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Effects of the phospholipid environment in the plasma membrane on receptor interaction with the adenylyl cyclase complex of intact cells

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In this study we have examined the effects of variations of the plasma membrane phospholipid and cholesterol content on the metabolic functions of the adenylyl cyclase complex in intact cells. Exposure of cells to 0.1 U/ml of sphingomyelinase led to the degradation of 75, 55 and 40% of the cellular total sphingomyelin mass in human skin fibroblasts (HSF), Chinese hamster lung fibroblasts (CHLF) and rat liver hepatocytes (RLH), respectively. Degradation of sphingomyelin in native cells led in turn to a reduction (within 60 min) of the plasma membrane cholesterol content (by 25, 15 and 10%, respectively). This manipulation of the plasma membrane lipid content did not affect the forskolin or prostaglandin E_1-induced activation of adenylyl cyclase (as measured from the conversion of [^{3}H]adenine via [^{3}H]ATP to [^{3}H]cAMP). These manipulations did, however, increase the basal rate of [^{3}H]cAMP formation in rat liver hepatocytes (but not in the fibroblast cell types). With Chinese hamster lung fibroblasts, transfected to express an α_2-adrenergic receptor, it was observed that the α_2-adrenergic receptor-induced inhibition of adenylyl cyclase activity was slightly (but significantly) diminished in sphingomyelin and cholesterol-depleted cells. With isolated rat liver hepatocytes it was observed that the glucagon (receptor) mediated activation of adenylyl cyclase was also reduced in sphingomyelinase-treated cells. In another set of experiments, CHLF and RLH cells were exposed for 2 h to vesicles prepared from dilauroylphosphatidylcholine, to increase the lateral packing density in the outer leaflet of the plasma membrane. In such treated cells, the receptor-coupling to adenylyl cyclase was markedly reduced both in CHLF (the α_2-adrenergic receptor) and RLH (the glucagon-receptor) cells. We conclude that the direct activation of adenylyl cyclase (i.e., by forskolin) is not markedly affected by manipulations outer leaflet phospholipid composition (either reduction of sphingomyelin or increase of phosphatidylcholine), whereas receptor-coupled events clearly are.

Introduction

The plasma membrane compartment of cells contains many integral receptor and enzyme functions which are associated with metabolic regulation and with the initiation of intracellular signaling events. Plasma membrane lipids can be viewed as regulating elements of these processes, since they provide the interface where many of the regulatory events take place, and since an increasing number of lipids and lipid metabolites are known to directly participate in signal initiation and transduction [1–4].

Cyclic adenosine monophosphate (cAMP) is one of the major intracellular second messenger. The enzyme responsible for the tightly controlled formation of cAMP, adenylyl cyclase, is a membrane-spanning protein consisting of 12 hydrophobic α-helical segments [5,6]. The receptor site of the enzyme is on the outer surface of the plasma membrane, while the catalytic site deliver the reaction product (cAMP) to the cytoplasm [7,8]. The enzyme is regulated by the guanine nucleotide binding proteins Gs (stimulatory) and Gi (inhibitory). The guanine nucleotide binding proteins are heterotrimers consisting of α-, β- and γ-subunits [9,10]. Membrane-spanning receptors may, after interaction with their respective agonists, convey stimulatory (e.g., α_2 glucagon-receptors via the Gs regulatory protein) or inhibitory (e.g., α_2-adrenergic receptor via the Gi regulatory protein) signals to the catalytic subunit of adenylyl cyclase [11–13]. The catalytic activity of adenylyl cyclase is known to be influenced by changes

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Abbreviations: DLPC, dilauroylphosphatidylcholine; HSF, human skin fibroblast; CHLF, Chinese hamster lung fibroblast; RLH, rat liver hepatocytes.

in membrane fluidity [14,15], and by alterations in membrane cholesterol [16–19] and phospholipid compositions [20].

We have previously made extensive studies on the effects of the hydrolysis of plasma membrane sphingomyelin on intracellular cholesterol distribution [21–23], and also examined how this treatment affects the catalytic properties of the membrane-bound Na^+/K^+-ATPase and adenylyl cyclase [24]. We observed that an almost complete degradation of plasma membrane sphingomyelin in baby hamster kidney cells led to a complete inactivation of the ouabain-sensitive Na^+/K^+-ATPase activity, whereas the forskolin-induced activation of adenylyl cyclase was unchanged [24]. This different response to such an extensive alteration of the plasma membrane lipid composition suggested that these two enzyme systems may be localized to widely different and specific domains in the plasma membrane, since Na^+/K^+-ATPase sensed the loss of sphingomyelin and cholesterol, whereas adenylyl cyclase did not.

The objective of this study was to further characterize the effects of changes in the plasma membrane phospholipid and cholesterol content on adenylyl cyclase activity in intact cells. We have examined the effects of lipid manipulations on both receptor-independent (forskolin) activation of adenylyl cyclase, and receptor-coupled responses (i.e., the glucagon-receptor in hepatocytes, and the α_2-adrenergic receptor in transfected Chinese hamster lung fibroblasts) in adenylyl cyclase activity.

Experimental procedures

Cell culture

Human skin fibroblasts (HSF; GM 8333) were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ), and were cultured in Dulbecco's modified Eagle's medium supplemented with 12% fetal calf serum.

Chinese hamster lung fibroblasts (CHLF), transfected to express the human platelet α_2-adrenergic receptor subtype α_2-C10 [25], originated from the laboratory of Dr. Regan (College of Pharmacy, University of Arizona, Tucson), and were cultured in Dulbecco's medium with 10% fetal calf serum. The growth medium for these cells also contained 150 μg/ml Geneticin (G418 sulfate). The expression vector pMAMneo (Clontech, Palo Alto, CA) that was used to transfect the cells contained a dexamethasone-sensitive inducible promotor [25]. Therefore, the cells were grown in the presence of 1 μM dexamethasone for 24 h prior to experiments. Cells for experiments were grown in 800 ml flasks (Nunclon, Nunc, Denmark), and were used at full confluency. The cells were washed with phosphate-buffered saline and harvested with EDTA. The experiments were performed in a TES buffer (20 mM at pH 7.5, containing 137 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM glucose and 0.5 mM EGTA).

Primary rat liver hepatocytes (RLH) were isolated from male Wistar rats (200–250 g) by a two step collagenase perfusion technique, as described by Seglen [26]. Hepatocytes to be used for adenylyl cyclase assays were kept in the suspension buffer (pH 7.6; 68 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2 \cdot 2H_2O$, 0.6 mM $MgCl_2 \cdot 6H_2O$, 1.1 mM KH_2PO_4, 0.7 mM Na_2SO_4, 30 mM Hepes, 30 mM Tes, 36 mM Tricine, 53 mM NaOH) supplemented with 1 % (w/w) bovine serum albumin, and 10 mM glucose. Hepatocytes to be used for cholesterol oxidase assays were suspended in HAM F12 medium.

Preparation of DLPC small unilamellar vesicles

Small unilamellar vesicles were prepared using dilauroylphosphatidylcholine. The lipid was dissolved in absolute ethanol, and a small volume of the stock solution (20–40 μl) was injected by a spring-loaded Hamilton syringe into the final buffer to give the desired final phospholipid concentration. The ethanol-injected DLPC SUVs had an average size of 60 nm.

Determination of sphingomyelin degradation

To determine the amount of cell sphingomyelin that was degradable by exogenously added sphingomyelinase in continuously cultured cells (i.e., the fibroblast cell lines), sphingomyelin (and phosphatidylcholine) was labeled with [*methyl*-^{3}H]choline chloride (75 Ci/mmol, Amersham) as follows: cells were cultured in growth medium supplemented with 2 μCi/ml of [*methyl*-^{3}H]choline chloride (28 μM final choline concentration) for 48 h. Cells were then rinsed (3 × 2 ml) with phosphate-buffered saline and fixed for 10 min at 4°C with 1% glutaraldehyde in phosphate-buffered saline. After the fixation, cells were rinsed (3 × 2 ml) and exposed to 0.1 U/ml of sphingomyelinase in phosphate-buffered saline for 40 min. The cellular content of [^{3}H]sphingomyelin in control and treated cells was determined by thin-layer chromatography of the total lipid extract.

To determine the extent of sphingomyelin degradation in isolated rat liver hepatocytes, cells in suspension ($3 \cdot 10^5$ cells per ml of HAM F12 medium) were exposed to 0.1 U/ml of sphingomyelinase for 40 min at 37°C. The extent of sphingomyelin degradation was determined from the total cell lipid extract by thin-layer chromatography and densitometric scanning of the stained plates.

Determination of plasma membrane cholesterol

We have previously shown that depletion of sphingomyelin by sphingomyelinase eventually results

in the translocation of a substantial amount of the plasma membrane cholesterol into intracellular sites [22,24,27]. To examine the extent of cholesterol translocation in these cell types, the cells were exposed to sphingomyelinase and the cholesterol content of plasma membranes was determined in control and sphingomyelinase-treated cells by a cholesterol oxidase assay.

2 days before the experiments, the fibroblast lines were incubated for 48 h in growth medium with 5% fetal calf serum containing [^{3}H]cholesterol (5–10 μCi/ml serum; 60 Ci/mmol, Du Pont New England Nuclear). After this 48 h incubation, the cells were further incubated in serum-free HAM F12 medium for 3–5 h. Cells were then exposed to 0.1 U/ml of sphingomyelinase for 60 min to induce cholesterol translocation, and the distribution of plasma membrane [^{3}H]cholesterol into intracellular membranes was determined. Confluent [^{3}H]cholesterol-labeled cells, pre-treated with sphingomyelinase, were rinsed once with ice-cold phosphate buffered saline and then fixed for 10 min (0°C) with 1% glutaraldehyde in phosphate buffered saline. The fixative was then removed and the cells rinsed (3 × 2 ml) with ice-cold phosphate buffered saline. After this rinse procedure, 1.0 ml HAM F-12 medium containing 1 U/ml of cholesterol oxidase (*Brevibacterium*; Beckman, Carlsbad, CA) and 0.1 U/ml of sphingomyelinase was added, and the cells were incubated at 37°C for 30 min. The second addition of sphingomyelinase was necessary in order to achieve optimal oxidation of control cells [21,28]. It should also be noted that sphingomyelinase does not induce cholesterol translocation in fixed cells, and hence does not affect the intracellular distribution of cholesterol (Slotte, unpublished observations). At the end of the oxidation, the dishes were chilled, rinsed with phosphate buffered saline and stored frozen (−20°C) until lipid analysis was performed.

The effects of sphingomyelin depletion of plasma membrane cholesterol translocation in isolated rat liver hepatocytes was performed as described above, except that the conversion of cholesterol to cholestenone was determined by gas-liquid chromatographic analysis of the cellular total lipid extract [21].

Experimental setup and assay for cAMP production

The relative activity of adenylyl cyclase in intact cells was determined from the conversion of [^{3}H]-adenine (via [^{3}H]ATP) to [^{3}H]cAMP. Confluent HSF cells in 35 mm cell culture dishes, or confluent CHLF cells in 800 ml flasks were incubated for 3 h in serum-free growth medium containing 5 μCi of [^{3}H]adenine (23.5 Ci/mmol; Amersham). Isolated hepatocytes in suspension (about 10^6 cells/ml) were incubated for 2 h in suspension buffer containing 5 μCi of [^{3}H]adenine. Sphingomyelinase (0.1 U/ml) or DLPC SUVs (25–150 μg/ml) was added to some sets of cells for the last 40 min (sphingomyelinase) or for the last 2 h (DLPC) of the labeling incubation. These treatments did not affect membrane integrity as assessed from the permeability to Trypan blue (data not shown). After the incubation with [^{3}H]adenine the cells were washed and pre-incubated for 15 min with experimental medium containing 0.5 mM 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor). In experiments with the α_2-adrenergic receptor agonists, the medium also contained 150 μM quinacrine (a phospholipase A_2 inhibitor), and 100 μM propranolol (a β-adrenergic receptor antagonist). Cells were then exposed for 15 min to agonists: either forskolin (20 μM), prostaglandin E_1 (20 μM), a combination of forskolin and norepinephrine (100 μM) or clonidine (100 μM), or glucagon (2 μM). Control cells received solvent alone (buffer or ethanol, final ethanol concentration less than 0.5%). The 15 min agonist exposure time was at the end of the linear range of the adenylyl cyclase response (or formation of cAMP). The conversion of [^{3}H]ATP to cyclic [^{3}H]AMP was determined by ion exchange chromatography from the cell-derived perchloroacid extracts [29]. The samples were passes sequentially over Dowex 50W (4% cross-linked, 200–400 dry mesh, Bio-Rad) to isolate [^{3}H]ATP, and alumina columns (Sigma) to isolate [^{3}H]cAMP. The loss of [^{3}H]cAMP during the extraction procedure was corrected for using an internal standard (about 1600 cpm of [^{14}C]cAMP). The extraction efficiency usually exceeded 70%.

Lipid analysis

Total cell lipids from control or sphingomyelinase-treated cells were extracted with hexane/2-propanol (3:2, v/v; 2 × 2 ml/dish for 20 min each). For phospholipid analysis, the organic solvent was evaporated, and the total lipids dissolved in chloroform and spotted on Kieselgel 60 thin-layer chromatography sheets (Merck, Germany). The plates were developed with chloroform/methanol/acetic acid/water (25:15:4:2, v/v [30]), air dried, and stained with iodine. The [^{3}H]sphingomyelin spots were cut and placed into scintillation vials and counted for radioactivity.

For the analysis of hepatocyte sphingomyelin mass, the total cell lipid extract was run on Kieselgel 60 glass plates, as described above. The plates were stained with 3 w% cupric acetate in 8 v% phosphoric acid [31], and developed by heating for 30 min to 150°C. The intensity of sphingomyelin was determined by scanning densitometry, and the sphingomyelin signal was normalized to the intensity of phosphatidylethanolamine (which is not a substrate of sphingomyelinase).

[^{3}H]Sterols in the total lipid extracts were separated on thin-layer chromatography sheets (Kodak Chromagram sheets) with hexane/diethyl ether/acetic acid (130:30:2, v/v) as developing solvent. Lipid spots

TABLE I

Sphingomyelin degradation and cholesterol translocation in native cells

The degradation of cell sphingomyelin was determined from the total cell lipid extract after exposure of the cells to 0.1 U/ml of sphingomyelinase for 40 min at 37°C (please refer to Experimental procedures). The extent of plasma membrane cholesterol translocation (e.g., reduction in plasma membrane cholesterol content) in native cells exposed for 60 min to 0.1 U/ml of sphingomyelinase was determined by a cholesterol oxidase-assay (as described under Experimental procedures). Values are averages from two separate, representative experiments ($n = 4$ for sphingomyelin degradation experiments, and $n = 6$–8 for cholesterol translocation assays: ±S.E.).

Cell type	Sphingomyelin degradation (%)	Reduction in plasma membrane cholesterol (%)
Human skin fibroblasts	75± 5	25±5
Chinese hamster lung fibroblasts	55±10	15±7
Rat liver hepatocytes	40± 5	10±2

were detected with I_2 staining. Spots for [^{3}H]cholesterol, [^{3}H]cholestenone and [^{3}H]cholesterol esters were identified from standards run in parallel. The appropriate spots were marked, the I_2 stain was removed and the spots cut into scintillation vials. The radioactivity was counted in an LKB RackBeta liquid scintillation counter.

The conversion of cholesterol to cholestenone following the oxidation of hepatocytes was determined from the total lipid extract by gas-liquid chromatography, as described previously [21].

Results

Degradation of cell sphingomyelin and cholesterol translocation

To determine the size of the cellular sphingomyelin pool that was degradable in glutaraldehyde-fixed cells by sphingomyelinase, cells were exposed to the enzyme (0.1 U/ml) for 40 min at 37°C, and the content of sphingomyelin in the cellular lipid extract was determined. The extent of sphingomyelin degradation varied somewhat between the three different cell lines (Table I). A substantial amount of the total cellular sphingomyelin in human skin fibroblasts was degradable, whereas a much lesser fraction of cell sphingomyelin was hydrolyzed in hepatocytes. The degradation of cell sphingomyelin in CHLF was intermediate to that observed in HSF and hepatocytes, respectively. It was further observed that the amount of sphingomyelin degradation was identical both in native and glutaraldehyde-fixed cells (tested for the fibroblast cells), indicating that fixation per se did not influence the degradation susceptibility (data not shown). These sphingomyelin hydrolysis results indicated clearly that the exposure of cells to sphingomyelinase led to a substantial change in the relative phospholipid composition of at least the plasma membrane compartment.

We have previously shown that the hydrolysis of cell sphingomyelin in native cells leads to the translocation of a significant amount of unesterified cholesterol from plasma membranes into intracellular sites [21,23]. This cholesterol translocation is observed in native cells exposed to sphingomyelinase, but not in fixed cells exposed to sphingomyelinase. The treatment of native cells with sphingomyelinase (for 60 min) did not render them permeable to Trypan blue (data not shown), indicating that the perturbations induced in the plasma membrane compartment were not severe enough to induce leakage of small molecules. To measure the extent of cholesterol translocation in the cells used in this study, we exposed native cells to sphingomyelinase for 60 min and determined the cholesterol content of the plasma membranes using cholesterol oxidase [21]. All three cell types showed a reduced content of plasma membrane cholesterol 60 min after exposure to sphingomyelinase (Table I). The reduction in unesterified cholesterol content ranged between 15 and 25% for the fibroblast cell lines, but was only about 10% in rat liver hepatocytes. Previous reports from this laboratory have indicated that the translocated cholesterol moves to intracellular sites, where some of it eventually is converted to cholesteryl esters by the action of acyl-CoA:cholesterol acyltransferase [21–24].

Forskolin-activated adenylyl cyclase activity

To determine the effects of the substantial reduction in plasma membrane sphingomyelin and cholesterol mass on the catalytic activity of adenylyl cyclase, native cells were exposed to sphingomyelinase for a total of 40 min, and the conversion of [^{3}H]adenine, via [^{3}H]ATP, to [^{3}H]cAMP was measured (both basal and forskolin-activated levels). With HSF cells, forskolin (20 μM, 15 min) was able to activate [^{3}H]cAMP formation about 5-fold over the basal rate. Treatment of HSF cells with sphingomyelinase altered neither the basal nor the forskolin-stimulated adenylyl cyclase activity (Fig. 1). A similar pattern was observed with CHLF cells. With rat liver hepatocytes the fold-increase in [^{3}H]cAMP levels after forskolin activation was higher than in the fibroblast cells. It also appeared that the basal rate of [^{3}H]cAMP formation was significantly higher in sphingomyelinase-treated hepatocytes compared to control hepatocytes (Fig. 1).

Prostaglandin E_1-induced activation of adenylyl cyclase

In addition to using forskolin to activate adenylyl cyclase, we also pursued other routes of affecting the over-all activity of adenylyl cyclase and to study possible effects of sphingomyelin degradation and cholesterol translocation on these mechanisms. Prostaglandin E_1 (PGE_1) also stimulates cAMP synthesis in cells.

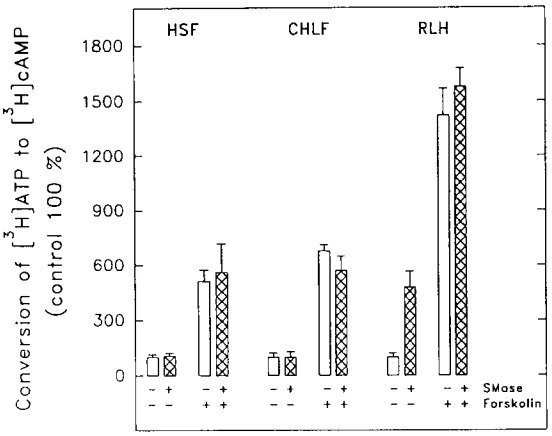

Fig. 1. Forskolin-activation of adenylyl cyclase activity in control and sphingomyelinase-treated cells. Cells on dishes (human skin fibroblasts, HSF; and Chinese hamster lung fibroblasts, CHLF) or in suspension (rat liver hepatocytes, RLH) were labeled for 2–3 h with [^{3}H]adenine, and during the last 40 min of this labeling period 0.1 U/ml of sphingomyelinase was added to selected sets of cells (hatched bars). At the end of the sphingomyelinase-exposure, cellular adenylyl cyclase was activated for 15 min with 20 mM forskolin (control cells received solvent alone), whereafter the formation of [^{3}H]cAMP was determined. Values are averages from at least three different experiments ($n = 12$) with each cell type (±S.E.). The radioactivity in [^{3}H]cAMP over the total [^{3}H]ATP activity in control cells (no sphingomyelinase) of each cell type was set to 100%, and the other values in the group were normalized to this number. Usually about 0.5–2% of the total [^{3}H]ATP activity was found as [^{3}H]cAMP in the cell extracts.

PGE_1 is believed to increase cAMP production by activating adenylyl cyclase via the stimulatory guanine nucleotide binding protein, Gs [32]. HSF cells, which were exposed for 15 min to PGE_1 (20 μM) increased their apparent adenylyl cyclase activity 6-fold (Fig. 2). Removal of sphingomyelin (virtually complete) and cholesterol (25%) from the plasma membranes did not

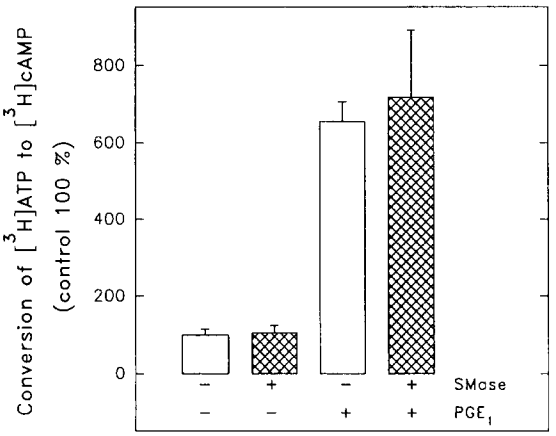

Fig. 2. Prostaglandin E_1-induced activation of adenylyl cyclase in human skin fibroblasts. Cells on dishes were treated as described in Fig. 1, except that 20 mM prostaglandin E_1 was used instead of forskolin to activate adenylyl cyclase. Values are averages of two separate experiments ($n = 6$; ±S.E.). The [^{3}H]cAMP content in control cells was set to 100%.

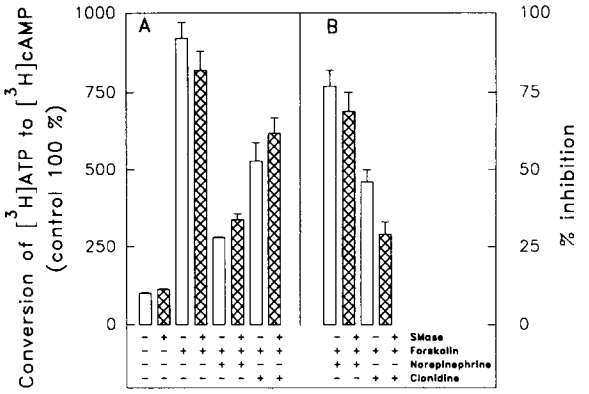

Fig. 3. α_2-Adrenergic receptor-mediated inhibition of forskolin-activated adenylyl cyclase activity in Chinese hamster lung fibroblasts. Cells on dishes were treated as described in Fig. 1, except that norepinephrine (100 μM) or clonidine (100 μM) were used as agonists to the α_2-adrenergic receptor (please refer to the detailed procedure under Experimental procedures). Values are averages from four different experiments ($n = 12$) ±S.E. The content of [^{3}H]cAMP in control cells was set to 100%. Bar pairs (±SMase) did not differ significantly from each other (A; $P > 0.05$). However, the degree of inhibition caused by the agonists (norepinephrine or clonidine), when normalized to the respective forskolin-activated control (B), was significantly different in sphingomyelinase-treated versus untreated cells ($P < 0.05$).

measurably affect the PGE_1-induced activation of adenylyl cyclase activity.

Inhibition of adenylyl cyclase through the α_2-adrenergic receptor

The activity of adenylyl cyclase can be inhibited with agonists to the α_2-adrenergic receptor in cells expressing this receptor [33]. This inhibition is thought to be mediated either directly through interaction of the $\beta\gamma$-subunits with Gi, or indirectly by interaction of the $\beta\gamma$-subunits with Gs [7,37]. To test whether the hydrolysis of plasma membrane sphingomyelin and the subsequent translocation of cell surface cholesterol had effects on the functions of the α_2-adrenergic receptor, the basal activity of adenylyl cyclase was stimulated with forskolin whereafter this stimulation was inhibited with norepinephrine (a non-specific agonist of the α_2-adrenergic receptor) or clonidine (a specific α_2-adrenergic receptor agonist). With native Chinese hamster lung fibroblasts, transfected to express the α_2-adrenergic receptor, norepinephrine caused a substantial inhibition (about 69% inhibition) of the forskolin-induced activation of adenylyl cyclase activity (Fig. 3). The extent of inhibition caused by norepinephrine in cells pre-exposed to sphingomyelinase was only slightly less (about 61% inhibition) than that observed in control cells. A similar trend was observed when the forskolin-induced activation of adenylyl cyclase was inhibited with clonidine in control (44% inhibition) and sphingomyelinase-treated (25% inhibition) CHLF cells (Fig. 3). The inhibition caused by both norepinephrine

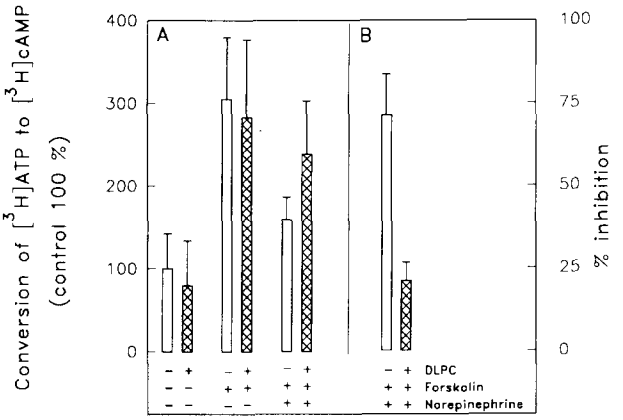

Fig. 4. Effect of DLPC treatment on α_2-adrenergic receptor mediated inhibition of forskolin-stimulated adenylyl cyclase activity in CHLF cells. Cells were labeled with [^{3}H]adenine (3 h) and DLPC (25 μg/ml; 2 h) as described under Experimental pocedures. Norepinephrine (100 μM) was used as an agonist for the receptor. Values are averages from four different experiments (with $n = 12$, $\pm$S.D.). A shows conversion values relative to the unstimulated basal level, whereas B shows the inhibition per centage. The norepinephrine induced inhibition in DLPC treated cells was not significantly different from its control (A; $P > 0.2$), whereas the degree of inhibition (B) was significantly different in DLPC-treated cells ($P < 0.005$).

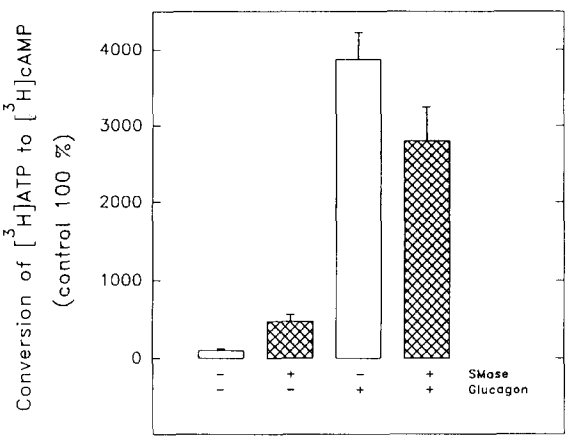

Fig. 5. Activation of adenylyl cyclase by glucagon in isolated rat liver hepatocytes. Cells in suspension were labeled with [^{3}H]adenine and treated with sphingomyelinase as described under Experimental procedures, and glucagon (2 mM) was used as the agonist. Values are averages from three separate experiments ($n = 9$) $\pm$ S.E. The content of [^{3}H]cAMP in control cells was set to 100%. Both bar pairs ($\pm$SMase) were significantly different from each other ($P < 0.05$).

and clonidine were significantly smaller in sphingomyelinase-treated cells compared to controls ($P < 0.05$). Taken together, the data indicate that the α_2-adrenergic receptor agonists were able to convey their inhibitory signal to the catalytic unit of the adenylyl cyclase irrespective of the selective loss of sphingomyelin and cholesterol from the plasma membranes of CHLF cells. The magnitude of the inhibitory effect appeared to be diminished in sphingomyelinase-treated cells.

Exposure of cells to small unilamellar vesicles (SUVs) of short-chain phospholipids (i.e., dilauroylphosphatidylcholine; DLPC) is known to lead to a rapid incorporation of DLPC into the external leaflet of the cell membrane [35–38]. When we exposed CHLF for 2 h to 25 μg/ml of DLPC SUVs, we observed a dramatic inhibition of the α_2-adrenergic receptor coupling to adenylyl cyclase (as determined from the reduced inhibition of the forskolin-activated adenylyl cyclase activity, Fig. 4). The DLPC exposure had no effects on either basal or forskolin-activated adenylyl cyclase activity per se.

Glucagon-stimulation of adenylyl cyclase in hepatocytes

In analogy with studies on the effects of sphingomyelinase-treatment on the function of α_2-adrenergic receptors in inhibiting adenylyl cyclase activity, we also examined the function of the glucagon-receptor in activating adenylyl cyclase in control and treated hepatocytes. Freshly isolated rat liver hepatocytes were exposed to sphingomyelinase for 40 min (control cells received no enzyme) and the conversion of [^{3}H]ATP to [^{3}H]cAMP was determined in the basal state and after activation by glucagon. Exposure of hepatocytes to glucagon gave an almost 40-fold activation (Fig. 5). The glucagon-induced activation of adenylyl cyclase was significantly less in sphingomyelinase-treated cells, suggesting that the stimulatory signal caused by glucagon was attenuated but not completely lost. This finding agrees with the analogous situation of sphingomyelinase-effects on the α_2-adrenergic receptor system in CHLF cells (Fig. 5).

Treatment of RLH for 90 min to increasing amounts of DLPC SUVs led to a concentration-dependent decrease of the glucagon-activated adenylyl cyclase activ-

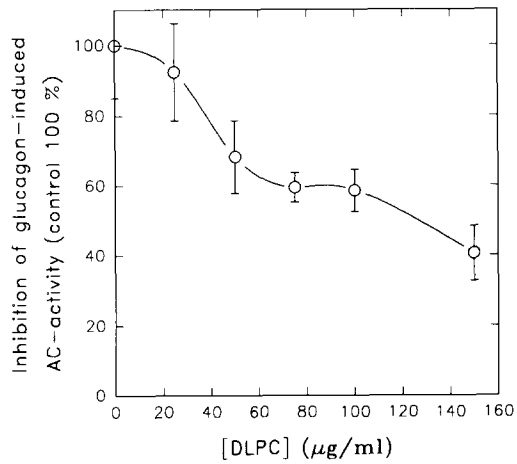

Fig. 6. Effect of DLPC treatment on glucagon-activated adenylyl cyclase activity in RLH. Cells in suspension were labeled with [^{3}H]adenine and exposed to indicated concentration of DLPC SUVs for 90 min before the 15 min exposure to 2 μM glucagon. Values for the relative adenylyl cyclase activity are averages from three different experiments ($n = 9$, $\pm$S.E.), with the stimulation in control cells set arbitrarily to 100%.

ity (Fig. 6), suggesting that the coupling of the glucagon-receptor to the adenylyl cyclase complex was hindered in DLPC treated RLH cells.

Discussion

Adenylyl cyclase is a transmembrane glycoprotein with 12 hydrophobic α-helical segments. It is thought that an interaction between several of the cytoplasmic domains is needed to create a functional catalytic subunit [5]. The coordinated regulation of cAMP formation involves a specific interaction between the catalytic subunit of adenylyl cyclase and guanine nucleotide regulatory proteins [11–13]. The signal which initiates (or inhibits) the catalytic function of adenylyl cyclase is mediated by membrane-spanning receptors for various hormones [33,39]. The interaction between the ligand (hormone) and the transmembrane receptor will eventually result in the formation of a multicomponent complex, involving the catalytic subunit of adenylyl cyclase, and resulting in the formation of cAMP. Since the individual components are able to diffuse laterally in the plane of the membrane, it is to be expected that manipulations of the lipid composition and/or fluidity parameters of the plasma membrane also will affect the formation of the multicomponent adenylyl cyclase complex and its catalytic function.

It has in fact been convincingly demonstrated that changes in membrane fluidity (through variations in membrane cholesterol content [14,17,18], or via variations in the phospholipid acyl chain composition [20]), and changes in membrane phospholipid head-group composition [20] markedly affect both basal-, agonist- (e.g., PGE_1), and receptor-activated adenylyl cyclase activity. Many of the previous studies on the effects of lipid variations on adenylyl cyclase activity have been performed with isolated plasma membranes from cultured cells or rat liver homogenates.

In our present study with intact cells, we observed consistently (save for the rat liver hepatocytes) that a treatment of cells with sphingomyelinase had very small or no effects on the basal, and forskolin-activated formation of cAMP (Fig. 1 and Ref. 24). With isolated rat liver hepatocytes, however, the exposure to sphingomyelinase led to a small but significant activation of the basal activity of adenylyl cyclase. But even in the hepatocyte, the forskolin-induced adenylyl cyclase activity was similar in control and sphingomyelinase-treated cells. Cholesterol enrichment of cellular plasma membranes has been reported to result in decreased basal adenylyl cyclase activities, both in rat kidney fibroblasts [16], and rat liver hepatocytes [17]. Our findings that sphingomyelin degradation and cholesterol translocation did not inhibit basal or forskolin-induced adenylyl cyclase activities in fibroblast cells (and in fact slightly stimulated basal adenylyl cyclase activity in rat liver hepatocytes), suggest that the treatment may not, after all, have lead to marked changes in the lipid environment proximal to the adenylyl cyclase complex, at least in the fibroblast cell types. Since sphingomyelin is located in the exofacial leaflet, its removal is not likely to directly affect the environment around the catalytic subunit of adenylyl cyclase in the endofacial leaflet. However, since the removal of sphingomyelin also leads to the redistribution of cholesterol mass, both within the plasma membrane and within the cell, the lack of response from the adenylyl cyclase complex (in fibroblast cells) may suggest that cholesterol did not partition to a significant extent into the vicinity of the catalytic subunit of adenylyl cyclase and its boundary lipids. The slight activation of the basal rate of adenylyl cyclase activity in rat liver hepatocytes after sphingomyelin degradation may indicate that the cholesterol concentration in the endofacial leaflet was not optimal from the adenylyl cyclase-point of view, and hence the adenylyl cyclase complex may have sensed the apparently small change in lipid composition and/or fluidity parameters brought about by the degradation of sphingomyelin.

Next we examined whether receptor-coupled activation of adenylyl cyclase activity could be affected by treatment of cells with sphingomyelinase. We looked at two opposite systems, one was the α_2-adrenergic receptor mediated inhibition of adenylyl cyclase activity, and the other was the glucagon-receptor mediated activation of adenylyl cyclase. In both cases, the receptor-mediated signal to the adenylyl cyclase complex was attenuated by sphingomyelinase-treatment, but in neither case was the signal completely lost. It appeared that the glucagon-receptor response was more affected by sphingomyelinase-treatment, and that the effects on the α_2-adrenergic receptor system were only marginal. It is not clear whether the effect of sphingomyelinase-treatment on the glucagon-receptor mediated activation of adenylyl cyclase activity resulted from the loss of sphingomyelin from the exofacial leaflet, or from the disturbed distribution of cholesterol. However, it is known that the glucagon-receptor activation of adenylyl cyclase in rat liver plasma membranes is markedly affected by a reduction in the plasma membrane content of cholesterol [18]. In an analogous study to ours, Rubalcava and Rodbell [40] reported that a phospholipase C-mediated (partial) degradation of phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin in rat liver plasma membrane homogenates only marginally affected the glucagon-stimulated adenylyl cyclase activity. On the other hand, they showed that a degradation of phosphatidylserine and phosphatidylinositol completely blocked the glucagon-activation of adenylyl cyclase [40]. In our system with intact cells, it is possible that the changes in lipid composition, as

induced by the sphingomyelinase-treatment, led to constraints in the lateral mobility of these transmembrane receptors. Changes in the lipid environment of the plasma membrane would be expected to have significant effects on the lateral diffusion of proteins (receptors). Constraints in the lateral mobility of receptors and adenylyl cyclase would consequently be expected to affect the degree of their interaction.

A way to test for this possibility would be to increase the lateral surface pressure in the plasma membrane of intact cells, and examine the effects of this manipulation on receptor coupling to the adenylyl cyclase system. It is well established that exposure of cells (erythrocytes) to small unilamellar vesicles containing short chain phospholipids (less than 15 carbon length) leads to a rapid transfer of short-chain phospholipids into the plasma membrane outer leaflet [35–38]. If the phospholipid type is phosphatidylcholine (PC), it will remain in the outer leaflet, since it does not spontaneously flip to the inner leaflet, and since there are no specific translocases for PC in the membrane [41]. The rapid transfer of PC from SUVs into cells will therefore lead to a marked increase in the lateral packing density of the plasma membrane outer leaflet. This has been verified by us in a separate study, in which we examined the degree of cholesterol exposure in cells treated by dilauroylPC (DLPC) (Slotte and Tenhunen, unpublished data). The exposure of CHLF to DLPC (25 μg/ml for 2 h) led to a significant reduction in the α_2-adrenergic receptor coupling to the adenylyl cyclase system (Fig. 4). This finding can be interpreted to support the notion that DLPC increased the lateral packing density of the outer leaflet of the plasma membrane, and thus also constrained the receptor-adenylyl cyclase interaction. This in turn could lead to the observed reduced inhibition of the forskolin-activated adenylyl cyclase activity. A similar finding was observed with the glucagon-receptor coupling to adenylyl cyclase in RLH exposed to DLPC (Fig. 6).

In conclusion, these experiments together indicate that receptor-coupling (either the α_2-adrenergic receptor or the glucagon-receptor) to adenylyl cyclase was clearly affected by manipulations of the plasma membrane phospholipid composition, whereas the direct activation of adenylyl cyclase by forskolin or PGE_1 was almost unaffected by these manipulations. The lack of effects of these manipulations on the direct activation of adenylyl cyclase (by, e.g., forskolin) may indicate that the immediate lipid environment surrounding the enzyme complex (including the boundary lipids) protected the function of the enzyme against acute changes in the overall lipid composition of the plasma membrane structure. Receptor coupling to the adenylyl cyclase, however, clearly sensed the changes in the phospholipid environment of the outer leaflet.

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