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Cholesterol Regulates μ-Opioid Receptor-Induced β-Arrestin 2 Translocation to Membrane Lipid Rafts[S]

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ABSTRACT

μ-Opioid receptor (OPRM1) is mainly localized in lipid raft microdomains but internalizes through clathrin-dependent pathways. 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 did affect 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 affect 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 modulate protein functions. For example, cholesterol is specifically required for the interaction between large- and intermediate-conductance Ca^{2+}-activated K channels (Romanenko et al., 2009). Cholesterol stabilizes the 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 regulate 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 -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 internalization of epidermal growth factor receptor via clathrin-coated pits is started from membrane rafts (Puri et al., 2005). Moreover, it has recently been reported that lipid rafts and clathrin cooperate in the internalization of the cellular prion protein (Sarnataro et al.,

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ABBREVIATIONS: OPRM1, μ-opioid receptor; MβCD, methyl-β-cyclodextrin; βArr, β-arrestin; HA, hemagglutinin; Ro-31-8425, bisindolylmaleimide X; GFP, green fluorescent protein; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; N2A, neuro2A neuroblastoma cell; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); ERK, extracellular signal-regulated kinase; TR, transferrin receptor; PKC, protein kinase C.

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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. As a large group of membrane proteins, the function of G protein-coupled receptors has also been broadly demonstrated to be regulated by cholesterol and lipid rafts (Chini and Parenti, 2004; Barnett-Norris et al., 2005). OPRM1 is shown to reside mainly in lipid raft microdomains, and its signaling can be either impaired or enhanced upon lipid raft disruption by cholesterol removal with methyl-β-cyclodextrin (MβCD) in different cell types (Huang et al., 2007; Zheng et al., 2008a; Levitt et al., 2009). 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 (Goodman et al., 1996; Ferguson, 2001; 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 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 B_{max} and K_d values for [^{3}H]diprenorphine were determined to be 1.9 pmol/mg protein and 0.30 ± 0.04 nM, respectively) were maintained in Dulbecco's modified Eagle's medium 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-Aldrich (St. Louis, MO). Simvastatin and bisindolylmaleimide X hydrochloride (Ro-31-8425) were purchased from EMD Biosciences. βArr2-GFP (in pEGFP-N1) was kindly provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA). Anti-βArr1 and βArr2 antibodies were kindly provided by Dr. Martin Oppermann (University of Göttingen, Göttingen, Germany).

Determination of Receptor Internalization by Fluorescence-Activated Cell Sorting Analysis. Receptor internalization was quantified by fluorescence-activated cell sorting analysis as described previously (Qiu et al., 2003). In brief, 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 Fluor 488 (Invitrogen, Carlsbad, CA)-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, San Jose, CA). 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 Fluor 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 (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. Then the cells were incubated for 15 min at 37°C and terminated by heating at 90°C for 8 min. The measurement of cAMP level in supernatant was performed by using AlphaScreen cAMP detection kit (PerkinElmer Life and Analytical Sciences, Waltham, 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°C. The reactions were terminated on ice. Cells were washed with phosphate-buffered saline at 4°C and subsequently lysed in 0.5 ml of lysis buffer [0.5% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, and 25 mM KCl, with 0.1 mM phenylmethylsulfonyl fluoride, 40 μg/ml Complete protease inhibitor mixture (Roche Applied Science, Indianapolis, IN), 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 Research Product, Princeton, NJ) and rProtein G agarose beads (Invitrogen) at 4°C 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-phospho-Ser^{375} of OPRM1 antibody (OPRM1phosphoSer^{375}; Cell Signaling Technology, Danvers, MA) and were normalized to the total immunoprecipitated receptors.

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, and 3.5 mM $MgCl_2$, pH 7.0) containing 0.1 mM phenylmethylsulfonyl fluoride, 40 μg/ml Complete protease inhibitor mixture. 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, and 0.05% SDS with protease inhibitors). Approximately equal amount of proteins was resolved by SDS-polyacrylamide gel electrophoresis 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

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described previously (Macdonald and Pike, 2005; Morris et al., 2008). In brief, 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 4°C 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 $MgCl_2$. After 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 Activation. Cells in six-well plates were treated with 1 μM morphine for 10 min, the medium was then aspirated, and cells were washed with phosphate-buffered saline 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). After centrifugation, the supernatant was transferred to a new tube, and SDS-polyacrylamide gel electrophoresis sample buffer was added. Approximately the 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 Technology) and was normalized to total ERKs detected by total ERK antibodies (Cell Signaling Technology).

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. 1, A and B). This inhibition was reversed by incubating with cholesterol after the MβCD treatment (Fig. 1B). The inhibition of receptor internalization was also manifested by treatment of cells with 5 μM simvastatin overnight (Supplemental Fig. 1A), which could lower the cholesterol level by blocking its synthesis. Likewise, 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. 1, C and D). The cellular cholesterol contents were demonstrated to be reduced by MβCD or simvastatin treatment, and the reduction was reversed by cholesterol replen-

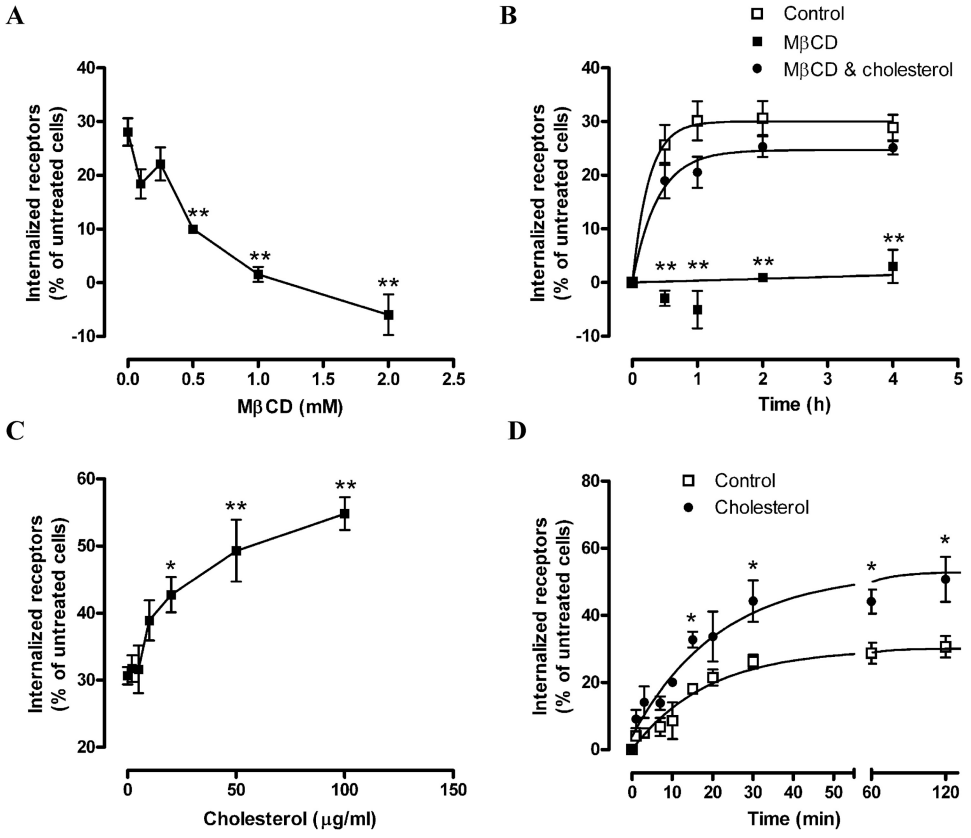

Fig. 1. Cholesterol reduction by MβCD decreased DAMGO-induced internalization of OPRM1, whereas excess cholesterol increased the internalization of OPRM1. Internalization of OPRM1 was quantified as the percentage loss of cell surface receptors in agonist-treated cells as described under *Materials and Methods*. A, N2A-OPRM1 cells were treated with different concentrations of MβCD for 3 h, then 1 μM DAMGO was added for further incubation for 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 and cholesterol), then cells were further incubated with 1 μM DAMGO for 0.5, 1, 2, and 4 h. C, N2A-OPRM1 cells were treated with different concentrations of cholesterol for 1 h, and 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 1, 3, 7, 10, 15, 20, 30, 60, and 120 min. 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 and D).

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ishment, 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. 2, A and B). 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; Supplemental Fig. 1B). Increasing the cellular cholesterol content by incubating the cells with 50 μg/ml cholesterol accelerated receptor desensitization (Fig. 2C). These data demonstrated that manipulation of cellular cholesterol level had similar effects on internalization and 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 the DAMGO potency and vice versa. 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-Arr2-OPRM1 Interaction. Because 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. 3, A and B, 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. 3, A and B). Without significant effect on receptor phosphorylation, the observed effects of cholesterol reduction on OPRM1 internalization could be a consequence of altered βArr-receptor interaction. When βArr2-GFP was overexpressed 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 imply 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. 5, A and B, 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

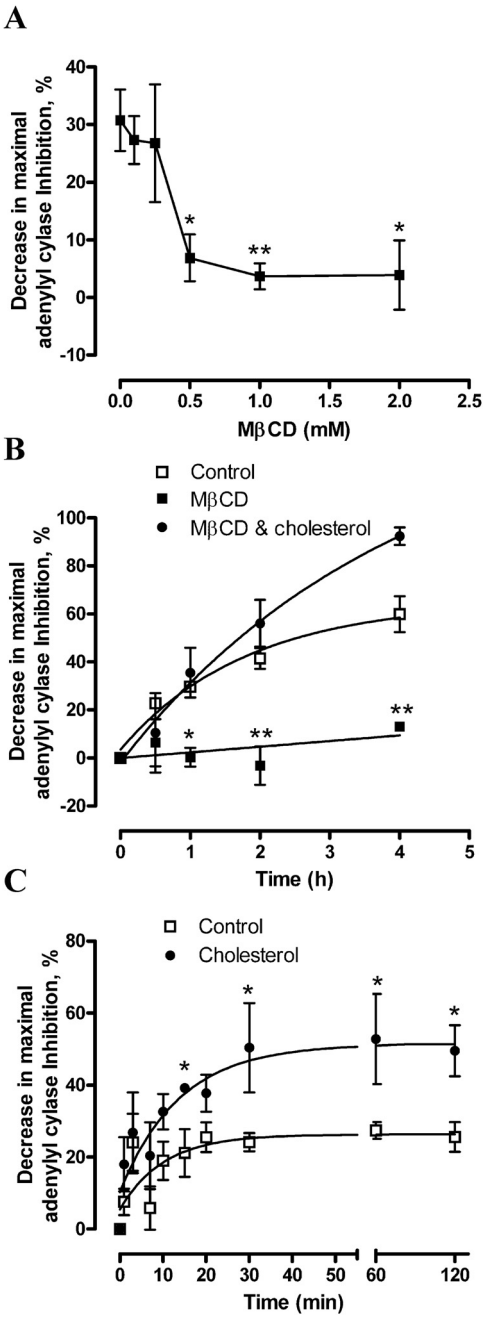

Fig. 2. Cholesterol reduction by MβCD decreased DAMGO-induced desensitization of OPRM1, whereas excess cholesterol increased the desensitization of OPRM1. Desensitization of OPRM1 was calculated as the percentage loss of the ability of 1 μM DAMGO to inhibit forskolin-stimulated intracellular cAMP production in agonist-treated cells as described under *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 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 and cholesterol); cells were then further incubated with 1 μM DAMGO for 0.5, 1, 2, and 4 h. 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 1, 3, 7, 10, 15, 20, 30, 60, and 120 min. 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 and C).

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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- versus 2.0 ± 0.24-fold over basal 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. Cholesterol, which has a high affinity for saturated lipids, is usually assumed to be the driving force of lipid raft domain formation in living cell membranes (Gómez 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 nonraft domains were separated as demonstrated by the maximal $G\alpha_q$ immunoreactivities (a lipid raft marker) in fractions 1 and 2 and the maximal transferrin receptor immunoreactivities (TR, a nonraft marker) in fractions 3 and 4 (Fig. 6A, left). Cytosolic proteins were the main 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. 6, A, left, and B), which is consistent with previous reports (Huang et al., 2007; Zheng et al., 2008a). A significant amount of OPRM1 translocated to nonraft fractions after 10 min of 1 μM DAMGO treatment (Fig. 6, A, left, and B). Cholesterol reduction by MβCD disrupted the lipid raft domains, shifting both $G\alpha_q$ and OPRM1 out of fractions 1 and 2 (Fig. 6, A, middle, and B). Treatment of DAMGO did not further shift the OPRM1 out of these fractions (Fig. 6, A, middle, and B). Increase in cholesterol level resulted in more $G\alpha_q$ 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). These data suggest that cholesterol could modify lipid raft domains. Excess cholesterol increased the distribution of OPRM1 to lipid raft fractions (Fig. 6, A, right, and B). By retaining more OPRM1 in raft domains, fewer receptors moved to nonraft domains after DAMGO treatment (43 ± 4.5 versus 50 ± 1.6% of control; Fig. 6, A, right, and B). 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. 6, A, right, and C). In cells without cholesterol manipulation, the distribution of βArr2 to membrane raft domains was increased minimally (Fig. 6, A, left, and C). The distribution of βArr2 in raft and nonraft fractions was unaltered by agonist activation in the presence of MβCD (Fig. 6, A, middle, and C). 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.

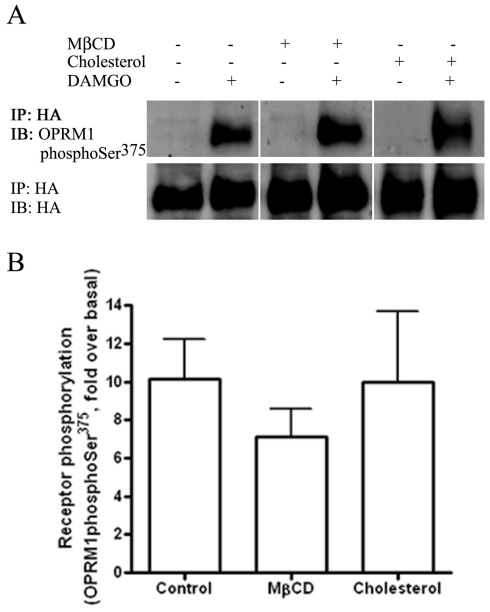

Fig. 3. Cholesterol reduction or incubating with cholesterol did not significantly affect receptor phosphorylation. N2A-OPRM1 cells were treated with 1 mM MβCD for 3 h or 10 μg/ml cholesterol for 1 h, then the cells were further incubated with 1 μM DAMGO for 10 min. Receptors were immunoprecipitated (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.

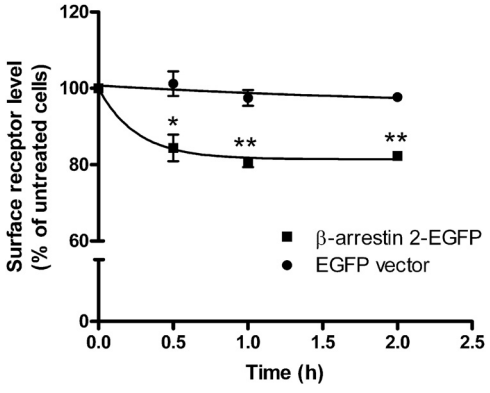

Fig. 4. Overexpression of βArr2 restored the agonist-induced internalization of OPRM1 blocked by MβCD. N2A-OPRM1 cells were transiently transfected with βArr2-GFP or pEGFP-N1 vector; 48 h later, cells were treated with 1 mM MβCD for 3 h and 1 μM DAMGO for 0.5, 1, and 2 h. The internalized receptors were detected and quantified as described under *Materials and Methods*. Data are mean ± S.E. of at least three independent experiments performed at least in duplicate. *, $P < 0.05$; **, $P < 0.01$ versus matched internalized receptors in cells expressing GFP.

Excess Cholesterol Evokes the Morphine-Induced Internalization of OPRM1 and Protein Kinase C-Independent ERK 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, incubating the N2A-OPRM1 cells with

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cholesterol in a concentration-dependent manner promoted morphine-induced OPRM1 internalization, suggesting that cholesterol facilitates the interaction between βArr and the activated receptors.

OPRM1-mediated ERK 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 a blockade of ERK activation using a general PKC inhibitor, Ro-31-8425 (Fig. 7B). However, when the N2A-OPRM1 cells were preincubated with cholesterol, morphine could still activate ERKs in the presence of Ro-31-8425 (Fig. 7B). Such observations imply that βArr-dependent signaling occurs for morphine in the presence of excess cholesterol.

Discussion

In the 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 indicate that the rates of receptor internalization and desensitization are 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 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 used the method developed by 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 (2005). The raft marker $G\alpha_q$ was much more enriched in the two fractions with the lightest densities (fractions 1 and 2), whereas nonraft marker TR was concentrated in the middle two fractions (fractions 3 and 4). Our data also showed that 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 $G\alpha_q$ distribution from the lightest density fractions to the middle fractions, whereas excess cholesterol resulted in more $G\alpha_q$ distribution into the lightest density fractions 1 and 2, especially fraction 1. Because 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 $G\alpha_q$ 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 cells with excess cholesterol. At the same time, excess cholesterol increased the

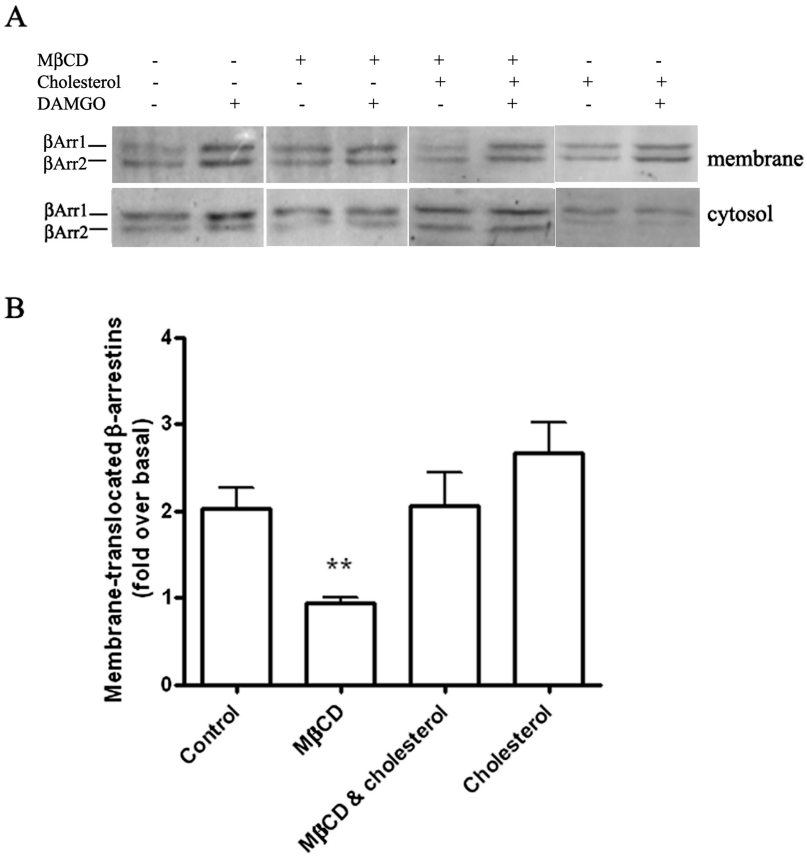

Fig. 5. Cholesterol reduction attenuates membrane translocation of βArr. N2A-OPRM1 cells were treated with 1 mM MβCD for 3 h or 1 mM MβCD for 3 h and then 10 μg/ml cholesterol for 1 h or 50 μg/ml cholesterol for 1 h, then cells were further incubated with 1 μM DAMGO for 10 min. βArr translocated to cell 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.

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amount of OPRM1 distributed in fractions 1 and 2 and reduced the agonist-induced OPRM1 translocation out of raft domains. The increase of OPRM1 in lipid rafts under the high cholesterol content probably facilitates the receptor-induced recruitment and translocation of βArr to raft domains. The inability to detect the βArr translocation in nontreated cells in the current study may be due to the fast translocation of OPRM1 to nonraft domains (Zheng et al., 2008a). Morphine, a poor inducer for βArr translocation, does not induce receptor internalization and activates ERKs through a 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 in the receptor's efficient recruitment of 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 (Stoddart et al., 2002; Puri et al., 2005). Second, the clathrin-dependent internalization of G protein-coupled cholecystokinin receptor can be inhibited by raft disruption (Harikumar et al., 2005). Third, the 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 nonraft microdomains after light activation (Nair et al., 2002). Therefore, the compartmentation of receptors in lipid rafts could be crucial for βArr recruitment. The direct interaction between the palmitate covalently attached to the C3.55(170) residue of OPRM1 and cholesterol has been demonstrated (H. Zheng, E. B. Poole, D. P. Hurst, Y. Zhang, J. Chu, P. H. Reggio, H. H. Loh, and P.-Y. Law, 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

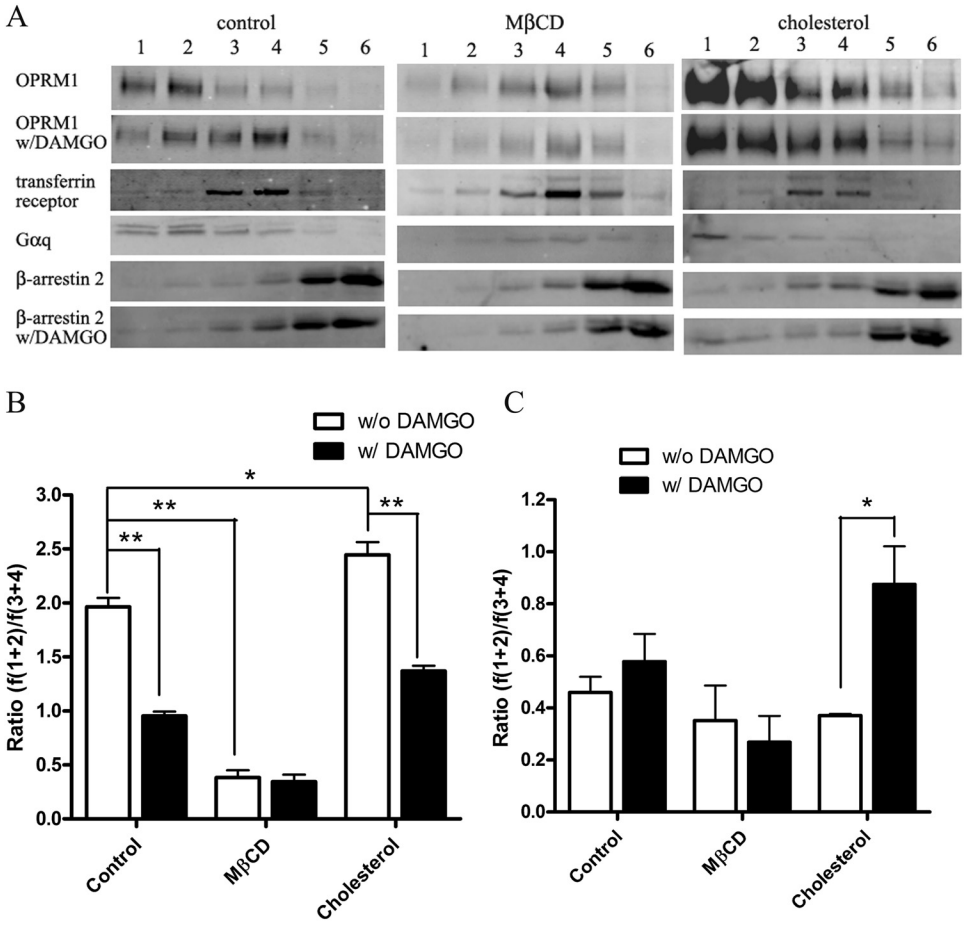

Fig. 6. Cholesterol reduction or incubating cells with cholesterol affected OPRM1 localization and the recruitment of βArr2 to membrane domains. A, distribution of OPRM1, TR, $G\alpha_q$, and βArr2 after MβCD or cholesterol treatment. N2A-OPRM1 cells were treated with 1 mM MβCD for 3 h or 50 μg/ml cholesterol 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. *, $P < 0.05$; **, $P < 0.01$.

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thereby 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. Because pathway-selective signaling determines the eventual cellular locations of the activated ERKs and the transcripts being regulated (Zheng et al., 2008b), the switching of the pathway selected in ERK activation under high cholesterol level could have implication in the eventual adaptational processes upon agonist exposure.

Taken together, the current study and others performed in our laboratory delineate the movement of receptor in membrane microdomains. Furthermore, 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 of modifying receptor trafficking and hence overall signaling of OPRM1—the molecular basis for opioid tolerance (Waldhoer et al., 2004)—by manipulating cholesterol level.

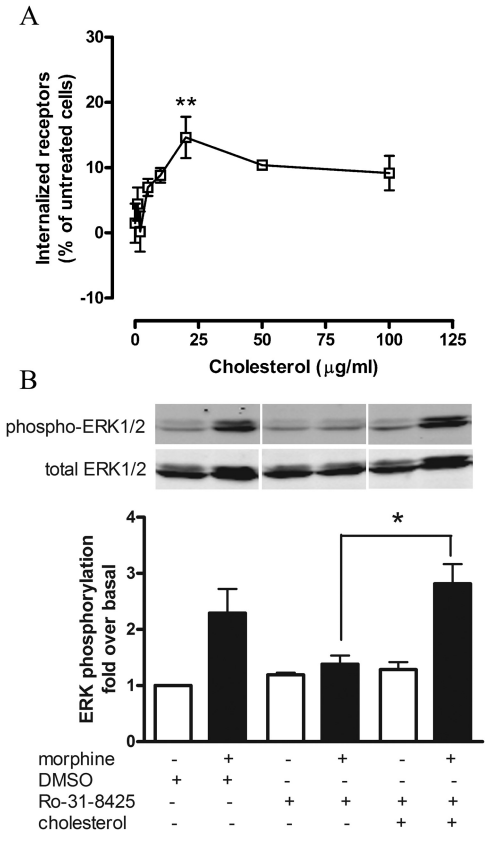

Fig. 7. Incubating cells with cholesterol evoked the morphine-induced internalization of OPRM1 and PKC-independent ERK activation. A, N2A-OPRM1 cells were treated with different concentration of cholesterol for 1 h, then 1 μM morphine was added for further incubation of 1 h. Data are mean ± S.E. of at 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.

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Authorship Contributions

Participated in research design: Qiu, Law, Chen, and Loh.
Conducted experiments: Qiu and Wang.
Performed data analysis: Qiu and Law.
Wrote or contributed to the writing of the manuscript: Qiu, Law, and Loh.

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