

Cholesterol Regulates Volume-Sensitive Osmolyte Efflux from Human SH-SY5Y Neuroblastoma Cells following Receptor Activation

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ABSTRACT

The ability of cholesterol to modulate receptor-mediated increases in the volume-dependent release of the organic osmolyte, taurine, has been examined. Depletion of cholesterol from SH-SY5Y neuroblastoma by preincubation of the cells with 5 mM methyl- β -cyclodextrin (CD) for 10 min resulted in a 40 to 50% reduction in cholesterol and an enhancement of the ability of proteinase-activated receptor (PAR) 1, muscarinic cholinergic receptor (mAChR), and sphingosine 1-phosphate receptor to stimulate taurine efflux, when monitored under hypoosmotic conditions. Basal (swelling-induced) release of taurine was also enhanced by cholesterol depletion, but less markedly. Both basal- and receptor-mediated increases in taurine efflux were mediated via a volume-sensitive organic osmolyte and anion channel in control and cholesterol-depleted cells. Studies with the PAR-1

and mAChR receptor subtypes indicated that the stimulatory effect of CD pretreatment could be reversed by incubation of the cells with either CD/cholesterol or CD/5-cholesten-3 α -ol donor complexes and that cholesterol depletion increased agonist efficacy, but not potency. The ability of cholesterol depletion to promote the PAR-1 receptor-mediated stimulation of osmolyte release was most pronounced under conditions of isotonicity or mild hypotonicity. In contrast to CD pretreatment, preincubation of the cells with cholesterol oxidase, a condition under which lipid microdomains are also disrupted, had no effect on either basal- or receptor-stimulated taurine efflux. Taken together, the results suggest that cholesterol regulates receptor-mediated osmolyte release via its effects on the biophysical properties of the plasma membrane, rather than its presence in lipid microdomains.

Volume regulation is a fundamental homeostatic mechanism used by cells and is of particular importance to the central nervous system because of restrictions of the skull. Even minor increases in brain volume can have adverse effects on cell-cell signaling events and more severe brain swelling, as may occur during episodes of hyponatremia, can lead to cerebral anoxia, ischemia, and ultimately death due to cardiac and respiratory arrest (Pasantes-Morales et al., 2000). In response to hypotonic stress, both neural and non-neural cells regulate their volume through the efflux of K⁺, Cl⁻, and "compatible" organic osmolytes such as taurine, glutamate, or myo-inositol. Loss of these osmolytes results in the exit of obligated water, thereby restoring cell volume, a process known as regulatory volume decrease. Organic os-

molutes and Cl⁻ are released from cells via a volume-sensitive organic osmolyte and anion channel (VSOAC), a channel that has been extensively characterized both electrophysiologically and pharmacologically, although its molecular structure remains unknown (Lang et al., 1998; Nilius and Droogmans, 2003).

Given the central role that maintenance of volume plays in the physiology of neural cells, mechanisms involved in its regulation assume major significance. In this context, we and others (Mongin and Kimelberg, 2002, 2005; Heacock et al., 2004, 2006; Cheema et al., 2005, 2007; Ramos-Mandujano et al., 2007) have recently demonstrated that the volume-dependent release of osmolytes from a variety of neural cells can be dramatically enhanced following activation of certain G-protein-coupled receptors. Receptor activation not only increases the extent of osmolyte release, but it also lowers the threshold osmolarity ("set-point") at which osmolytes are released, thereby permitting cells to respond to small, physiologically relevant, reductions in osmolarity. This observation

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ABBREVIATIONS: VSOAC, volume-sensitive organic osmolyte and anion channel; PAR, proteinase-activated receptor; mAChR, muscarinic cholinergic receptor; S1P, sphingosine 1-phosphate; CD, methyl- β -cyclodextrin; epicholesterol, 5-cholesten-3 α -ol; DCPIB, 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid; DMEM, Dulbecco's modified Eagle's medium; ANOVA, analysis of variance; IP, inositol phosphate.

raises the possibility that, in vivo, receptor activation may be a prerequisite for the efficient volume regulation of cells. Although volume-dependent osmolyte release from cells is mediated via a VSOAC under both basal (swelling-induced)- and receptor-stimulated conditions (Abdullaev et al., 2006; Cheema et al., 2007), separate mechanisms underlie these two responses, as is evident from their differential sensitivities to Ca^{2+} availability and activation of protein kinase C (Mongin and Kimelberg, 2005; Heacock et al., 2006).

Cholesterol availability represents an additional means whereby the activity of VSOAC may be regulated. In some non-neural cells, depletion of cholesterol facilitates the activity of VSOAC in response to hypotonicity, an effect that is reversed by increases in the cholesterol content of the cells (Levitan et al., 2000; Romanenko et al., 2004; Klausen et al., 2006; Byfield et al., 2006; Lim et al., 2006). As previously observed for receptor activation, cholesterol depletion is reported to optimally facilitate basal VSOAC activity under conditions of limited reductions in osmolarity. However, whether cholesterol availability can further regulate receptor-stimulated osmolyte release under physiologically relevant conditions has yet to be addressed. In addition, the mechanism(s) underlying cholesterol regulation of basal VSOAC activity has not been established. In endothelial cells, cholesterol dependence of VSOAC activity has been attributed to changes in membrane fluidity and/or a negative regulation of VSOAC by segregation of the channel into membrane lipid domains such as rafts or caveolae (Romanenko et al., 2004; Byfield et al., 2006). In contrast, in other cell types, VSOAC activity appears to be dependent upon the integrity of caveolae (Trouet et al., 1999, 2001; Ullrich et al., 2006). Since multiple receptor subtypes and associated signaling proteins have been localized to caveolae and/or lipid rafts (Allen et al., 2007), it is conceivable that cholesterol availability may differentially influence swelling-induced and receptor-activated VSOAC activity.

In the present study, we have addressed the possibility that cholesterol availability can regulate receptor-mediated VSOAC activity by monitoring the effects of cholesterol depletion and readdition on volume-dependent taurine release from human SH-SY5Y neuroblastoma cells. We have previously demonstrated that these cells possess several pharmacologically distinct receptors that couple to osmolyte efflux, including proteinase-activated receptor (PAR) 1, muscarinic cholinergic receptor (mAChR), and sphingosine 1-phosphate (S1P; Heacock et al., 2004, 2006; Cheema et al., 2007). The results indicate that cholesterol depletion, induced by methyl- β -cyclodextrin (CD), synergistically enhances the ability of all three receptors to increase taurine efflux above basal levels and that this potentiation can be reversed by administration of cholesterol or its stereoisomer, 5-cholesten-3 α -ol (epicholesterol). Cholesterol depletion appears to exert its most significant regulatory influence on receptor-stimulated taurine efflux under conditions of isotonicity or mild hypotonicity. Thus, in SH-SY5Y neuroblastoma cells, both cholesterol availability and receptor activation may act in concert to enable the cells to respond to small changes in osmolarity.

Materials and Methods

Materials. [1,2- ^3H]Taurine (1.15 TBq/ml) was obtained from Amersham Biosciences (Piscataway, NJ). Thrombin, oxotremorine-M,

sphingosine 1-phosphate, methyl- β -cyclodextrin, trypan blue, cholesterol (5-cholesten-3 β -ol), and cholesterol oxidase were purchased from Sigma-Aldrich (St. Louis, MO). DCPIB was obtained from Tocris Biosciences (Ellisville, MO). Epicholesterol was purchased from Steraloids (Newport, RI). Amplex Red Cholesterol Assay kit was from Molecular Probes, Inc. (Eugene, OR). Dulbecco's modified Eagle's medium (DMEM) and 50 \times penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum was obtained from Cambrex Bio Science, Inc. (Walkersville, MD). Tissue culture supplies were obtained from Corning Glassworks (Corning, NY), Starstedt (Newton, NC), and BD BioSciences (Franklin Lakes, NJ). Universol was obtained from Valeant Pharmaceuticals (Costa Mesa, CA).

Cell Culture Conditions. Human SH-SY5Y neuroblastoma cells (passages 70–89) were grown in tissue culture flasks (75 cm²/250 ml) in 20 ml of DMEM supplemented with 10% (v/v) of fetal bovine serum with 1% penicillin/streptomycin. The osmolarity of the medium was 330 to 340 mOsm. Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂. The medium was aspirated, and the cells were detached from the flask with a trypsin-versene mixture (Cambrex Bio Science, Inc., Walkersville, MD). Cells were then resuspended in DMEM/10% fetal bovine serum with penicillin/streptomycin and subcultured into 35-mm, six-well culture plates for 5 to 6 days. Experiments were routinely conducted on cells that had reached 70 to 90% confluency.

Measurement of Taurine Efflux. Osmolyte efflux from SH-SY5Y neuroblastoma cells was monitored essentially as previously described (Heacock et al., 2004; Cheema et al., 2007). In brief, cells were prelabeled to isotopic equilibrium with 18.5 kBq/ml [^3H]taurine at 37°C for 24 h. After prelabeling, the cells were washed twice with 2 ml of isotonic buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 3.6 mM NaHCO₃, 1 mM MgCl₂, and 30 mM HEPES, pH 7.4, 1 mg/ml D-glucose; approximately 340 mOsm). Cells were then allowed to incubate in 2 ml of hypotonic buffer A (295–200 mOsm; rendered hypotonic by a reduction in NaCl concentration) in the absence or presence of the agonists. In some experiments, buffer A was made hypertonic (370 and 450 mOsm) by the addition of NaCl. Osmolarities of buffer A were monitored by means of an Osmette precision osmometer (PS Precision Systems, Sudbury, MA). At times indicated, aliquots (200–400 μl) of the extracellular medium were removed and radioactivity determined after the addition of 5 ml of Universol scintillation fluid. The reactions were terminated by rapid aspiration of the buffer, and cells were lysed by the addition of 2 ml of ice-cold 6% (w/v) trichloroacetic acid. Taurine efflux was calculated as a fractional release, i.e., the radioactivity released in the extracellular media as a percentage of the total radioactivity present initially in the cells. The latter was calculated as the sum of radioactivity recovered in the extracellular medium and that remaining in the lysate at the end of the assay. Because SH-SY5Y cells had been labeled to isotopic equilibrium with [^3H]taurine, measurement of radioactivity in the extracellular medium reflects changes in the mass of the osmolyte. Throughout this study, "basal" release of taurine is defined as that which occurs at a specified osmolarity in the absence of an agonist.

Modulation of Cellular Cholesterol Content and Substitution of Cholesterol with Epicholesterol. SH-SY5Y neuroblastoma were either depleted of cholesterol by incubation of the cells for 10 min at 37 °C with 5 mM CD alone or alternatively enriched with cholesterol by CD complexed with cholesterol dissolved in serum-free media for 1 h at 37°C. Cholesterol was complexed with CD as described previously (Romanenko et al., 2004). Briefly, a small amount of cholesterol was dissolved in chloroform/methanol (1:1, by volume) in a glass tube, and the solvent was evaporated. Then, a 5 mM CD solution in serum-free DMEM was added to the dried cholesterol. The tube was then vortexed, sonicated, and incubated overnight in a shaking bath at 37°C. CD was complexed with cholesterol at a saturating ratio of 1:8 (0.625 mM cholesterol; Christian et al., 1997). In preparation for an experiment, cells prelabeled with [^3H]taurine

were first depleted of cholesterol by exposing them to CD for 10 min in a humidified CO₂ incubator at 37°C. Cells were then washed with isotonic buffer A and exposed to a cholesterol/CD (1:8) donor complex for 1 h and then returned to the incubator. After 1 h, cells were washed once more with isotonic buffer A before measurement of taurine efflux. Control cells were treated in a similar way with serum-free DMEM. An epicholesterol/CD (1:8) complex was prepared as described for the cholesterol/CD complex above (Romanenko et al., 2002). The effects of CD on cell viability were routinely assessed by means of monitoring the ability of cells to exclude the dye, trypan blue.

Cellular Cholesterol Content. The Amplex Red-based cholesterol assay was conducted in a 96-well microplate using a 100- μ l reaction volume per well. Cells were lysed with 1 \times reaction buffer containing 0.1 mM potassium phosphate, pH 7.4, 0.05 mM NaCl, 5 mM cholic acid, and 0.1% Triton X-100. The cells were then sonicated for 20 s before 10- μ l aliquots of the lysates were diluted into 40 μ l of 1 \times reaction buffer. The cholesterol detection assay was initiated by the addition of 50 μ l/well of 300 μ M Amplex Red Reagent working solution containing 2 U/ml horseradish peroxidase, 2 U/ml cholesterol oxidase, and 0.2 U/ml cholesterol esterase. The reaction mixtures were incubated at 37°C for 30 min, and the fluorescence intensities were measured using a fluorescence microplate reader equipped with a filter set for excitation and emission at 560 \pm 10 and 590 \pm 10 nm, respectively (FLUOstar Optima BMG LabTech, Durham, NC). The cholesterol values were compared with a cholesterol standard curve from (0–8 μ g/ml). Protein assay was performed with a bicinchoninic acid protein assay reagent kit obtained from Pierce (Rockford, IL). Cholesterol values quoted are normalized to the protein content of the cell lysates.

Measurement of Phosphoinositide Turnover. To monitor phosphoinositide turnover, SH-SY5Y cells that had been prelabeled with 111 KBq/ml [³H]inositol for 48 h were incubated in hypotonic buffer A (230 mOsM) that contained 5 mM LiCl. The accumulation of radiolabeled inositol phosphates present in the trichloroacetic acid cell lysates was determined as previously described (Thompson and Fisher, 1990).

Data Analysis. Experiments were performed in triplicate and repeated at least three times. Values quoted are given as means \pm S.E.M. for the number (*n*) of independent experiments indicated. A two-tailed Student's *t* test (paired or unpaired) was used to evaluate differences between two experimental groups (level of significance, *p* < 0.05). Ordinary or repeated measures analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used for statistical significance of differences between multiple groups. EC₅₀ values were obtained using Prism 4.0a (GraphPad Software Inc., San Diego, CA).

Results

Cholesterol Depletion Enhances Both Basal- and Thrombin-Stimulated Taurine Efflux. CD, a water-soluble cyclic oligosaccharide, provides an effective and reproducible method for extracting cholesterol from a variety of cells, thereby disrupting lipid microdomains (Christian et al., 1997; Brown and London, 2000). However, since this approach has not previously been employed for SH-SY5Y cells, initial studies were directed at determining the optimal conditions for cholesterol extraction while limiting the exposure of cells to CD, as recommended by Zidovetzki and Levitan (2007). When SH-SY5Y cells were pretreated with increasing concentrations of CD (1–5 mM) for 10 min at 37°C, a dose-dependent decrease in total cholesterol content was observed (Fig. 1A). Measurement of cell viability by means of trypan blue exclusion indicated that cells retained their integrity under these conditions. More prolonged incubation of the

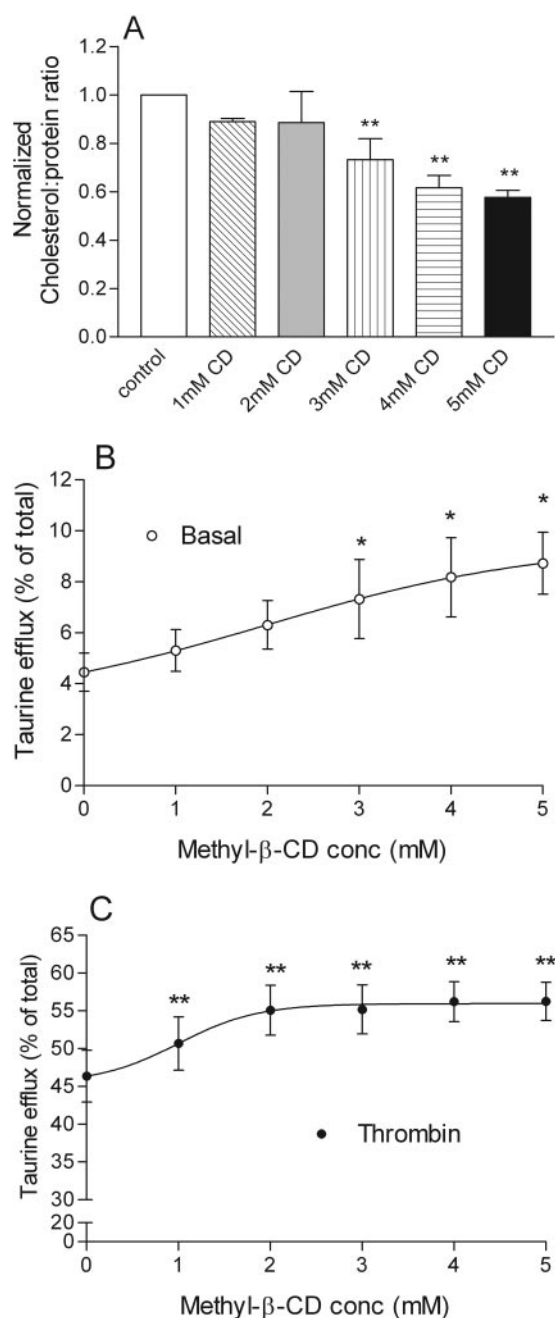


Fig. 1. Cholesterol depletion enhances both basal- and thrombin-stimulated taurine efflux. A, SH-SY5Y neuroblastoma cells were pretreated for 10 min at 37°C with either serum-free media (control) or with increasing concentrations of CD (1–5 mM) dissolved in serum-free media. The cells were then washed twice with 2 ml of isotonic buffer A, and cholesterol content was measured by the Amplex Red assay (see *Materials and Methods*). Cell viability following CD pretreatment was routinely assessed by trypan blue exclusion. Cholesterol values, calculated as micrograms per milligram of protein, have been normalized to the value of cholesterol in control cells (1.0) to account for interexperimental variation. **, different from control, *p* < 0.01 (by ordinary ANOVA followed by Dunnett's multiple comparisons test). B and C, cells prelabeled with [³H]taurine were treated under similar conditions as in (A) and then incubated in 230 mOsM buffer A in the absence (Basal) or presence of 1.25 nM thrombin. Reactions were terminated after 5 min, and release of taurine was monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means \pm S.E.M. for three to seven independent experiments. *, different from basal control, *p* < 0.05; **, different from thrombin control, *p* < 0.01 (by repeated measures ANOVA followed by Dunnett's multiple comparisons test).

cells with CD resulted in cell rounding and a loss of morphology. In SH-SY5Y cells, total cellular cholesterol (predominantly nonesterified) was $17.04 \pm 3.0 \mu\text{g}/\text{mg}$ protein ($n = 10$). A maximum reduction in cholesterol content (43%) was observed following pretreatment of the cells with 5 mM CD for 10 min. Both basal (swelling-activated) and thrombin-stimulated taurine efflux monitored under hypotonic conditions (230 mOsM) were significantly enhanced by depletion of cholesterol. A significant effect on basal taurine release was observed at 3 mM CD with a maximum effect observed at a 5 mM concentration. Under the latter conditions, efflux increased from 4.5 to 8.7% of the total taurine pool, a net increase of 4.2%; Fig. 1B). CD pretreatment also significantly potentiated thrombin-stimulated taurine release at all concentrations of CD tested (1–5 mM) with a maximum observed at CD concentrations >2 mM. Following pretreatment of the cells with 5 mM CD, taurine efflux increased from 46 to 56% of the total taurine pool, a net increase in the efflux of 10% (Fig. 1C). In subsequent experiments, a 10-min pretreatment with 5 mM CD was routinely utilized so that changes in both basal- and receptor-stimulated taurine efflux could be readily evaluated.

Time Course of Volume-Dependent Taurine Efflux following Cholesterol Depletion. When SH-SY5Y cells that had been prelabeled with [^3H]taurine were exposed to hypotonic buffer A (230 mOsM; $\sim 30\%$ reduction in osmolarity), there was a time-dependent release of the radiolabeled amino acid from the cells, as previously reported (Cheema et al., 2007). This basal release was enhanced by ~ 2 -fold (2.8–7.0% of the total taurine pool) when the cells were pretreated with 5 mM CD for 10 min at 37°C (Fig. 2). Pretreatment of cells with CD also significantly enhanced the ability of thrombin (0.25U/ml, equivalent to 1.25 nM) to facilitate tau-

rine efflux at all time points examined (Fig. 2). Since the CD-mediated increases in the magnitude of both basal- and receptor-stimulated release of taurine were maximal at 5 min of incubation or thereafter, osmolyte efflux was subsequently routinely monitored following a 5-min incubation.

Cholesterol Depletion Facilitates Taurine Efflux Elicited by Activation of Multiple Receptors. We have previously demonstrated that SH-SY5Y cells express mAChR, PAR-1, and S1P receptors, the activation of which significantly increase taurine release during hypotonic stress (Loveday et al., 2003; Heacock et al., 2006; Cheema et al., 2007). To determine whether the ability of cholesterol depletion to enhance taurine release is receptor-specific, the effect of CD pretreatment on the ability of all three receptors to stimulate taurine efflux was evaluated. It was observed that CD pretreatment facilitated the ability of thrombin, Oxo-M, and S1P to enhance the volume-dependent release of taurine (net increases in efflux of 13.4, 14.8, and 19.9% of the total taurine pool, respectively). In contrast, the corresponding value for the net increase in basal release of taurine observed following cholesterol depletion was 5.6% (Fig. 3). Moreover, the increase in receptor-mediated efflux of taurine from cholesterol-depleted cells was greater than that due to the increase in basal release alone, i.e., a synergistic, rather than additive, enhancement of receptor-mediated osmolyte efflux was observed for all three receptors ($p < 0.01$; paired Student's t test). Because previous studies of SH-SY5Y cells have extensively characterized the coupling of PAR-1 and mAChRs to volume-dependent taurine efflux (Heacock et al., 2004, 2006; Cheema et al., 2007), in the current study, emphasis has been placed on monitoring the effects of cholesterol depletion and repletion on the activity of these receptor subtypes. In addition, the effects (if any) of CD on signal transduction events can readily be assessed by monitoring mAChR-stimulated phosphoinositide turnover in these cells.

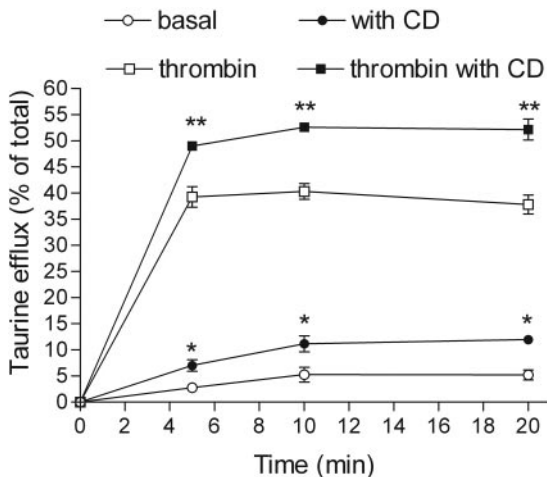


Fig. 2. Time course of volume-dependent taurine efflux after cholesterol depletion. SH-SY5Y neuroblastoma cells that had been prelabeled with [^3H]taurine were pretreated for 10 min at 37°C with serum-free media or 5 mM CD dissolved in serum-free media. The cells were then washed twice with 2 ml of isotonic buffer A and then incubated in 230 mOsM buffer A in the presence or absence of 1.25 nM thrombin. Reactions were terminated at the times indicated, and taurine release was measured. Results are expressed as basal taurine efflux (—○—, without CD; —●—, with CD) or thrombin-stimulated taurine efflux (—□—, without CD; —■—, with CD) (percentage of total soluble radioactivity) and are the means \pm S.E.M. for three independent experiments performed in triplicate. *, different from basal efflux without CD, $p < 0.05$; **, different from thrombin-stimulated efflux without CD, $p < 0.05$ (by repeated measures ANOVA followed by Dunnett's multiple comparisons test).

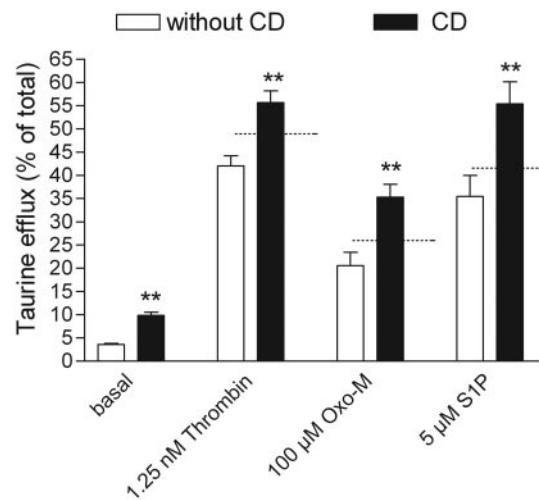


Fig. 3. Cholesterol depletion facilitates taurine efflux elicited by the activation of multiple receptors. Cells prelabeled with [^3H]taurine were pretreated for 10 min at 37°C with either serum-free media or 5 mM CD. The cells were then washed twice with 2 ml of isotonic buffer A and incubated in 230 mOsM buffer A for 5 min in the presence or absence of the receptor agonists at the concentrations indicated. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means \pm S.E.M. for three to four independent experiments. The dotted lines indicate additive effects on taurine efflux. * and **, different from without CD, $p < 0.05$; $p < 0.01$ (by paired Student's t test and ordinary ANOVA followed by Dunnett's multiple comparisons test).

CD Pretreatment Increases Agonist Efficacy for Taurine Efflux but Not Agonist Potency. To determine whether cholesterol depletion enhances agonist potency and/or efficacy, dose-response curves for taurine efflux were constructed for thrombin and Oxo-M, for both control cells and those pretreated with CD. Cholesterol depletion increased agonist efficacy over the range of concentrations of thrombin or Oxo-M tested. However, agonist potency was unaffected by CD pretreatment. Thus, EC_{50} values were 105 and 60 pM for thrombin and 0.9 and 1.9 μ M for Oxo-M in the absence or presence of CD pretreatment, respectively (Fig. 4, A and B).

Blockade of VSOAC with DCPIB Attenuates the CD-Induced Facilitation of Taurine Release. DCPIB, an agent that is considered highly selective for VSOAC (Decher et al., 2001; Best et al., 2004; Abdullaev et al., 2006), was

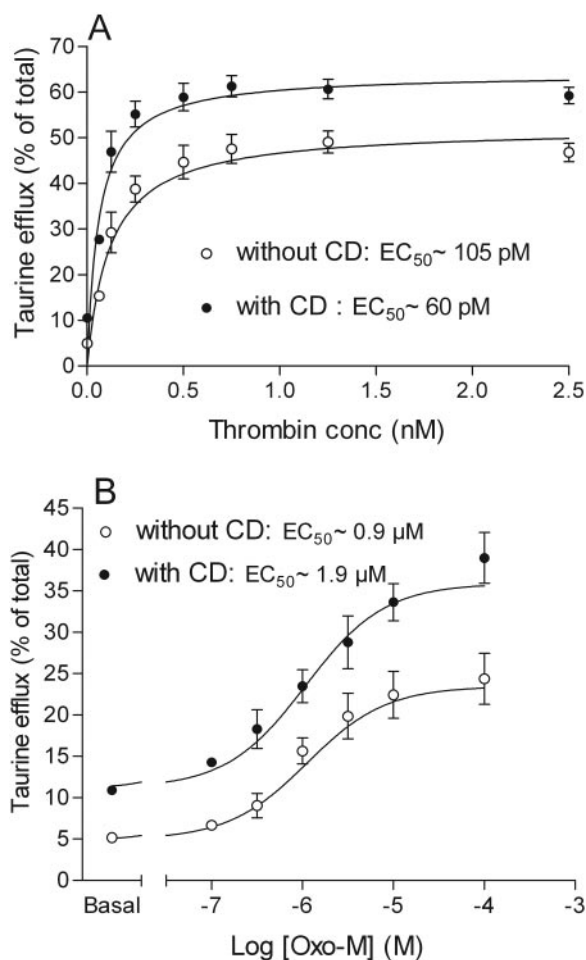


Fig. 4. CD pretreatment increase agonist efficacy for taurine efflux but not agonist potency. Cells prelabeled with [3 H]taurine were pretreated for 10 min at 37°C with either serum-free media or 5 mM CD. The cells were then washed twice with 2 ml of isotonic buffer A and incubated in 230 mOsM buffer A in the presence or absence of thrombin (A) or Oxo-M (B) at the concentrations indicated. Reactions were terminated after 5 min, and taurine efflux was monitored. Results are expressed as percentage of total soluble radioactivity released and are the means \pm S.E.M. for three to four independent experiments. Where error bars are not shown, the values fell within the symbol. The calculated EC_{50} values for stimulated taurine efflux for thrombin were 105 and 60 pM (in the absence and presence of CD, respectively) and 0.9 and 1.9 μ M for Oxo-M (in the absence and presence of CD, respectively). At all concentrations of either thrombin or Oxo-M, taurine release was greater for the CD-pretreated cells than for control cells [$p < 0.05$; by ordinary (thrombin) or repeated measures (Oxo-M) ANOVA followed by Dunnett's multiple comparisons test].

examined for its ability to inhibit the CD-induced increases in taurine efflux. Inclusion of 20 μ M DCPIB significantly inhibited basal taurine efflux monitored under both control and CD-pretreated conditions (56 and 67% inhibition, respectively). Similarly, DCPIB also inhibited thrombin-stimulated taurine efflux monitored in either control or CD-pretreated cells (94 and 76% inhibition, respectively; Fig. 5).

Osmolarity Dependence of Basal- and Thrombin-Stimulated Taurine Efflux under Conditions of Cholesterol Depletion. Previously, we have demonstrated that receptor-mediated enhancement of osmolyte release is dependent on the degree of hypo-osmotic stress in SH-SY5Y cells (Heacock et al., 2004, 2006; Cheema et al., 2007). Thus, the ability of cholesterol depletion to potentiate taurine release at different osmolarities was examined. SH-SY5Y cells were pretreated with 5 mM CD and both basal- and thrombin-stimulated release of taurine was monitored under conditions of isotonicity (340 mOsM; defined by the osmolarity of the DMEM/fetal calf serum medium in which the cells were grown), mild to moderate hypotonicity (295–200 mOsM), or hypertonicity (370–450 mOsM). The basal release of taurine was not appreciably increased until the osmolarity had been reduced to 200 mOsM. Although CD pretreatment of cells further increased the basal release of taurine when monitored under conditions of hypotonicity, this effect only became significant following a 30% decrease in osmolarity (a net increase in efflux of 5.9% of the total taurine pool at 230 mOsM). As previously reported (Cheema et al., 2007), the addition of thrombin to control cells resulted in a significant increase in taurine efflux under either mild or moderate hypotonic conditions (Fig. 6A). CD pretreatment of the cells further enabled thrombin to enhance taurine efflux under

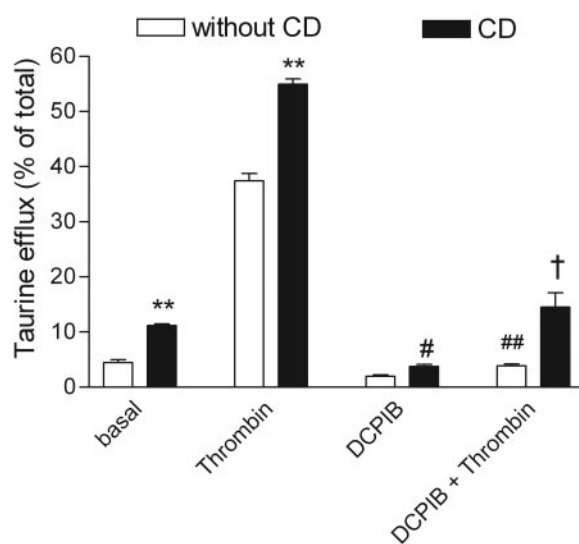


Fig. 5. Blockade of VSOAC with DCPIB attenuates the CD-induced facilitation of taurine release. Cells prelabeled with [3 H]taurine were pretreated with 20 μ M DCPIB in either serum-free media or 5 mM CD for 10 min before incubation in hypotonic buffer A (230 mOsM) containing 20 μ M DCPIB in the absence (open bars) or presence (black bars) of 1.25 nM thrombin. Reactions were terminated after 5 min, and efflux of taurine was monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means \pm S.E.M. for three independent experiments, each performed in triplicate. **, different from respective controls, $p < 0.01$; #, different from basal plus CD, $p < 0.01$; ##, different from thrombin alone, $p < 0.01$; †, different from thrombin plus CD, $p < 0.01$ (by repeated measures ANOVA followed by Dunnett's multiple comparisons test).

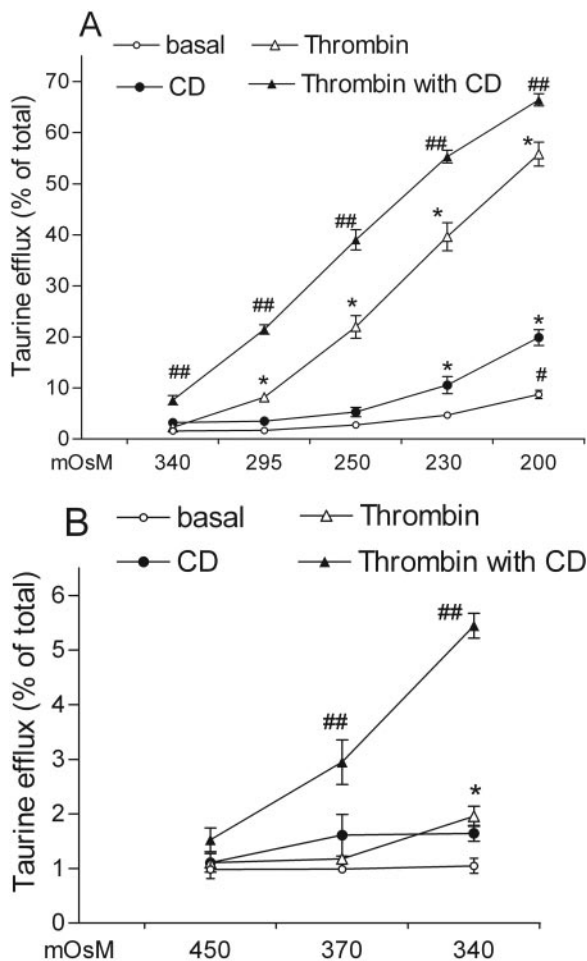


Fig. 6. Osmolarity dependence of basal- and thrombin-stimulated taurine efflux under control and cholesterol-depleted conditions. Cells prelabeled with [3 H]taurine were first pretreated for 10 min at 37°C with either serum-free media (open symbols) or 5 mM CD (closed symbols). The cells were then washed twice with 2 ml of isotonic buffer A and incubated in hypo-osmolar buffers for 5 min (A) or hyperosmolar buffers for 10 min (B) at the osmolarities indicated in the absence (basal, ○, ●) or presence (△, ▲) of 1.25 nM thrombin. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means \pm S.E.M. for three independent experiments, each performed in triplicate. *, different from basal, $p < 0.05$ (by repeated measures ANOVA followed by Dunnett's multiple comparison test); #, different from isotonic 340 mOsM, $p < 0.01$; ##, different from thrombin-stimulated efflux without CD, $p < 0.05$ (by ordinary ANOVA followed by Dunnett's multiple comparisons test).

isotonic, as well as mild to severe hypotonic, conditions. However, the stimulatory effect of cholesterol depletion on PAR-1-mediated taurine release was most marked under conditions of either isotonicity (340 mOsM) or mild hypotonicity (295 mOsM). Thus, although little or no increase in taurine efflux was observed following the addition of thrombin to control cells incubated in isotonic buffer A, a significant increase in osmolyte release was obtained from CD-pretreated cells under isotonic conditions (a net increase in efflux of 5.3% of the total taurine pool). Similarly, cholesterol depletion increased the ability of thrombin to stimulate taurine release from 8.2 to 21.5% of the total taurine pool, when monitored at 295 mOsM (Fig. 6A). When thrombin-stimulated taurine release for cholesterol-depleted cells was calculated (as a percentage) relative to control cells, the values were 332, 262, 178, and 139% of control at 340, 295, 250, and 230 mOsM, respectively. The ability of cholesterol depletion

to enhance thrombin-stimulated taurine efflux was reduced under conditions of mild hypertonicity (370 mOsM) and was abolished under more severely hypertonic conditions (450 mOsM; Fig. 6B).

Enhancement of Taurine Efflux by CD Is Reversed by the Readdition of Either Cholesterol or Epicholesterol. The ability of CD pretreatment to facilitate either thrombin- or Oxo-M-mediated taurine efflux under hypoosmotic conditions could be fully reversed by preincubation of the cells for 1 h in the presence of a saturating (1:8) cholesterol/CD complex (0.625 mM cholesterol). In fact, following cholesterol administration, the efflux of taurine monitored under basal-, thrombin-, and Oxo-M-stimulated conditions was significantly inhibited (61–77%) when compared with control values (Fig. 7A). Concurrent measurements of the cholesterol content of SH-SY5Y cells revealed that a 65% increase in cholesterol content over control cells was observed after the cells were exposed to the cholesterol:CD complex (Fig. 7B). The addition of a 1:8 cholesterol:CD complex to control cells also resulted in an increase in cholesterol content and a reduction in both basal- and thrombin-stimulated taurine release (data not shown). The specificity with which cholesterol administration can reverse the stimulatory effects of CD pretreatment was further tested by determining whether epicholesterol, a stereoisomer of cholesterol, could substitute for cholesterol. Epicholesterol and cholesterol have previously been employed to discriminate between effects due to sterol-protein interactions and those due to changes in the physical properties of the membrane lipid bilayer (Zidovetzki and Levitan, 2007). As observed for cholesterol readdition, administration of an epicholesterol/CD complex (1:8) reversed the stimulatory effects of cholesterol depletion and resulted in an inhibition of agonist-stimulated taurine efflux (25–29%) but did not inhibit basal release.

Alterations in Cholesterol Content or Replacement with Epicholesterol Do Not Influence the Extent of mAChR-Stimulated Phosphoinositide Turnover. In some cells, CD pretreatment results in an attenuation of cell signaling events (Burger et al., 2000). To evaluate the possibility that modulation of cholesterol content alters receptor signaling events within SH-SY5Y cells, basal- and mAChR-stimulated phosphoinositide turnover was monitored under control, cholesterol-depleted, and cholesterol- or epicholesterol-supplemented conditions. Neither CD pretreatment (Fig. 8A) nor supplementation with either cholesterol or epicholesterol had any significant effect on either basal or mAChR-stimulated phosphoinositide turnover (Fig. 8, B and C).

Pretreatment of SH-SY5Y Cells with Cholesterol Oxidase Has No Effect on Basal- or Receptor-Stimulated Taurine Efflux. In many cell types, a reduction in cholesterol content can also be achieved following incubation of the cells with cholesterol oxidase, an enzyme that converts cholesterol into 4-cholesten-3-one, a sterol that exhibits membrane fluidity characteristics similar to those of cholesterol (Gimpl et al., 1997). Preincubation of SH-SY5Y cells with cholesterol oxidase (1.5 U/ml) in serum free media for 1 h had no effect on basal-, mAChR-, or PAR-1-stimulated taurine efflux under hypotonic conditions (Fig. 9A), although the cholesterol content of SH-SY5Y cells was reduced by 65% (Fig. 9B).

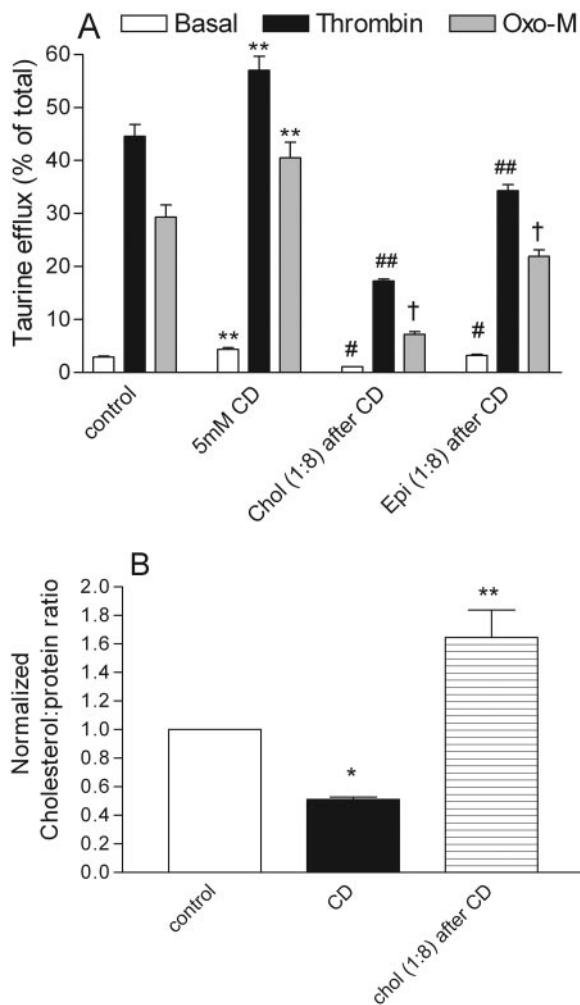


Fig. 7. Enhancement of taurine efflux by CD is reversed by the readdition of cholesterol or epicholesterol. Cells that had been prelabeled overnight with [3 H]taurine were treated with either serum-free media or 5 mM CD for 10 min at 37°C. The cells were washed once with 2 ml of isotonic buffer A and incubated with serum-free media, cholesterol:CD (1:8 ratio) or epicholesterol/CD (1:8 ratio) for 1 h. The cells were washed once more with isotonic buffer A before incubation for 5 min with hypotonic buffer A (230 mOsM) in the absence (open bars) or presence of thrombin (1.25 nM; black bars) or Oxo-M (100 μ M; gray bars). Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means \pm S.E.M. for three to nine independent experiments, each performed in triplicate. **, different from control efflux in the absence of CD, $p < 0.01$; #, different from basal efflux plus CD, $p < 0.05$; ##, different from thrombin-stimulated efflux, $p < 0.05$; †, different from Oxo-M-stimulated efflux after CD, $p < 0.01$ (by ordinary ANOVA followed by Dunnett's multiple comparisons test). B, cells were treated the same way, and cholesterol content was measured by Amplex Red kit. Results (means \pm S.E.M) are normalized as cholesterol/protein ratios from four independent experiments. * and **, different from control cells, $p < 0.05$, $p < 0.01$ (by repeated measures ANOVA followed by Dunnett's multiple comparisons test).

Discussion

Cholesterol availability has been reported to modulate the activity of several different ion channels (Bolotina et al., 1989; Jennings et al., 1999; Martens et al., 2001; Lockwich et al., 2000), in addition to that of VSOAC. In agreement with previous studies of non-neural cells, depletion of cholesterol from SH-SY5Y cells by pretreatment with CD under relatively mild conditions (5 mM for 10 min) facilitated the basal release of taurine in response to hypotonicity. However, the

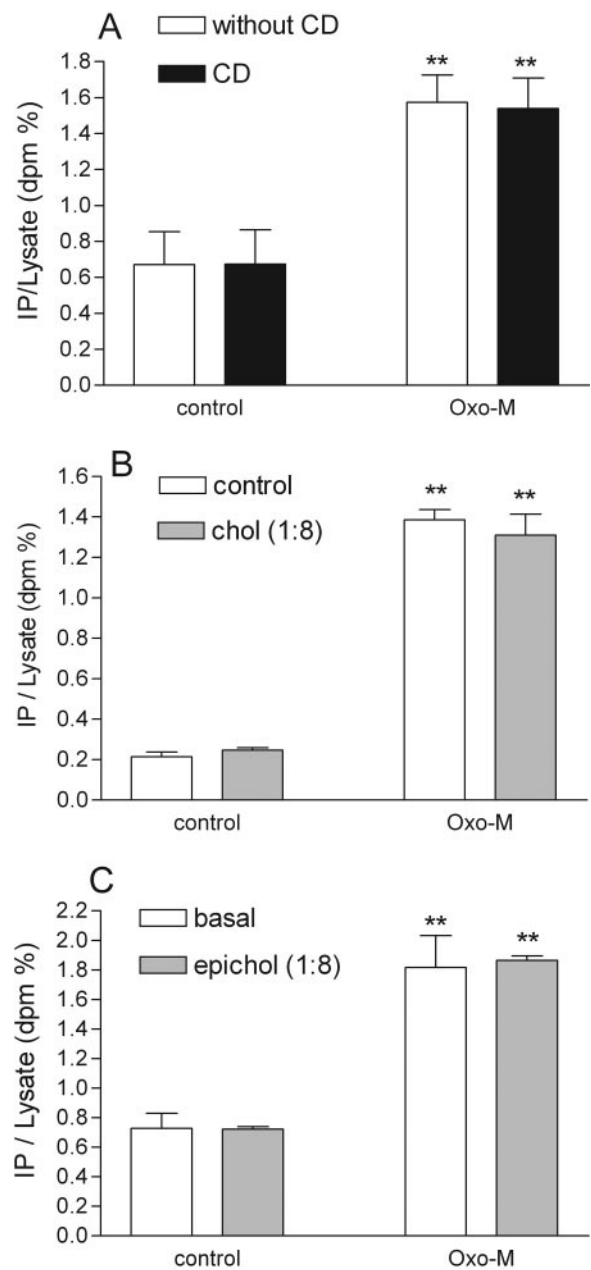


Fig. 8. Alterations in cholesterol content or replacement with epicholesterol do not modulate mAChR-stimulated phosphoinositide turnover. Cells that had been prelabeled for 48 h with [3 H]inositol were pretreated with either serum-free media or 5 mM CD for 10 min (A) or pretreated with serum-free media or CD for 10 min followed by incubation with a cholesterol/CD complex (1:8) for 1 h (B) or pretreated with serum-free media or CD for 10 min followed by incubation with an epicholesterol/CD complex (1:8) for 1 h (C). The cells were then washed with isotonic buffer A and incubated for 10 min in hypotonic buffer A (230 mOsM) in the presence or absence of Oxo-M (100 μ M). Reactions were terminated by the addition of trichloroacetic acid, and the accumulation of radiolabeled inositol phosphates (IPs) was monitored as an index of stimulated phosphoinositide turnover. Results are expressed as IP release/total soluble radioactivity in cell lysates (IP/lysate; disintegrations per minute percentage) and are the means \pm S.E.M. for three to four independent experiments. **, different from respective controls, $p < 0.001$ (by ordinary ANOVA followed by Dunnett's multiple comparisons test).

more significant observation was that depletion of cholesterol resulted in a further potentiation of receptor-stimulated osmolyte efflux, such that, following CD pretreatment, a synergistic, rather than additive, release of taurine above

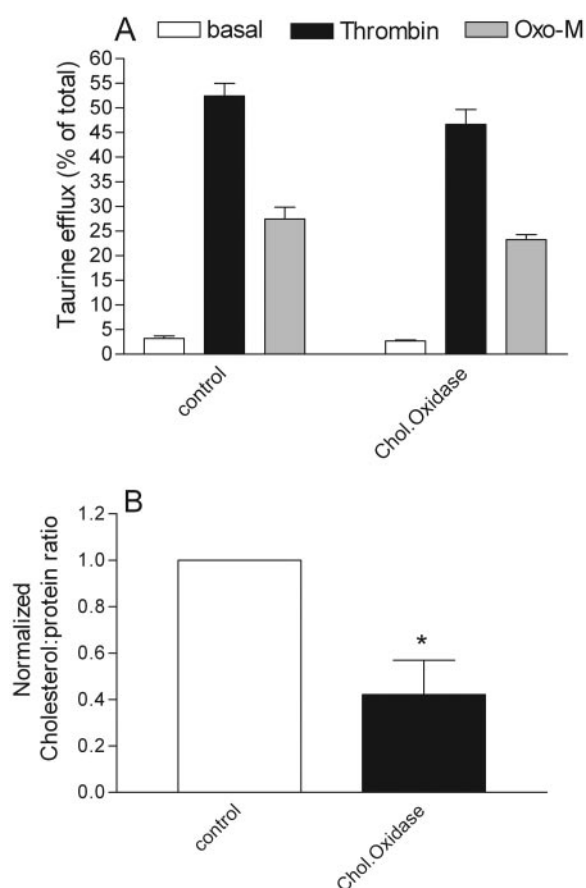


Fig. 9. Pretreatment with cholesterol oxidase has no effect on basal- or receptor-stimulated taurine efflux. Cells that had been prelabeled overnight with [^3H]taurine were treated with either 1 ml of serum-free media or 1.5 U/ml cholesterol oxidase for 1 h at 37°C. The cells were then washed twice with 2 ml of isotonic buffer A and incubated in hypotonic buffer A (230 mOsm) for 5 min in the absence (open bars) or presence of either thrombin (1.25 nM; black bars) or Oxo-M (100 μM ; gray bars). Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means \pm S.E.M. for three to four independent experiments. B, unlabeled cells were treated as above, and cholesterol content was measured by Amplex Red kit. Results (means \pm S.E.M.) are normalized as cholesterol/protein ratios from three experiments, each performed in triplicate. *, different from control, $p < 0.05$ (by unpaired Student's t test).

controls was observed (Fig. 3). The ability of cholesterol depletion to enhance receptor-stimulated taurine efflux was observed for all three receptors examined (mAChR, PAR-1, and S1P), even though they operate via different signal transduction mechanisms. Although cholesterol depletion has been reported to alter ligand binding to cell surface receptors (Burger et al., 2000), CD pretreatment of SH-SY5Y cells increased the efficacy of thrombin- or Oxo-M-mediated taurine release but had no effect on agonist potency (Fig. 4). In addition, because cholesterol depletion had no effect on the extent of mAChR-stimulated phosphoinositide turnover (Fig. 8), this result argues against either nonspecific effects of CD or increases in second messenger production as causes for enhancement of receptor-stimulated taurine release. Taken collectively, the results suggest that the ability of cholesterol depletion to potentiate receptor-stimulated osmolyte efflux occurs independently of any changes in either ligand binding or monitored signal transduction events and that cholesterol has a modulatory role at the VSOAC channel itself or, alternatively, at an associated membrane domain.

Further evidence for a role of cholesterol in the regulation of receptor-stimulated taurine efflux was obtained from experiments in which CD, complexed with cholesterol, was used as a donor to restore cholesterol to CD-pretreated cells. When this approach was employed, it was observed that the CD-mediated potentiation of both PAR-1- and mAChR-stimulated taurine release could be reversed when the cells were presented with a CD/cholesterol (1:8) complex (Fig. 7). Under these conditions, cellular cholesterol concentrations were increased by 65%, and this was accompanied by a significant inhibition (51–70%) of both basal- and receptor-stimulated taurine efflux. Administration of epicholesterol, a chiral analog of cholesterol, was similarly able to reverse the stimulatory effects of cholesterol depletion on taurine release and also reduced efflux to below control levels. Since administration of either cholesterol- or epicholesterol-CD complexes to SH-SY5Y cells did not result in any change in the extent of mAChR-stimulated phosphoinositide turnover, an effect of these sterols on signaling events appears unlikely. The more pronounced ability of cholesterol (than epicholesterol) to inhibit agonist-stimulated taurine efflux (61–77% versus 25–29%) could reflect either a more limited rate of exchange of epicholesterol into the cells or, alternatively, differential abilities of cholesterol and epicholesterol to form sterol-protein interactions (Zidovetzki and Levitan, 2007). Since elevated cholesterol concentrations have been reported to inhibit basal VSOAC activity in endothelial and intestinal 407 cells (Levitan et al., 2000; Lim et al., 2006) but not in ascites cells (Klausen et al., 2006), the degree of cholesterol regulation of VSOAC activity may be cell-type specific.

One of the most salient features of volume-dependent osmolyte efflux monitored following receptor activation is that the threshold osmolarity for release is significantly reduced, thereby allowing osmolyte release at more physiologically relevant osmolarities (Mongin and Kimelberg, 2002; Heacock et al., 2004, 2006; Cheema et al., 2007). A major conclusion to emanate from the present study is that cholesterol depletion further increases this ability of receptor activation to facilitate osmolyte efflux and, moreover, that the effect is most marked under conditions of isotonicity or mild hypotonicity (295 mOsm; a 13% reduction in osmolarity, Fig. 6). From these results, we conclude that cholesterol depletion and receptor activation can act in concert to promote osmolyte efflux and, by inference, cell volume regulation under conditions of minimal reductions in osmolarity. A limited degree of facilitation of taurine efflux by thrombin in cholesterol-depleted cells was even detected under mildly hypertonic conditions (370 mOsm) but not under conditions of more severe hypertonicity, a result that indicates that removal of cholesterol permits the VSOAC channel to remain partially open under conditions of mild hypertonicity. In this context, Levitan et al. (2000) have proposed that cholesterol content alters the equilibrium between the closed and open states of the VSOAC, with cholesterol depletion favoring the existence of an open channel. Since VSOAC, via the release of glutamate, another organic osmolyte, has also been implicated in cell-cell signaling events under isotonic conditions (Mulligan and MacVicar, 2006), regulation of channel activity by cholesterol availability could be of physiological significance under conditions of both isotonicity and hypotonicity. Although cholesterol depletion also enhanced the basal release of taurine from SH-SY5Y cells in a volume-dependent manner, the

stimulatory effect was less marked than that observed for receptor activation, and only reached significance at an osmolarity of 230 mOsm. In contrast to previous studies obtained for non-neural cells in which the stimulatory effects of cholesterol depletion on VSOAC activity were abrogated by larger reductions in osmolarity (Levitan et al., 2000; Klausen et al., 2006; Lim et al., 2006), cholesterol depletion of SH-SY5Y cells was observed to facilitate the basal efflux of taurine even under conditions of a 40% reduction in osmolarity. As observed for control cells, inclusion of DCPIB attenuated the increases in both basal- and receptor-stimulated efflux of taurine from CD-pretreated SH-SY5Y cells. Thus, the increased release of osmolytes from cholesterol-depleted cells is also mediated by a VSOAC.

The functional relationship between alterations in cholesterol content and VSOAC activity remains uncertain. Two major roles for cholesterol in the regulation of VSOAC have previously been proposed, i.e., that the channel is localized to lipid rafts or caveolae (Trouet et al., 1999, 2001; Ullrich et al., 2006) or, alternatively, that cholesterol-induced changes in the biophysical properties of the membrane result in an altered VSOAC activity (Levitan et al., 2000; Romanenko et al., 2004; Byfield et al., 2006). In SH-SY5Y cells, depletion of cholesterol with CD, a treatment documented to disrupt lipid microdomains (Brown and London, 2000; Zidovetzki and Levitan, 2007), resulted in an enhancement of both basal- and receptor-stimulated taurine efflux. Although one interpretation of this result is that the VSOAC channel is located in a lipid microdomain, under a negative constraint, this explanation seems unlikely since preincubation of SH-SY5Y cells with cholesterol oxidase (or sphingomyelinase or filipin; data not shown), conditions under which lipid rafts are also disrupted (Samsonov et al., 2001), did not facilitate the release of osmolytes under either basal- or receptor-stimulated conditions (Fig. 9A). Changes in the concentration of cell cholesterol are also documented to exert a major impact on the physical properties of the membrane lipid bilayer, with a reduction resulting in an increase in membrane fluidity, whereas elevated concentrations of cholesterol drive the fluid membrane bilayer into a more rigid state (Xu and London, 2000). In SH-SY5Y cells, the ability of cholesterol depletion to facilitate VSOAC activity, whereas cholesterol replenishment inhibits channel activity, is consistent with the possibility that VSOAC is regulated by changes in membrane fluidity. This conclusion is strengthened by the observation that epicholesterol, a chiral analog of cholesterol that exhibits similar properties as cholesterol in terms of its effect on bulk membrane fluidity (Gimpl et al., 1997), essentially mimicked the ability of cholesterol to reverse the stimulation of taurine efflux elicited by cholesterol depletion. In addition, although cholesterol oxidase treatment, like that of CD, resulted in a loss of ~50% of cell cholesterol, the oxidation product, namely 4-cholesten-3-one, exhibits the same membrane fluidity properties as cholesterol (Gimpl et al., 1997), and this may account for the absence of change in either the basal- or receptor-stimulated osmolyte release. Taken collectively, the results obtained for SH-SY5Y cells suggest that a change in membrane fluidity, rather than disruption of a lipid microdomain, is the major determinant of PAR- and mAChR-stimulated VSOAC activity, even though both receptors and attendant signaling proteins have previously been localized to lipid rafts (Allen et al., 2007; Carlisle-Klusacek

and Rizzo, 2007). Cholesterol availability may also regulate membrane-cytoskeleton adhesion (Sun et al., 2007).

Alterations in cholesterol content within the central nervous system occur in conditions associated with genetic deficits in cholesterol synthesis/trafficking (Maxfield and Tabas, 2005), following dietary modification (Foot et al., 1982) or after administration of lipophilic statins (Eckert et al., 2001). Since these changes in free cholesterol content are similar in magnitude to those observed to regulate VSOAC activity in the present study, cholesterol could serve an important modulatory role for VSOAC activity in vivo. In this context, it is relevant to note that VSOAC activity is markedly reduced in the fibroblasts of patients with Niemann-Pick's disease, a condition in which cholesterol content is elevated (Lim et al., 2006). The present results, obtained for SH-SY5Y neuroblastoma cells, are also consistent with the possibility that cholesterol availability, acting in concert with receptor activation, provides an additional means whereby volume regulation in neural cells is modulated.

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