Defect of receptor-G protein coupling in human gallbladder with cholesterol stones

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Xiao, Zuo-Liang, Qian Chen, Joseph Amaral, Piero Biancani, and Jose Behar. Defect of receptor-G protein coupling in human gallbladder with cholesterol stones. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G251–G258, 2000.—Human gallbladders with cholesterol stones (ChS) exhibit an impaired muscle contraction and relaxation and a lower CCK receptor-binding capacity compared with those with pigment stones (PS). This study was designed to determine whether there is an abnormal receptor-G protein coupling in human gallbladders with ChS using [35S]labeled guanosine 5'-O-(3-thiotriphosphate) ([35S]GTPγS) binding, 125I-labeled CCK-8 autoradiography, immunoblotting, and G protein quantitation. CCK and vasoactive intestinal peptide caused significant increases in [35S]GTPγS binding to Ga\textsubscript{13} and G\textsubscript{αα}, respectively. The binding was lower in ChS than in PS (P < 0.01). The reduced [35S]GTPγS binding in ChS was normalized after the muscles were treated with cholesterol-free liposomes (P < 0.01). Autoradiography and immunoblots showed a decreased optical density (OD) for CCK receptors, an even lower OD value for receptor-G protein coupling, and a higher OD for uncoupled receptors or Ga\textsubscript{13} protein in ChS compared with PS (P < 0.001). G protein quantitation also showed that there were no significant differences in the Ga\textsubscript{13} and G\textsubscript{αα} content in ChS and PS. We conclude that, in addition to an impaired CCK receptor-binding capacity, there is a defect in receptor-G protein coupling in muscle cells from gallbladder with ChS. These changes may be normalized after removal of excess cholesterol from the plasma membrane.

MUSCLE STRIPS AND CELLS FROM gallbladders with cholesterol stones (ChS) exhibit an impaired contraction and relaxation in response to agonists that act on transmembrane proteins (5, 7, 17, 35). These abnormal responses, however, were normalized when membrane receptors were bypassed by the G protein activator guanosine 5'-O-(3-thiotriphosphate) (GTPγS), second messengers inositol 1,4,5-trisphosphate and 2,3-diphosphate-d-glycerate, the enzyme calmodulin, or nitric oxide (2, 34, 36). Plasma membranes of muscle from human gallbladders with ChS and prairie dogs on a high-cholesterol diet (1.2%) also showed a high cholesterol content and cholesterol-to-phospholipid ratio (33). These abnormalities were reversed to normal after these defective muscle cells were incubated with cholesterol-free liposomes that leach out the excess cholesterol from the plasma membrane (8, 33). Thus excessive cholesterol incorporation in plasma membranes may affect the signal-transduction cascade across them, which may account for the muscle dysfunction associated with ChS.

Recent studies in our laboratory showed that the binding capacity of CCK receptors in muscle membranes from human gallbladders with ChS was impaired. These findings suggest that the functions of these membrane proteins are affected by excessive cholesterol incorporation (32). This defect may be located in the extracellular domain (for ligand binding), in the intracellular domain (for G protein coupling) of CCK receptors, or in the G proteins themselves (6, 34).

It is known that the interaction of the intracellular domain of receptors and related G proteins can be modulated by the extracellular domain of the receptors (37). Likewise, the binding properties of these receptors can also be influenced by other factors, such as guanine nucleotides (9). G proteins bound to guanine nucleotides decrease receptor-G protein coupling in detergent solutions and in conditions in which the dissociation of receptors from G proteins clearly fails to occur (20).

The mechanisms whereby excess membrane cholesterol affects receptors are not fully understood. The normal distribution of cholesterol in the plasma membrane is asymmetric. Membrane proteins are located in cholesterol-rich domains and cholesterol-poor domains and are even directly associated with cholesterol (27). A normal level of membrane cholesterol is essential for the optimal functional activity of many membrane proteins, such as receptors and ion channels (11). Changes of the cholesterol content in plasma membranes may contribute to the impairment of their functions (12, 19).

CCK acts on CCK-A receptors that activate Ga\textsubscript{13} protein to cause gallbladder muscle contraction (1, 6). Vasoactive intestinal peptide (VIP) relaxes the gallbladder muscle through receptors that couple with G\textsubscript{α0} (6, 14). However, the functional integrity of CCK or VIP receptor coupling to G proteins across the plasma membrane has yet to be demonstrated in human gallbladders with gallstones. Furthermore, there is no direct evidence of how CCK receptors and related G proteins associate in the cell membrane milieu, nor is it...
known whether and how excessive membrane cholesterol affects receptor-G protein coupling.

The aim of these studies therefore was to examine receptor-G protein coupling to further investigate whether the functions of CCK and VIP receptors were affected in muscle cells with excessive membrane cholesterol incorporation from human gallbladders with ChS.

METHODS

Patients. Human gallbladders were obtained by elective laparoscopic cholecystectomy performed for gallstone diseases. None of the patients had a history or clinical evidence of acute cholecystitis. Gallstones were classified as ChS or PS according to their gross appearance and chemical analysis. The gallbladders were kept in ice-cold oxygenated Krebs solution (in mM: 116.6 NaCl, 3.4 KCl, 21.9 NaHCO3, 1.2 NaH2PO4, 2.5 CaCl2, 1.2 MgCl2, and 5.4 glucose). After removal of the serosa and mucosa under a dissecting microscope, the muscle layer was carefully cleaned by further removing the remaining connective tissue and small blood vessels and was cut into strips for further use.

Preparation of cholesterol-free liposomes. Cholesterol-free liposomes were prepared by using egg phosphatidylcholine (3, 16). Phosphatidylcholine (3 ml; 20 mg/ml in chloroform) in a glass test tube was dried under a stream of nitrogen. The dried lipids were suspended in 3 ml of normal saline and sonicated for 30 min with a Branson 2200 sonicator (Branson Ultrasonics, Danbury, CT). The suspension was then centrifuged at 10,000 g for 30 min to sediment the undispersed lipids. Two milliliters of the supernatant and eight milliliters of 0.2% BSA-HEPES buffer (in mM: 24 HEPES, pH 7.4, 112.5 NaCl, 5.5 KCl, 2.0 KH2PO4, 1.9 CaCl2, 0.6 MgCl2, and 10.8 glucose) were mixed to make cholesterol-free liposomes.

Preparation of enriched plasma membranes. Enriched plasma membranes from muscle strips were prepared and purified by sucrose gradient centrifugation (28, 32). Muscle strips were homogenized in sucrose-HEPES buffer using a tissue tearer (Biospec Products, Racine, WI). This sucrose-HEPES buffer contained 0.25 M sucrose, 10 mM HEPES, pH 7.4, 0.01% soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM 1,10-phenanthroline, and 1 mM 2-mercaptoethanol. The homogenates were centrifuged at 600 g for 5 min, and the supernatant was collected in a clean tube and again centrifuged at 150,000 g for 45 min. The pellet was resuspended in sucrose-HEPES buffer, layered over a linear 9–60% sucrose gradient, and centrifuged once more at 90,000 g for 3 h. The plasma membranes were collected at ~24% sucrose and stored at ~70°C.

35S-labeled GTPγS binding and immunoprecipitation. [35S]GTPγS binding was assayed by immunoprecipitation (23). Plasma membranes were solubilized for 1 h at 4°C with 1% CHAPS. Solubilized membranes at a concentration of 2.5 mg protein/ml were incubated with 60 nM [35S]GTPγS in the presence or absence of agonists (CCK or VIP) at 37°C for 10 min in a solution containing (in mM) 10 HEPES, pH 7.4, 0.1 EDTA, and 10 MgCl2. For nonspecific binding, 6 µM GTP was used. The reaction was stopped with 10 volumes of ice-cold Tris buffer (100 mM Tris–HCl, pH 8.0, 10 mM MgCl2, 100 mM NaCl, and 20 µM GTP). Aliquots (200 µl) of the reaction mixture were added to pre-treated ELISA wells. The ELISA wells were initially coated with an anti-rabbit immunoglobulin antibody (1:2,000) followed by specific G protein subunit antibodies (1:2,000) at 4°C for 1 h each. The specific G protein subunit antibodies of anti-Gi1,2, Gi3, Goi, and Gα were used. After incubation at 4°C for 2 h, the wells were washed three times with phosphate buffer (in mM: 1 K2HPO4, pH 7.4, 10 Na2HPO4, 137 NaCl, and 2.7 KCl) containing 0.05% Tween-20. The radioactivity of each well was counted by beta counter. Data were expressed as percent increase over basal levels (without agonist).

Solubilization of the CCK receptor-G protein complex. Membrane (1 mg; 200 µl) was incubated with 280 pM 125I-labeled CCK-8 in phosphate-magnesium buffer (1 mM MgSO4 and 50 mM Na3PO4, pH 7.4) at room temperature for 30 min (total volume 500 µl) (14, 25). The bound complex was obtained by addition of 0.5 ml of phosphate-magnesium buffer and centrifuged at 15,000 rpm (Microcentrifuge, model 235C, Fisher Scientific) for 15 min at 4°C. The pellet was washed with the same buffer and resuspended (250 µl). The crosslinking agent disuccinimidyl suberate (DSS) in DMSO was added (final concentration 5 mM) and incubated at room temperature for 30 min. Then the reaction was quenched by adding 1 M Tris (pH 7.5, final concentration 20 mM) and incubating at room temperature for 10 min. After centrifugation at 15,000 rpm for 5 min, the pellet was washed twice with HEPES buffer and solubilized with 1% Triton X-100 at 4°C for 30 min and again spun at 15,000 rpm for 30 min. The resulting supernatant was incubated with 5 mM bis(sulfosuccinimidyl) suberate (BS3) (crosslinking agent) in HEPES buffer at room temperature for 1 h. The mixture was diluted with SDS-loading buffer (2% SDS, 62.5 mM Tris, pH 6.8, 1% 2-mercaptoethanol, 10% glycerol, and 0.01% bromphenol blue) and separated on 9% SDS-PAGE (Mini-Protein II cell, Bio-Rad, Hercules, CA). Autoradiography was performed to locate the radiolabeled proteins by exposing the gels to a Kodak film for 1–3 days at ~70°C. The desired protein bands were determined by densitometric scanning using the NIH Image analysis system (National Institutes of Health, version 1.44).

Immunoblotting analysis of receptor-G protein coupling. Immunoblotting analysis was performed by a slight modification of a method reported by Kermode et al. (14). Membranes were incubated with 1 µM CCK-8 at room temperature for 30 min and centrifuged at 15,000 rpm (Microcentrifuge, model 235C) for 15 min at 4°C. After the pellet was washed and resuspended (250 µl), the crosslinking agent DSS was added (final concentration 5 mM) and this was incubated at room temperature for 30 min. The reaction was quenched by adding 1 M Tris (pH 7.5, final concentration 20 mM) and incubating at room temperature for 10 min. The mixture was centrifuged at 15,000 rpm for 5 min; the pellet was washed twice and solubilized with 1% Triton X-100, incubated at 4°C for 30 min, and again spun at 15,000 rpm for 30 min. The supernatant was incubated with 5 mM BS3 at room temperature for 1 h. The reaction mixture was diluted with SDS-loading buffer and separated on 10% SDS-PAGE (Mini-Protein II cell). Immunoblotting was performed to locate the desired proteins using a specific antibody against Gi1,3. The Gi protein bands were identified by using enhanced chemiluminescence reagents (ECL kit; Amersham International, Amersham, UK) and quantitated by densitometric scanning using NIH Image 1.44.

G protein quantitation. The contents of Goi,3 and Gα were determined by using a G protein quantitation kit (Cytosignal, Irvine, CA) (15, 21). Membranes were solubilized on ice for 30 min with 1% sodium cholate. The suspension was centrifuged at 13,000 g for 5 min and the supernatant was mixed with SDS-loading buffer (100 µg membrane protein/lane). Pure Goi,3 and Gα subunit standards (5, 10, 20, and 40 ng/lane) were also prepared in the same manner. The samples were boiled and subjected to 10% SDS-PAGE (Mini-Protein II cell). The separated proteins were electrically trans-
ferred to nitrocellulose membrane (Bio-Rad). The membrane was blocked with blocking solution, which consists of 5% nonfat dried milk, 0.01% antiform A, 0.02% sodium azide, and 0.02% Tween-20 in PBS (in mM: 80 Na2HPO4, pH 7.5, 20 NaH2PO4, and 100 NaCl) at room temperature for 1 h. Then the membrane was incubated with anti-G protein subunit antibodies (anti-Go1,3 and anti-Go, separately, 1:2,000 dilution) in a blocking solution at room temperature for another hour. The membranes were washed three times with a blocking solution without nonfat dried milk and incubated with horseradish peroxidase-conjugated protein A (1:2,000 dilution) in blocking solution at room temperature for 1 h. The G protein bands were identified by using the ECL kit. Quantitation of the immunoblots was performed by densitometric scanning of the bands by means of NIH Image 1.44. The standard curves of G proteins for Go1,3 and Go were obtained by plotting the band densities against standard G protein concentrations. The contents of Go1,3 and Go in muscle membranes were then calculated from the band densities of the standard curves and expressed as nanograms per milligram of membrane protein.

Protein determination. Protein content in plasma membranes was measured by using the Bio-Rad protein assay kit. Values are means of triplicate measurements of each sample.

Materials. Bolton-Hunter-labeled 125I-CCK-8 (2,200 Ci/mmol) and [35S]GTPγS (1,250 Ci/mmol) were from DuPont NEN; CCK-8 and VIP were from Bachem (Torrance, CA); DSS and BS3 were from Pierce (Rockford, IL); G protein subunit antibodies and G protein quantitation kit were purchased from CytoSignal (Irving, CA); horseradish peroxidase-conjugated protein A and ECL kit were from Amersham; soybean trypsin inhibitor was from Worthington Biochemicals (Freehold, NJ); egg phosphatidylcholine and other reagents were purchased from Sigma Chemical (St. Louis, MO).

Statistical analysis. Results are expressed as means ± SE. Statistical significance was evaluated by using Student’s t-test for unpaired and paired values. P < 0.05 was considered significantly different.

RESULTS

In agreement with previous reports (22), Western blots showed the presence of a full complement of G protein subunits Go1,2, Go11, Go1,3, and Go, in gallbladder muscle (data not shown). The immunoprecipitation of [35S]GTPγS-G protein complex using specific G protein subunit antibodies revealed similar basal levels (without agonist stimulation) of [35S]GTPγS activity bound to different G protein subunits in muscle membranes from human gallbladders with ChS and PS (Fig. 1).

The interaction between receptors and their G proteins was first determined by [35S]GTPγS binding. CCK-8 at 1 µM increased the binding of [35S]GTPγS to Go1,3 up to 77.8 ± 2.7% in muscle membranes from gallbladders with PS. The magnitude of [35S]GTPγS bound to Go1,3 was significantly lower in gallbladders with ChS (32.8 ± 8.5%) than in those with PS (Fig 2, P < 0.01). The CCK receptor only coupled to Go1,3 because there was no change in [35S]GTPγS bound to Go1,2, Go11, or Go. VIP at 1 µM also increased the [35S]GTPγS binding to Go in gallbladders with PS by 88.2 ± 9.0%, compared with 36.5 ± 9.4% in gallbladders with ChS (Fig. 3, P < 0.01). These findings suggest that receptor-G protein activation stimulated by agonists is impaired in human gallbladders with ChS.

As shown in our previous studies (8), muscle membranes from human gallbladders with ChS exhibit excessive cholesterol content and abnormal cholesterol-to-phospholipid ratio. To determine whether the defective receptor-G protein activation was related to excessive membrane cholesterol content, muscle strips from human gallbladders with ChS were pretreated with cholesterol-free liposomes. CCK-induced [35S]GTPγS bound to Go1,3 was significantly increased, from 30.5 ± 5.7% (untreated) to 54.9 ± 5.4% after treatment with cholesterol-free liposomes (Fig 4, P < 0.01). Similarly, the [35S]GTPγS binding to Go induced by VIP was also increased (from 43.2 ± 2.3% to 66.3 ± 1.2%) (Fig 5, P < 0.01). These findings support our hypothesis that the impaired receptor-G protein coupling induced by agonists in human gallbladders with ChS is due to excessive membrane cholesterol content.

Fig. 1. Basal 35S-labeled guanosine 5’-O-(3-thiotriphosphate) (35S)GTPγS binding level (without agonist stimulation) of G proteins in muscle membranes from gallbladders with cholesterol stones (ChS) (open bars, n = 4) and pigment stones (PS) (closed bars, n = 3). Membranes from both groups were incubated with [35S]GTPγS. Bound components were precipitated by specific G protein subunit (Go1,2, Go11, Go1,3, and Go) antibodies and counted in beta counter. There were no significant differences in [35S]GTPγS binding activity between these two groups.

Fig. 2. CCK-induced [35S]GTPγS binding in gallbladder muscle with ChS (open bars, n = 4) and PS (closed bars, n = 4). Membranes from both groups were incubated with [35S]GTPγS and 1 µM CCK-8. Bound [35S]GTPγS was precipitated by specific G protein subunit antibodies and counted in beta counter. Data were expressed as percent increase of binding from basal level. CCK receptor coupled with Go1,3 and CCK-induced [35S]GTPγS binding were significantly lower in human gallbladders with ChS than those with PS. Values are means ± SE; *P < 0.01.
To examine the nature of this impairment, covalent crosslinking of \( ^{125}\)I-CCK-8 to its receptors was performed using the crosslinking agent DSS and followed by electrophoretic analysis and autoradiography (Fig. 6A). A single radioactive band of 92 kDa for CCK receptors was found in human gallbladders with PS and ChS. The density of the band from gallbladder with ChS was lower than that from PS (lanes 1 and 3). These bands were eliminated completely by the addition of 1 \( \mu \)M unlabeled CCK-8 (lanes 2 and 4).

The interaction of CCK receptors with their related G proteins was examined by adding the crosslinking reagent BS\(^3\) after the membranes were solubilized with Triton X-100 (G proteins are insoluble in regular buffer) (Fig. 6B). Two bands of 92 kDa for CCK receptors and 136 kDa for the ligand receptor-G protein complex were detected in each lane (lanes 1–3). A higher density of the 136-kDa band and a lower density of the 92-kDa band (uncoupled with G proteins) was 12.4 in ChS, which is higher than that for PS (3.4 after the membranes were treated with the crosslinking agent BS\(^3\) (Fig. 7B), the OD of the 136-kDa band (ligand receptor-G protein complex) was 3.0 ± 2.4 for ChS (lane 3), which is lower than that for PS (lane 1) (15.4 ± 0.2, \( P < 0.001 \)). In contrast, the OD of the 92-kDa band (uncoupled with G proteins) was 12.4 ± 1.4 for ChS, which is higher than that for PS (3.4 ± 1.1, \( P < 0.001 \)). These findings suggest lower receptor-G protein coupling in muscle membranes from gallbladders with ChS. Pretreatment of plasma membranes with GTP\(\gamma\)S (lane 2) also affected the receptor-G protein interaction in PS (OD of 136-kDa band decreased from 15.4 ± 0.2 to 10.7 ± 1.4, \( P < 0.05 \)).

Moreover, the assumption that less receptor-G protein coupling occurred in ChS was further supported by the fact that the addition of GTP\(\gamma\)S caused a significant decrease in the density of the 136-kDa band in PS (lane 2). These results are in complete agreement with previous studies (9).

The optical density (OD) of these bands is shown in Fig. 7. The OD of the 92-kDa band for the ligand receptor complex was 20.3 ± 4.3 for ChS, which was lower than that for PS (45.7 ± 3.2, \( P < 0.01 \)) (Fig. 7A). After the membranes were treated with the crosslinking agent BS\(^3\) (Fig. 7B), the OD of the 136-kDa band (ligand receptor-G protein complex) was 3.0 ± 2.4 for ChS (lane 3), which is lower than that for PS (lane 1) (15.4 ± 0.2, \( P < 0.001 \)). In contrast, the OD of the 92-kDa band (uncoupled with G proteins) was 12.4 ± 1.4 for ChS, which is higher than that for PS (3.4 ± 1.1, \( P < 0.001 \)). These findings suggest lower receptor-G protein coupling in muscle membranes from gallbladders with ChS. Pretreatment of plasma membranes with GTP\(\gamma\)S (lane 2) also affected the receptor-G protein interaction in PS (OD of 136-kDa band decreased from 15.4 ± 0.2 to 10.7 ± 1.4, \( P < 0.05 \)).

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immunoblotting analysis (Fig. 8). Similar density of the band for G\(_{\alpha i-3}\) alone (42 kDa) was determined both in ChS and PS (Fig. 8A); however, a lower density of the ligand receptor-coupled G\(_{\alpha i-3}\) band (136 kDa) and a higher uncoupled G\(_{\alpha i-3}\) band (42 kDa) were observed in muscle membranes from gallbladders with ChS compared with those in muscle membranes from gallbladders with PS (Fig. 8B). A density scan (Fig. 9) showed a similar OD value of the G\(_{\alpha i-3}\) band in ChS and PS (Fig. 9A) but a much lower OD value of 3.2 ± 1.4 for ligand receptor-G protein complex and a higher OD of 8.4 ± 2.4 for uncoupled G\(_{\alpha i-3}\) in ChS compared with that in PS (14.4 ± 3.2 and 2.4 ± 1.1; \(P < 0.001\) and \(P < 0.05\) vs PS, respectively) (Fig. 9B). These results indicate that the receptor-G protein interaction after agonist stimulation is impaired in muscle membranes from human gallbladders with ChS.

To determine if this impaired receptor-G protein interaction was caused by quantitative changes of the G proteins, the contents of G\(_{\alpha i-3}\) and G\(_{\alpha \alpha}\) proteins were measured. Recombinant G\(_{\alpha i-3}\) (43.2 kDa) and G\(_{\alpha \alpha}\) (48.5 kDa) His-tagged proteins were used as the standards to calculate the contents of G\(_{\alpha i-3}\) and G\(_{\alpha \alpha}\). The immunoblots clearly showed one 42-kDa band for G\(_{\alpha i-3}\) (Fig. 10) and two bands of 47-kDa and 45-kDa for G\(_{\alpha \alpha}\) (Fig. 11). These immunoblots also showed a dose-dependent increase in the band density, with increased concentrations of G protein standards. The G protein contents were calculated from standard curve (OD/mg protein) and OD value of the desired G protein band (Fig. 12). No significant differences in the contents of G\(_{\alpha i-3}\) and G\(_{\alpha \alpha}\) were observed in ChS and in PS.

**DISCUSSION**

Although previous studies have shown an impaired muscle contraction and relaxation in human and animal gallbladders with ChS (1, 5, 18, 24), the mechanisms responsible for this defect are still not fully understood. We have shown that muscle cells from gallbladders with ChS have an abnormal CCK receptor-binding capacity (32) and a normal signal-transduction cascade distal to the activation of G proteins (34, 36). This abnormal CCK receptor-binding capacity may be due to the presence of an excessive cholesterol content, high cholesterol-to-phospholipid ratio, and decreased membrane fluidity (8), which may directly or indirectly
those from gallbladders with PS. The decrease in CCK-8 or VIP was significantly reduced in muscle muscle with ChS. These abnormalities are not confined to a functional defect of the intracellular domain of the receptors where G protein couples, since there was no decreased receptor-G protein coupling is probably due to the reduced receptor-binding capacity because the magnitude of the receptor-G protein coupling was even lower than that of ligand receptor binding in ChS compared with those from gallbladders with PS. The decrease in CCK-8-induced $[^{35}\text{S}]$GTP$\gamma$S binding cannot be completely explained by the reduced receptor-binding capacity because the magnitude of the receptor-G protein coupling was even lower than that of ligand receptor binding in ChS compared with those in PS. Second, the decreased receptor-G protein coupling is probably due to a functional defect of the intracellular domain of the receptors where G protein couples, since there was no significant change in G protein content of gallbladder muscle with ChS. These abnormalities are not confined to the receptor-G protein cascade that mediates muscle contraction, since VIP-induced GTP$\gamma$S bound to G$\alpha$ that mediates muscle relaxation was also affected. These abnormal receptor-G protein couplings appear to be related to the presence of excessive cholesterol incorporation in the plasma membrane because they were normalized after incubation with cholesterol-free liposomes.

Like other G protein-coupled receptors, the actions of CCK and VIP are mediated through their receptors and related G proteins. The peptide sequences of these receptors have seven putative transmembrane domains that are largely constituted of hydrophobic amino acids and three hydrophilic loops. The COOH-terminal portion of the third cytoplasmic loop (Ci-3) of these receptors contains a stretch of charged residues that are thought to form an amphipathic $\alpha$-helical extension of the sixth transmembrane domain in a critical orientation for G protein activation (13, 30). Moreover, the first intracellular loop of these receptors may also play a role in receptor-G protein coupling (31). Although receptors may contact with both the $\omega$-subunit and $\beta$-dimer of the G protein, the $\alpha$-subunit of G protein was shown to play a critical role in determining the specificity of receptor-G protein coupling by interacting with certain motifs within a cavity formed by the third intracellular loop of the receptors (4).

The mechanisms whereby cholesterol-lipid or cholesterol-protein interactions might cause these alterations in the muscle membrane are not completely clear. However, there are a number of observations that provide insights that may be relevant to our findings. Incorporated free cholesterol is inserted in the bilayer of the plasma membrane and can move freely in the plasma membrane or be physically associated with sphingomyelin (29). Gimpl et al. (11) reported that ligand binding of CCK receptors was strongly dependent on the level of the membrane fluidity. Excessive cholesterol incorporation of plasma membrane affects not only the membrane fluidity but also a variety of other properties of the membrane bilayer (thickness, curvature, dipole potential, and so forth) as well as membrane protein functions (25). It is conceivable that excessive cholesterol incorporation may interact directly with transmembrane proteins by fitting between the grooves of the $\alpha$-helices and may restrict the conformational changes or increase the distance between transmembrane domains of the receptor (10). Therefore, the signal-transduction cascade through receptor and G protein could become more difficult. These may explain the decreased CCK receptor-G protein coupling in muscle membranes from human gallbladders with ChS. These results are in complete agreement with our previous findings in gallbladder muscle from specimens with ChS and PS (1, 2, 7, 8, 33, 35, 36).

The present studies also showed that the impairment of agonist-induced $[^{35}\text{S}]$GTP$\gamma$S binding could be reversed to normal when the muscle strips were incubated with cholesterol-free liposomes. Our previous studies demonstrated that cholesterol-free liposomes

![Fig. 11. Quantitation of G$\alpha$ in muscle membranes from human gallbladders with PS and ChS. Purified recombinant G$\alpha$ (lane 1, 5 ng; lane 2, 10 ng; lane 3, 20 ng; and lane 4, 40 ng; lane 5 and lane 7 were avoided) were subjected to SDS-PAGE along with membranes from PS (lane 6) and ChS (lane 8). Samples were subsequently immunoblotted for G$\alpha$ using anti-G$\alpha$ antibody. Arrows indicate molecular mass of G$\alpha$ (left: recombinant standard, 48.5 kDa; right: in tissue, 47 and 45 kDa).](http://ajpgi.physiology.org/10.1152/ajpgi.00034.2006)
may leach out excess cholesterol from plasma membranes, resulting in normal cholesterol content, cholesterol-to-phospholipid ratio, and membrane fluidity as well as agonist-induced muscle cell contraction and relaxation (8, 33). These findings, therefore, suggest that, although the membrane receptors are quantitatively normal, a percentage of them are functionally defective because of the excessive amount of cholesterol or because of increased membrane stiffness. The findings that the gallbladder muscle contraction and relaxation are reversible may be due to the recovery of receptor functions. Thus impairments of membrane receptor functions by excessive cholesterol incorporation may be the key factor for the defective muscle contraction and relaxation in human gallbladders with ChS.

In conclusion, our study showed that the interaction of membrane receptor and related G protein was impaired in human gallbladders with ChS compared with those with P5. These changes may be due to membrane receptor dysfunction caused by excessive membrane cholesterol incorporation since the G protein content is not affected. Defective membrane receptor functions might be the leading cause of the impaired muscle contraction and relaxation of human gallbladders with ChS. Removal of excessive membrane cholesterol by cholesterol-free liposomes normalized the impaired functions of receptor-G protein coupling and muscle contraction and relaxation.

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