts and how this is regulated. One possible mechanism is variations in plasma membrane cholesterol levels, a particularly interesting hypothesis because it is known that cholesterol accumulates in cells under various conditions. For example, the cholesterol content increases as endothelial cells reach confluency and differentiate (5, 10) and at the end of pregnancy in human myometrium cells (13). It has also been known for about a century that cholesterol and other fatty acids may accumulate in solid tumors (49), and the results of many studies of human subjects and animal models support the existence of a relationship between cholesterol and carcinogenesis and/or cancer progression. However, we found that a mere increase in cholesterol levels in HEK-293 cells was unable to shift the antiproliferative effect of human OTRs into a mere increase in cholesterol levels in HEK-293 cells was unable to shift the antiproliferative effect of human OTRs into a proliferative response, which suggests that the level of lipid raft integrity within lipid rafts will unfortunately remain elusive until a more molecular determinants affecting the “avidity” of OTRs to lipid rafts. There must therefore be other molecular determinants affecting the “avidity” of OTRs for lipid rafts and thus regulating their coupling specificity.

In conclusion, our data confirm the role of lipid raft integrity in OTR signaling, as previously demonstrated in the case of other GPCRs, such as ANG-1 (46), endothelin ETA and ETB (20, 44), GnRH (28), and bradykinin B2 receptors (21). However, much work remains to be done to establish the role of lipid rafts and cholesterol in regulating the specific outcome of human OTR responses under pathophysiological conditions.

ACKNOWLEDGMENTS
We thank Prof. N. Borgese and Dr. G. Gimpl for helpful suggestions concerning cholesterol determinations, and Dr. D. Caldara for technical help.

GRANTS
This study was supported by a grant from the Italian Association for Cancer Research (AIRC 2005) and the Fondazione Cariplo Grant 2004/1419: “New GRANTS.

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