Activation of cardiac muscarinic acetylcholine receptors (mACHR) on cultured chick heart cells results in a decrease in cellular cAMP levels and a stimulation of phosphoinositide breakdown. A serum-free culture system has been used to investigate the regulation of mACHR number and function by purified serum high density lipoprotein (HDL). Administration of HDL purified from rooster serum to chick heart cells cultured in defined medium results in an attenuation of the ability of muscarinic agonist to inhibit forskolin-stimulated cAMP accumulation, with no change in its ability to stimulate phosphoinositide hydrolysis or to mediate down-regulation of receptor number. The inclusion of HDL in the culture medium did not result in appreciable changes in mACHR number or affinity, nor were the levels of the inhibitory guanine nucleotide-binding regulatory proteins (G-proteins) altered. However, the ability of guanine nucleotides to inhibit forskolin-stimulated adenylate cyclase activity was reduced by HDL treatment, suggesting that HDL interferes with the capacity of G-proteins to interact with adenylate cyclase. In order to determine which component of native HDL mediates the decreased effectiveness of carbachol, the ability of lipid and apoprotein fractions to mimic the effect of HDL was tested. HDL lipid fractions were able to mimic the effect of native HDL, while protein fractions were not. This result suggests that the ability of HDL to attenuate muscarinic receptor function is mediated by its lipid constituents. The effect of HDL and LDL lipid fractions were not correlated with changes in membrane cholesterol content.

Cardiac muscarinic acetylcholine receptors (mACHR) mediate the decreased rate and force of contraction observed in response to stimulation of the vagus nerve. The decrease in heart rate following mACHR activation is due in part to the opening of a receptor-gated inward rectifying potassium channel, while the decrease in cardiac contractility arises from diminished calcium entry (Loffelholz and Pappano, 1985). Biochemical consequences of mACHR activation include an increase in cellular cGMP, a decrease in cellular cAMP, and an increase in phosphoinositide hydrolysis (reviewed in Nathanson, 1987). Receptor-mediated changes in cellular cAMP and cGMP have been implicated in the mediation of the negative inotropic response caused by muscarinic agonists (Fischmeister and Hartzell, 1986; Hartzell and Fischmeister, 1986). Stimulation of phosphoinositide hydrolysis results in the production of additional second messengers, including diacylglycerol, which activates protein kinase C, and inositol triphosphate, which causes the release of intracellular calcium (Berridge and Irvine, 1984). The mACHR produces these biochemical effects via interactions with the guanine nucleotide regulatory proteins (G-proteins) G\textsubscript{i}, G\textsubscript{o}, and the as yet unidentified G-protein which couples to phospholipase C (Fiorio and Sternweis, 1985; Hepler and Harden, 1986; Martin et al., 1988).

Chick heart cells represent a convenient model system in which to study the regulation of cardiac mACHR function because they are easily cultured, because they readily respond to muscarinic agonists, and because their environment can be controlled. We have previously demonstrated that chick heart cells can be cultured in fully defined, serum-free medium with retention of mACHR-mediated responses (Subers and Nathanson, 1988). Thus, cells cultured in defined medium respond to the agonist carbachol with an inhibition of cAMP accumulation, a stimulation of phosphoinositide metabolism, and a down-regulation of mACHR number. This culture system allows for the investigation of the role of serum components in the regulation of mACHR function, without the complications arising from the use of undefined serum-supplemented culture medium. The sensitivity to the muscarinic agonist carbachol can vary over two orders of magnitude depending on the serum lot used to supplement the medium in which the cells are cultured (Hunter and Nathanson, 1986, 1986) indicating that serum constituents may regulate mACHR function. In addition, cells cultured in serum-free defined medium tend to show a greater inhibition of cAMP accumulation and a greater stimulation of phosphoinositide metabolism in response to mACHR activation than do cells cultured in the presence of 5% fetal calf serum (Subers and Nathanson, 1988). More specifically, Renaud et al. (1982) demonstrated that chick ventricular cells grown in fetal calf serum depleted of lipoproteins responded to the muscarinic agonist oxotremorine with a decrease in beating rate, while those cultured in medium supplemented with complete fetal calf serum did not. Haigh et al. (1988) reported that the increased negative chronotropic response of chick atrial cells cultured in lipoprotein-deficient serum could be reversed by...
the addition of bovine LDL. These results suggested that serum lipoproteins may decrease mAChR responsiveness in cultured chick heart cells. In order to test the role of serum lipoproteins on cardiac mAChR function in a defined, homologous system, we determined the effect of purified chicken lipoproteins on mAChR number and function in chick heart cells cultured in serum-free defined medium, using biochemical assays, which are more defined and specific than physiologic measurements such as beating rate responses. In this report we demonstrate that serum high density lipoprotein (HDL) decreases the ability of carbobito to inhibit forskolin-stimulated cAMP accumulation, without affecting its ability to stimulate phosphoinositide metabolism or agonist-mediated loss of cell surface receptors. HDL-mediated inhibition of mAChR responsiveness appears to result from a diminished ability of Gt to couple with adenylate cyclase in membranes prepared from HDL-treated cells. This effect of HDL was mimicked by the lipid fraction of native HDL and not by the apoprotein fraction, suggesting that alterations in membrane lipid composition may mediate the decrease in mAChR responsiveness observed after HDL treatment.

**EXPERIMENTAL PROCEDURES**

**Materials**—White leghorn chicken eggs were obtained from College Biological Supply (Bothell, WA) and maintained in a humidified 38 °C incubator until the ninth incubation day. White leghorn roosters, age 20–22 weeks, were obtained from H & N International (Redmond, WA), and fed a standard laboratory diet ad libitum, until the day before use at which time they were restricted to water only. Tissue culture materials were obtained as described previously (Subers and Nathanson, 1988). [3H]-N-methylscopolamine ([3H]-NMS, 72–95 Ci/mmol), [3H]quinuclinyl benzilate ([3H]QNB, 35–44 CI/mmol), and [3H]myo-inositol (16–20 CI/mmol) were obtained from Amersham Corp. [3H]Acetate and [3H]ATP (3000 Ci/mmol) were from Du Pont-New England Nuclear. The ion exchange resin AG50W-X4 (200–400 mesh, hydrogen form) and AG1-X8 (100–200 mesh, formate form), used in cAMP accumulation and adenylate cyclase assays or in phosphoinositide hydrolysis assays, respectively, were from Bio-Rad. Reagents for the development of Western blots were obtained as described by Luetje et al. (1987). Antisera AS7 and RV3 were a generous gift of Dr. Allen Spiegel (National Institutes of Health). All other materials were purchased as described previously (Halvorsen and Nathanson, 1986; Luetje et al., 1987).

**Cell Culture**—Chick heart cells were cultured in serum-free defined medium from 9-day embryonic chickens as described previously (Subers and Nathanson, 1988). The medium, based on that of Libby (1984) and referred to as ITs, was M199, supplemented with penicillin-streptomycin (100 units/ml and 100 μg/ml final concentrations, respectively), insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (5 μg/ml), vitamin B12 (10 μg/ml), and triliodothyronine (3 nM). The medium was changed on the third day in culture, and experiments were performed on the fourth. In general, four dozen hearts were dissected, dissociated, and plated on either eight 100-mm plates for cAMP accumulation assays, or sixty to seventy 35-mm plates for phosphoinositide and intact cell binding assays.

**Purification of Lipoproteins**—Roosters were anesthetized with 2–3 ml of pentobarbital and 30–50 ml of ice cold solution was drawn from the jugular vein. Serum was prepared from clotted blood, and high density lipoproteins were purified from serum by flotation on KBr-NaCl gradients by the method of Chapman (1981) as described by Hermier et al. (1985). Fractions containing HDL or LDL were identified by silver-stained SDS-polyacrylamide gel electrophoresis as described previously (Halvorsen and Nathanson, 1984). The medium, based on that of Libby (1984) and referred to as ITs, was M199, supplemented with penicillin-streptomycin (100 units/ml and 100 μg/ml final concentrations, respectively), insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (5 μg/ml), vitamin B12 (10 μg/ml), and triliodothyronine (3 nM). The medium was changed on the third day in culture, and experiments were performed on the fourth. In general, four dozen hearts were dissected, dissociated, and plated on either eight 100-mm plates for cAMP accumulation assays, or sixty to seventy 35-mm plates for phosphoinositide and intact cell binding assays.

**Phosphoinositide Hydrolysis Assays**—Cells were incubated overnight in 1 ml of growth medium containing 1 μCi of [3H]myo-inositol on the third culture day, and experiments were performed the following day as described previously (Subers and Nathanson, 1985). Briefly, cells were rinsed three times with physiological saline solution. (118 mM NaCl, 4.7 mM KCl, 3 mM CaCl2, 1.2 mM KH2PO4, 10 mM glucose, 0.3 mM EDTA, 20 mM HEPES, pH 7.4) at 37 °C, preincubated with the same containing 10 mM LiCl for 30 min, treated as indicated for an additional 15 min, and the inositol phosphates (Ins-
P's produced were isolated and quantitated as described by Masters et al. (1984).

**Quantitative Immunoblot Assays**—Culture plates were rinsed, scraped, and homogenized on ice with 20 mM NaPO₄, 150 mM NaCl containing 0.4 mM phenylmethylsulfonyl fluoride, 1 mM 1,10-phenanthroline, 1 mM iodoacetamide, and 1 μM pepstatin A. Membranes were prepared as described above and stored at -70°C until use. Aliquots of membrane samples were pelleted in a microcentrifuge, resuspended in SDS sample buffer, and run on 9% SDS-polyacrylamide gels according to the method of Laemmli (1970). G-protein standards were run in duplicate on the same gels as were triplicate samples of membranes from control and treated cells. Proteins were then electrophoretically transferred to nitrocellulose by the method of Towbin et al. (1979). The nitrocellulose was allowed to dry, and then either stained with Amido Black to determine the total protein present, or incubated in 10% bovine hemoglobin for 1 h. Hemoglobin-blocked blots were rinsed with distilled water and incubated overnight at room temperature with affinity purified primary antisera diluted with 100 μl of 1 M Tris-HCl, pH 8.0, containing 0.2 mg/ml p-nitrophenyl phosphate. After 10-20 min, color development was stopped by the addition of 100 μl of K₂HPO₄, and the optical density determined at 410 nm. By comparing the optical density of known amounts of G-protein subunit standards to that of the samples, the amount of G-protein subunit in the sample could be determined.

**Membrane Cholesterol Assays**—Membranes were prepared in 50 mM NaPO₄ buffer as described above and stored at -20°C until the day of use. Cholesterol content was determined by the cholesterol oxidase method as described by Oram (1986).

**RESULTS**

**Purification of HDL**—Lipoprotein fractions containing apoprotein A-I, the major apoprotein in HDL, were identified by SDS-gel electrophoresis and pooled to yield the final HDL or LDL preparation. The apoprotein content of these preparations was then analyzed by silver-stained SDS-polyacrylamide gels electrophoresis as shown in Fig. 1. Panel A demonstrates that the major HDL apoprotein band migrates at Mr 24,300-25,700 on a 15% gel, agreeing with the reported Mr of rooster apoA-I of 27,000-28,000 obtained in a different gel system (Hermier et al., 1985). The density of the fractions pooled for the final HDL preparation ranged from 1.08 to 1.17 g/ml. Minor bands are observed at Mr 14,000-15,000, reported previously to be apolipoproteins present in HDL fractions (Hermier et al., 1985). Faint bands are seen at Mr > 60,000 but are likely to be contaminants in the gel buffers as they appear in many gel lanes, regardless of the sample content. These results indicate that HDL was purified to near homogeneity from rooster serum. Panel B, lane 1, shows the typical band profile for pooled LDL fractions, which ranged in density from 1.04 to 1.06 g/ml. The major LDL apoprotein is apoprotein B-100 (arrow), which has a reported Mr of 410,000 (Hermier et al., 1985). HDL apoprotein A-I is present in the pooled LDL fractions. Lane 2 shows that the HDL preparations are free of contaminating LDL, as no apoprotein B-100 is observed in the HDL lane. LDL preparations devoid of contaminating HDL could not be obtained using this purification scheme.

**Effect of HDL on mAChR-mediated Responses**—Renaud et al. (1982) observed that chick heart cells cultured in the absence of lipoproteins responded to the agonist oxotremorine with a decrease in beating rate, while cells cultured in their presence did not. This result suggests that serum lipoproteins can inhibit mAChR-mediated responses. More recently, Haigh et al. (1988) demonstrated that LDL derived from fetal calf serum could reverse the increased responsiveness of chick heart cells cultured in lipoprotein-deficient serum. We wanted to determine whether purified serum lipoproteins could alter mAChR responsiveness in a defined culture system that was amenable to biochemical manipulation. Purified LDL or HDL was added to defined medium and the ability of the agonist carbachol to inhibit forskolin-stimulated cAMP accumulation was tested. The final concentrations of LDL and HDL tested, 13 μg/ml and 25-26 μg/ml, respectively, were chosen because these would be equivalent to the final concentrations which would be attained if the cells were cultured in medium supplemented with 5% rooster serum. The results, shown in Fig. 2, indicate that both serum HDL and LDL inhibit the maximal response to carbachol and increase the concentration necessary for half-maximal inhibition (EC₅₀). Because LDL was somewhat less effective than HDL in attenuating the response to carbachol, and because LDL was not available free of contaminating HDL, subsequent experiments were performed using HDL. Concentration-response curves for carbachol demonstrate that the maximum inhibition of cAMP accumulation is 62% in control cells, and 38% in HDL-treated cells, while the EC₅₀ for carbachol in control and HDL-treated cultures are 0.3 and 3 μM, respectively (Fig. 3). In order to determine whether the ability of HDL to attenuate mAChR-mediated inhibition of cAMP accumulation arose from the increased protein content of the HDL-supplemented culture medium, the effect of the addition of a nonspecific protein, hemoglobin, was tested. When equivalent amounts of bovine hemoglobin were added to the culture medium, no significant attenuation in carbachol-mediated inhibition of forskolin-stimulated cAMP accumulation was observed (data not shown). This result indicates that a general increase in the protein content of the culture medium does not explain the capacity of serum lipoproteins to inhibit mAChR responsiveness. Cultured chick heart cells respond to mAChR activation with an increase in phosphoinositide metabolism. In order to
The observed differences in response to carbachol for cells cultured in the presence and absence of HDL were statistically significant at all concentrations of carbachol tested (p < 0.05).

determine whether this pathway was also affected by HDL treatment of the cells, we measured the ability of carbachol to stimulate PI turnover in cells that had been cultured in the presence and absence of HDL. The maximum increases in [3H]Ins-Ps produced by cells cultured in the absence and presence of HDL were 378 and 26 pg of HDL/ml medium (0), respectively (Fig. 4). These results suggest that mACHR-mediated inhibition of phosphoinositide turnover or down-regulation in control and in HDL-treated cultures was examined. The maximum decrease in cell surface mACHR number, as measured by [3H]NMS binding to intact cells, was 68% in control cultures and 67% in HDL-treated cells, and the EC50 for carbachol was also equivalent (Fig. 5). These results indicate that HDL treatment does not alter the ability of agonist to regulate mACHR number.

The observations that HDL treatment does not affect carbachol-mediated stimulation of phosphoinositide turnover or down-regulation suggest that HDL treatment does not cause a general decrease in receptor-mediated function. Thus, HDL appears to selectively attenuate mACHR-mediated inhibition of cAMP accumulation.

**Determination of the Site of Action of HDL—Muscarinic Receptor-mediated inhibition of cAMP accumulation requires the functional coupling of mACHR and G protein G, and of G, adenylyl cyclase (Murayama and Ui, 1983; Martin et al., 1985). Thus, HDL could inhibit signal transduction by decreasing mACHR number or affinity for agonist, decreasing the ability of mACHR to interact with G, decreasing the level
of G\textsubscript{i}, or decreasing the ability of G\textsubscript{i} to couple with adenylate cyclase.

The possibility that mAChR-mediated inhibition of cAMP accumulation is attenuated in HDL-treated cells due to a decrease in mAChR number was tested by comparing total receptor number and antagonist affinity in cells cultured in the absence and presence of HDL. As shown in Fig. 6, saturation binding curves for the antagonist [\(\text{H}\)]QNB are similar in membranes prepared from treated and untreated cells. The maximum number of [\(\text{H}\)]QNB-binding sites is 196 ± 29 fmol/mg membrane protein in control membranes and 192 ± 17 fmol/mg in membranes prepared from HDL-treated cells. The inset, a Scatchard plot of the data, demonstrates that there is no difference in mAChR affinity for antagonist: the \(K\_D\) for [\(\text{H}\)]QNB binding is 63 pm in control membranes and 54 pm in membranes prepared from HDL-treated cells.

A decrease in mAChR affinity for agonist could explain the increase in the \(EC_{50}\) for carbachol-mediated inhibition of cAMP accumulation in HDL-treated cells. We therefore tested the ability of carbachol to compete for [\(\text{H}\)]NMS binding to intact heart cells that had been cultured in the presence and absence of HDL. The results, shown in Fig. 7, demonstrate that carbachol competed for [\(\text{H}\)]NMS binding identically in cells cultured under both conditions, indicating that mAChR affinity for agonist was the same in HDL-treated and control cells.

The affinity of mAChR for agonist can be controlled by the degree to which the receptor and the G-proteins with which it interacts are coupled. Thus, guanine nucleotides and their analogs have been shown to cause a decrease in agonist affinity (Florio and Sternweis, 1985), and the magnitude of this shift in affinity has been taken as a measure of coupling between G-proteins and mAChR. The decreased ability of carbachol to inhibit cAMP accumulation resulting from HDL treatment could arise from an HDL-mediated decrease in coupling between the receptor and G\textsubscript{i}. In order to determine whether HDL treatment results in a diminished coupling between mAChR and the G-proteins with which it interacts, we determined the ability of carbachol to compete for [\(\text{H}\)]QNB binding in the presence and absence of the non-hydrolyzable GTP analog, GppNHp, in membranes prepared from HDL-treated and control cells. The results shown in Fig. 8 demonstrate that carbachol competition for [\(\text{H}\)]QNB binding in the absence of GppNHp is similar in membranes prepared from HDL-treated and control cultures. The \(EC_{50}\) for carbachol inhibition of [\(\text{H}\)]QNB binding in the absence of GppNHp in membranes from control and HDL-treated cells are 3 and 5.6 \(\mu\)M, respectively. The \(EC_{50}\) for carbachol inhibition of [\(\text{H}\)]QNB binding in the presence of 10 \(\mu\)M GppNHp in membranes from control and HDL-treated cells are 18 and 70 \(\mu\)M, respectively. Therefore, GppNHp shifted the \(EC_{50}\) for carbachol 6-fold in control membranes, and 12.5-fold in HDL-treated membranes. These data indicate that the ability of GppNHp to regulate agonist binding is not diminished in HDL-treated cells and that the coupling between the mAChR and G-proteins is somewhat greater for HDL-treated cells.

Because the mAChR is known to interact with a variety of G-proteins in heart cells (Martin et al., 1985), it is possible that changes in coupling of mAChR with G\textsubscript{i} specifically might not be apparent in binding studies such as those performed above. Therefore, we measured the actual level of G\textsubscript{i} present in membranes prepared from control and HDL-treated cells using monospecific antibodies which recognize G\textsubscript{i}. Previous studies by Luetje et al. (1987) demonstrated that two forms...
of $G_{\alpha_3}$, one of $M$, 39,000 and one of $M$, 41,000, were present in chick heart. The 39,000 form of $G_{\alpha_3}$ co-migrates with $G_{\alpha_4}$ but is immunologically distinct from $G_{\alpha_4}$ (Luetje et al., 1987). Quantitative immunoblot analysis using monospecific antisera demonstrates that the amounts of both the 41,000 and 39,000 forms of $G_{\alpha_3}$ are similar in membranes prepared from control and HDL-treated cells (Table I). These results suggest that alterations in the level of $G_{\alpha}$ do not occur in response to HDL treatment and are unlikely to account for the diminished ability of carbachol to mediate inhibition of cAMP accumulation. Reports that certain responses could be due in part to the release of $G_{\alpha}$ which could then act on effector proteins (Logothetis et al., 1987; Kim et al., 1989) raised the possibility that HDL might act by altering the level of $G_{\alpha}$ in the membrane. However, membranes prepared from control and HDL-treated cells contained the same amount of $G_{\alpha_3}$, making this hypothesis untenable.

Because HDL did not appear to act by altering receptor number, affinity, or coupling or by altering $G_{\alpha_4}$ or $G_{\alpha_5}$ levels, it was possible that $G_{\alpha}$ coupling with adenylate cyclase was less effective in HDL-treated cells. This theory was tested by analyzing the ability of GppNHp to inhibit, via activation of $G_{\alpha}$, forskolin-stimulated adenylate cyclase activity in membranes prepared from control and HDL-treated cells. Because forskolin increases adenylate cyclase activity independently of stimulatory receptor activation, this protocol has been extensively used to quantitate $G_{\alpha}$-adenylate cyclase coupling (Seamon and Daly, 1981; Halvorsen and Nathanson, 1984; Hunter and Nathanson, 1984; Martin et al., 1987; Luetje et al., 1987). GppNHp may also interact with $G_{\alpha}$ to stimulate adenylate cyclase, resulting in an upward swing of a concentration-response curve at higher concentrations of GppNHp. Inhibition of adenylate cyclase activity by GppNHp occurs at lower concentrations of GppNHp than does activation because the $K_i$ for GppNHp binding to $G_{\alpha}$ is thought to be greater than that to $G_{\alpha}$ (Seamon and Daly, 1982). As demonstrated in Fig. 9, GppNHp was less effective in inhibiting adenylate cyclase activity in membranes prepared from HDL-treated cells than in control membranes. The maximum inhibition of cyclase activity by GppNHp was 32% in membranes from control cells and 23% in membranes from HDL-treated cells, while the EC50s for GppNHp were 10 and 20 nM, respectively. The reversal of GppNHp-mediated inhibition of adenylate cyclase at 0.1 µM or greater GppNHp is most likely a reflection of the ability of GppNHp to stimulate adenylate cyclase activity via $G_{\alpha}$ activation. These changes in GppNHp effectiveness are qualitatively similar to those observed for carbachol-mediated inhibition of forskolin-stimulated cAMP accumulation and could account for the decreased responsiveness seen in HDL-treated cells.

### Determination of the Component of HDL Necessary for Inhibition of mACHR Responsiveness—A number of reports suggest that the effect of native lipoproteins can be accounted for in some cases by the apoprotein (Chen et al., 1986; Tourrier et al., 1984; Wu et al., 1988) and in others by the lipid (Van Sickle et al., 1986; Cuthbert and Lipsky, 1986a) subfractions. We therefore tested each of these HDL subfractions for the ability to attenuate carbachol-mediated inhibition of forskolin-stimulated cAMP accumulation. Cells cultured in the presence of HDL apoprotein were as sensitive to carbachol as were control cells indicating that HDL apoprotein alone is not sufficient to explain the action of native HDL (Fig. 104).

| G<sub>α3</sub> (pmol G-protein/mg membrane protein) | G<sub>α4</sub> (pmol G-protein/mg membrane protein) | G<sub>α5</sub> (pmol G-protein/mg membrane protein) |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Control                                      | 20 ± 3                                        | 39 ± 5                                        | 68 ± 10                                      |
| HDL                                          | 21 ± 6                                        | 41 ± 9                                        | 62 ± 16                                      |
In contrast, cells cultured in the presence of the HDL lipid subfraction were less responsive to carbachol (Fig. 10B). The maximum inhibition of cAMP accumulation caused by carbachol was 61% in control cells and 41% in cells treated with HDL lipid. This diminution of the maximum response is similar to that seen with native HDL (cf. Figs. 2 and 3), suggesting that HDL lipid can fully account for the decrease in mACHr responsiveness arising from HDL treatment. If the ability of HDL to attenuate carbachol-mediated inhibition of cAMP accumulation arises from a decreased effectiveness of G, to inhibit adenylate cyclase and if HDL lipid is responsible for the effect of native HDL, then HDL lipid should also diminish the ability of GppNHP to inhibit adenylate cyclase. The results shown in Fig. 11 demonstrate that GppNHP was less effective in inhibiting forskolin-stimulated adenylate cyclase activity in membranes prepared from HDL lipid-treated cells than in those prepared from control cells. This result is consistent with the hypothesis that HDL acts to inhibit G-adenylate cyclase interaction via its lipid content.

It has been suggested that the increased mACHr responsiveness in heart cells cultured in lipoprotein-depleted serum result from decreases in the level of cellular cholesterol (Haigh et al., 1988). Because HDL is known to alter the cholesterol level of cultured cells (Karlin et al., 1987), we wanted to determine if this could account for the effect of HDL on mACHr-mediated responses. The membrane cholesterol content of chick heart cells which had been cultured in the absence and presence of either native HDL or of HDL lipid on GppNHP-mediated inhibition of cAMP accumulation and a stimulation of PI metabolism (Subers and Nathanson, 1988). Renaud et al. (1982) and, more recently, Haigh et al. (1988) observed that cultured chick heart cells have a greater negative chronotropic response to muscarinic agonist when the cells were cultured in the absence of serum lipoproteins. This result suggests that serum lipoproteins can regulate the sensitivity of cultured chick heart cells to mACHr activation. Using a fully defined culture system, the ability of HDL purified from rooster serum to alter mACHr function was investigated. Consistent with the results of Renaud et al. (1982), we observed that cells cultured in the presence of HDL were less sensitive to muscarinic agonists than were untreated cells. Interestingly, this diminished sensitivity to mACHr activation was restricted to carbachol-mediated inhibition of cAMP accumulation; carbachol stimulation of PI turnover and of agonist-induced down-regulation were unaffected by exposure of the cells to HDL (Figs. 3–5). This result suggests that HDL selectively decreases the function of the signal transduction pathway responsible for mACHr-mediated inhibition of cAMP accumulation, without reduction of other mACHr-coupled pathways. Recent reports that a decrease in intracellular cAMP is involved in the mACHr-mediated regulation of the pacemaker current, \( I_f \) (DiFrancesco and Tromba, 1988), suggest that the observed ability of lipoproteins to decrease the negative chronotropic response of cultured chick heart cells (Renaud et al., 1982) could be related to our observation that HDL can decrease the capacity of muscarinic agonist to cause a decrease of cAMP accumulation.

The mACHr, the inhibitory guanine nucleotide-binding regulatory protein of adenylate cyclase, \( G_i \), and the enzyme adenylate cyclase, must be present and functional for the decrease in cellular cAMP levels produced by mACHr activation. One goal of this work was to determine where in this signal transduction pathway HDL exerted its effect. The possible sites of action of HDL include, but are not restricted to, a decrease in mACHr number or affinity, a decrease in membrane \( G_i \) content, a decrease in the ability of \( G_i \) to interact with the receptor, or a decrease in the ability of \( G_i \) to inhibit adenylate cyclase activity. Each of these potential sites of action of HDL was investigated.

Agonist and antagonist binding data indicate that HDL did not cause a decrease in either mACHr number or affinity for agonist, eliminating the possibility that HDL treatment caused a decrease in mACHr sensitivity by altering receptor binding properties (Figs. 6 and 7). Agonist binding to cell surface receptors on intact chick heart cells was measured because this assay most closely duplicates the conditions under which functional properties of mACHr were studied. The ability of guanine nucleotides to shift agonist binding curves in membrane homogenates can be taken as a measure of the coupling between the receptor and the G-proteins with which it interacts. The non-hydrolyzable GTP analog, GppNHP, produced greater shifts in competition curves between carbachol and \([H]QNB\) in membranes from HDL-treated cells, suggesting that mACHr was more efficiently coupled to the G-proteins with which it interacts following HDL treatment. Thus, a decreased efficiency of interaction between mACHr and \( G_i \) is unlikely to account for the diminished ability of carbachol to inhibit cellular cAMP accumulation in HDL-treated cells.

Alternatively, an HDL-mediated decrease in the level of \( G_i \) in the membrane could explain the diminished responsiveness of HDL-treated cells. When the amount of \( G_{ina} \) and \( G_i \) were measured by quantitative immunoblot analysis in membranes prepared from control and HDL-treated cells, no difference
was observed (Table I). This result suggests that neither a
decrease in G\textsubscript{\textalpha} nor in G\textsubscript{\gamma} can account for the effect of HDL.

In order to determine whether HDL acted to reduce the
efficiency of coupling between G\textsubscript{\alpha} and adenylyl cyclase, a
comparison of the ability of GppNHp to inhibit forskolin-
stimulated adenylyl cyclase activity in membranes prepared
from control and HDL-treated cells was performed. GppNHp-
induced inhibition of adenylyl cyclase activity was attenu-
ated in membranes prepared from HDL-treated cells (Fig.
9), indicating that the coupling between G\textsubscript{\alpha} and adenylyl cyclase was reduced by HDL-treatment. These experiments
suggest that HDL causes a decrease in mACHR responsiveness
by interfering with the interaction between G\textsubscript{\alpha} and adenylyl cyclase.

Aipoprotein fractions of HDL were unable to cause a de-
creased sensitivity to carbachol in cultured chick heart cells,
suggesting that HDL apoproteins cannot substitute for native
HDL. However, when the ability of lipid fractions of HDL to
decrease mACHR responsiveness was examined, it was found
that they could mimic the effect of native HDL. Because the
degree of attenuation of the response to carbachol was similar
for native HDL and its lipid fractions, it appears that the
effect of the lipid fractions of HDL can fully account for the
effect of native HDL. Addition of HDL lipid fractions to the
culture medium also decreased GppNHp-mediated inhibition
of adenylyl cyclase activity, which is consistent with the
conclusion that HDL acts to decrease the efficiency of cou-
pling between G\textsubscript{\alpha} and adenylyl cyclases.

Because the ability of G\textsubscript{\alpha} to inhibit adenylyl cyclase and
adenylyl cyclase activity itself is sensitive to the membrane
lipid composition (Engelhard et al., 1978; Murphy, 1986; Mur-
phy et al., 1987), it seems likely that HDL acts to alter cellular
membrane lipid composition, thus inhibiting mACHR-me-
diated reductions in cellular cAMP accumulation. In a related
study, Haigh et al. (1988) reported that removal of lipoproteins
from fetal calf serum used to supplement the medium resulted
in an increased sensitivity to carbachol in cultured chick atrial cells exposed to muscarinic agonists. The in-
creased sensitivity to mACHR activation was reversed when
bovine LDL, but not bovine HDL, was added back to the
culture medium. The heightened mACHR responsiveness
was correlated with decreases in cellular cholesterol content, sug-
gestig that the increased level of cholesterol in atrial cells
can decrease mACHR responsiveness. The bovine HDL used in
that study did not alter membrane cholesterol content,
suggesting that it did not interact effectively with chick heart
cells. In contrast, the data presented here demonstrate that
both chicken HDL and LDL decrease mACHR sensitivity in
heart cell cultures. Because HDL is able to stimulate bidirec-
tional cholesterol flux in cultured cells (Karlin et al., 1987),
the effect of HDL observed here could have been due to an
increase in membrane cholesterol content, analogous to those
reported by Haigh et al. (1988). However, the results reported
here indicate that treatment of cultured chick heart cells with
rooster HDL reduces membrane cholesterol content. More-
ever, the lipid fraction of HDL did not alter membrane cholesterol but did mimic the effect of native HDL on mACHR
sensitivity to agonist, suggesting that changes in membrane
cholesterol do not mediate the effect of native HDL. Haigh et
al. (1988) also observed a significant increase in mACHR number and in G\textsubscript{\textalpha} subunit levels in atrial cells cultured in
serum in the absence of lipoproteins, which could explain the
increased sensitivity of these cells to mACHR activation. It
appears that lipoproteins can regulate mACHR function by
more than one mechanism because addition of HDL to cells
cultured in defined medium changed neither mACHR nor G\textsubscript{\textalpha}
levels (Fig. 6, Table I). It should be noted that Haigh et al.
(1988) measured only the ability of mACHR to mediate a
decrease in beating rate following the removal of lipoproteins;
mACHR-mediated inhibition of cAMP accumulation and
stimulation of phosphoinositide metabolism were not exam-
ined. Here, we clearly demonstrate that lipoproteins do not
affect all mACHR signal transduction pathways, so that it is
possible that different pathways, e.g. beating rate response
and cAMP response, are regulated by different lipoproteins
with different mechanisms of action.

The work presented here shows that mACHR function is
regulated by serum lipoproteins, especially by HDL. HDL
duces a decrease in sensitivity of carbachol to inhibit
cAMP accumulation in cultured chick heart cells, with no
change in its ability to stimulate phosphoinositide turnover,
indicating that a general decrease in cellular, membrane, or
receptor function does not occur. HDL apparently produces
this effect by interfering with the ability of G\textsubscript{\alpha} to inhibit
adenylyl cyclase activity. The lipid fraction of HDL can mimic
the effect of native HDL, suggesting that HDL may exert its effect by altering cellular lipid content.

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