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Cholesterol regulates μ-opioid receptor induced β-arrestin 2 translocation to membrane lipid rafts

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Running title: Cholesterol-regulated β-arrestin 2 translocation

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Abbreviations: OPRM1, μ-opioid receptor; GPCRs, G protein-coupled receptors; MβCD, Methyl-β-cyclodextrin; DAMGO, [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin; N2A, neuro2A neuroblastoma cell; βArr, β-arrestin; βArr1, β-arrestin 1; βArr2, β-arrestin 2; TR, transferrin receptor; PKC, protein kinase C; ERKs, extracellular signal-regulated kinases; IP, immunoprecipitation; IB, immunoblot.

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Abstract

μ-opioid receptor (OPRM1) is mainly localized in lipid raft microdomains but internalizes through clathrin-dependent pathway. Our previous studies demonstrated that disruption of lipid rafts by cholesterol-depletion reagent blocked the agonist-induced internalization of OPRM1 and G protein-dependent signaling. The present study demonstrated that reduction of cholesterol level decreased and culturing cells in excess cholesterol increased the agonist-induced internalization and desensitization of OPRM1 respectively. Further analyses indicated that modulation of cellular cholesterol level did not affect agonist-induced receptor phosphorylation, but rather membrane translocation of β-arrestins. The translocation of β-arrestins was blocked by cholesterol reduction and the effect could be reversed by incubating with cholesterol. OptiPrep gradient separation of lipid rafts revealed that excess cholesterol retained more receptors in lipid raft domains and facilitated the recruitment of β-arrestins to these microdomains upon agonist activation. Moreover, excess cholesterol could evoke receptor internalization and protein kinase C-independent extracellular signal-regulated kinases activation upon morphine treatment. Therefore, these results suggest that cholesterol not only can influence OPRM1 localization in lipid rafts, but also can effectively enhance the recruitment of β-arrestins and thereby affecting the agonist-induced trafficking and agonist-dependent signaling of OPRM1.

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Introduction

Cholesterol, a major constituent of membrane lipids, plays critical roles in structure and function of membrane proteins. Cholesterol can directly interact with membrane proteins and thus modulates protein functions. For example, cholesterol is specifically required for the interaction between large-conductance (maxi-K) and intermediate-conductance (IK1) Ca^{2+}-activated K channels (Romanenko et al., 2009). Cholesterol stabilizes oxytocin receptor to maintain the high-affinity state of the receptor for agonists (Gimpl et al., 2008). Moreover, cholesterol can interact with sphingolipids and other lipids to segregate into dynamic microdomains in the cell membranes (Lingwood et al., 2009). Lipid rafts are such microdomains that can cluster specific membrane proteins and thus regulating the protein functions (Allen et al., 2007).

Internalization (endocytosis) is an important biological process essential for many functions including cell growth and differentiation, pathogen entry, receptor signaling and down-regulation. The internalization of membrane proteins can be mediated by clathrin-dependent and clathrin-independent, lipid raft-dependent pathways (Le Roy and Wrana, 2005). Cholesterol is shown to be essentially required in the formation of clathrin-coated endocytic vesicles (Rodal et al., 1999; Subtil et al., 1999). However, several lines of evidence suggest that the effect of cholesterol is far reaching. Cholesterol and lipid rafts are more profoundly involved in the clathrin-dependent internalization pathway. Cholesterol depletion which releases epidermal growth factor receptor from lipid rafts inhibits agonist-induced receptor internalization without impairing receptor function (Pike and Casey, 2002). Further study indicates that the

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internalization of epidermal growth factor receptor via clathrin-coated pits is started from membrane rafts (Puri et al., 2005). Moreover, it is recently reported that lipid rafts and clathrin cooperate in the internalization of the cellular prion protein (Sarnataro et al., 2009). The internalization of the integral membrane protein CD317 which resides in the lipid rafts but internalizes through clathrin-coated pits is inhibited by dissociating it with the rafts (Rollason et al., 2007).

μ-opioid receptor (OPRM1) belongs to the superfamily of G protein-coupled receptors (GPCRs). As a large group of membrane proteins, the function of GPCRs has also been broadly demonstrated to be regulated by cholesterol and lipid rafts (Barnett-Norris et al., 2005; Chini and Parenti, 2004). OPRM1 is shown to reside mainly in lipid raft microdomains and its signaling either can be impaired or enhanced upon lipid raft disruption by cholesterol removal with methyl-β-cyclodextrin (MβCD) in different cell types (Huang et al., 2007; Levitt et al., 2009; Zheng et al., 2008a). Moreover, the agonist-induced internalization of OPRM1 is shown to be blocked by MβCD treatment (Zhao et al., 2006). Because OPRM1 internalizes through clathrin-coated pits (Minnis et al., 2003; von Zastrow, 2003), how cholesterol depletion blocks the clathrin-dependent internalization is unclear. The internalization of GPCRs is initiated by receptor phosphorylation and subsequent recruitment of β-arrestins (βArr) which couple receptors to the clathrin-coated pits (Ferguson, 2001; Goodman et al., 1996; von Zastrow et al., 2003) and uncouple receptors from G proteins to terminate receptor signaling (desensitization) (Lefkowitz and Shenoy, 2005), indicating that receptor internalization and desensitization share common pathways. Thus, whether cholesterol manipulation can affect OPRM1 desensitization

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needs to be clarified.

Therefore, we carried out the current study to investigate the role of cholesterol in OPRM1 internalization and desensitization. Our results showed that cholesterol manipulation by incubating with cholesterol-depletion reagents or with excess cholesterol could decrease or increase the agonist-induced internalization and desensitization of OPRM1. These effects could be attributed to the compartmentation of the receptor and recruitment of βArr to lipid raft microdomains.

Materials and methods

Cells and materials

Murine neuroblastoma Neuro2A (N2A) cells stably expressing hemagglutinin-tagged μ-opioid receptor (HA-OPRM1) (the *Bmax* and *Kd* values for [^{3}H]diprenorphine were determined to be 1.9 pmol/mg protein and 0.30 ± 0.04 nM, respectively) were maintained in DMEM supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml G418 in a 10% CO_2 incubator at 37 °C. MβCD and cholesterol were purchased from Sigma. Simvastatin and Ro-31-8425 were purchased from EMD Biosciences. β-arrestin 2-GFP (βArr2-GFP) (in pEGFP-N1) was kindly provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA). Anti-β-arrestin 1 (βArr1) and β-arrestin 2 (βArr1) antibodies were kindly provided by Dr. Martin Oppermann (University of Göttingen, Germany).

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Determination of receptor internalization by FACS analysis

Receptor internalization was quantified by FACS analysis as previously described (Qiu et al., 2003). Briefly, after incubation with 1 μM agonist for the indicated time intervals, cells were chilled on ice to terminate receptor trafficking, and cell surface receptors were visualized by incubating the cells with anti-HA antibody (1:1000), followed by incubation with the Alexa 488-conjugated anti-mouse IgG antibody (1:1000). Surface receptor staining intensity of the antibody-labeled cells was analyzed using fluorescence flow cytometry (FACScan, BD Biosciences). To exclude the possible effects of cholesterol manipulation on cell surface receptor level or antibody immunoreactivities, control cells without agonist treatment were treated with the same tested concentrations of MβCD or cholesterol. Receptor internalization was quantified as the percentage loss of cell surface fluorescence in agonist-treated cells. For cells transfected with βArr2-GFP or pEGFP-N1 vector, the cell surface receptors were labeled with Alexa 633-conjugated anti-mouse IgG antibody and cells expressing GFP were gated to determine agonist-induced receptor internalization.

Determination of receptor desensitization by intracellular cAMP assay

The intracellular cAMP level was measured as described previously (Zhao et al., 2006). Cells in 96-well plates were exposed to agonist for the indicated time intervals. The medium was then removed and replaced with 100 μl of Krebs-Ringer-HEPES buffer (KRHB, 110 mM NaCl, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$, pH 7.4) with 0.5 mM 3-isobutyl-1-methylxanthine, 10 μM forskolin and with or without agonist.

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Then the cells were incubated for 15 min at 37 ℃ and terminated by heating at 90ºC for 8 min. The measurement of cAMP level in supernatant was performed by using AlphaScreen™ cAMP detection kit (PerkinElmer Life and Analytical Sciences, Boston, MA). Receptor desensitization was calculated as the percentage loss of the ability of agonist to inhibit forskolin-stimulated intracellular cAMP production in agonist-treated cells.

Receptor phosphorylation assay

Cells cultured in 100 mm dishes were incubated with 1 μM DAMGO for 30 min at 37 ℃. The reactions were terminated on ice. Cells were washed with phosphate-buffered saline at 4 ºC and subsequently lysed in 0.5 ml lysis buffer (0.5% TritonX-100, 10 mM Tris pH 7.4, 150 mM NaCl, 25 mM KCl with 0.1 mM phenylmethylsulfonyl fluoride, 40 μg/ml Complete® protease inhibitors mixture, and with 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and 0.1 mM sodium vanadate as phosphatase inhibitors). After centrifugation at 12,000 ×*g* for 5 min, the supernatant was immunoprecipitated with 1 μl of mouse anti-HA (Covance, NJ) and rProtein G agarose beads (Invitrogen, CA) at 4 ℃ overnight. Then the beads were washed six times with cell lysis buffer and were extracted with SDS-PAGE sample buffer. Approximately equal amount of proteins was resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The phosphorylated OPRM1 receptors were detected by anti-phosphoSer^{375} of OPRM1 antibody (OPRM1phosphoSer^{375}, Cell Signaling, MA) and were normalized to the total immunoprecipitated receptors.

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β-arrestin translocation assay

The agonist-induced translocation of endogenous βArr to the cell membrane was analyzed as described previously (Huttenrauch et al., 2002). Cells cultured in 150 mm dishes were incubated with 1 μM [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) for 10 min at 37 °C. The cells were then placed on ice and scraped into 3 ml of buffer A (10 mM PIPES, 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, pH 7.0) containing 0.1 mM phenylmethylsulfonyl fluoride, 40 μg/ml Complete® protease inhibitors mixture (Roche Applied Science). The cells were homogenized and sonicated and subjected to centrifugation at 1000 ×*g* for 20 min. The supernatant was loaded on a discontinuous gradient of 50, 35, and 20% sucrose in buffer A and centrifuged at 160,000 ×*g* and 4 °C for 2 h. The supernatant (cytosol) was removed. The 35/50% sucrose interface (membrane) was collected and diluted in 3 ml of buffer A, and centrifuged at 160,000 ×*g* and 4 °C for 15 min again. The pellet was resuspended in 40 μl of detergent buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.05% SDS with protease inhibitors). Approximately equal amount of proteins was resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. βArr1 and βArr2 were detected by monoclonal anti-βArr1 and βArr2 antibodies (1:500) and determined with the analysis software ImageQuant (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Lipid raft separation

Separation of the lipid rafts from other membrane domains by OptiPrep density gradient was carried out as described previously (Macdonald and Pike, 2005; Morris et al., 2008). Briefly,

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cells were collected with base buffer (20 mM Tris, pH 7.5, at 25 °C (pH ~7.8), 250 mM sucrose, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) at 4 °C and centrifuged for 2 min at 250 ×*g* (4 °C). Cells were resuspended with 800 μl of base buffer with protease inhibitors. The raft extraction was performed by slowly passing the cell solution 15 times through a 1 ml syringe with a 22 gauge, 3 inch stainless steel needle. Samples were then centrifuged (10 min, 1000 ×*g*, 4 °C). The crude lipid raft extract was collected and mixed with an equal volume of 50% OptiPrep in base buffer at 4ºC and underlaid beneath an OptiPrep density gradient: 0% (0.5 ml), 5% (1 ml), 10% (1 ml), 15% (1 ml), and 20% (1 ml) in base buffer without $CaCl_2$ and $MgCl_2$. Following ultracentrifugation in a swinging bucket rotor (90 min, 52,000 ×*g*, 4 °C), 1 ml fractions across the density interfaces were carefully collected from the top. The collected fractions were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The amount of proteins of interest was monitored by specific antibodies and determined with the analysis software ImageQuant.

Measurement of extracellular signal-regulated kinases (ERKs) activation

Cells in 6-well plates were treated with 1 μM morphine for 10 min, then the medium was aspirated and cells were washed with PBS at 4°C twice and lysed with 0.1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 0.1% Triton X-100, 50 mM NaF, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM sodium pyrophosphate, 10 mM sodium vanadate, and Complete™ protease inhibitor cocktail (Roche)). After centrifugation, the supernatant was transferred to a new tube, and

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SDS-polyacrylamide gel electrophoresis sample buffer was added. Approximately same amount of protein was resolved by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The amount of phosphorylated ERKs was monitored by a monoclonal antibody for phosphorylated ERKs (Cell Signaling) and was normalized to total ERKs detected by total ERK antibodies (Cell Signaling).

Statistical Analysis

Data are presented as mean ± S.E. of at least three independent experiments. Either unpaired Student's *t* test (two-tailed) or one-way ANOVA was performed for statistical comparisons. When one-way ANOVA was used and when this analysis indicated significance ($p < 0.05$), Dunnett's multiple comparison test was used to determine which conditions were significantly different from the controls.

Results

Cholesterol manipulation regulates agonist-induced internalization of OPRM1

N2A cells are originated from neuronal cells and are used as models in the studies of neuronal functions. By treating the N2A cells stably expressing OPRM1 with MβCD, we observed a concentration- and time-dependent inhibition of the receptor internalization induced by 1 μM DAMGO, with 1 mM MβCD almost totally blocking the DAMGO-induced receptor internalization (Fig. 1A and Fig. 1B). This inhibition was reversed by incubating with cholesterol after the MβCD treatment (Fig. 1B). The inhibition of receptor internalization was also

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manifested by treatment of cells with 5 μM of simvastatin overnight (supplemental Fig. 1A), which could lower the cholesterol level by blocking its synthesis. Similarly, the effects of simvastatin could also be offset by the addition of cholesterol. To further investigate the role of cholesterol in OPRM1 internalization, the cells were incubated with various amount of cholesterol and then receptor internalization was investigated. DAMGO-induced receptor internalization was concentration- and time-dependently increased by incubating cells with excess cholesterol (Fig. 1C and 1D). The cellular cholesterol contents were demonstrated to be reduced by MβCD or simvastatin treatment and the reduction was reversed by cholesterol replenishment, while incubating the cells with cholesterol increased cellular cholesterol level (Supplemental Fig. 2). Thus, the regulation of OPRM1 internalization by treatment with MβCD, simvastatin and/or cholesterol could be correlated to their effects on cellular cholesterol level.

Cholesterol manipulation regulates agonist-induced OPRM1 desensitization

When the DAMGO inhibition of forskolin-stimulated intracellular cAMP accumulation was measured, MβCD could concentration- and time-dependently attenuate the DAMGO-induced receptor desensitization (Fig. 2A and 2B). Reducing the cholesterol level by simvastatin also inhibited receptor desensitization (supplemental Fig. 1B). Replenishment of cholesterol reversed the effects of MβCD and simvastatin on receptor desensitization (Fig. 2B and supplemental Fig. 1B). Increasing the cellular cholesterol content by incubating the cells with 50 μg/ml of cholesterol accelerated receptor desensitization (Fig. 2C). These data demonstrated that manipulation of cellular cholesterol level had similar effects on internalization and

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desensitization of OPRM1.

Because cholesterol depletion could reduce MOR signaling in some cell lines (Huang et al., 2007; Zheng et al., 2008a), the above effects of cholesterol manipulation could be due to the changes of receptor function. As shown in supplemental Table 1 and supplemental Fig. 3, maximum inhibition of forskolin-induced cAMP accumulation by DAMGO was not attenuated by treatment with the concentrations of MβCD tested, even though the potencies of DAMGO were reduced ≤ 2-fold. Incubation of the cells with cholesterol at concentrations > 50 μg/ml reduced the maximal inhibition to some extent. But the effects of cholesterol on potencies were mixed. Incubating the cells with low concentrations of cholesterol reduced while incubating with high cholesterol concentrations increased the DAMGO potency. Thus the slight changes of receptor signaling by cholesterol manipulation did not parallel with the alteration in receptor internalization and desensitization.

Cholesterol manipulation does not affect receptor phosphorylation but alters β-arrestin 2-OPRM1 interaction

Since receptor phosphorylation can affect the rate of OPRM1 internalization and desensitization (Qiu et al., 2003), the observed effects of cholesterol on receptor internalization and desensitization could be a direct result of alteration in receptor phosphorylation. As shown in Fig. 3A and 3B, cholesterol reduction by MβCD did not significantly influence DAMGO-induced receptor phosphorylation of Ser^{375}. Increase of the cellular cholesterol level did not affect receptor phosphorylation either (Fig. 3A and 3B). Without significant effect on

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receptor phosphorylation, the observed effects of cholesterol reduction on OPRM1 internalization could be a consequence of altered βArr-receptor interaction. When βArr2-GFP was over-expressed in N2A-OPRM1 cells, DAMGO-induced receptor internalization was restored in cells after MβCD treatment (Fig. 4). Because βArr is absolutely required for agonist-induced OPRM1 internalization (Whistler and von Zastrow, 1998), these data implicate that cholesterol directly modulates the interaction between receptor and βArr.

Cholesterol reduction attenuates membrane translocation of β-arrestins

The ability of cholesterol to modulate βArr-receptor interaction suggests probable effects of the sterol on the membrane translocation of βArr. As shown in Fig. 5A and 5B, treatment of N2A-OPRM1 cells with 1 μM DAMGO induced ~ 2-fold (2.0 ± 0.24) increase of the association of βArr1 and βArr2 with the membrane fraction. When the cholesterol level was reduced by 1 mM MβCD treatment, complete attenuation of the membrane translocation of βArr1 and βArr2 was observed. When the cells were replenished with cholesterol after MβCD treatment, the membrane translocation of βArr1 and βArr2 induced by DAMGO was restored. Increase of the cholesterol level by incubating the cells with excess cholesterol increased the amount of βArr1 and βArr2 translocated to the membranes in the presence of DAMGO to some extent (2.7 ± 0.35 fold over basal *versus* 2.0 ± 0.24 fold in the absence of cholesterol treatment).

Cholesterol manipulation regulates the distribution of OPRM1 in membrane domains and the recruitment of β-arrestin 2 to lipid raft domains upon agonist activation

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Cholesterol, has a larger affinity for saturated lipids, is usually assumed to be the driving force of lipid raft domain formation in living cell membranes (Gomez et al., 2008). Thus, we further investigated whether the observed effects of cholesterol manipulation on OPRM1 desensitization and internalization were mediated through its modulation on membrane raft domains. Using the OptiPrep density gradient, the lipid raft and non-raft domains were separated as demonstrated by the maximal Gαq immunoreactivities (a lipid raft marker) in fractions 1 and 2 and the maximal transferrin receptor immunoreactivities (TR, a non-raft marker) in fractions 3 and 4 (Fig. 6A, left panel). Cytosolic proteins composed of mainly contents in fraction 5 as demonstrated by immunoreactivities to clathrin heavy chain. Fraction 6 was a mix of cytosolic proteins and nuclear proteins, as demonstrated by its immunoreactivities to both clathrin heavy chain and lamin A/C (data not shown). OPRM1 was localized mainly in lipid raft fractions (Fig. 6A, left panel, 6B), which is consistent with previous reports (Huang et al., 2007; Zheng et al., 2008a). A significant amount of OPRM1 translocated to non-raft fractions after 10 min of 1 μM DAMGO treatment (Fig. 6A, left panel, 6B). Cholesterol reduction by MβCD disrupted the lipid raft domains, shifting both Gαq and OPRM1 out of fractions 1 and 2 (Fig. 6A, middle panel and 6B). Treatment of DAMGO did not further shift the OPRM1 out of these fractions (Fig. 6A, middle panel and 6B). Increase in cholesterol level resulted in more Gαq distributed to lipid raft fractions, especially fraction 1. However, the TR immunoreactivities were not influenced by the increase of cellular cholesterol content (Fig. 6A, right panel). These data suggest that cholesterol could modify lipid raft domains. Excess cholesterol increased the distribution of OPRM1 to lipid raft fractions (Fig. 6A, right panel and 6B). By retaining more OPRM1 in raft domains, there was

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less receptors moved to non-raft domains after DAMGO treatment (43 ± 4.5% *versus* 50 ± 1.6% of control, Fig. 6A, right panel and 6B). When the immunoreactivity of βArr2 was determined in these gradient fractions, most of βArr2 was found in fraction 5 and 6, demonstrating it mainly localized in the cytosol. Significant amount of βArr2 was demonstrated to translocate to lipid raft domains after agonist treatment when excess cholesterol was presented (Fig. 6A, right panel and 6C). In cells without cholesterol manipulation, the distribution of βArr2 to membrane raft domains was increased minimally (Fig. 6A, left panel and 6C). The distribution of βArr2 in raft and non-raft fractions was unaltered by agonist activation in the presence of MβCD (Fig. 6A, middle panel and 6C). All these data suggest that cholesterol modulates the translocation of OPRM1 in lipid rafts and subsequent recruitment of βArr2 by affecting the composition of lipid raft domains in cell membranes.

Excess cholesterol evokes the morphine-induced internalization of OPRM1 and protein kinase C (PKC)-independent ERKs activation

The failure of morphine to induce OPRM1 internalization was attributed to the low level of receptor phosphorylation induced by morphine and subsequent failure to induce βArr translocation (Whistler and von Zastrow, 1998; Zhang et al., 1998). The absence of morphine effect can be rescued by the over-expression of either G protein-coupled receptor kinase 2 or βArr2. Our current data indicate that cholesterol facilitates βArr recruitment. If this is the case, then by increasing the amount of βArr associated with the receptor, the increase in cholesterol level should facilitate morphine-induced receptor internalization. As shown in Fig. 7A,

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incubating the N2A-OPRM1 cells with cholesterol in a concentration-dependent manner was able to promote the morphine-induced OPRM1 internalization, suggesting that cholesterol facilitates the interaction between βArr and the activated receptors.

OPRM1-mediated ERKs activation has been shown to be pathway-selective and agonist-dependent. Morphine activates ERKs via a PKC-dependent pathway (Zheng et al., 2008b), which can be easily demonstrated with the blockade of ERKs activation using a general PKC inhibitor, Ro-31-8425 (Fig 7B). However, when the N2A-OPRM1 cells were pre-incubated with cholesterol, morphine still could activate ERKs in the presence of Ro-31-8425 (Fig. 7B). Such observations implicate the occurrence of βArr-dependent signaling for morphine in the presence of excess cholesterol.

Discussion

In current study, we demonstrated that reducing cellular cholesterol level by MβCD or simvastatin could attenuate the agonist-induced internalization and desensitization of OPRM1, whereas raising cellular cholesterol content increased the internalization and desensitization of the receptor. The data indicated that the rates of receptor internalization and desensitization were positively correlated to cellular cholesterol level. Further analyses indicated that modulation of cellular cholesterol level did not affect agonist-induced receptor phosphorylation but membrane translocation of βArr was blocked by cholesterol reduction. Furthermore, replenishment of cholesterol restored the βArr translocation to the membranes. Thus, the modulation of βArr

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recruitment by cholesterol manipulation contributes to its effects on internalization and desensitization of OPRM1.

Cholesterol plays a crucial regulatory role in controlling the lipid raft domains on plasma membrane (Simons and Ehehalt, 2002), thus the effects cholesterol has on OPRM1 function may be attributable to its role in lipid rafts. In current study, we utilized the method developed by Morris *et al* (Morris et al., 2008) to extract lipid rafts except that the gradient was run in the buffer without calcium and magnesium. We found that the distribution pattern of various proteins in fractions was similar to that in the linear OptiPrep gradient used by Macdonald and Pike (Macdonald and Pike, 2005). The raft marker Gαq was much more enriched in the two fractions with the lightest densities (fractions 1 and 2), whereas non-raft marker TR was concentrated in the middle two fractions (fractions 3 and 4). Our data also showed the cytosolic and nuclear proteins were located in another two fractions with the highest densities (fraction 5 and 6). Furthermore, our gradient separation of lipid rafts demonstrated that cholesterol reduction could shift Gαq distribution from the lightest densities fractions to the middle fractions, whereas excess cholesterol resulted in more Gαq distribution into the lightest densities fractions 1 and 2, especially fraction 1. Since the fraction of the membranes in the *lo* phase (lipid rafts) is directly proportional to the cholesterol concentration in phospholipid mixtures (Crane and Tamm, 2004), the alteration in the Gαq distribution in the gradient fractions under different cholesterol levels should represent the altered raft domains. Using this method, we monitored the translocation of proteins into and out of lipid rafts and demonstrated that βArr2 tended to translocate into the lipid raft fractions after agonist treatment. This βArr2 translocation was more pronounced in

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cells with excess cholesterol. At the same time, excess cholesterol increased the amount of OPRM1 distributed in fractions 1 and 2 and reduced the agonist-induced OPRM1 translocation out of raft domains. Probably, the increase of OPRM1 in lipid rafts under the high cholesterol content facilitates the receptor-induced recruitment and translocation of βArr to raft domains. The inability to detect the βArr translocation in non-treated cells in current study may be due to the fast translocation of OPRM1 to non-raft domains (Zheng et al., 2008a). Morphine, a poor inducer for βArr translocation, does not induce receptor internalization and activates ERKs through PKC-dependent pathway. However, with excess cholesterol, we were able to demonstrate that morphine induced receptor internalization and activated ERKs in a PKC-independent manner. These observations further support the view that lipid rafts played a role for receptor to efficiently recruit βArr.

For GPCRs that reside in the lipid rafts but internalize through clathrin-coated pits, it is generally postulated that receptors move out of lipid rafts before internalizing through clathrin-coated pits (Chini and Parenti, 2004; Morris et al., 2008). But accumulating evidence challenges this hypothesis. First, the role of lipid rafts in clathrin-dependent internalization of membrane proteins other than GPCRs has been well recognized. Clustering of the anthrax toxin receptor into rafts is necessary to trigger efficient clathrin-dependent internalization (Abrami et al., 2003). Ligand binding of the B-cell antigen receptor and epidermal growth factor receptor recruits clathrin and clathrin-coated pits assembly proteins to the raft microdomains (Puri et al., 2005; Stoddart et al., 2002). Second, the clathrin-dependent internalization of G protein-coupled cholecystokinin receptor can be inhibited by raft disruption (Harikumar et al., 2005). Third, the

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dependence of βArr recruitment on cholesterol has been indicated in several studies. The βArr translocation to the membrane after activation of *N*-formyl peptide receptor was not observed in cholesterol-depleted cells (Xue et al., 2004). Our study with OPRM1 and a study with the neurokinin-1 receptor (Kubale et al., 2007) demonstrate that agonist-induced βArr recruitment is substantially attenuated by the disruption of lipid rafts. Moreover, translocation of βArr to lipid raft domains has been observed with the lipid raft-located rhodopsin receptor that does not translocate to non-raft microdomains after light activation (Nair et al., 2002). Therefore, the compartmentation of receptors in lipid rafts could be crucial for βArr recruitment. Recently, the direct interaction between the palmitate covalently attached to the C3.55(170) residue of OPRM1 and cholesterol has been demonstrated (*unpublished results*). Cholesterol is trapped at the interface of OPRM1 homodimer and stabilizes the homodimerization which is important for G protein coupling and lipid raft location. Thus the increase in cholesterol level also could contribute to the stabilization of the OPRM1 homodimer, and subsequent βArr-receptor interaction. Our previous report demonstrates that agonist-induced OPRM1 translocation out of lipid raft domains requires the binding of the receptor with βArr (Zheng et al., 2008a). Thus we hypothesize that cholesterol stabilizes OPRM1 in lipid rafts and thus promotes G protein coupling and βArr recruitment, with the recruited βArr translocating the receptor out of lipid rafts. In addition, the efficient recruitment of βArr to OPRM1 complex enables the switching of the pathway-selected receptor signaling, as demonstrated by the ability of morphine to activate ERKs in the presence of PKC inhibitor Ro-31-8425. Since the consequence of pathway-selective signaling is the eventual cellular locations of the activated ERKs and the transcripts being

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regulated (Zheng et al., 2008b), the switching of the pathway selected in ERKs activation under high cholesterol level could have implication in the eventual adaptational processes upon agonist exposure.

Taken together, the current study and others in our laboratory delineate the movement of receptor in membrane microdomains. Further, our study demonstrates that cholesterol contributes to the compartmentation of OPRM1 to lipid rafts and facilitates the efficient recruitment of βArr. Our study provides the possibility to modify the receptor trafficking and hence the overall signaling of OPRM1—the molecular basis for opioid tolerance (Waldhoer et al., 2004)— by manipulating cholesterol level.

Acknowledgements

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Authorship Contributions

Participated in research design: Qiu, Law, Chen, Loh

Conducted experiments: Qiu, Wang

Performed data analysis: Qiu, Law

Wrote or contributed to the writing of the manuscript: Qiu, Law, Loh

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Footnotes

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Figure legends

Fig. 1 Cholesterol reduction by MβCD decreased DAMGO-induced internalization of OPRM1, while excess cholesterol increased the internalization of OPRM1. Internalization of OPRM1 was quantified as the percentage loss of cell surface receptors in agonist-treated cells as described in "Materials and Methods". A. N2A-OPRM1 cells were treated with different concentrations of MβCD for 3 h, then 1 μM DAMGO was added for further incubation of 2 h. B. N2A-OPRM1 cells were treated with 1 mM MβCD for 3 h (MβCD) or 1 mM MβCD for 3 h and then 10 μg/ml cholesterol for 1 h (MβCD & cholesterol), then cells were further incubated with 1 μM DAMGO for indicated time intervals. C. N2A-OPRM1 cells were treated with different concentrations of cholesterol for 1 h, then 1 μM DAMGO was added for further incubation of 1 h. D. N2A-OPRM1 cells were treated with 50 μg/ml cholesterol (cholesterol) for 1 h, then cells were further incubated with 1 μM DAMGO for indicated time intervals. Data are mean ± S.E. of at least three independent experiments performed at least in duplicate. *, $P < 0.05$; **, $P < 0.01$ *versus* cells without MβCD (A) or cholesterol (C) incubation or matched internalized receptors in controls (B, D).

Fig. 2 Cholesterol reduction by MβCD decreased DAMGO-induced desensitization of OPRM1, while excess cholesterol increased the desensitization of OPRM1. Desensitization of OPRM1 was calculated as the percentage loss of the ability of 1 μM DAMGO to inhibit forskolin-stimulated intracellular cAMP production in agonist-treated cells as described in "Materials and Methods". A. N2A-OPRM1 cells were treated with different concentrations of

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MβCD for 3 h, then 1 μM DAMGO was added for further incubation of 1 h. B. N2A-OPRM1 cells were treated with 1 mM MβCD for 3 h (MβCD) or 1 mM MβCD for 3 h and then 10 μg/ml cholesterol for 1 h (MβCD & cholesterol), then cells were further incubated with 1 μM DAMGO for indicated time intervals. C. N2A-OPRM1 cells were treated with 50 μg/ml cholesterol (cholesterol) for 1 h, then cells were further incubated with 1 μM DAMGO for indicated time intervals. Data are mean ± S.E. of at least three independent experiments performed at least in triplicate. *, $P < 0.05$; **, $P < 0.01$ *versus* cells without MβCD treatment (A) or matched receptor desensitization in controls (B, C).

Fig. 3 Cholesterol reduction or incubating with cholesterol did not significantly affect receptor phosphorylation. N2A-OPRM1 cells were treated with 1 mM MβCD for 3 h or 10 μg/ml cholesterol for 1 h, then the cells were further incubated with 1 μM DAMGO for 10 min. Receptors were immunoprecipitated (IP) and receptor phosphorylation was immunoblotted (IB) by OPRM1phosphoSer^{375} antibody (A) and quantified (B) as described under "Materials and Methods". Data are mean ± S.E. of at least three independent experiments.

Fig. 4 Over-expression of βArr2 restored the agonist-induced internalization of OPRM1 blocked by MβCD. N2A-OPRM1 cells were transiently transfected with βArr2-GFP or pEGFP-N1 vector, 48 h later, cells were treated with 1 mM MβCD for 3 h and 1 μM DAMGO for indicated time intervals. The internalized receptors were detected and quantified as described under "Materials and Methods". Data are mean ± S.E. of at least three independent

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experiments performed at least in duplicate. *, $P < 0.05$; **, $P < 0.01$ *versus* matched internalized receptors in cells expressing GFP.

Fig. 5 Cholesterol reduction attenuates membrane translocation of βArr. N2A-OPRM1 cells were treated with 1 mM MβCD for 3 h or 1 mM MβCD for 3 h and then 10 μg/ml cholesterol for 1 h or 50 μg/ml cholesterol for 1 h, then cells were further incubated with 1 μM DAMGO for 10 min. βArr translocated to cell membrane were assayed (A) and quantified (B) as described under "Materials and Methods". Data are mean ± S.E. of at least three independent experiments. **, $P < 0.01$ *versus* control.

Fig. 6 Cholesterol reduction or incubating cells with cholesterol affected OPRM1 localization and the recruitment of βArr2 to membrane domains. A. Distribution of OPRM1, TR, Gαq and βArr2 after MβCD or cholesterol treatment. N2A-OPRM1 cells were treated with 1mM MβCD for 3 h or 50 μg/ml cholesterol for 1 h, then cells were further incubated with 1 μM DAMGO for 10 min. Membrane lipid raft separation and immunoblot of proteins of interest were performed as described under "Materials and Methods". B. Quantitative analysis of OPRM1 distribution in fractions (1+2) *versus* fractions (3+4). C. Quantitative analysis of βArr2 distribution in fractions (1+2) *versus* fractions (3+4). Data are mean ± S.E. of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

Fig. 7 Incubating cells with cholesterol evoked the morphine-induced internalization of

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OPRM1 and PKC-independent ERKs activation. A. N2A-OPRM1 cells were treated with different concentration of cholesterol for 1 h, then 1 μM morphine was added for further incubation of 1 h. Data are mean ± S.E. of at least three independent experiments performed at least in duplicate. B. N2A-OPRM1 cells were treated with DMSO or 5 μM Ro-31-8425 for 2 h, then 25 μg/ml cholesterol was added for further incubation of 1 h. 1 μM morphine was added for 10 min and then cells were lysed and phosphorylated ERKs were detected and quantified as described under "Materials and Methods". Data are mean ± S.E. of at least three independent experiments. *, $P < 0.05$, **, $P < 0.01$ *versus* cells without cholesterol incubation or as denoted.

Fig. 1

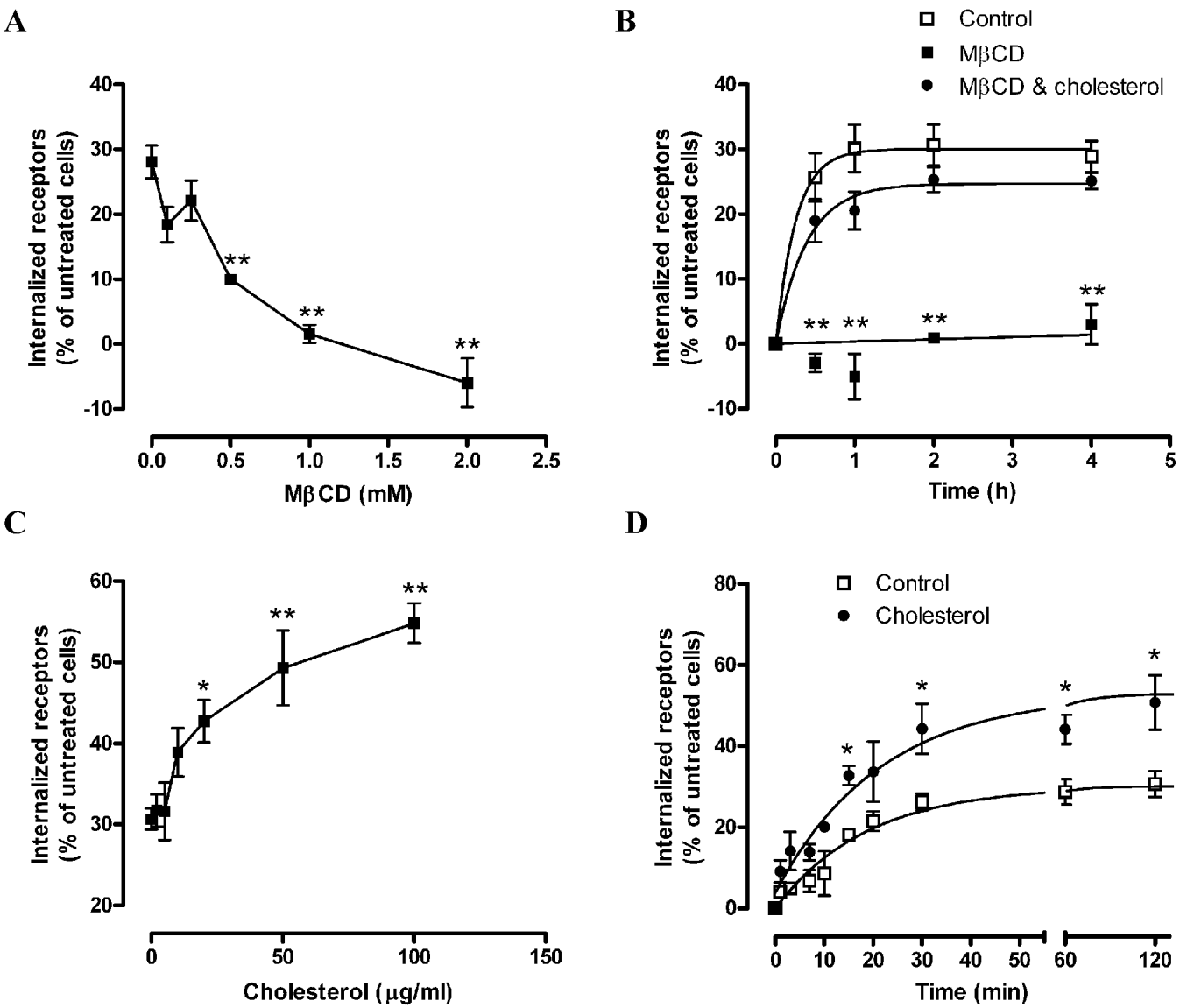

A
Internalized receptors (% of untreated cells)
MβCD (mM)
B
Control
MβCD
MβCD & cholesterol
Internalized receptors (% of untreated cells)
Time (h)
C
Internalized receptors (% of untreated cells)
Cholesterol (μg/ml)
D
Control
Cholesterol
Internalized receptors (% of untreated cells)
Time (min)

Fig. 2

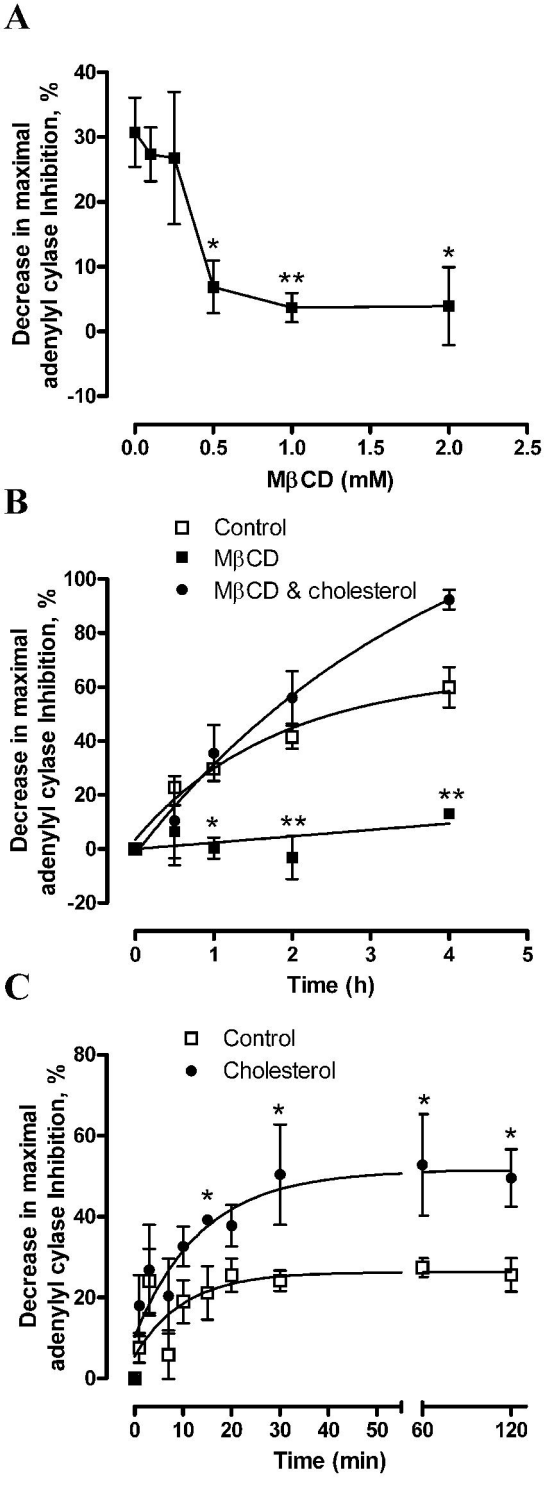
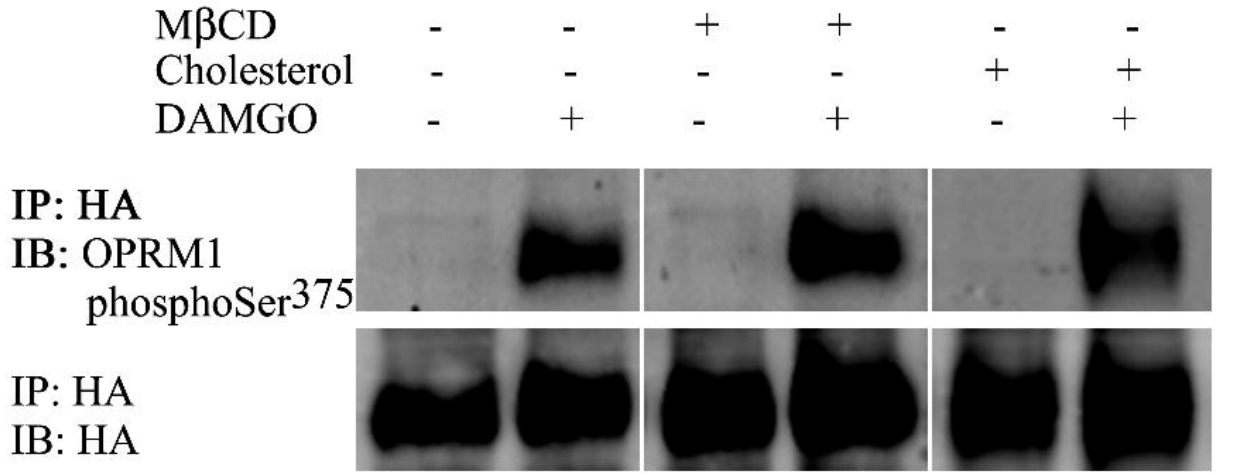

A
Decrease in maximal adenylyl cylase Inhibition, %
40
30
20
10
0
-10
*
**
*
0.0
0.5
1.0
1.5
2.0
2.5
MβCD (mM)
B
Control
MβCD
MβCD & cholesterol
Decrease in maximal adenylyl cylase Inhibition, %
100
80
60
40
20
0
-20
*
**
**
0
1
2
3
4
5
Time (h)
C
Control
Cholesterol
Decrease in maximal adenylyl cylase Inhibition, %
80
60
40
20
0
*
*
*
*
0
10
20
30
40
50
60
120
Time (min)

Fig. 3

A


MβCD - - + + - -
Cholesterol - - - - + +
DAMGO - + - + - +
IP: HA
IB: OPRM1
phosphoSer375
IP: HA
IB: HA

B

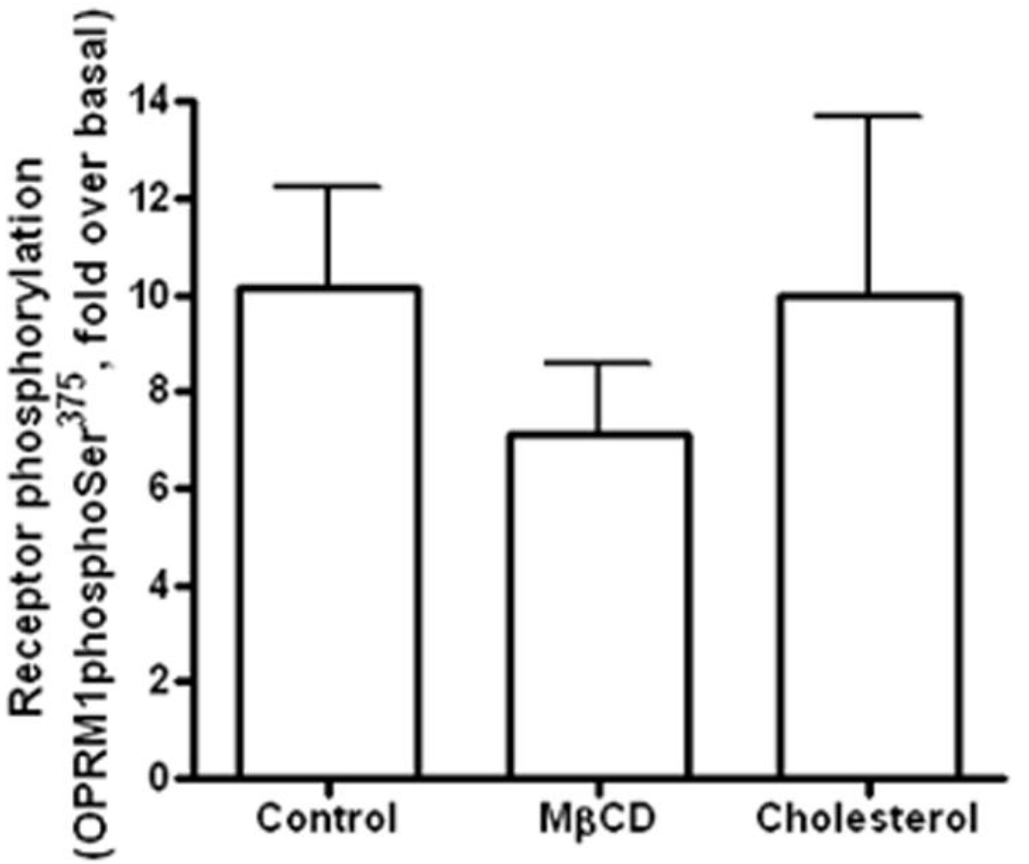

Receptor phosphorylation
(OPRM1phosphoSer375, fold over basal)
0
2
4
6
8
10
12
14
Control
MβCD
Cholesterol

Fig. 4

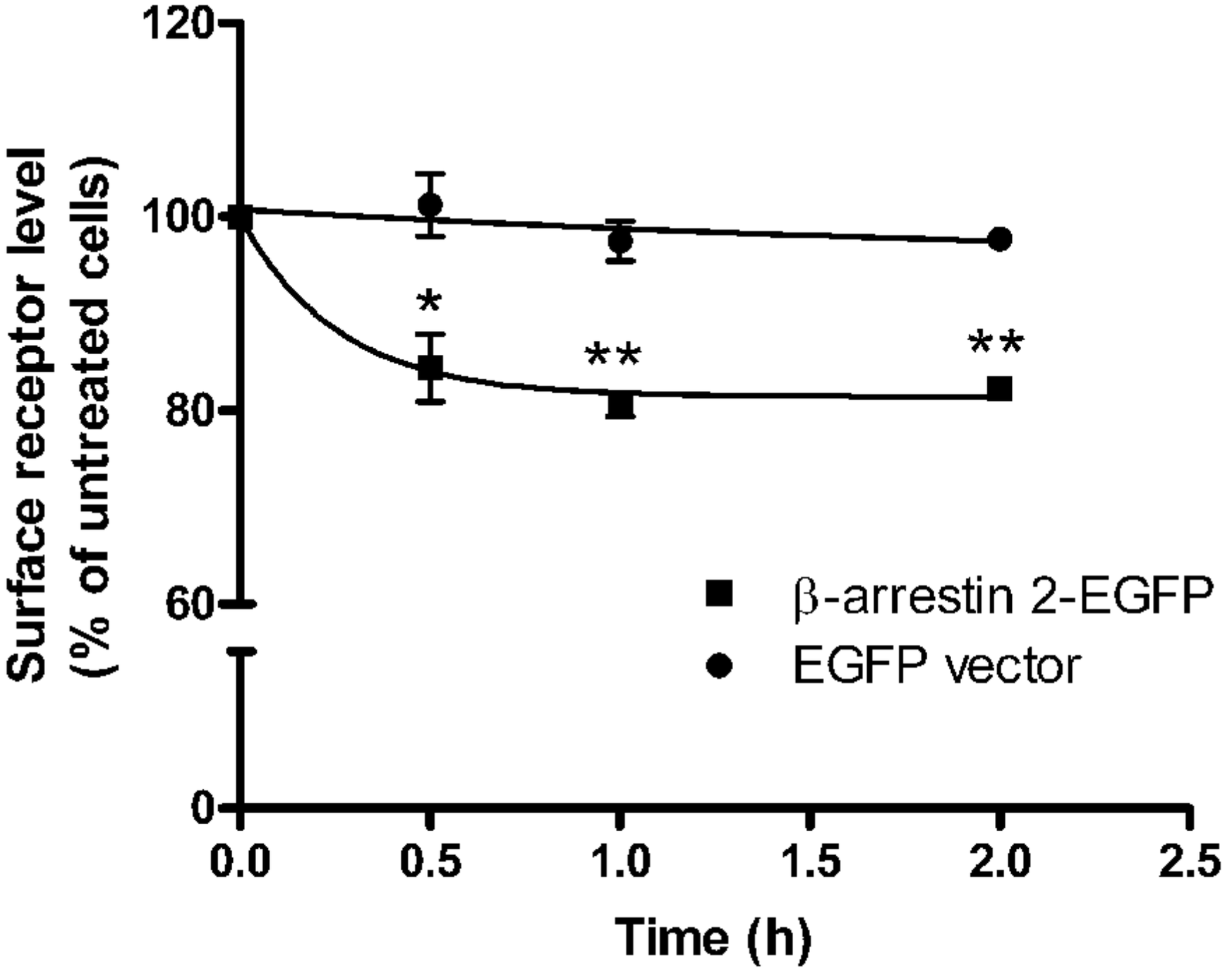
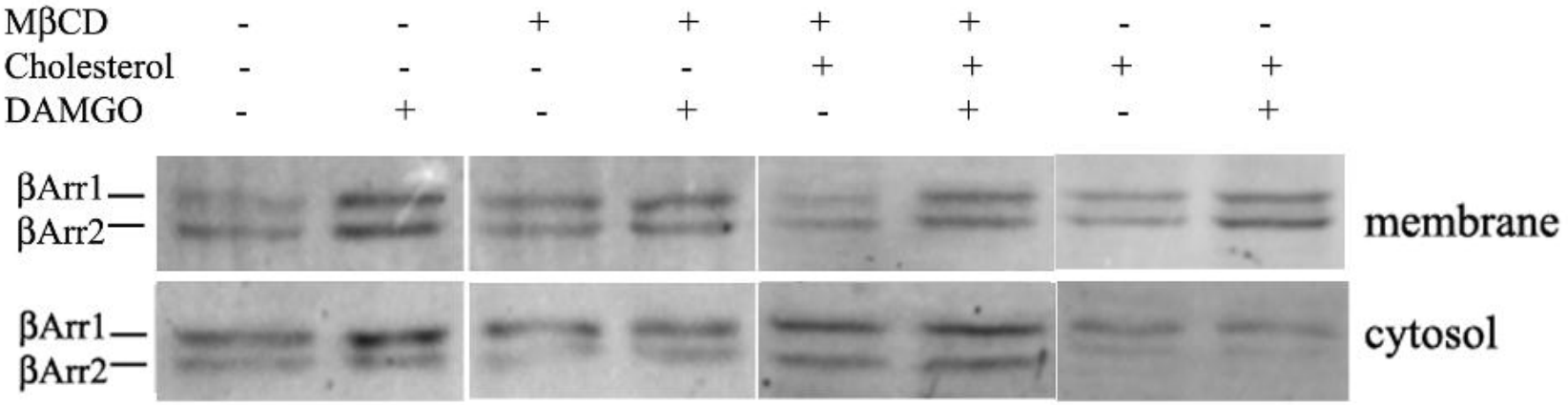

120
100
80
60
0
Surface receptor level
(% of untreated cells)
*
**
**
β-arrestin 2-EGFP
EGFP vector
0.0
0.5
1.0
1.5
2.0
2.5
Time (h)

Fig. 5

A


MβCD - - + + + + - -
Cholesterol - - - - + + + +
DAMGO - + - + - + - +
βArr1
βArr2
membrane
βArr1
βArr2
cytosol

B

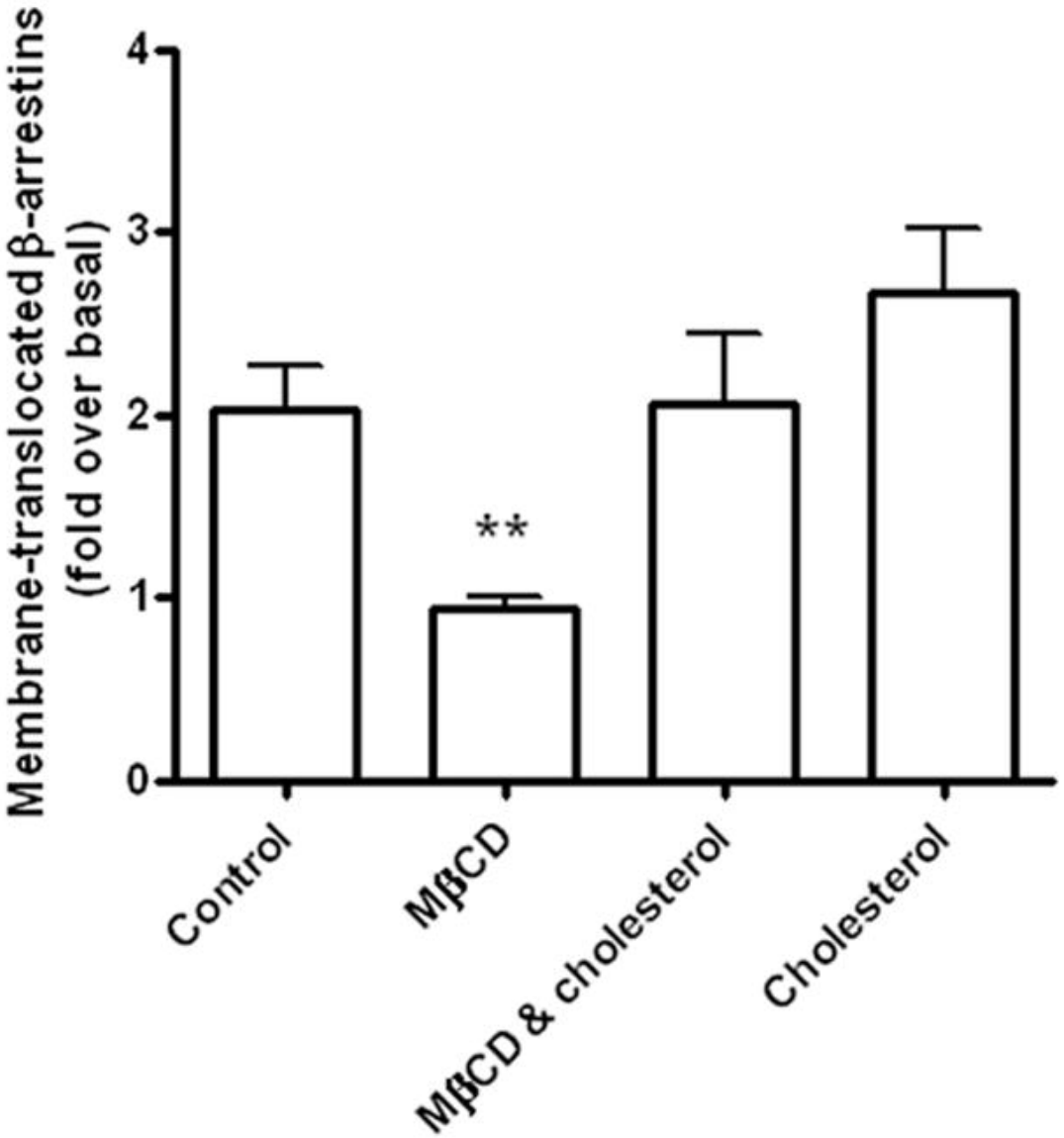

Membrane-translocated β-arrestins (fold over basal)
0
1
2
3
4
**
Control
MβCD
MβCD & cholesterol
Cholesterol

Fig. 6

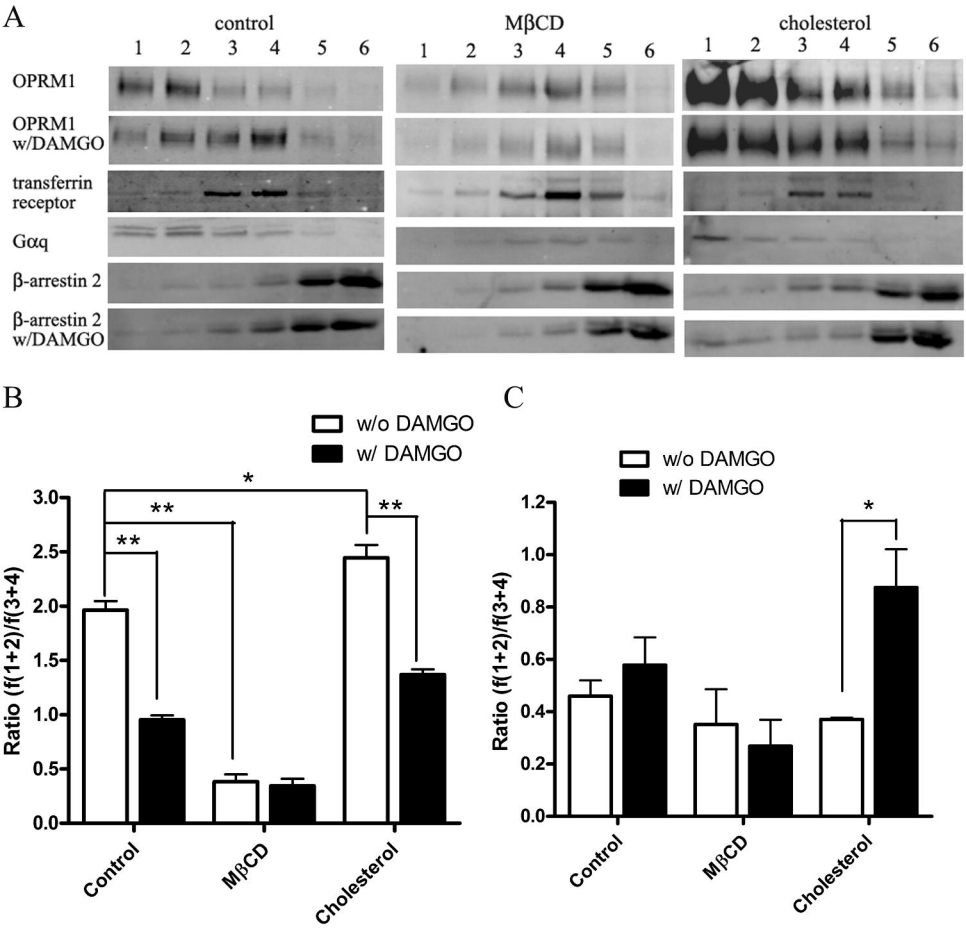
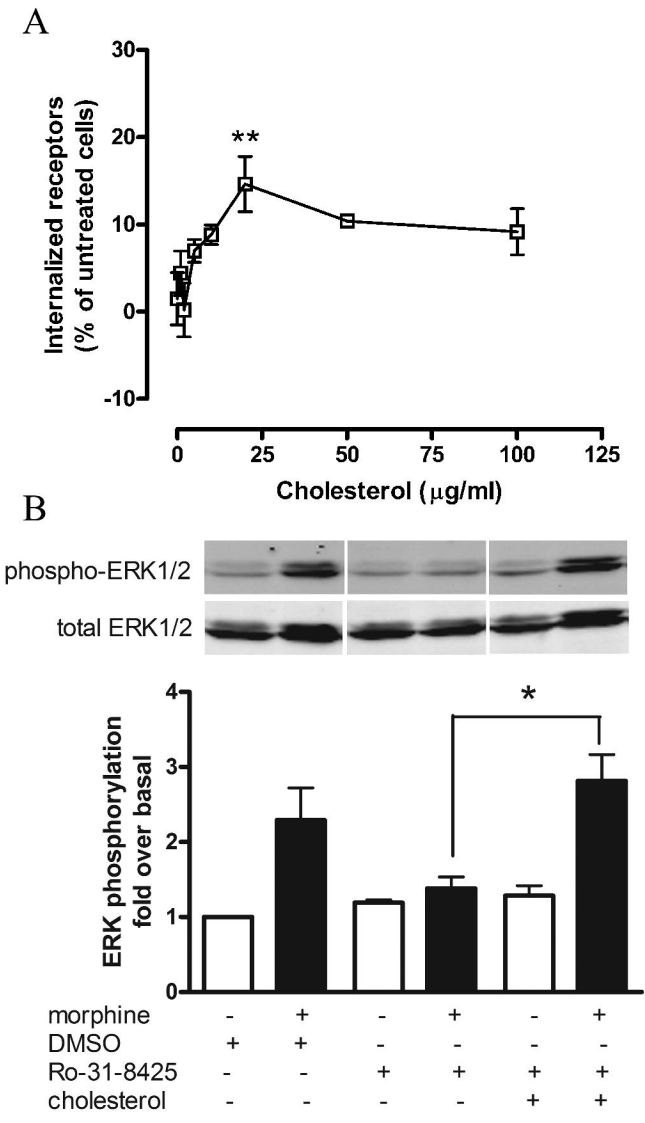

A
control
MβCD
cholesterol
1 2 3 4 5 6
OPRM1
OPRM1 w/DAMGO
transferrin receptor
Gαq
β-arrestin 2
β-arrestin 2 w/DAMGO
B
w/o DAMGO
w/ DAMGO
Ratio (f(1+2)/f(3+4)
Control
MβCD
Cholesterol
C
w/o DAMGO
w/ DAMGO
Ratio (f(1+2)/f(3+4)
Control
MβCD
Cholesterol

Fig. 7


A
Internalized receptors (% of untreated cells)
30
20
10
0
-10
**
0
25
50
75
100
125
Cholesterol (μg/ml)
B
phospho-ERK1/2
total ERK1/2
ERK phosphorylation fold over basal
4
3
2
1
0
*
morphine - + - + - +
DMSO + + - - - -
Ro-31-8425 - - + + + +
cholesterol - - - - + +

Supplemental data

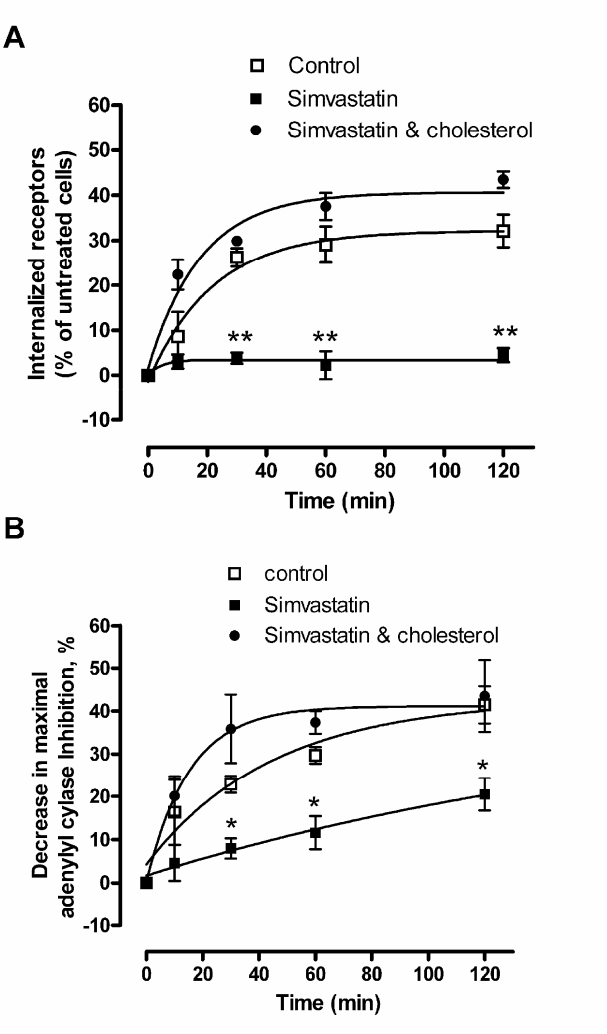
Molecular Pharmacology

Cholesterol regulates μ-opioid receptor induced β-arrestin 2 translocation to membrane lipid rafts. Yu Qiu, Yan Wang, Ping-Yee Law, Hong-Zhuan Chen, and Horace H. Loh

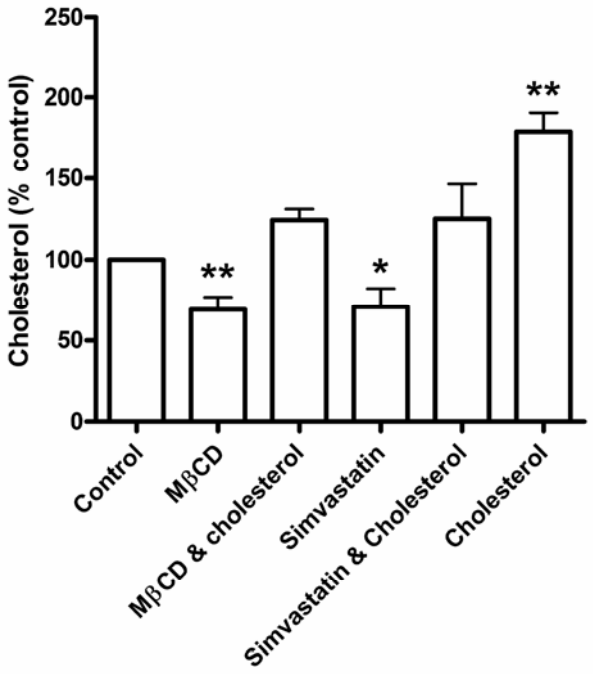
Supplemental Table 1 Characterization of cholesterol manipulation on the ability of DAMGO to inhibit the forskolin-induced cAMP accumulation

	IC_{50}, nM	Maximal inhibition, %
control	8.3 ± 2.4	46 ± 3.0
MβCD 0.25 mM	17 ± 9.5	46 ± 4.3
MβCD 0.5 mM	17 ± 14	45 ± 7.3
MβCD 1 mM	14 ± 3.0	45 ± 3.3
MβCD 2 mM	12 ± 0.4	43 ± 5.6
Cholesterol 2 μg/ml	22 ± 10	53 ± 11
Cholesterol 5 μg/ml	21 ± 6.8	44 ± 9.8
Cholesterol 10 μg/ml	7.4 ± 3.7	42 ± 10.9
Cholesterol 20 μg/ml	18 ± 10	46 ± 10
Cholesterol 50 μg/ml	8.0 ± 3.8	32 ± 6.6
Cholesterol 100 μg/ml	3.6 ± 2.2	27 ± 5.9

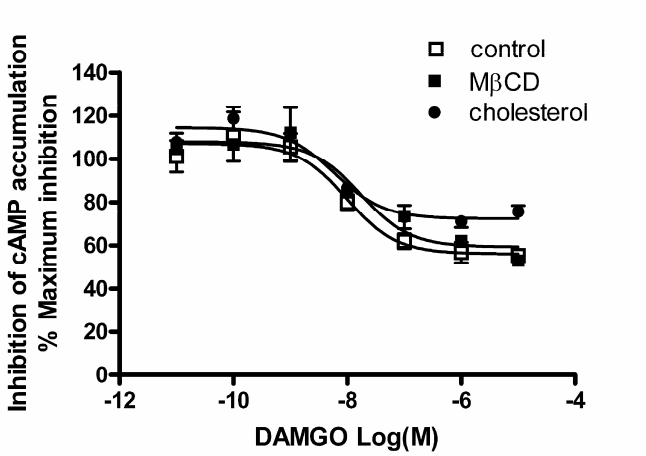
The abilities of DAMGO to inhibit 10 μM forskolin-stimulated intracellular cAMP production in N2A-MOR cells treated with various concentrations of MβCD for 3 h or cholesterol for 1 h were determined. IC_{50} represents the concentration of DAMGO to produce 50% of the maximal inhibition. Data are mean ± S.E. of at least three independent experiments performed at least in triplicate.



Supplemental Fig. 1 Simvastatin decreased DAMGO-induced internalization and desensitization of OPRM1. N2A-OPRM1 cells were treated with 5 μM simvastatin overnight and then treated with 10 μg/ml cholesterol for 1 h or not. Then cells were further incubated with 1 μM DAMGO for indicated time intervals. Receptor internalization (*A*) and desensitization (*B*) were measured as described in "Materials and Methods". *, $p < 0.05$; **, $P < 0.01$ *versus* matched the internalization or desensitization rate of controls. Data are mean ± S.E. of at least three independent experiments performed at least in triplicate.



Supplemental Fig. 2 MβCD and simvastatin decreased while incubating with cholesterol increased cellular cholesterol level. N2A-OPRM1 cells were treated with 1 mM MβCD for 3 h or 5 μM simvastatin for overnight and then treated with 10 μg/ml cholesterol for 1 h or not, or treated with 50 μg/ml cholesterol for 1 h. Then the cells were lysed in lysis buffer. Cholesterol concentrations in total lysate were determined by an Amplex Red Cholesterol Assay Kit (Invitrogen, Carlsbad, CA) and were normalized to protein contents. *, $p < 0.05$; **, $P < 0.01$ *versus* control. Data are mean ± S.E. of at least three independent experiments.



Supplemental Fig. 3 The effects of cholesterol manipulation on the abilities of DAMGO to inhibit the forskolin-induced cAMP accumulation. N2A-OPRM1 cells were treated with 1mM MβCD for 3 h (MβCD) or 50 μg/ml cholesterol for 1 h (cholesterol), then the abilities of various concentrations of DAMGO to inhibit 10 μM forskolin-stimulated intracellular cAMP production were determined. Data are mean ± S.E. of at least three independent experiments performed at least in triplicate.