Membrane Cholesterol Modulates Galanin−GalR2 Interaction

Ling Pang, Michael Graziano, and Suke Wang*

Department of CNS/CV Biological Research, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, New Jersey 07033

Received January 29, 1999; Revised Manuscript Received July 20, 1999

ABSTRACT: The neuropeptide galanin mediates a number of diverse physiological and pathophysiological actions via interaction with membrane-bound receptors. The role that membrane cholesterol plays in modulating the interaction between galanin and one of the three cloned galanin receptor subtypes (GalR2) expressed in Chinese hamster ovary (CHO) cells was examined. Reduction of membrane cholesterol by treatment with methyl-β-cyclodextrin (CD) or by culturing cells in lipoprotein-deficient serum markedly decreased galanin binding to the receptor. Addition of cholesterol back to CD-treated, cholesterol-depleted membranes restored galanin binding to control levels. Hill analysis suggests that the GalR2 binds multiple molecules of cholesterol ($n \geq 3$) in a positively cooperative manner. This interaction appears to be cholesterol-specific as only cholesterol and a limited number of cholesterol analogues were able to rescue galanin binding. The inability of some of these analogues to rescue the binding activity also suggests that binding of galanin to GalR2 is independent of membrane fluidity as, like cholesterol, cholesterol analogues generally rigidize membranes. In addition, treatment of the membranes with other modulators of membrane fluidity, e.g. ethanol, did not affect galanin binding to the GalR2. In contrast, treatment of membranes, with filipin, a molecule that clusters cholesterol within the membranes, or with cholesterol oxidase resulted in markedly reduced galanin binding. Incubation of membranes with 100 μM GTP-γ-S did not alter the IC_{50} for CD in the prebinding assay treatment suggesting that the effect of cholesterol was independent of G protein interaction. Preincubation of intact cells with CD also drastically impaired the ability of galanin to activate intracellular inositol phosphate accumulation in GalR2-transfected CHO cells. These data detail a new mechanism for the regulation of galanin receptor signaling which may link altered functions of GalRs with abnormal cholesterol metabolism.

Many neuronal functions are regulated by neuropeptides through binding to specific membrane-bound receptors. The neuropeptide galanin is a 29−30-amino acid peptide (*1*) that is ubiquitously expressed in both the peripheral and central nervous systems. Galanin has been implicated in the regulation of endocrine function, e.g. release of growth hormones and insulin (*2, 3*), and in digestive functions, e.g. gastric acid secretion and intestinal motility (*4*−*12*). In the central nervous system (CNS) galanin modulates a wide range of behaviors including feeding, anxiety, nociception, and learning and memory (*13*−*17*).

The biological actions of galanin are mediated through binding to multiple subtypes of galanin receptors (GalRs).[1] To date three galanin receptor subtypes have been cloned. All three receptors are predicted to have the seven transmembrane domain (TM) structure which is typical for members of the G protein-coupled receptor (GPCR) superfamily (*18*−*22*). While all three GalRs bind to galanin with high affinity ($K_D = \sim 1$ nM) they show a relatively low level of sequence homology, i.e. 36−54% (*22*). The coding region of GalR1 consists of three exons: the first exon encodes the N-terminus through TM5, the second exon encodes the third intracellular loop, and the third encodes the region covering TM6 through the C-terminus (*23*). The coding regions of GalR2 comprise two exons that are separated by a single intron located at the junction of TM3 and intracellular loop 2 (*24, 25*). The molecular definition of the galanin receptors will aid in elucidating the roles that these subtypes play in the divergent physiological functions of galanin (*12*). For example, GalR2-selective agonists regulate jejunal smooth muscle contraction but do not appear to have effect on feeding behavior (*12*).

Galanin signaling is regulated at several levels. Northern blot analysis shows the three GalR subtypes to be differently expressed in tissues. Expression of GalR1 is restricted to the CNS with significant mRNA levels seen primarily in brain and spinal cord (*23, 26*). In contrast, GalR2 mRNA is abundantly expressed in the CNS and many peripheral tissues (*20, 21*). GalR3 mRNA is expressed at low levels in several peripheral tissues and brain regions (*22, 27*). The galanin receptor subtypes have different affinities for galanin analogues (*22*). The N-terminal Gly residue is important for galanin to bind GalR1 but not GalR2 and GalR3; residues 17−19 of galanin are required for high-affinity binding of GalR3 (*22*). These data imply that differential processing of galanin by endogenous protease activities could result in

* Corresponding author. Tel: 908-740-3949. Fax: 908-740-2383. E-mail: suke.wang@spcorp.com.

[1] Abbreviations: CHO, Chinese hamster ovary; CD, methyl-β-cyclodextrin; PC, phosphatidylcholine; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; Ch-CD, cholesterol- containing CD; IP, inositol phosphate; PBS, phosphate-buffered saline; GalR, galanin receptor; GPCR, G protein-coupled receptor.

10.1021/bi990227a CCC: $18.00
Published on Web 08/20/1999

subtype-specific activation of the GalR subtypes (*28*−*31*). Modified galanin peptides have been used to define molecular interactions responsible for the binding of galanin to its receptor(s) (*32*, *33*).

On the basis of the data from recombinant expression systems, the GalR subtypes appear to use diverse signal transduction pathways. Activation of GalR1 leads to inhibition of cAMP production or to stimulation of MAP kinase activity. GalR1 is coupled to a Gi type G protein through both the α and $\beta\gamma$ subunits (*34*). In contrast, GalR2 is more permissive and is coupled to Gq/11, Go, and Gi types of G proteins, leading to inositol phosphate accumulation, MAP kinase activation, and inhibition of cAMP (*34*, *35*). GalR3 appears to be linked to Gi/Go proteins, activation of which leads to enhanced inward K^+ in xenopus oocytes (*27*). In addition to the mechanisms described above, other regulatory mechanisms may also exist for activation of the cloned GalRs. Upon activation of GalR1 by galanin, the receptor undergoes a rapid ($t_{2/1}$ = 20 min) and extensive (>80%) internalization via an energy-requiring endocytotic process (*36*).

Lipid−receptor interaction has been shown to modulate ligand−receptor interaction and receptor activation of the nicotinic acetylcholine receptor, transferrin receptor, oxytocin receptor, and rhodopsin receptor (*37*−*40*). Despite the recent advances in defining the molecular and biochemical aspects of the GalRs signaling, the effect that membrane lipids may play in the regulation of ligand interaction and activation is unknown. In the present study, we test the hypothesis that membrane cholesterol may play a direct modulatory role in the binding and activation of GalRs using CHO cells stably expressing the GalR2 receptor.

MATERIALS AND METHODS

Materials. ^{125}I-Labeled human galanin (2200 Ci/mmol), myo[^{3}H]inositol, and [1,2,6,7-^{3}H(N)]cholesterol (75.1 Ci/mmol) were purchased from DuPont/New England Nuclear (Boston, MA). Methyl-β-cyclodextrin (CD), cholesterol, 5-pregnen-3β-ol-20-one, 4-cholesten-3-one, 5-cholestene, 5-cholesten-3-one, cholesterol assay kit (cat. no. 352-50), filipin, and lipoprotein-deficient serum (LPDS) were purchased from Sigma (St. Louis, MO). Superfect transfection agent was purchased from Qiagen (Chatsworth, CA). Rat galanin was purchased from Peninsula Laboratories (Belmont, CA). Phosphatidylcholine was determined using an assay kit from Biochemical Diagnostic, Inc. (NY) (cat. no. 990-54009).

Membrane Preparation. CHO cells expressing rat GalR2 were prepared as described previously (*34*). CHO cells were grown in F12 medium supplemented with 10% fetal calf serum (FCS) and 300 μg/mL G418. For experiements involving reduction of membrane cholesterol in cultured cells, CHO cells were cultured in F12 medium containing LPDS (5% v/v) for 24 h prior to the experiment. Cell membranes were prepared as follows: cells in 100-mm dishes were washed twice with cold PBS (without Ca^{2+}/Mg^{2+}); 5 mL of hypotonic buffer (5 mM Hepes, pH 7.4, 5 mM EDTA, 0.1 mM PMSF) was added to the cell monolayer. After incubation at room temperature for 15 min, the cells were scraped off with a 32-cm cell scraper and centrifuged at 15 000 rpm in a Sorvall SS-34 rotor for 30 min. The membrane pellets were washed once with the same buffer. The washed pellet was suspended in 1 mL of buffer containing 25 mM Tris-HCl, pH 7.4, 1 mM EDTA. The protein concentration was determined using BCA Protein Assay Reagent (Pierce, MD). The membranes were aliquoted into 1-mL Eppendorf tubes, snap-frozen on dry ice, and stored at −80 °C. When required, ∼50 and ∼150 μg of membranes were used for phosphatidylcholine and cholesterol determinations, respectively.

[^{125}I]Galanin Binding Assay. Binding of [^{125}I]human galanin to CHO cell membranes was performed as described previously (*21*). Saturation binding was performed at room temperature using 10 μg of membrane protein in a total volume of 200 μL of assay buffer (25 mM Tris-Cl, pH 7.4, 1% BSA, 0.1% bacitracin, 2 μg/mL leupeptin, 0.1 mM PMSF, and 10 mM $MgCl_2$) containing 0−3600 pM of [^{125}I]-human galanin. Nonspecfic binding was determined in the presence of 5 μM galanin. Binding of [^{125}I]human galanin in competition with galanin was performed in a total volume of 200 μL containing 10 μg of membrane protein, 0.3 nM [^{125}I]galanin, and 0−1 μM galanin. All binding data were analyzed by nonlinear regression using the Prism program (GraphPad, San Diego, CA). K_i values were calculated by the method of Cheng and Prusoff (*41*).

Treatment of Membranes with CD and Membrane Modulators. CHO membranes (500 μg of protein) were incubated in 500 μL of assay buffer with various concentrations of CD (0−100 mg/mL). The samples were incubated at room temperature for 30 min with shaking. The mixture was centrifuged at 16000*g* at 4 °C for 10 min (*39*). The resulting pellets were washed by suspension in 500 μL of assay buffer followed by centrifugation. The washed pellets were resuspend in 200 μL of assay buffer (*39*).

Pretreatment of the membrane with filipin is similarly performed. Filipin in methanol at 20 mM was added to 200 μL of membranes (100:1 dilution) and incubated at room temperature for 10 min. The membrane was washed and resuspended as described above. Ethanol (100%) was added to membranes directly to a final concentration up to 5% (v/v) (*46*), and binding assays were performed similarly.

Incorporation of Cholesterol into Cholesterol-Depleted Membranes . Membranes were treated with 100 mg/mL CD, as described above to remove the endogenous cholesterol; 250 μL of cholesterol-deplete membranes (1 mg of protein/mL) was mixed and incubated with various concentrations of cholesterol-CD complex (Ch-CD) (0−1.2 mM cholesterol) for 30 min at room temperature. The membranes were centrifuged and washed once with assay buffer. The washed membranes were resuspended in 250 μL of assay buffer for ligand binding assays. The Ch-CD complexes were prepared as previously described (*42*). Cholesterol (50 μL of 50 mg/mL in chloroform:methanol, 1:1, v:v) and 5 μL of [^{3}H]-cholesterol (s.a. 75.1 Ci/mmol; NEN, cat. #NET725) were mixed, and the solvent was evaporated under a gentle stream of nitrogen. Eighty milligrams of CD (Sigma, cat. #C4555) was dissolved in 10 mL of PBS without Mg^{2+}/Ca^{2+}. The solution was then added to the cholesterol (CD:cholesterol = ∼10:1, mol/mol). The tubes were vortexed and then sonicated at 70% output (Heat Systems Ultrasonic, model W-385) for 1−3 min. The Ch-CD solutions were incubated with vigorous stirring overnight in a water bath at 80 °C. The solution was filtered through a 0.45-μm filter prior to use.

Incorporation of Steroids into Cholesterol-Depleted Membranes. Incorporation of steroids into the cholesterol-depleted membranes was performed as described above for incorporation of cholesterol. Steroid-CD complexes were prepared as described previously (*39*). Briefly, 12 mg of steroid was dissolved in 80 μL of 2-propanol:chloroform (2:1). CD (200 mg) was dissolved in 2.2 mL of water and heated to 80 °C with stirring on a hot plate. Steroid was added in small aliquots, and the solution was stirred until clear. The final concentration of steroid in the solution was 13.6 mM (CD:sterol = ~ 5:1, mol/mol).

Treatment of Membranes with Cholesterol Oxidase. Membranes (10 μg/reaction) were pretreated at 37 °C for 1 h with cholesterol oxidase (Sigma, cat. #C8153; stock solution prepared at 200 units/mL in a buffer containing 100 mM Mes, pH 6.0, and 3 M NaCl) at various concentrations in 100 μL of buffer containing 20 mM Hepes, pH 7.0, 5 mM $MgCl_2$, 10 mM mannitol, and 1 munit/mL of sphingomyelinase (Sigma, cat. #S5383; stock solution prepared at 0.04 unit/mL in a buffer containing PBS, pH 7.4, and 50% (v/v) glycerol). Binding of [^{125}I]human galanin was determined as described above by addition of assay buffer (100 μL) containing 0.3 nM radioligand.

Inositol Phosphate Turnover Assay. COS-7 cells were cultured to 80% confluence in 6-well plates; 2 μg of an expression plasmid harboring the rat GalR2 cDNA (pCR3.1-rGalR2) was transfected into the cells using the SuperFect agent (Qiagen). Three days post-transfection, the cells were washed with PBS and cultured for 24 h in 1 mL of minimal essential media (Gibco) supplemented with 0.5% FCS and 2 μCi/mL myo[^{3}H]inositol (DuPont/NEN:NET114A). The cells were washed with PBS (37 °C) and then incubated in 0.5 mL of PBS containing various concentrations of CD for 0.5 h at 37 °C. The cells were washed twice with 3 mL of PBS and then incubated with 200 nM galanin in PBS (without Ca^{2+}/Mg^{2+}) containing 20 mM LiCl and 1 mM $CaCl_2$ for 60 min at 37 °C. The reaction was terminated by removing the media and adding 1 mL of 0.4 M perchloric acid to each well. Following incubation at 4 °C for 10 min the solution was removed and 500 μL of neutralizing solution (0.72 M KOH/0.6 M $KHCO_3$) was added to each well. Dowex anion-exchange columns were prepared using 1 mL of 50:50 slurry of Dowex (formate form); 1 mL of supernatant and 3 mL of water were applied to each column which was then washed twice with 10 mL of water. Inositol phosphates were eluted using 3.5 mL of 0.1 M formic acid/1 M ammonium formate and quantified by liquid scintillation spectroscopy.

RESULTS

The cholesterol content of membranes prepared from CHO cells overexpressing the rat GalR2 (GalR2/CHO) was altered by incubation with CD. CD removed cholesterol from GalR2/CHO membranes in a dose-dependent manner (Figure 1); 80−85% of the membrane cholesterol was removed by treating the GalR2/CHO membranes with 50−100 mg/mL CD (Figure 1). The binding of [^{125}I]galanin to CD-treated membranes was quanitified. Treatement of GalR2/CHO membranes with 100 mg/mL CD resulted in a subsequent 91% reduction of [^{125}I]galanin (experiment A, Table 1). [^{125}I]-Galanin binding to the GalR2/CHO membranes declined in proportion to the decrease of membrane cholesterol, indicating that cholesterol is critical for galanin binding. To ensure that CD selectively removed cholesterol, protein and phosphatidylcholine concentrations of the CD-treated membranes were also measured. The protein content of the membranes was not altered by treatment with CD in a concentration range of 0−100 mg/mL. There was a small decrease of phosphatidylcholine by CD (~10% at the highest CD

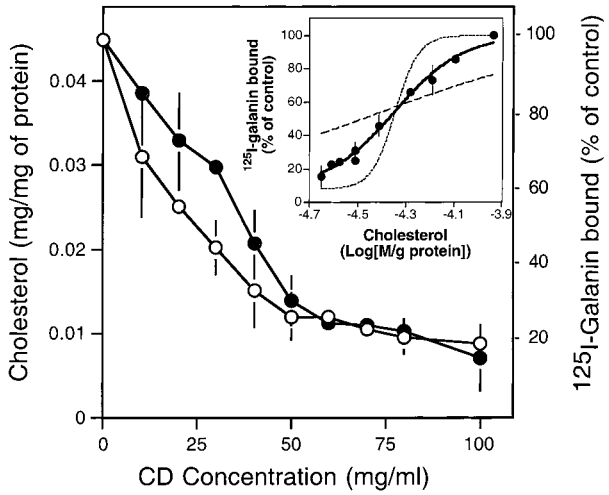

FIGURE 1: [^{125}I]Galanin binding to membranes treated with various concentrations of CD. Membranes from GalR2/CHO cells were incubated with increasing amounts of CD. After removal of CD, membrane pellets were suspended in a buffer containing 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 mM PMSF. [^{125}I]Galanin binding was determined by using 10 μg of membrane at a [^{125}I]-galanin concentration of 0.3 nM (●). Cholesterol levels were determined by using a cholesterol oxidase assay kit with 150 μg of each treated membrane (○). Values are mean ± SD (n = 3−6). Inset: Hill plot analysis of the binding data. Curves indicate the fit: Hill coefficient (H) = 3.1 (solid line), 1 (dashed line), and 10 (dotted line).

Table 1: Effect of Membrane Cholesterol on [^{125}I]Galanin Binding to Rat GalR2[a]

treatment	galanin bound (dpm)	% of control
Experiment A		
a. control	14898 ± 387	100
b. control + 5 μM galanin	799 ± 264	0
c. treated with CD[b]	1858 ± 312	9
d. CD + 5 μM galanin	582 ± 88	0
Experiment B		
a. control	6778 ± 1319	100
b. treated with CD	1584 ± 59	19
c. b + Ch-CD[c]	6493 ± 342	95
d. control + 5 μM galanin	403 ± 109	0
Experiment C[d]		
a. medium with 10% FCS	4253 ± 173	100
b. a + 5 μM galanin	528 ± 31	0
c. medium with 5% LPDS	1937 ± 184	40
d. c + 5 μM galanin	461 ± 21	0

[a] All experiments were performed in radioligand binding assays with 0.3 nM [^{125}I]human galanin and membranes prepared from GalR2/CHO cells (*21*). "+ 5 μM galanin" indicates the use of cold galanin in the binding assays to quantify nonspecific binding. [b] Membranes were treated with CD (100 mg/mL) prior to radioligand binding assays. [c] CD-treated membranes were treated with Ch-CD complex (at 1.2 mM cholesterol concentration) prior to radioligand binding assays. [d] Membranes prepared from cells grown in medium supplemented with either 10% FCS or 5% LPDS were used in the binding assays. Data are mean ± SD from 3 to 6 determinations.

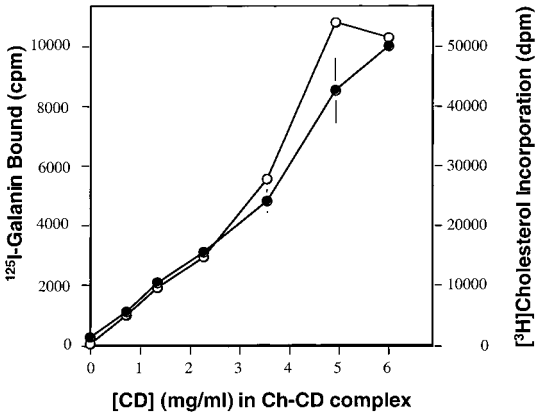

FIGURE 2: Restoration of [^{125}I]galanin binding to cholesterol-depleted GalR2/CHO membranes by adding Ch-CD complex. 5 mg of GalR2/CHO membranes was pretreated with 100 mg/mL CD, and aliquots of the cholesterol-depleted membranes (250 μg) were inoculated with increasing amount of Ch-CD inclusion complex (equivalent of 0−1.2 mM cholesterol in CD complex) labeled with [^{3}H]cholesterol. [^{125}I]Galanin binding assay was performed by using 10 μg of membrane (●). [^{3}H]Cholesterol incorporation into membranes was determined by liquid scintillation counting of 10 μg of resuspended membranes (○). Data are mean ± SD, $n = 3$.

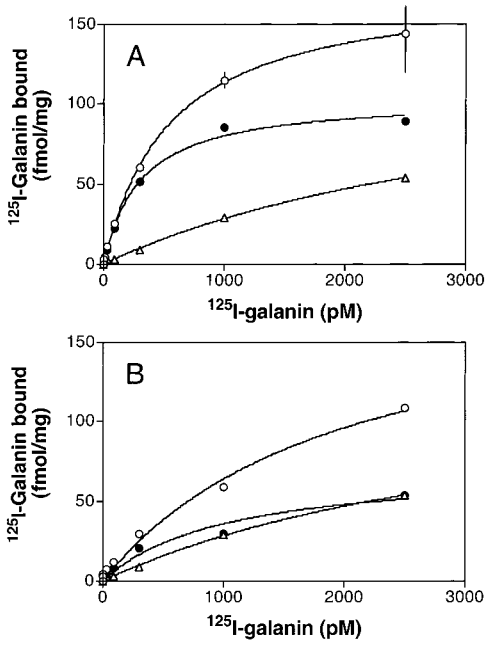

FIGURE 3: Saturation [^{125}I]galanin binding to membranes prepared from GalR2/CHO cells grown in FCS−containing (A) or LPDS-containing (B) medium. 10 μg of membranes was used in each assay. Total (○) and nonspecific binding (△) (determined in the presence of 5 μM unlabeled galanin) are shown. The specific binding was calculated as the difference of the total and nonspecific counts (●). Data are mean ± SD, $n = 3$.

concentration 100 mg/mL). Because the removal of cholesterol was more drastic by CD treatment (>90%), the decrease of phosphatidylcholine was small compared to that of cholesterol. Thus the cholesterol-to-phosphatidylcholine ratio declined linearly from 0.44 to 0.15 when CD was increased from 0 to 100 mg/mL (not shown). These measurements demonstrate that treatment with CD selectively decreased cholesterol concentration in the membranes over protein and other lipids.

Hill analysis to obtain cooperativity of cholesterol binding to GalR2 was performed by fitting the data to eq 1:

$$\%\ \text{binding of control} = \text{bottom} + [(\text{top} - \text{bottom})/(1 + 10^{(\log EC_{50} - [\text{Ch}])H})] \quad (1)$$

where *top* is the total binding defined as 100%, *bottom* is the nonspecific binding defined as 0%, [Ch] is the cholesterol concentration in membranes (M/g of protein), and *H* is the Hill coefficient. Equation 1 is a slightly modified form of the Bjerrum plot which allows quantification of cooperativity of ligand binding based on the broadness of isotherm curves by direct nonlinear regression analysis. Analysis of the results shown in Figure 1 using regression defined by eq 1 yields a Hill coefficient (*H*) of 3.1 and an EC_{50} (concentration that gives a 50% of maximum binding) of 51 ± 4 μM/mg of protein (inset, Figure 1) (correlation coefficient $r^2 = 0.96$). Fixing the value of *H* at 1 or 10 in the analyses resulted in poorer fitting ($r^2 = 0.66$ or 0.84, respectively) (inset, Figure 1).

The reduction of [^{125}I]galanin binding to cholesterol-depleted CHO membranes expressing GalR2 was reversible. Incubation with 5−6 mg/mL Ch-CD complex (1.0−1.2 mM cholesterol) resulted in a membrane cholesterol level similar to that of the untreated control membranes (~45 μg/mg of protein, Figure 2). Incubation of the cholesterol-deprived membranes with Ch-CD restored [^{125}I]galanin binding to 95% of control levels (experiment B, Table 1). The restoration of [^{125}I]galanin binding correlated with an increase in membrane cholesterol. Maximum restoration of binding occurred following incubation with 5 or 6 mg/mL Ch-CD, which was about 100% of the specific binding to the native membrane (Figure 2). The data presented in Figures 1 and 2 also suggest that the effect of CD pretreatment of the membranes on galanin binding is not a result of nonspecific, direct interaction between GalR2 and CD that may be carried over in residual amounts through washes of the membranes into the binding assays.

Cellular cholesterol levels were modulated by culturing GalR2/CHO cells in LPDS. Membranes prepared from these cells showed a 60% reduction in [^{125}I]galanin binding (experiment C, Table 1). The cholesterol content in the membranes (18 μg/mg of protein) decreased to 40% from that of control membranes (45 μg/mg of protein). This level of cholesterol present in membranes prepared from cells cultured in LPDS likely results from intracellular cholesterol biosynthesis (*43*). Saturation ligand binding assays revealed comparable B_{max} values (maximum number of specific binding sites) between the normal and cholesterol-reduced membranes (103 ± 8 vs 72 ± 22 fmol/mg of protein) (Figure 3). In contrast, the K_d value (equilibrium dissociation constant) of [^{125}I]galanin was 3-fold higher in membranes prepared from LPDS-treated cells, i.e. $K_d = 1.0 \pm 0.3$ nM vs 0.29 ± 0.07 nM for untreated membranes (Figure 3). Galanin affinity was decreased to a similar degree, i.e. K_i (dissociation constant of competing molecule) = 21 ± 7 nM vs 4.4 ± 1.2 nM for control membranes determined in radioligand competition assays (Figure 4).

The effect of CD on reduction of cholesterol contents in the GalR2/CHO membranes and the subsequent effect of the reduction on K_D and B_{MAX} were assessed by [^{125}I]galanin saturation binding (Table 2). Pretreatment with CD caused a drastic decrease of cholesterol in the membranes (Table

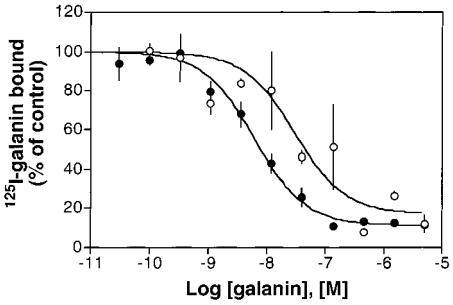

FIGURE 4: Competition [^{125}I]galanin binding to membranes prepared from GalR2/CHO cells grown in FCS− or LPDS-containing medium. 10 μg of membranes prepared from cells cultured in FCS (●) or LPDS (○) and 0.3 nM [^{125}I]galanin was used in each assay. Data are expressed as percent of total binding (2453 ± 45 and 1342 ± 471 cpm (n = 3) for control and LPDS membranes, respectively).

Table 2: Saturation of [^{125}I]Galanin Binding to GalR2/CHO Membranes with Reduced Cholesterol by Treatment with CD[a]

CD (mg/mL)	cholesterol (% of control)	K_D (nM)	B_{MAX} (fmol/mg)
0	100 ± 4	0.35 ± 0.09	112 ± 9
30	53 ± 6	0.55 ± 0.08	124 ± 7
60	39 ± 7	0.93 ± 0.20	131 ± 16

[a] Membranes were treated with CD (0, 30, or 60 mg/mL) to modify the cholesterol contents as described in Materials and Methods. Cholesterol concentrations were determined after the CD treatment (n = 3). The cholesterol content for the control (CD = 0 mg/mL) was 45 μg/mg. Saturation binding experiments to obtain K_D and B_{MAX} were performed using 10 μg of membrane protein/assay, and the data are means ± SD from 2 independent assays each performed in duplicate.

2). Consistent with the results obtained with membranes with reduced cholesterol by growing cells in LPDS-containing medium, the membranes pretreated with CD also displayed a decreased affinity, as shown by increased K_d values of 0.35, 0.55, and 0.93 nM responding to CD pretreatments at 0, 30, and 60 mg/mL, respectively (Table 2). In contrast, the CD treatment of membranes did not cause significant changes for the B_{MAX} values (Table 2).

The factors that influence membrane fluidity include membrane content of cholesterol or a cholesterol analogue and modulators of membrane physical states. First, the effect that a number of agents known to modulate the physical state of biomembranes would have on [^{125}I]galanin binding to GalR2/CHO membranes was assessed. Incubation with ethanol (2%, v/v), known to fluidize biological membranes (*44*−*46*), did not affect binding of [^{125}I]galanin to GalR2/CHO membranes (Figure 5). Preincubation with filipin, known to form filipin−cholesterol complexes in membranes (*47*), effectively inhibited [^{125}I]galanin binding to GalR2/CHO membranes (Figure 5). When the same assay was employed to test a range of concentrations for ethanol, no significant changes for galanin−GalR2 interaction were observed (Table 3) as compared to the sharp response of galanin binding with GalR2 to cholesterol modulation shown in Figures 1 and 2.

Since cholesterol analogues have profound effect on membrane fluidity (*48*), the potential of a number of steroids to restore [^{125}I]galanin binding to cholesterol-depleted membranes was then assessed. Different steroid-CD complexes were generated and incubated with cholesterol-depleted GalR2/CHO membranes (pretreated with 100 mg/mL CD). Steroids 5-cholestene, 5-pregnen-3β-ol-20-one, and 4-cholesten-3-one had no effect on restoring galanin binding to the membranes (Figure 6). Among the agents tested only 5-cholesten-3-one was able to reverse the cholesterol depletion-mediated inhibition of [^{125}I]galanin binding (Figure 6). Like cholesterol, these cholesterol analogues are able to restore the rigidity of biomembranes that have been deprived of cholesterol (*48*). Thus, these results again indicate that GalR2−galanin binding is not dependent on the fluidity of the membranes. Rather, the results suggest that the binding is dependent on the structure of cholesterol since only a limited number of steroids could rescue ligand binding (Figure 6).

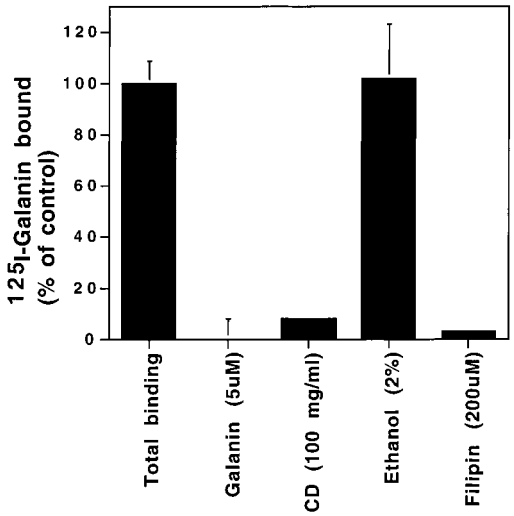

FIGURE 5: Effect of various agents on galanin−GalR2 interaction. [^{125}I]Galanin (0.3 nM) was incubated with 10 μg of GalR2/CHO membranes that had been treated with the indicated concentrations of the agents shown. Total binding = 3412 ± 471 (100%); nonspecific binding = 436 ± 114 dpm (0%) (n = 3).

Table 3: Effect of Ethanol on [^{125}I]Galanin Binding to GalR2/CHO Membranes[a]

ethanol concn (v/v)	relative binding
0	100 ± 13
1.25%	95 ± 5
2.5%	98 ± 14
5%	120 ± 15

[a] 10 μg of GalR2/CHO membranes was incubated with ethanol at indicated concentrations for 20 min at room temperature before addition of 0.3 nM [^{125}I]galanin (final concentration) to start the binding assays (see Materials and Methods for details). Data represent mean ± SD from 2 independent experiments performed in triplicate. The total binding for controls (in the absence of ethanol) was 3146 ± 279 dpm and defined as 100%. The nonspecific binding, determined in the presence of 2 μM unlabeled galanin, was 1033 ± 22 dpm and defined as 0%.

Cholesterol oxidase converts the accessible pool of membrane cholesterol to 4-cholesten-3-one. [^{125}I]Galanin binding to rat GalR2 was quantified in GalR2/CHO membranes pretreated with cholesterol oxidase. In contrast to CD treatment, cholesterol oxidase modifies but does not remove membrane cholesterol. Given that the total amount of cholesterol and 4-cholesten-3-one does not change in the membrane, the fluidity of the membrane remains relatively constant (*48*). Pretreatment of GalR2/CHO membranes with cholesterol oxidase resulted in a dose-dependent decrease in [^{125}I]galanin binding to GalR2 (Figure 7). Under the conditions employed, a 45% decrease in [^{125}I]galanin binding

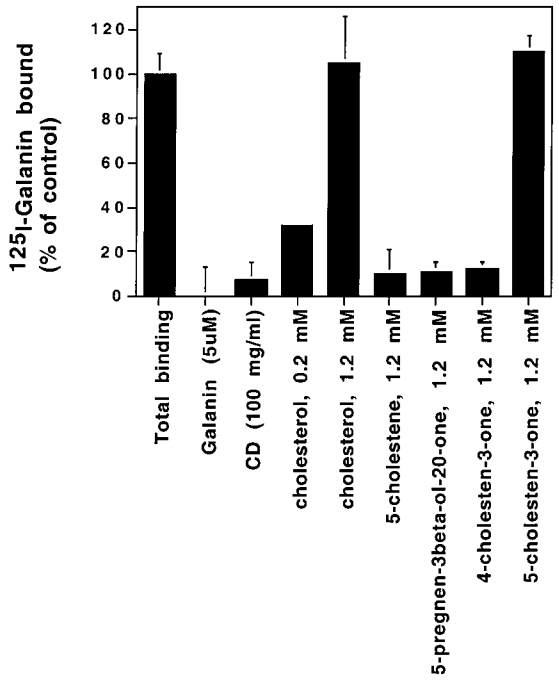

FIGURE 6: Effect of different steroids on the binding activity of cholesterol-depleted GalR2/CHO membrane. Membranes pretreated with 100 mg/mL CD were incubated with 0.2 and 1.2 mM Ch-CD (cholesterol concentration, ratio of steroid to CD = 1:5) or 1.2 mM steroids as indicated. The membranes were centrifuged and washed once with PBS. Galanin binding was determined in membranes using the [^{125}I]galanin binding assay. Data are expressed as percentage of specific binding to control membranes (total binding = 2927 ± 205 dpm (100%); nonspecific binding = 593 ± 192 (0%) (n = 3)).

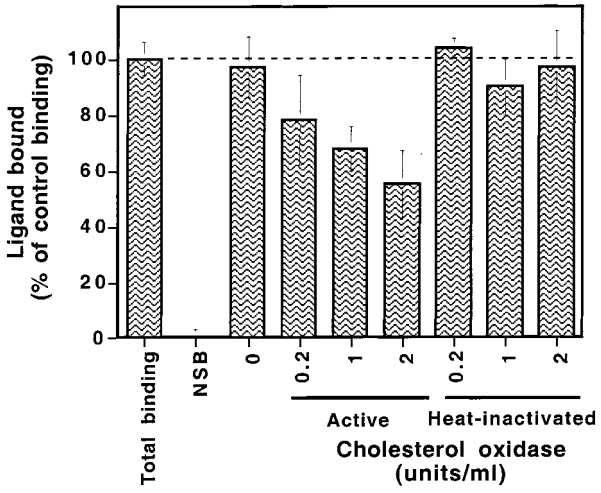

FIGURE 7: Effect of cholesterol oxidation on [^{125}I]galanin binding to GalR2/CHO cell membranes. Freshly dissolved cholesterol oxidase, active or inactivated by boiling for 15 min, was incubated with 10 μg of GalR2 membranes in the presence of 1 mU/mL sphingomyelinase followed by measurement of [^{125}I]galanin binding. Data (mean ± SD, n = 3) are expressed as a percentage of control binding (100%, 1852 ± 102 dpm, n = 3) over the nonspecific binding determined in the presence of 5 μM unlabeled galanin (NSB = 0%, 425 ± 60 dpm, n = 3).

was obtained after pretreament of the membranes with 2 U/mL cholesterol oxidase. (Figure 7). In contrast, incubation with heat-inactivated oxidase did not alter the level of [^{125}I]-galanin binding (Figure 7). Sphingomyelinase, used to facilitate the conversion of cholesterol to 4-cholesten-3-one

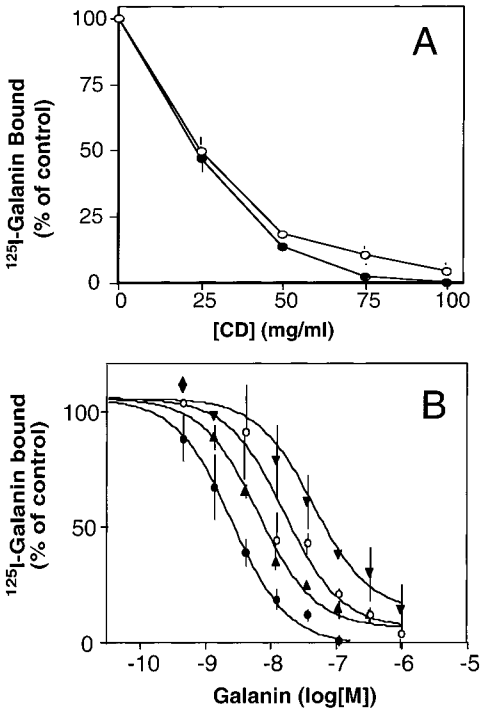

FIGURE 8: Effect of cholesterol and GTP-γ-S on GalR2−galanin binding activity. (A) Radioligand binding to membranes pretreated with CD in the presence (●) or absence (○) of 100 μM GTP-γ-S. 10 μg of membranes and 0.3 nM [^{125}I]galanin were used in the assay. The control bindings in the absence and presence of GTP-γ-S are 2979 ± 62 and 1329 ± 28 cpm (n = 3), respectively. (B) Competition assays to determine the affinity of rat galanin for normal GalR2/CHO membranes (●), membranes pretreated with 20 mg/mL CD (▲), membranes in the presence of 100 μM GTP-γ-S (○), or membranes with both CD and GTP-γ-S (▼).

(*49*), did not affect the ligand binding when used alone (third bar in Figure 7, oxidase at 0 U/mL).

The affinity of a ligand for a G protein-coupled receptor may depend on the functional coupling of the receptor to a G protein (*50*). The affinity of galanin for GalR2/CHO membranes pretreated with CD at various concentrations was measured in the presence of 100 μM GTP-γ-S, a nonhydrolyzable GTP analogue which functionally uncouples receptor from its G protein. Since varying CD creates a cholesterol gradient in the membranes, the experiments aimed to assess the effect of GTP-γ-S (via G protein uncoupling) on GalR2−cholesterol interaction (Figure 8A). The presence of GTP-γ-S did not alter the ability of cholesterol (displayed by CD concentrations in the preincubation) to inhibit [^{125}I]galanin binding to GalR2/CHO membranes (at EC_{50} ~ 23 mg/mL CD) (Figure 8A). In separate experiments, the effects of reduction of membrane cholesterol, GTP-γ-S, and the combination of both on galanin binding to GalR2 were tested in radioligand competition assays. The K_i values of galanin for control GalR2/CHO membranes, for CD-pretreated membranes (20 mg/mL CD which removes 40% of the endogenous cholesterol), and for membranes incubated with GTP-γ-S in the binding assays were 1.8, 4.8, and 11.9 nM, respectively (Figure 8B). The K_i of galanin was 33 nM for membranes with both 20 mg/mL CD pretreatment and 100 μM GTP-γ-S incubation (Figure 8A). These data suggest that the effect of cholesterol on galanin−GalR2 binding is unlikely related to GalR2 coupling with G protein.

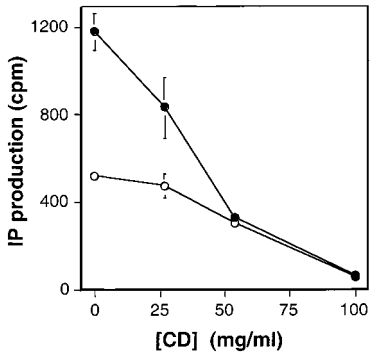

FIGURE 9: Effect of membrane cholesterol on IP production in COS-7 cells. COS-7 cells transfected with rat GalR2 cDNA were treated with CD and stimulated with 200 nM galanin. Galanin-stimulated (●) and basal level of (○) IP production in the cells were determined. Data are mean ± SD ($n = 3$).

Activation of GalR2 results in an increase in intracellular IP production through a pathway involving activation of the Gq/G_{11} G protein(s) (*34*, *35*). The effect of membrane cholesterol on GalR2-mediated IP production in COS-7 cells transiently transfected with rat GalR2 cDNA was determined. In the absence of CD pretreatment, galanin activation of GalR2 results in a ~3-fold increase of intracellular IP over the basal level (Figure 9). CD pretreatment drastically reduced the levels of galanin-mediated increase in intracellular IP. The CD treatment-mediated inhibition was dose-dependent, and the stimulated IP production was completely inhibited at a CD concentration of 50 mg/mL (Figure 9).

DISCUSSION

Ligand-stimulated signaling through G protein-coupled receptors is mediated on a number of different levels. In the present study, we have used GalR2 as a prototypic GalR to demonstrate that membrane cholesterol is crucial for galanin−GalR interaction and GalR-mediated signaling. Three independent approaches were employed to manipulate the cholesterol content of the membranes: removal of membrane cholesterol by preincubation with CD, addition of cholesterol to cholesterol-depleted membranes, and reduction of cholesterol by culturing cells in medium supplemented with LPDS (Table 1). In all three cases, membrane cholesterol is altered and results in a modulation of galanin binding to the GalR2 receptor (Figures 1, 2, and 4; Table 1).

Several lines of evidence suggest that the phenomenon appears to be cholesterol structure-specific but not related to the physical state of the membranes. (A) As major modulators of membrane fluidity, cholesterol analogues added back to cholesterol-deprived membranes were not able to uniformly rescue galanin binding to GalR2 (Figure 6); these analogues were demonstrated to have a general ability to restore the rigidity of CD-treated membranes (*48*). (B) Nonsteroid membrane fluidity modulators, such as ethanol, had no significant effects on galanin binding (Figure 5 and Table 3). In contrast, filipin, which may sequester cholesterol into patches, reduced the level of ligand binding (Figure 6). (C) Oxidation of cholesterol with cholesterol oxidase markedly decreased galanin binding to the receptor (Figure 7). This treatment of cholesterol maintains membrane fluidity by keeping the total amount of cholesterol and 4-cholesten-3-one constant. 4-Cholesten-3-one is the product of the cholesterol oxidation mediated by cholesterol oxidase and is able to sustain membrane fluidity like cholesterol (*48*). Taken together, these results suggest specific structural elements in cholesterol that are critical for its interaction with the GalR2. The effect of cholesterol on GalR1 or GalR3 activity is unknown but may not be excluded.

Cholesterol affects galanin−GalR2 interaction in a positively cooperative manner. A Hill slope of 3.1 suggests that (1) there are multiple binding sites for cholesterol (≥ 3) on the GalR2 and (2) sequential binding of cholesterol to the GalR2 results in a conformational change in GalR2 leading to high-affinity galanin binding. In contrast loss of cholesterol from the membrane sensitively results in an inactive conformation of the GalR2 receptor. One may speculate that the active GalR2 protein species is a homodimer, as is the case with the dimerized estrogen receptor that possesses positive cooperativity for estradiol (*51*). Cholesterol is highly hydrophobic and resides within the membrane bilayer. Cholesterol probably interacts with amino acid residues in the TMs of the GalR2 receptor, separate from the site of G protein coupling (Figure 6) which is thought to involve the intracellular loops (*52*).

The level of membrane cholesterol affects galanin-mediated signaling via GalR2. Reduction of cholesterol by preincubation of cells with CD resulted in decreased intracellular IP production (Figure 9). The data support the concept that cholesterol can regulate cellular response to GalR2 activation by affecting ligand binding. The observation that reduction of membrane cholesterol only affects the affinity of galanin for GalR2, not the numbers of surface GalR2 (Figures 1−4), suggests that the site of cholesterol action on GalR2 is in the plasma membrane and that the modulation of cholesterol levels does not affect synthesis or transport of GalR2 to the plasma membrane. The observation that CD preincubation inhibits galanin binding and galanin-stimulated IP turnover with similar EC_{50} values (25 mg/mL) suggests that cholesterol is critical for maintaining an active GalR2 conformation. However, recent studies have suggested a role for cholesterol in maintaining signaling proteins in cholesterol-rich domains which may be required for proper function of receptor-stimulated IP turnover (*53*). Thus the effect of cholesterol on GalR2 signaling may be a result of the combination of ligand−receptor interaction and phosphatidylinositol bisphosphate delocalization.

The data suggest that modulation of cholesterol metabolism could alter receptor functions such as galanin receptors. Recent studies have implicated roles of abnormal cholesterol metabolism in pathological phenotypes, such as in Alzheimer's disease (AD), which may be caused by one or two apoE4 alleles (*54*, *55*), and in holoprosencephaly, which may result from exposure to vetratrum alkaloids and distal inhibitors of cholesterol synthesis (*56*, *57*). A direct correlation of in vivo galanin activity and cholesterol metabolism has not been established, but galanin hyperinnervation of the basal forebrain and highly elevated density of galanin-immunoreactive axons and terminals on the cholinergic nucleus basalis of Meynert (*58*−*60*) and reduced cholesterol level (*61*, *62*) in AD patients have been observed. In addition, as compared to normal brain, the inhibitory effect of galanin on the Na,K-ATPase activity in tumor brain tissue was reduced to one-half of the control value while the cholesterol-to-phospholipid ratio was doubled (*63*). The present study

has for the first time directly demonstrated that cholesterol can have profound modulatory effects on ligand interaction and receptor activation for one of the defined GalR subtypes and will likely lead to further studies to understand the physiological roles of cholesterol in regulating functions of GalR2 and other neuropeptide receptors.

REFERENCES

1. Tatemoto, K., Rokaeus, A., Jornwall, H., McDonald, T. J., and Mutt, V. (1983) *FEBS Lett. 164*, 124−128.
2. Maiter, D. M., Hooi, S. C., Koenig, J. I., and Martin, J. B. (1990) *Endocrinology 126* (2), 1216−1222.
3. McDonald, T. J., Dupre, J., Tatemoto, K., Greenberg, G. R., Radziuk, J., and Mutt, V. (1985) *Diabetes 34* (2), 192−196.
4. Brown, D. R., Hildebrand, K. R., Parsons, A. M., and Soldani, G. (1990) *Peptides 11*, 497−500.
5. Fox, J. E., Brooks, B., McDonald, T. J., Barnett, W., Kostolanska, F., Yanaihara, C., Yanaihara, N., and Rokaeus, A. (1988) *Peptides 9*, 1183−1189.
6. Katsoulis, S., Clemens, A., Morys-Wortmann, C., Schworer, H., Schaube, H., Klomp, H. J., Folsch, U. R., and Schmidt, W. E. (1996) *Scand. J. Gastroenterol. 31*, 446−451.
7. Maggi, C. A., Patacchini, R., Santicioli, P., Giuliani, S., Turini, D., Barbanti, G., Giachetti, A., and Meli, A. (1990) *Naunyn-Schmiedebergs Arch. Pharmacol. 341*, 256−261.
8. Kwok, Y. N., Verchere, C. B., McIntosh, C. H., and Brown, J. C. (1988) *Eur. J. Pharmacol. 145*, 49−54.
9. Rossowski, W. J., Rossowski, T. M., Zacharia, S., Ertan, A., and Coy, H. H. (1990) *Peptides 11*, 333−338.
10. Schepp, W., Prinz, C., Tatge, C., Hakanson, R., Schusdziarra, V., and Classen, M. (1990) *Am. J. Physiol. 258* (4 Pt 1), G596−G602.
11. Yagci, R. V., Alptekin, N., Rossowski, W. J., Brown, A., Coy, D. H., and Ertan, A. (1990) *Scand. J. Gastroenterol. 25*, 853−858.
12. Wang, S., Ghibaudi, L., Hashemi, T., He, C., Strader, C., Bayne, M., Davis, H., and Hwa, J. J. (1998) *FEBS Lett. 434* (3), 277−282.
13. Crawley, J. N., Austin, M. C., Fiske, S. M., Martin, B., Consolo, S., Berthold, M., Langel, U., Fisone, G., and Bartfai, T. (1990) *J. Neurosci. 10*, 3695−3700.
14. Crawley, J. N. (1996) *Life Sci. 58*, 2185−2199.
15. Wiesenfeld-Hallin, Z., Xu, X. J., Langel, U., Bedecs, K., Hokfelt, T., and Bartfai, T. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89* (8), 3334−3337.
16. Fuxe, K., Ogren, S. O., Jansson, A., Cintra, A., Harfstrand, A., and Agnati, L. F. (1988) *Acta Physiol. Scand. 133* (4), 579−581.
17. Bartfai, T., Bedecs, K., Land, T., Langel, U., Bertorelli, R., Girotti, P., Consolo, S., Xu, X. J., Wiesenfeld-Hallin, Z., Nilsson, S., and et al. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88* (23), 10961−10965.
18. Wang, S., and Parker, E. (1998) *Exp. Opin. Ther. Patents 8* (10), 1225−1235.
19. Habert-Ortoli, E., Amiranoff, B., Loquet, I., Laburthe, M., and Mayaux, J. F. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9780−9783.
20. Howard, A. D., Tan, C., Shiao, L.-L., Palyha, O. C., McKee, K. K., Weiger, D. H., Feighner, S. D., Cascieri, M. A., Smith, R. G., Van Der Ploeg, L. H. T., and Sullivan, K. A. (1997) *FEBS Lett. 405*, 285−290.
21. Wang, S., Hashemi, T., He, C., Strader, C., and Bayne, M. (1997) *Mol. Pharmacol. 52*, 337−343.
22. Wang, S., He, C., Hashemi, T., and Bayne, M. (1997) *J. Biol. Chem. 272*, 31949−31952.
23. Wang, S., He, C., Maguire, M., Clemmons, A., Burrier, R., Guzzi, M., Strader, C., Parker, E., and Bayne, M. (1997) *FEBS Lett. 411*, 225−230.
24. Fathi, Z., Battaglino, P. M., Iben, L. G., Li, H., Baker, E., Zhang, D., McGovern, R., Mahle, C. D., Sutherland, G. R., Iismaa, T. P., Dickinson, K. E. J., and Antal Zimanyi, I. (1998) *Brain Res. Mol. Brain Res. 58* (1−2), 156−169.
25. Pang, L., Hashemi, T., Lee, H.-J. J., Maguire, M., Graziano, P. M., Bayne, M., Hawes, B., Wong, G., and Wang, S. (1998) *J. Neurochem. 71*, 2252−2259.
26. Gustafson, E. L., Smith, K. E., Durkin, M. M., Gerald, C., and Branchek, T. A. (1996) *Neuroreport 7*, 953−957.
27. Smith, K. E., Walker, M. W., Artymyshyn, R., Bard, J., Borowsky, B., Tamm, J. A., Yao, W. J., Vaysse, P. J. J., Branchek, T. A., Gerald, C., and Jones, K. A. (1998) *J. Biol. Chem. 273* (36), 23321−23326.
28. Land, T., Langel, U., and Bartfai, T. (1991) *Brain Res. 558*, 245−250.
29. Bedecs, K., Langel, U., Xu, X. J., Z., W.-H., and Bartfai, T. (1994) *Eur. J. Pharmacol. 259*, 151−156.
30. Harling, H., and Holst, J. J. (1992) *Am. J. Physiol. 262* (1 Pt 1), E52−57.
31. Jureus, A., Lindgren, M., Langel, U., and Bartfai, T. (1998) *Neuropeptides 32* (5), 453−460.
32. Kask, K., Berthold, M., Kahl, U., Nordvall, G., and Bartfai, T. (1996) *EMBO J. 15*, 236−244.
33. Berthold, M., Kahl, U., Jureus, A., Kask, K., Nordvall, G., Langel, U., and Bartfai, T. (1997) *Eur. J. Biochem. 249*, 601−606.
34. Wang, S., Hashemi, T., Fried, S., Clemmons, A. L., and Hawes, B. E. (1998) *Biochemistry 37* (19), 6711−6717.
35. Fathi, Z., Cunningham, A. M., Iben, L. G., Battaglino, P. B., Ward, S. A., Nichol, K. A., Pine, K. A., Wang, J., Goldstein, M. E., Iismaa, T. P., and Zimanyi, I. A. (1997) *Brain Res. Mol. Brain Res. 51* (1−2), 49−59.
36. Wang, S., Clemmons, A., Strader, C., and Bayne, M. (1998) *Biochemistry 37* (26), 9528−9535.
37. Narayanaswami, V., and McNamee, M. G. (1993) *Biochemistry 32* (46), 12420−12427.
38. Nunez, M. T., and Glass, J. (1982) *Biochemistry 21* (17), 4139−4143.
39. Klein, U., Gimpl, G., and Fahrenholz, F. (1995) *Biochemistry 34* (42), 13784−13793.
40. Boesze-Battaglia, K., and Albert, A. D. (1990) *J. Biol. Chem. 265* (34), 20727−20730.
41. Cheng, Y., and Prusoff, W. H. (1973) *Biochem. Pharmacol. 22*, 3099−3108.
42. Christian, A. E., Haynes, M. P., Phillips, M. C., and Rothblat, G. H. (1997) *J. Lipid Res. 38* (11), 2264−2272.
43. Balasubramaniam, S., Goldstein, J. L., Faust, J. R., Brunschede, G. Y., and Brown, M. S. (1977) *J. Biol. Chem. 252* (5), 1771−1779.
44. Steiner, M. (1981) *Biochim. Biophys. Acta 640* (1), 100−105.
45. Moran, J., Salazar, P., and Pasantes-Morales, H. (1987) *Exp. Eye Res. 45* (6), 769−776.
46. Dave, J. R., and Witorsch, R. J. (1986) *Biochem. Biophys. Res. Commun. 134* (3), 1122−1128.
47. Bittman, R., Clejan, S., and Rottem, S. (1983) *Yale J. Biol. Med. 56* (5−6), 397−403.
48. Gimpl, G., Burger, K., and Fahrenholz, F. (1997) *Biochemistry 36* (36), 10959−10974.
49. el Yandouzi, E. H., and Le Grimellec, C. (1992) *Biochemistry 31* (2), 547−551.
50. Kim, M. H., and Neubig, R. R. (1987) *Biochemistry 26*, 3664−3672.
51. Notides, A. C., Lerner, N., and Hamilton, D. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78* (8), 4926−4930.
52. Saar, K., Valkna, A., Soomets, U., Rezaei, K., Zorko, M., Zilmer, M., and Langel, U. (1997) *Biochem. Soc. Trans. 25* (3), 1036−1040.
53. Pike, L. J., and Miller, J. M. (1998) *J. Biol. Chem. 273* (35), 22298−22304.
54. Snipes, G. J., and Suter, U. (1997) *Subcellular Biochemistry* (Bittman, R., Ed.) 28, Plenum Press, New York.
55. Pitas, R. E., Boyles, J. K., Lee, S. H., Foss, D., and Mahley, R. W. (1987) *Biochim. Biophys. Acta 917* (1), 148−161.
56. Cooper, M. K., Porter, J. A., Young, K. E., and Beachy, P. A. (1998) *Science 280* (5369), 1603−1607.
57. Tint, G. S., Irons, M., Elias, E. R., Batta, A. K., Frieden, R., Chen, T. S., and Salen, G. (1994) *N. Engl. J. Med. 330* (2), 107−113.

58. Chan-Palay, V. (1988) *J. Comp. Neurol. 273* (4), 543−557.
59. Mufson, E. J., Cochran, E., Benzing, W., and Kordower, J. H. (1993) *Dementia 4* (5), 237−250.
60. Beal, M. F., MacGarvey, U., and Swartz, K. J. (1990) *Ann. Neurol. 28* (2), 157−161.
61. Montine, T. J., Montine, K. S., and Swift, L. L. (1997) *Am. J. Pathol. 151* (6), 1571−1575.
62. Mason, R. P., Shoemaker, W. J., Shajenko, L., and Herbette, L. G. (1993) *Ann. N. Y. Acad. Sci. 695,* 54−58.
63. Laasik, J., Sillard, R., Langel, U., and Zilmer, M. (1992) *Acta Physiol. Scand. Suppl. 607,* 249−251.

BI990227A