Articles

Relationship between $Fc_{\gamma 2b}$ Receptor and Adenylate Cyclase of a Murine Macrophagelike Cell Line, $P388D_1$†

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ABSTRACT: The relationship between Fc receptor specific for IgG_{2b} ($Fc_{\gamma 2b}R$) and membrane adenylate cyclase was investigated. The specific binding of IgG_{2b} immune complexes to $P388D_1$ cell surface $Fc_{\gamma 2b}R$ was found to inhibit the basal, forskolin-stimulated, and NaF-stimulated activities of membrane adenylate cyclase by 53%, 57%, and 31%, respectively. On the other hand, the binding of IgG_{2a} immune complexes to cell surface $Fc_{\gamma 2a}R$ increased the basal activity about 2.5-fold and the forskolin- and NaF-stimulated activities slightly. The fusion of liposomes containing $Fc_{\gamma 2b}R$, which was obtained as phosphatidylcholine (PC) binding protein as previously described, with the cyc^- membrane preparations resulted in the marked suppression of membrane adenylate cyclase, whereas the fusion of liposomes containing $Fc_{\gamma 2a}$, which was obtained as IgG-binding protein, led to about a 2.7-fold increase. The $Fc_{\gamma 2b}R$-mediated inhibition of adenylate cyclase may be due to the temporary change of the lipid environment caused by the action of phospholipase A_2, which was previously shown to be associated with $Fc_{\gamma 2b}R$, since (1) addition of snake venom phospholipase A_2 or cholate-solubilized PC-binding protein to $P388D_1$ membrane was found to inhibit adenylate cyclase in a dose-dependent manner, (2) prior treatment of snake venom phospholipase A_2 or PC-binding protein with a specific inhibitor, *p*-bromophenacyl bromide, significantly reduced their inhibitory action, and (3) a product of phospholipase A_2 action, arachidonic acid, was found to be an effective inhibitor of membrane adenylate cyclase, whereas the other product, lysophosphatidylcholine, was much less inhibitory than arachidonic acid. Arachidonic acid appeared to interfere with the functions of both guanine nucleotide-binding stimulatory (Gs) protein and the catalytic subunit of adenylate cyclase, since exogenously added arachidonic acid significantly suppressed the GTPase activity of $P388D_1$ membrane and the forskolin response of the adenylate cyclase activity of Gs protein deficient cyc^- membrane. The primary site of action of lysophosphatidylcholine is not clear but may be other than Gs protein and/or the catalytic subunit, since it did not change either GTPase activity of $P388D_1$ membrane or the response to forskolin of adenylate cyclase of cyc^- membrane. The $Fc_{\gamma 2b}R$/phospholipase A_2 mediated inhibition of adenylate cyclase would be a transient event in viable cells, since phospholipase A_2 did not inhibit adenylate cyclase in the presence of microsomal fraction, mitochondria, and coenzyme A, suggesting the occurrence of rapid acylation of CoA and reacylation of lysolecithin.

Murine macrophagelike cell lines such as $P388D_1$ carry on their surface at least two biochemically distinct Fc receptors, one specific for IgG_{2a} ($Fc_{\gamma 2a}R$)[1] and another for IgG_{2b} ($Fc_{\gamma 2b}R$) (Walker, 1976; Heusser et al., 1977; Unkeless, 1977; Anderson & Grey, 1978; Suzuki et al., 1982). $Fc_\gamma R$ plays an essential role in the antibody-dependent cell-mediated cytotoxicity (Perlman et al., 1972), the suppression of humoral immune response (Uhr and Möller, 1968) or B cell differentiation (Kölsch et al., 1980) by circulating immune complexes, or the triggering of prostaglandin synthesis by macrophages (Passwell et al., 1979, 1980; Bonney et al., 1979). Two types of $Fc_\gamma R$ present on the surface of macrophages then could transmit, upon binding of specific ligands, signals unique to each type, which trigger and maintain a complex program of biochemical events involving the generation, amplification, and propagation of a series of signals leading to the modulation of cellular functions. We have previously shown that a signal to trigger the arachidonic acid metabolic cascade through the cyclooxygenase pathway is transmitted by $Fc_{\gamma 2b}R$, but not by $Fc_{\gamma 2a}R$ (Nitta & Suzuki, 1982a), probably by activating phospholipase A_2 activity, associated with this type of Fc receptor, within the lipid bilayer (Suzuki et al., 1982). Phospholipase A_2 catalyzes the cleavage of the ester bond at the *sn*-2 position of the glycerol backbone of phospholipids, a component of the plasma membrane, leading to the formation of free fatty acid and lysolecithin. Thus, the activation of phospholipase A_2 associated with $Fc_{\gamma 2b}R$ could result in the rapid and transient alteration of the membrane integrity, which may be reflected in the changes in the activities of membrane enzymes. This

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[1] Abbreviations: BSA, bovine serum albumin; cAMP, adenosine cyclic 3′,5′-monophosphate; CoA, coenzyme A; DTT, dithiothreitol; EA_{2a} and EA_{2b}, sheep erythrocytes coated with monoclonal anti-sheep erythrocyte antibodies of IgG_{2a} and IgG_{2b} subclasses, respectively; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $Fc_{\gamma 2a}R$ and $Fc_{\gamma 2b}R$, Fc receptors specific for IgG_{2a} and IgG_{2b}, respectively; Gs protein, guanine nucleotide-binding stimulatory protein; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IgG, immunoglobin G; *p*-BPB, *p*-bromophenacyl bromide; PC, *rac*-1-(9-carboxynonyl)-2-hexadecyl-*sn*-glycero-3-phosphocholine; PE, PI, and PS, phosphatidylethanolamine, -inositol, and -serine, respectively; PMSF, phenylmethanesulfonyl fluoride; PG, prostaglandin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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possibility was examined by investigating the effects of the specific binding of immune complexes to viable cell surface $Fc_{\gamma 2b}R$ or $Fc_{\gamma 2a}R$ and of phosphatidylcholine (PC) binding proteins isolated from $P388D_1$ cell lysate or snake venom phospholipase A_2 on the membrane adenylate cyclase system. Results presented in this paper will demonstrate that membrane adenylate cyclase is inhibited by the specific binding of immune complexes to cell surface $Fc_{\gamma 2b}R$, but not to $Fc_{\gamma 2a}R$. The data suggesting that this inhibition is probably due to the activation of phospholipase A_2 activity associated with $Fc_{\gamma 2b}R$ and may be a transient event in the viable cells will be also presented.

Materials and Methods

Cells. The murine macrophagelike cell line $P388D_1$, derived from methylcholanthrene-induced neoplasm of a DBA/2 mouse (Dawe & Potter, 1957), was a gift of Dr. H. Koren of the U.S. Environment Protection Agency at Research Triangle Park, NC. This cell line has been shown to possess characteristics typical for macrophages, such as adhesion on glass and plastic surface, mediation of antibody-dependent cell-mediated cytolysis, and expression of receptors for the Fc portion of IgG and for C3, but of neither surface immunoglobulins nor Thy-1,2 antigens, on the cell surface (Koren et al., 1975). $P388D_1$ cells were grown as monolayer in plastic flasks (Falcon 3024, Oxnard, CA) at 37 °C in an atmosphere containing 5% CO_2 in the culture medium consisting of RPMI 1640, heat-inactivated (56 °C, 30 min) fetal calf serum (10%), streptomycin (100 μg/mL), and penicillin (100 units/mL) (all from Hazelton Dutchland, Denver, PA), unless otherwise stated. Cell density was maintained between approximately 5×10^5 and 2×10^6 cells/mL. Under these culture conditions, the generation time was about 24 h.

A murine T lymphoma cell line (S49) and its genetic variant (cyc^-) were a gift of Dr. A. G. Gilman of the University of Texas Health Sciences Center of Dallas. S49 cell line, which was established from a lymphoma in a BALB/c/st mouse by phage and oil induction (Horibata & Harris, 1970), retains many of the thymocyte properties, such as expression of Thy-1,2, TL, and $H\text{-}2^d$ antigens on the surface of cells, and possesses a typical hormone-sensitive adenylate cyclase system, consisting of cell surface receptors, Gs protein, and a catalytic unit (Ross et al., 1977). A genetic variant cell line, cyc^-, which was selected by Bourne et al. (1975) from S49 cells, grown in soft agar containing isoproterenol and a phosphodiesterase inhibitor, Ro-20-1724, possesses receptors for various hormones and the intact catalytic unit of adenylate cyclase but lacks Gs protein (Bourne et al., 1975; Ross & Gilman, 1977; Naya-Vigne et al., 1978). By EA rossetting technique, we have shown that S49 cells (about 32 and 44%) express both $Fc_{\gamma 2a}R$ and $Fc_{\gamma 2b}R$, respectively, whereas cyc^- cells (about 39%) express only $Fc_{\gamma 2b}R$ (Fernandez-Botran & Suzuki, 1986). S49 and cyc^- cells were grown in plastic flasks (Falcon 3024) at 37 °C in an atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium, containing heat-inactivated (56 °C, 30 min) horse serum (10%), streptomycin (100 μg/mL), and penicillin (100 units/mL) (all from Hazelton Dutchland). Cell density was maintained between 5×10^5 and 2×10^6 cells/mL.

Biosynthetic Radiolabeling of $P388D_1$ Cells. Cells (5×10^8) were biosynthetically radiolabeled with [^{3}H]leucine (0.33 μCi/mL, Schwarz/Mann, Spring Valley, NY) for 16 h in the RPMI 1640 culture medium from which leucine was deleted, as described (Fernandez-Botran & Suzuki, 1985).

Preparation of Lysate. Metabolically radiolabeled cells were washed 3 times with cold Hank's balanced salt solutions without Ca^{2+} and Mg^{2+} (HBSS; Flow Laboratories, Rockville, MD) and were lysed at 0 °C with 0.5% Triton X-100, made in Tris-HCl buffer (0.15 M, pH 8), containing 1 mM each of phenylmethanesulfonyl fluoride (PMSF) and ethylenediaminetetraacetate (EDTA) (buffer I). After being stirred for 1 h at 0 °C, the lysate was centrifuged for 60 min at 4 °C at 10000 rpm in a Beckman J21 refrigerated centrifuge to remove nuclei, unlysed cells, and other debris. The clear supernatant obtained was immediately subjected to affinity chromatography.

Affinity Chromatography and Isolation of IgG- and PC-Binding Proteins. PC–Sepharose 4B used to extract PC-binding proteins was prepared by coupling *rac*-1-(9-carboxynonyl)-2-hexadecyl-*sn*-glycero-3-phosphocholine (Calbiochem, LaJolla, CA) to AH-Sepharose 4B (Pharmacia, Uppsala, Sweden) in the presence of carbodiimide (Rock & Snyder, 1975), as described in detail (Suzuki et al., 1980). Heat-aggregated IgG was coupled to CNBr-activated Sepharose 4B, as described (Suzuki et al., 1980). These were packed in glass columns connected in tandem and were equilibrated against buffer I. Isolation and purification of PC- and IgG-binding proteins were carried out, as described (Suzuki et al., 1982). Briefly, the detergent lysates of $P388D_1$ cells were immediately subjected to affinity chromatography over the columns of PC– and IgG–Sepharose 4B connected in tandem in this order. After thorough washing with buffer I, the columns were disconnected. The materials bound were separately eluted with 6 M deionized urea, made in 0.2 ionic strength Tris-HCl buffer (pH 8), containing 1 mM each of PMSF and EDTA (buffer II). The eluted materials were separately passed through a column (4.5 × 60 cm) of Sephadex G-100, which was previously equilibrated against buffer II, unless otherwise stated. Sephadex G-100 gel filtration of IgG-binding proteins obtained by affinity chromatography usually led to the separation of a fraction excluded from the gel and another that follows the first peak as broad shouldered material. These representing about 55% and 45% of the materials applied to the column were denoted as IgG-B1 and -B2 proteins, respectively (Suzuki & Fernandez-Botran, 1986; Fernandez-Botran & Suzuki, 1986). They were then separately passed again through the same column of Sephadex G-100 to minimize cross-contamination. IgG-B1 proteins were then dialyzed against deionized water and electrofocused in a pH gradient, formed with carrier ampholyte pH 5–10 in the presence of 6 M urea, with the use of an LKB ampholine column, as described (Suzuki et al., 1982). The electrofocused IgG-B1 proteins were exhaustively dialyzed against buffer I. The IgG-B1 proteins thus obtained have been shown in our previous studies to bind specifically to the Fc portion of IgG_{2a} (Suzuki et al., 1982) and to activate, in cholate-solubilized form, the catalytic subunit of cyc^- cell membrane adenylate cyclase (Fernandez-Botran & Suzuki, 1986).

About 90% of PC-binding proteins applied to Sephadex G-100 gel filtration were excluded from the gel. They were dialyzed against deionized water and subjected to isoelectric focusing, as described above. The PC-binding proteins that focused at pH near 5.8 were collected and exhaustively dialyzed against buffer I. Our previous studies showed that the PC-binding proteins thus obtained are associated with phospholipase A_2 activity and bind specifically to the Fc portion of IgG_{2b} (Suzuki et al., 1982).

Plasma Membranes. Membranes of S49 and cyc^- cells were prepared, as described by Ross et al. (1977). Briefly, cells (5×10^8) were first washed twice with cold HBSS at 4 °C, resuspended, and held for 15 min at 4 °C in 15 mL of 20 mM Na-HEPES buffer, pH 8.0, containing 2 mM $MgCl_2$ and 1

mM EDTA (buffer III). Cells were then homogenized in a Dounce homogenizer with a tight-fitting pestle (20 strokes), and the homogenate was centrifuged for 5 min at 900*g* at 4 °C. The supernatant was then centrifuged for 20 min at 43000*g* at 4 °C. The resultant pellet was resuspended and homogenized in 3 mL of 10% sucrose in buffer III in a Dounce homogenizer with a loose-fitting pestle, layered over the top of a discontinuous concentration gradient, formed with 20, 30, and 40% sucrose in buffer III, and centrifuged for 90 min at 100000*g* at 4 °C. The bands sedimenting on top of the 30 and 40% sucrose concentration were collected by aspiration, pooled, diluted 3-fold with buffer III, and centrifuged for 40 min at 100000*g* at 4 °C. The pellet obtained was resuspended in buffer III containing 1 mM DTT and used as partially purified plasma membranes. Membranes of $P388D_1$ cells were prepared by the methods of Warren et al. (1966) and Nachman et al. (1971), as described (Fernandez-Botran & Suzuki, 1984).

Microsomal and Mitochondrial Preparations. These were prepared by differential centrifugation, essentially as described by Hogeboom (1955), as follows. The supernatant that was obtained by centrifugation (900*g*, 5 min, at 4 °C) of the homogenate of $P388D_1$ cells (5×10^8) was first centrifuged for 10 min at 5000*g* at 4 °C. The pellet obtained was resuspended in 5 mL of 0.25 M sucrose made in buffer III and centrifuged for 10 min at 24000*g* at 4 °C. The resultant pellet was again resuspended in 5 mL of 0.25 M sucrose made in buffer III and centrifuged for 10 min at 24000*g* at 4°C. The pellet obtained was used as mitochondria. The supernatants obtained from two succesive centrifugations were pooled and centrifuged for 60 min at 54000*g* at 4 °C. The pellet obtained was used as crude microsomal fraction.

Preparation of Liposomes and Insertion of IgG-B1 or PC-Binding Proteins into Liposome. The preparation of liposomes consisting of phosphatidylcholine and phosphatidylethanolamine and the insertion of IgG-B1 or PC-binding proteins into the liposomes were carried out as previously described (Fernandez-Botran & Suzuki, 1986). Our previous studies showed that about 35–37% of PC-binding proteins and about 17–20% of IgG-B1 proteins could be inserted into liposomes. Both IgG-B1 and PC-binding proteins have been demonstrated to be inserted into liposomes in a proper orientation, since the liposome-inserted proteins were found to bind to the Fc portion of the appropriate IgG subclasses as expected.

Adenylate Cyclase Assay. This was performed by the method of Salomon (1979), as described (Fernandez-Botran & Suzuki, 1984). Briefly, assay was initiated by addition of partially purified plasma membranes (15 μg of protein), in the presence or absence of snake venom phospholipase A_2 (*Naja naja*, Sigma) or various lipids, to an assay mixture: 50 μL of 25 mM Tris-HCl buffer, pH 7.8, containing creatine phosphate (5 mM), creatine phosphokinase (50 units/mL), magnesium acetate (10 mM), ATP (0.5 mM), 3′,5′-cAMP (0.05 mM), GTP (0.01 mM), dithiothreitol (DTT, 1 mM), bovine serum albumin (BSA, 0.1 mg/mL), 3′-isobutyl-1-methylxanthine (1 mM), and [α-^{32}P]ATP [(2–6) × 10^6 cpm]. All of the chemicals, except radioactive ATP, in the assay mixture were obtained from Sigma (St. Louis, MO). [α-^{32}P]ATP (>400 Ci/mmol) was from ICN (Irvine, CA). After the incubation for 30 min at 37 °C, the reaction was stopped by addition of 100 μL of stopping solution consisting of sodium dodecyl sulfate (SDS, 2%, Sigma), ATP (45 mM), cAMP (1.3 mM), and a trace of [2,8-^{3}H]-3′,5′-cAMP (30–50 Ci/mmol, New England Nuclear). Each sample was heated for 3 min at 100 °C to solubilize membranes. [^{32}P]cAMP was separated from the reaction mixtures by sequential chromatography on columns of Dowex AG 50W-X4 (Bio-Rad) and of neutral alumina WN-3 (Sigma). [^{32}P]cAMP with internal standard, [^{3}H]cAMP, thus obtained was processed for liquid scintillation counting. When the effects of exogenously added fatty acids or lysophopholipids were examined, these lipids were dissolved in dimethyl sulfoxide prior to the addition to membranes. Final concentration of dimethyl sulfoxide in the reaction mixtures including the control was 1%. Adenylate cyclase activity was expressed as picomoles of cAMP formed per milligram of protein per 30 min. The results of triplicate assays were presented as means ± standard errors and compared by the Student's *t* test.

GTPase Assay. This was carried out by the method of Cassell and Selinger (1976, 1977). Briefly, membranes of $P388D_1$ cells were incubated for 15 min at 37 °C, in the presence or absence of varying doses of lipids, in a final volume of 100 μL of 50 mM imidazole hydrochloride buffer, pH 6.7 containing 0.25 μM [γ-^{32}P]GTP (30 mCi/μmol, New England Nuclear), 5 mM $MgCl_2$, 0.2 mM 5′-adenylyl imidodiphosphate, 0.1 mM ATP, 2 mM creatine phosphate, 30 units of creatine phosphokinase, 1 mM DTT, and 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). The reaction was stopped by addition of 0.1 mL of 2.5% SDS. The cleavage product $^{32}PO_4^{3-}$ was separated from nucleotide-bound phosphate by a small column (0.7 × 7 cm) of activated charcoal (Norit A) and counted in a scintillation counter.

Radiolabeling with [^{3}H]Arachidonic Acid. $P388D_1$ cells (5×10^7) were cultured for 48 h in medium containing 2 μCi of [^{3}H]arachidonic acid (60 μCi/mmol, Amersham, Arlington Heights, IL). Cells were then washed 3 times with the culture medium to remove free [^{3}H]arachidonic acid and were cultured for an additional 24 h. At this stage, about 98% of [^{3}H]arachidonic acid was found to be in the lipids extracted by chloroform/methanol (2/1 v/v) from the membrane prepared from the radiolabeled $P388D_1$ cells. The analysis of lipids in the extract was carried out by thin-layer chromatography on a silica gel G-25 coated class plate with the use of a solvent system consisting of chloroform/methanol/ammonium hydroxide (13/7/1 v/v/v), as described by Hsueh et al. (1979). The result showed that 36% of the radioactivity was associated with phosphatidylethanolamine, 13% with phosphatidylcholine, and 24% with the combined fraction of phosphatidylserine and -inositol; about 26% of the radioactivity was found in the free fatty acid and the neutral lipid fraction, respectively.

Preparation of Immune Complexes. IgG_{2a} and IgG_{2b} subclass monoclonal anti-bovine Ig light chain antibodies were obtained from HyClone (Logan, UT). Aliquots containing 200 μg of the antibodies of each isotype were separately incubated for 30 min at 37 °C and then for 16 h at 4 °C with 200 μg of $F(ab')_2$ fragments of bovine IgG (Cappel, Malvern, PA) in phosphate-buffered saline (0.15 M, pH 7.4), prior to addition to membranes.

IgG_{2a} and IgG_{2b} subclass monoclonal anti-sheep erythrocyte (SRBC) antibodies were obtained by affinity chromatography on protein A–Sepharose (Pharmacia) of the culture supernatants of mouse–mouse hybridoma cell lines S-S1 and N-S.8.1 (both from American Type Culture Collection, Rockville, MD), respectively. Monoclonal antibodies isolated were dialyzed against phosphate-buffered saline (0.15 M, pH 7.4) and heated for 30 min at 56 °C to inactivate complement components possibly present in the preparations. Sheep erythrocytes coated with antibodies (EA_{2a} or EA_{2b}) were

Table I: Effects of Immune Complex Binding to $Fc_\gamma R$ on Membrane Adenylate Cyclase[a]

stimuli	incubation with E	EA_{2a}	EA_{2b}	adenylate cyclase activity: pmol mg^{-1} $(30\ min)^{-1}$	% control
none	+	–	–	83.5 ± 4.6	100
	–	+	–	208.8 ± 27.9[d]	250
	–	–	+	39.0 ± 10.4[d]	47
forskolin	+	–	–	431.7 ± 44.1	100
	–	+	–	529.4 ± 27.5[b]	123
	–	–	+	184.5 ± 22.4[e]	43
NaF	+	–	–	544.7 ± 42.3	100
	–	+	–	673.8 ± 37.3[b]	124
	–	–	+	376.5 ± 87.9[c]	69

[a] $P388D_1$ cells (2×10^8) were cultured for 180 min at 37 °C with E, EA_{2a}, or EA_{2b} (1×10^9 erythrocytes) in plastic flasks in tissue culture media. After the incubation period, cells were harvested, exposed for a few second to hypotonic saline to lyse sheep erythrocytes, and washed 3 times with HBSS. Plasma membranes were prepared from the washed cells and were assayed for adenylate cyclase in the presence or absence of 50 μM forskolin or 10 mM NaF as described under Materials and Methods. Results presented are the mean ± SEM of triplicate determinations. [b] $p < 0.05$. [c] $p < 0.02$. [d] $p < 0.01$. [e] $p < 0.001$.

prepared by incubating erythrocytes (5% in HBSS) for 60 min at 37 °C with an equal volume of nonagglutinating doses of monoclonal antibodies. EA preparations were washed 3 times with HBSS and resuspended at 2×10^8/mL in HBSS.

Other Methods. Isoelectric focusing in the presence of 6 M urea was carried out as described (Suzuki et al., 1980, 1982). Protein concentration was estimated by Lowry's (1951) method.

Results

Effects of the Immune Complex Binding to Fc_γ Receptors on Membrane Adenylate Cyclase. Our previous studies showed the association of phospholipase A_2 activity with the $Fe_\gamma R$ specific for IgG_{2b}, but not with that specific for IgG_{2a}, present on the surface of $P388D_1$ cells as well as peritoneal macrophages (Suzuki et al., 1982; Nitta & Suzuki, 1982a; Nitta et al., 1984). The activation of phospholipase A_2, as a result of the specific binding of immune complexes to the cell surface $Fc_{\gamma 2b}R$ (Nitta & Suzuki, 1982a; Rhodes et al., 1985), could affect the activity of membrane adenylate cyclase, since this enzyme must act on phospholipids, a component of the lipid bilayer, in which the adenylate cyclase system is embedded. This possibility was first examined by assaying for the adenylate cyclase activities of the membranes of $P388D_1$ cells, which were cocultured for 180 min with sheep erythrocytes (E), EA_{2a}, or EA_{2b}. As summarized in Table I, the incubation of $P388D_1$ cells with EA_{2b} caused about 53% inhibition of the basal adenylate cyclase activity in comparison to the incubation with E. As reported previously (Suzuki & Fernandez-Botran, 1986; Fernandez-Botran & Suzuki, 1986), the incubation of $P388D_1$ cells with EA_{2a} resulted, on the other hand, in about a 2.5-fold increase in the basal activity. Forskolin- and NaF-stimulated activities were also significantly inhibited by the incubation of $P388D_1$ cells with EA_{2b} (59 and 31%, respectively) and were enhanced by the incubation with EA_{2a} (about 1.2-fold), in comparison to the control.

Effects of PC-Binding Proteins on the Catalytic Activity of Adenylate Cyclase. In order to investigate the mechanism of $Fc_{\gamma 2b}R$-mediated inhibition of adenylate cyclase, PC-binding proteins were isolated from the detergent lysate of $P388D_1$ cells and purified, as described earlier (Suzuki et al., 1982; Nitta et al., 1984). They were made free of Triton X-100 by SM2 bead adsorption and were inserted into liposomes, consisting of phosphatidylcholine and phosphatidylethanolamine (1:1 w/w). Liposomes containing about 10 μg of PC-binding proteins were fused with 2×10^7 cyc^- cells with the use of poly(ethylene glycol) as described previously (Fernandez-Botron & Suzuki, 1986). The membrane prepared from resultant hybrid was assayed for the adenylate cyclase activity in the presence or absence of NaF (10 mM) or of soluble immune complexes, IC_{2b} (10 μg/mL) or IC_{2a} (10 μg/mL), which were prepared as described under Materials and Methods. Results showed, as summarized by Table II, that the fusion of liposomes containing PC-binding proteins with cyc^- cells resulted in the significant inhibition (about 80%) of adenylate cyclase activity, particularly in the presence of IC_{2b}.

Table II: Effects of PC-Binding Proteins on Catalytic Activity of cyc^- Adenylate Cyclase[a]

treatment with NaF	IC_{2a}	IC_{2b}	adenylate cyclase activity [pmol mg^{-1} $(30\ min)^{-1}$] with liposome containing: no protein	PC-binding protein	% change
–	–	–	43.1 ± 18.5	49.3 ± 16.6	+14.4
+	–	–	50.6 ± 15.1	28.6 ± 14.2	−45.5
–	+	–	34.1 ± 9.9	19.9 ± 6.5	−58.4[b]
–	–	+	36.5 ± 10.0	7.2 ± 3.1	−80.3[c]

[a] Liposome containing about 10 μg of PC-binding proteins was fused with 2×10^7 cyc^- cells as described (Fernandez-Botran & Suzuki, 1986). The membrane adenylate cyclase activity of the hybrid was assayed in triplicate in the presence or absence of NaF (10 mM) or of soluble immune complexes, IC_{2a} or IC_{2b} (10 μg/mL), which were prepared as described under Materials and Methods. The results of the fusion of IgG-binding protein (1 and 1.5 μg) containing liposome with 2×10^7 cyc^- cells peerformed simultaneously were reported earlier and showed the 1.5- and 2.8-fold increase in the adenylate cyclase activities, respectively, when assayed in the presence of IC_{2a} (10 μg/mL) (Fernandez-Botran & Suzuki, 1986). [b] $p < 0.05$. [c] $p < 0.02$.

Table III: Effects of Cholate-Solubilized PC-Binding Proteins on S49 Adenylate Cyclase[a]

PC-binding proteins (μg/mg of membrane)	adenylate cyclase activity [pmol mg^{-1} $(30\ min)^{-1}$]	% inhibition
0	335 ± 53	0
0.005	326 ± 15	2.7
0.05	204 ± 32	39.1[b]
0.5	120 ± 36	64.2[c]
5.0	95 ± 10	71.6[c]

[a] S49 membrane preparations (1 mg of protein/mg) were incubated for 60 min at 10 °C with various amounts of the cholate-solubilized PC-binding proteins. At the end of incubation period, adenylate cyclase activities of the mixtures were assayed in triplicate. [b] $p < 0.05$. [c] $p < 0.01$.

Next, the question of whether or not the PC-binding protein in soluble form could affect the adenylate cyclase system was investigated. The electrofocused PC-binding proteins were first extensively dialyzed against 25 mM cholate–HME buffer (pH 8). An aliquot (40 μL) containing various amounts (5 ng to 100 μg/mg of membrane protein) of the cholate-solubilized PC-binding proteins was incubated at 10 °C for 60 min with 60 μL of S49 cell membrane preparation (1 mg of protein/mL). At the end of the incubation period, adenylate cyclase activities of the mixtures were assayed. Results summarized by Table III show that the addition of PC-binding proteins to S49 membranes inhibited the basal adenylate cyclase activities of the membranes in a dose-dependent manner and that a significant inhibition could result from the addition of 0.5–5 μg of PC-binding proteins/mg of membrane protein.

Effects of Phospholipase A_2 on Adenylate Cyclase. The noted inhibition of membrane adenylate cyclase, by the specific binding of immune complexes to the cell surface $Fc_{\gamma 2b}R$ or

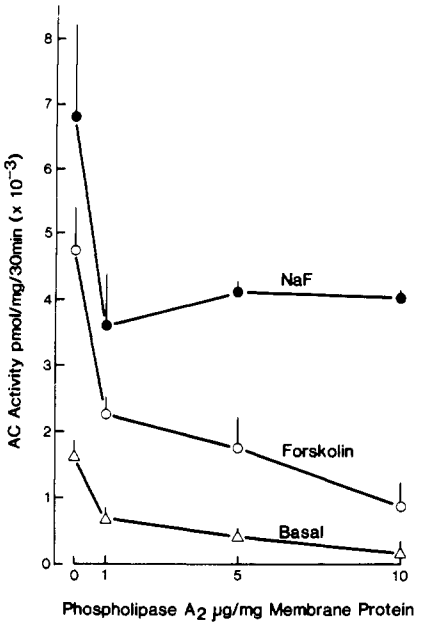

FIGURE 1: Effects of exogenously added snake venom phospholipase A_2 on the basal and forskolin- and NaF-stimulated adenylate cyclase activities of P388D$_1$ membrane. Snake (*Naja naja*) venom phospholipase A_2 (800 units/mg, Sigma) was first activated by incubation for 60 min at 37 °C in 10 mM Tris-HCl buffer (pH 8) containing 5 mM Ca^{2+} and then dialyzed for 16 h at 4 °C against four changes of a 1000-fold excess volume of the same buffer from which Ca^{2+} was removed. P388D$_1$ membrane preparations were incubated for 30 min at 30 °C with varying doses (1–100 μg/mg of membrane protein) of the preactivated snake venom phospholipase A_2 and then were assayed for the adenylate cyclase activities in the presence or absence of 10 mM NaF or 50 μM forskolin. Each point represents the mean ± SEM of triplicate determinations.

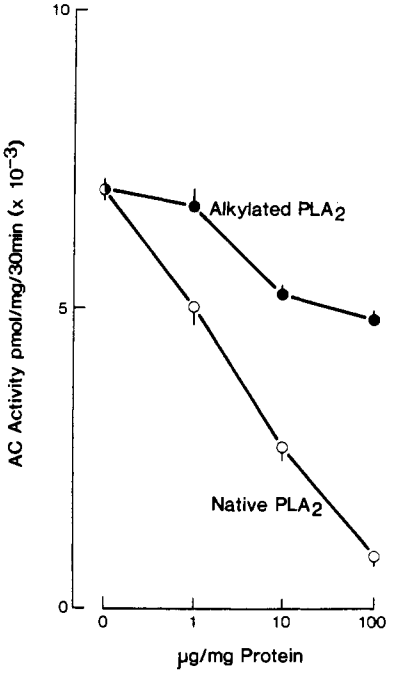

FIGURE 2: Effects of phospholipase A_2 alkylated with *p*-bromophenacyl bromide on P388D$_1$ membrane adenylate cyclase. Snake venom phospholipase A_2 (1 mg/mL) was treated for 1 h at 37 °C at pH 8 with 10 μM *p*-BPB and was then dialyzed for 16 h at 4 °C against four changes of a 1000-fold excess volume of Tris-HCl buffer (10 mM, pH 8). The alkylated enzyme at 1 μg/mg of membrane protein catalyzed the release of about 10% of [^{3}H]arachidonate associated with phosphatidylethanolamine and -choline of the membrane prelabeled with radioactive arachidonate, whereas the native enzyme at the same concentration catalyzed the release of about 90% of [^{3}H]arachidonate from the same membrane. The effects of alkylated phospholipase A_2 and untreated (native) enzyme on the basal activity of P388D$_1$ membrane adenylate cyclase were examined as described in Figure 1. Each point represents the mean ± SEM of triplicate determinations.

by fusion of liposome containing the isolated PC-binding proteins, could be due to the phospholipase A_2 mediated alteration of the membrane lipid environment in which the adenylate cyclase system is embedded, since PC-binding proteins have been shown to possess phospholipase A_2 activity (Suzuki et al., 1982). If this is the case, treatment of membrane with snake venom phospholipase A_2 should also result in the inhibition of adenylate cyclase. To test this, snake (*Naja naja*) venom phospholipase A_2 (800 units/mg of protein, Sigma, MO) was first activated by incubation at 37 °C for 60 min in 10 mM Tris-HCl buffer (pH 8) containing 5 mM Ca^{2+} and then dialyzed for 16 h at 4 °C against four changes of a 1000-fold excess volume of the same buffer from which Ca^{2+} was removed. P388D$_1$ membrane preparations were incubated with varying doses (1–100 μg/mg of membrane protein) of the preactivated snake venom phospholipase A_2 or with the same volume of the last dialyzate (control) for 30 min at 30 °C and then assayed for their basal adenylate cyclase activities. Results illustrated by Figure 1 clearly show that phospholipase A_2 inhibited the membrane adenylate cyclase activities in a dose-dependent manner, causing 50% inhibition of the basal activity with about 1–5 μg of phospholipase A_2/mg of membrane protein. The adenylate cyclase activities in response to forskolin or NaF were also significantly reduced by the treatment of the membranes with 1 μg of phospholipase A_2. If the noted inhibition of adenylate cyclase activities is due to the action of phospholipase A_2, the alkylation of snake venom phospholipase A_2 with *p*-bromophenacyl bromide (*p*-BPB), an inhibitor of the lipase (Volwerk et al. 1974), should abrogate the inhibitory action of phospholipase A_2. To this end, the snake venom phospholipase A_2 preparation (1 mg/mL) was treated for 1 h at 37 °C at pH 8 with 10 μM *p*-BPB and was dialyzed at 4 °C for 16 h against four changes of a 1000-fold excess volume of Tris-HCl buffer (10 mM, pH 8). The effects of varying doses of alkylated phospholipase A_2 on the basal adenylate cyclase activity were then compared to those of the native enzyme. As seen in the Figure 2, the prior treatment of snake venom phospholipase A_2 with an inhibitor of the enzyme abrogated partially its inhibitory activity. Similarly, the prior treatment of cholate-solubilized PC-binding proteins with *p*-BPB abrogated their inhibitory activity by about 50% (data not shown).

Effects of Fatty Acids and Lysophospholipids on Membrane Adenylate Cyclase. The *sn*-2 position of phospholipids is often occupied by unsaturated fatty acids such as arachidonate. If the noted inhibition of adenylate cyclase by PC-binding proteins or snake venom phospholipase A_2 is, indeed, due to the cleavage of phospholipids, the exogeneously added fatty acids, particularly unsaturated ones, or lysophospholipids should exert a profound influence on the adenylate cyclase activity. In order to examine this possibility, the question of whether or not the exogeneously added phospholipase A_2 or PC-binding proteins indeed catalyzes the hydrolysis of membrane phospholipids was first examined as follows. The membranes were prepared from P388D$_1$ cells that were labeled with [^{3}H]arachidonate as described under Materials and Methods. They were incubated for 60 min at 10 °C and for an additional 30 min at 37 °C in 25 mM Tris-HCl buffer (pH 8) with native or *p*-BPB-treated snake venom phospholipase A_2 or with cholate-solubilized PC-binding protein that was previously purified from the detergent lysate of P388D$_1$ cells as described (Suzuki et al., 1982) and was preactivated with

Table IV: Effects of Snake Venom Phospholipase A_2 or Cholate-Solubilized PC-Binding Protein on $P388D_1$ Membrane Phospholipids[a]

treatment (per milligram of membrane protein) with	radioactivity (cpm × 10^{-3}) in		
	fatty acid	PC	PE
none	5.2 ± 0.09	2.3 ± 0.04	7.7 ± 0.7
native phospholipase A_2 (1 μg)	16.2 ± 0.8	0.6 ± 0.07	1.1 ± 0.1
alkylated phospholipase A_2 (1 μg)	6.7 ± 0.8	1.8 ± 0.2	6.0 ± 0.8
alkylated phospholipase A_2 (10 μg)	13.5 ± 0.2	1.4 ± 0.01	3.4 ± 0.8
PC-binding protein (3 μg)	5.6 ± 0.4	2.0 ± 0.2	7.4 ± 1.3
PC-binding protein (30 μg)	11.9 ± 0.3	0.04 ± 0.01	1.5 ± 0.2

[a] $P388D_1$ membrane was prepared from the cells (10^8) that were previously radiolabeled with [^{3}H]arachidonate as described under Materials and Methods. The membranes were incubated with native or *p*-BPB-treated phospholipase A_2 or with cholate-solubilized PC-binding protein for 60 min at 10 °C and then for 30 min at 37 °C in 25 mM Tris-HCl buffer (pH 8) with constant stirring. At the end of the incubation period, lipids were extracted with cold chloroform/methanol (2:1 v/v), fractionated by thin-layer chromatography, and counted by a scintilation counter as described under Materials and Methods.

Ca^{2+} as described above. After the incubation period, lipids were extracted from the reaction mixtures and fractionated by thin-layer chromatography as described under Materials and Methods. As shown by Table IV, the treatment of the membrane with native phospholipase A_2 at 1 μg/mg of membrane protein resulted in the cleavage of arachidonate from the *sn*-2 position of phosphatidylcholine (PC) and -ethanolamine (PE), since almost all of the radioactivities associated with these phospholipids was lost, whereas the radioactivities in the free fatty acid fraction were concomitantly increased. The treatment of the membrane with *p*-BPB-treated phospholipase A_2 at the concentration (1 μg/mg of membrane protein) that did not cause a significant reduction in the basal adenylate cyclase activity (see Figure 2) did not release radioactive arachidonate from either PE or PC. At higher concentration (10 μg/mg of membrane protein) of alkylated phospholipase A_2, which caused some 26% inhibition of the basal adenylate cyclase (Figure 2), presumably due to incomplete alkylation of lipase, more than 50% of arachidonate associated with PE and PC were found to be cleaved. Similarly, the treatment of the membrane with the cholate-solubilized PC-binding proteins at 30 μg/mg of membrane protein resulted in the release of most of arachidonate associated with PE and PC with the concomitant increase of the radioactivities in the fatty acid fraction. These data thus showed that the treatment of $P388D_1$ membrane with phospholipase A_2 active materials indeed results in the cleavage of the ester bond at the *sn*-2 position of endogenous PE and PC at the concentrations of lipases that cause a significant reduction of the membrane adenylate cyclase activity.

Next, the question of whether or not the cleavage products of phospholipase A_2 directly affect the adenylate cyclase activity was examined by assaying the adenylate cyclase activities of $P388D_1$ membranes in the presence or absence of various fatty acids or lysophospholipids. As illustrated by Figure 3, all fatty acids examined at the concentration below 50 μg/mg of membrane protein were found to increase the basal activities about 2–3-fold, regardless their chain length or number of unsaturated bonds. Only arachidonate at the highest concentration tested (100 μg/mg of membrane protein) significantly inhibited the basal enzymatic activity (about 70% reduction). Oleate was found to be the best stimulator of the basal activities and enhanced the basal activities almost 3-fold at the concentration of 50 and 100 μg/mg of membrane protein. The effects of exogenously added palmitate were

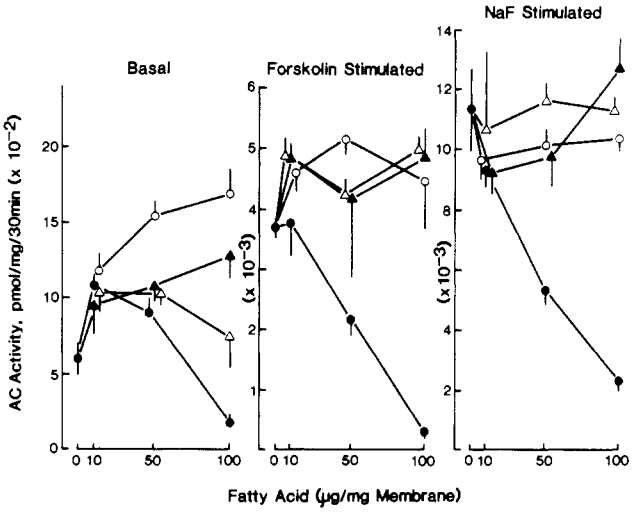

FIGURE 3: Effects of fatty acids on adenylate cyclase of $P388D_1$ membrane. $P388D_1$ membrane preparations were incubated for 60 min at 10 °C in the presence or absence of varyihng doses (10–100 μg/mg of membrane protein) of arachidonate (●), oleate (○), palmitate (▲), or steartate (△), which were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the experimental and control mixtures was 1%. At the end of the incubation period, adenylate cyclase activities of the mixtures were assayed at 37 °C in the presence or absence of 50 μM forskolin or 10 mM NaF. Each point represents the mean ± SEM of triplicate determinations.

similar to but less than those of oleate. The effects of stearate at the concentration of 10 and 50 μg/mg of membrane protein were similar to those of arachidonate and of palmitate.

The responses of the adenylate cyclase system of $P388D_1$ membrane to forskolin were found to be drastically reduced by the presence of arachidonate at the concentration of 50 and 100 μg/mg of membrane protein, to about 50 and 10% of the control level, respectively. The effects of oleate at a concentration below 50 μg/mg of membrane protein were found to be again stimulatory, but to a lesser degree than the enhancement of the basal activities. The higher concentration of oleate appeared to lose its stimulatory activity, although the effects were not statistically significant, when compared to the control. Both palmitate and stearate exerted similar and modest enhancing effects, some of which were statistically insignificant over the control. The responses to NaF were also affected by exogenously added fatty acids to a varying degree. But, only significant and drastic inhibitory effects were noted when the enzyme assays were carried out in the presence of arachidonate. Reduction in the NaF response by arachidonate thus increased, in a dose-dependent manner, to about 30% of the control level at a concentration of 100 μg/mg of membrane protein.

As shown by Figure 4, among three different lysophospholipids tested, only lysophosphatidylcholine significantly inhibited the basal activities, in a dose-dependent manner, to about 50% of the control level at the highest concentration tested (100 μg/mg of membrane protein). The responses to forskolin or NaF of adenylate cyclase were found to be reduced by only lysophosphatidylcholine at its highest concentration tested. None of the lysophospholipids were found to stimulate the basal activities or the responses to forskolin or NaF of adenylate cyclase at any concentration examined.

Effects of Lipids on GTPase Activity of $P388D_1$ Membrane. The specific binding of immune complexes to the cell surface $Fc_{\gamma 2b}R$ or the exogenous additions of the isolated PC-binding proteins or snake venom phospholipase A_2 thus all resulted in the inhibition of adenylate cyclase not only in its basal activity but also its responses to NaF, which stimulates Gs protein

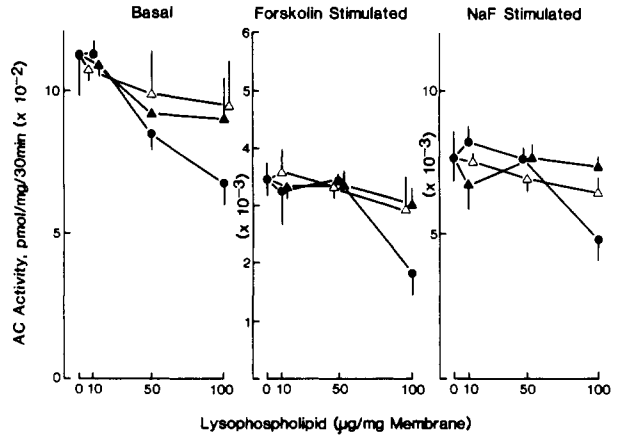

FIGURE 4: Effects of lysophospholipids on adenylate cyclase of $P388D_1$ membrane. $P388D_1$ membranes were incubated for 60 min at 10 °C with or without varying doses (10–100 μg/mg of membrane protein) of lysophosphatidylcholine (●), -ethanolamine (△), or -serine (▲) and then assayed for adenylate cyclase in the presence or absence of 50 μM forskolin or 10 mM NaF as described in Figure 1. All lipids were dissolved in dimethyl sulfoxide prior to the addition to the membranes. The final concentration of dimethyl sulfoxide in the experimental and control mixtures was kept at 1%.

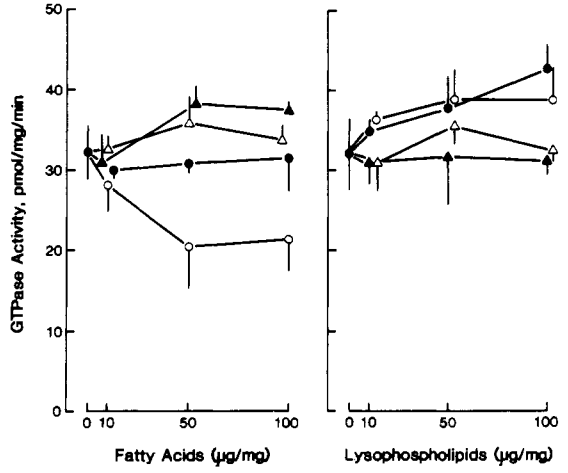

FIGURE 5: Effects of fatty acids [left, arachidonate (○), oleate (●), palmitate (△), and stearate (▲)] and lysophospholipids [right, lysophosphatidylinositol (○), -choline (●), -serine (△), and -ethanolamine (▲)] on the GTPase activity of $P388D_1$ membrane. $P388D_1$ membranes were incubated for 60 min at 10 °C with varying doses of fatty acids or lysophospholipids as in Figures 3 and 4. The GTPase activities of the mixtures were assayed at 37 °C by the methods of Cassel and Selinger (1976, 1977) as described under Materials and Methods. Each points represents the mean ± SEM of triplicate determinations.

function (Ross et al., 1978; Downs et al., 1980), and to forskolin, which directly stimulates the catalytic subunit of the enzyme (Pfeuffer et al., 1983; Smigel, 1986). It follows that the inhibition of adenylate cyclase caused by the action of phospholipase A_2 active materials may be due to the inactivation of Gs functions and/or the catalytic subunit activity. In order to examine this possibility, the effects of various lipids on the GTPase activity of $P388D_1$ cell membrane were next investigated, since GTPase activity has been demonstrated to be a function of Gs protein (Ross & Gilman, 1980). Figure 5 illustrates the results of GTPase assays carried out in the presence of various free fatty acids or lysophopholipids. It is clear that only arachidonate at a concentration greater than 50 μg/mg of membrane protein exerted statistically significant inhibition (about 40%) of the GTPase activity.

Effects of Lipids on the Catalytic Subunit Activity. The effects of various lipids on the catalytic subunit function were next investigated by assaying the adenylate cyclase activities

Table V: Effects of Lipids on Forskolin-Stimulated Adenylate Cyclase Activity of cyc^- Membrane[a]

exogenously added material	adenylate cyclase activity [pmol mg^{-1} $(30\ min)^{-1}$]	% control
none	121.0 ± 13.0	100
fatty acids		
C20:4	44.9 ± 6.1[c]	37
C18:1	98.3 ± 5.4[b]	81
C18:0	146.7 ± 29.4	121
C16:0	165.2 ± 23.1[b]	137
lysophosphatidylcholine	134.4 ± 3.9	111
lysophosphatidylethanolamine	129.1 ± 26.8	107
lysophosphatidylserine	128.8 ± 3.4	106

[a] cyc^- membranes were incubated for 60 min at 10 °C with or without various lipids (100 μg/mg of membrane protein), which were dissolved in dimethyl sulfoxide prior to the addition. The final concentration of dimethyl sulfoxide in the mixtures including the control was 1%. At the end of the incubation period, adenylate cyclase activity was assayed in triplicate in the presence of 50 μM forskolin. [b] $p < 0.05$. [c] $p < 0.001$.

of cyc^- membranes in response to forskolin, since the cyc^- cell membrane has been shown to lack Gs protein but to possess a functional catalytic subunit of adenylate cyclase (Bourne et al., 1975; Ross & Gilman, 1977; Naya-Yigne et al., 1978; Ross et al., 1978). Results summarized by Table V show that the incubation of cyc^- membrane with arachidonate (100 μg/mg of membrane protein) reduced adenylate cyclase activity in response to forskolin from the control value of 121 ± 13 pmol mg^{-1} $(30\ min)^{-1}$ to 44.9 ± 6.1 pmol mg^{-1} $(30\ min)^{-1}$ (63% inhibition). The incubation with oleate also resulted in the inhibition of the enzymatic activity, with a considerably lesser effect (19% inhibition) than that obtained by arachidonate. The incubation of cyc^- membrane with either palmitate or stearate resulted in a slight increase of the enzymatic activities. Three types of lysophospholipids had essentially no effect on the enzymatic activities of cyc^- membrane.

Reversal of Phospholipase A_2 Induced Inhibition of Adenylate Cyclase. The above results suggested that the activation of phospholipase A_2 associated with $Fc_{\gamma 2b}R$ due to the specific binding of immune complexes leads to the inhibition of membrane adenylate cyclase as a consequence of the generation of unsaturated fatty acids, particularly arachidonate, and lysophospholipids within the lipid bilayer. Such inactivation may be a rapid, transient event in the viable cells, because cells may be able to maintain their membrane integrity by converting fatty acids to acyl-CoA, which could be reutilized for reacylation of lysophospholipids catalyzed by acyltransferase. In order to examine this possibility, the adenylate cyclase activities of the $P388D_1$ membranes (15 μg of protein per assay) in the presence of preactivated snake venom phospholipase A_2 (10 μg/mg of membrane protein) were assayed in the presence or absence of the reacylation system, which is composed of mitochondria (5 μg of protein/mg of membrane protein, asa source of acyl-CoA synthetase), microsomal fraction (5 μg of protein/mg of membrane protein, as a source of acyl-CoA:lysophosphatidylcholine acyltransferase), and CoA (2 ng/mg of membrane protein). As shown by Figure 6, the treatment of $P388D_1$ membrane with phospholipase A_2 in the absence of the reacylation system (experiment B) caused more than 50% reduction in the basal adenylate cyclase activity and more than 70% reduction in the response to forskolin, when compared to the control (experiment A). The simultaneous addition of the reacylation system (experiment C) clearly prevented the action of phospholipase A_2 to reduce the basal activity of membrane adenylate cyclase, although the reversal was not complete. This reversal is not due to the adenylate

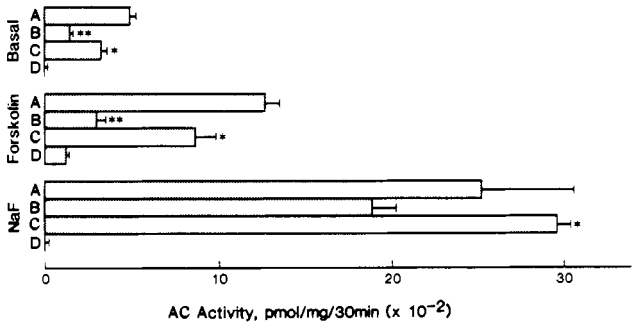

FIGURE 6: Effects of microsomal fraction, mitochondria, and coenzyme A on the phospholipase A_2 induced inhibition of adenylate cyclase. $P388D_1$ membranes (15 μg of protein/assay) were incubated for 60 min at 10 °C with (experiment B) or without (experiment A) preactivated snake venom phospholipase A_2 (10 μg/mg of membrane protein). The basal and forskolin- (50 μM) or NaF- (10 mM) stimulated adenylate cyclase activities of the mixtures were assayed at 37 °C in the presence (experiment C) or absence (experiment B) of microsomal (5 μg/mg of membrane protein) and mitochondrial (5 μg/mg of membrane protein) fractions and CoA (2 ng/mg of membrane protein). Experiment D represents the results of adenylate cyclase assays of the mixture of microsomal and mitochondrial fractions and CoA, which did not include $P388D_1$ membranes. Each bar represents the mean ± SEM of triplicate determinations. (*) $p < 0.001$, compared to experiment B; (**) $p < 0.01$, compared to experiment A.

cyclase activities potentially associated with the microsomal fraction and/or mitochondria, since the reacylation system alone (experiment D) did not show any measurable basal or forskolin- or NaF-stimulated enzymatic activity. The significant inhibition of the responses of membrane adenylate cyclase to forskolin by phospholipase A_2 in the absence of the reacylation system was also removed by the addition of the reacylation system. The inhibition of the response of adenylate cyclase to NaF by phospholipase A_2 in the absence of the reacylation was small and statistically insignificant. In the presence of the reacylation system, phospholipase A_2 clearly did not inhibit the response of adenylate cyclase to NaF.

Discussion

The data presented in this paper show that the specific binding of immune complexes to $Fc_{\gamma 2b}R$ present on the surface of $P388D_1$ cells leads to the inhibition of the basal and NaF- or forskolin-stimulated activities of membrane adenylate cyclase. This inhibition is probably due to the changes in the lipid environment surrounding the adenylate cyclase system, which are caused by the generation of unsaturated fatty acid, particularly arachidonate, within the lipid bilayer due to the action of phospholipase A_2, which is found to be associated with $Fc_{\gamma 2b}R$ (Suzuki et al., 1982) and activated in response to the binding of immune complexes to the receptor (Nitta & Suzuki, 1982a; Rhodes et al., 1985). Consistent with this hypothesis is the findings of the inhibition of membrane adenylate cyclase by exogenously added phospholipase A_2 active materials, snake venom enzyme (Figure 1 and 2), and the isolated PC-binding proteins in liposome-inserted or cholate-solubilized forms (Tables II and III). The findings of the loss of inhibitory activity by prior alkylation of phospholipase A_2 with its specific inhibitor, *p*-BPB (Figure 2), also support the hypothesis. Furthermore, the data of Table IV showing the almost complete release of [^{3}H]arachidonate from endogenous [^{3}H]arachidonate-containing PC and PE by the phospholipase A_2 active materials and the data of Figures 3 and 4 showing the marked inhibitory activities of exogenously added arachidonate and lysolecithin support the basic concept that adenylate cyclase activities will be influenced by the changes in the lipid environment in which the enzyme is embedded. The data of Figure 5 and Table V show that the inhibitory effects of arachidonate are exerted on the functions of both Gs protein and the catalytic subunit of adenylate cyclase. The role of lysolecithin in the inhibition of adenylate cyclase is less clear, on the other hand, because exogenously added lysolecithin did not appear to affect functions of either Gs protein or the catalytic subunit. The possibility that remains to be examined is that lysolecithin may inhibit adenylate cyclase by interfering with the ADP-ribosylation site of the α subunit of Gs protein or with the interaction between Gs protein and the catalytic subunit, which is prerequisite for the activation of the catalytic activity, or activating the guanine nucleotide-binding inhibitory protein.

We have previously shown that the PC-binding protein isolated from the detergent lysate of $P388D_1$ cells specifically binds to the Fc portion of IgG_{2b} but not to IgG_{2a} (both presented as EA) and is associated with phospholipase A_2 activity, whereas the IgG_{2a}-binding protein isolated from the same lysate does not bind to IgG_{2b} and is not associated with phospholipase A_2 activity (Suzuki et al., 1982). The isolated PC/IgG_{2b}-binding protein has been shown to share common antigenic determinants with $Fc_{\gamma 2b}R$ recognized by monoclonal antibody (2.4G2), which is presumably directed against cell surface $Fc_{\gamma 2b}R$ (Unkeless, 1979), whereas the isolated IgG_{2a}-binding protein does not react with this monoclonal antibody (Fernandez-Botran & Suzuki, 1985). We have also shown that $P388D_1$ cells respond to the specific binding of EA_{2b} to the cell surface $Fc_{\gamma 2b}R$ by the triggering of the arachidonic acid metabolic cascade (Nitta & Suzuki, 1982a). Rhodes et al. (1985) reported that murine peritoneal macrophages also utilize $Fc_{\gamma 2b}R$, but not $Fc_{\gamma 2a}R$, to signal arachidonic acid release from endogenous phospholipids. We then showed that the $Fc_{\gamma 2b}R$-triggered release of arachidonate into the media, which occurs instantaneously, is followed by gradual accumulation of intracellular cAMP, which reaches a plateau in about 90 min (Nitta & Suzuki, 1982b). This delay in the $Fc_{\gamma 2b}R$-mediated rise of intracellular cAMP is probably due in part to the time required to convert arachidonate into prostaglandins (PG), whose concentration must reach a certain level to promote the activation of PG receptor coupled adenylate cyclase. Indeed, the increase in the concentration of the cell-bound PGE following the binding of EA_{2b} to cell surface $Fc_{\gamma 2b}R$ has been shown to require more than 30 min (Nitta & Suzuki, 1982b). The present studies suggest that the activation of phospholipase A_2 associated with $Fc_{\gamma 2b}R$ may also contribute to the delay of the response of adenylate cyclase to the increase synthesis of PGs, because of the temporary inhibition of the adenylate cyclase activity. This inhibition, if it occurs in situ, may be a transient event, because in viable cells the structural and functional integrity of the lipid bilayer must be maintained. One way to achieve this would be to convert arachidonate into arachidonyl-CoA, which can be then utilized in the reacylation of lysolecithin. The data presented by Figure 6 are consistent with this hypothesis, since they show the prevention of phospholipase A_2 mediated inhibition of membrane adenylate cyclase by supplementing membrane preparations with mitochondria, microsomal fraction, and CoA. However, direct evidence for the occurrence of the formation of arachidonyl-CoA and of the reacylation of lysophosphatidylcholine within the membrane requires further experiments. Also unclear is the precise relationship with our findings and those of Ding-E Young et al. (1983a,b), who reported the ligand-dependent, cation-selective channel formation triggered by the membrane $Fc_{\gamma 2b}R$ isolated as 2.4G2

antibody-binding protein and inserted into liposome.

All components that constitute the membrane adenylate cyclase system—cell surface receptor, Gs protein, and the catalytic subunit—have been shown to be embedded in the lipid bilayer at least partially [reviewed by Ross and Gilman (1980)]. Any alteration of the lipid environment of such integral membrane enzymes as adenylate cyclase may be expected to affect their activities. Rodbell and his colleagues first reported that the treatment of rat liver plasma membranes with either digitonin or phospholipase A_2 almost completely destroyed the glucagon-sensitive adenylase cyclase activity (Birnbaumer et al., 1969) and that such inhibition could be at least partially prevented by supplementig exogenous phospholipids (Pohl et al., 1971), thus suggesting the importance of phospholipids as a regulator of adenylate cyclase. Several other investigators also utilized exogenous phospholipase A_2 to study the relationship between adenylate cyclase and its lipid environment in various tissues including liver (Rethy et al., 1971; Rubalcava & Rodbell, 1973; Lad et al., 1979), heart (Lefkowitz, 1975), thyroid (Yamashita & Field, 1973), erythrocytes (Limbird & Lefkowitz, 1976), and brain (Anand-Srivastava & Johnson, 1981; Reese & Hoss, 1983). In many of these studies, the membrane adenylate cyclase activities in response to either hormone or NaF stimulation have been reported to be reduced by phospholipase A_2 treatment. However, Reese and Hoss (1983) reported that the treatment of rat brain membrane with phospholipase A_2 dramatically increases the adenylate cyclase activity even after maximal stimulation by NaF. The authors attributed this stimulation of adenylate cyclase to the generation of lysophosphatidylcholine within the immediate lipid environment of adenylate cyclase, since exogenously added lysophosphatidylcholine stimulated the adenylate cyclase of this tissue, whereas free fatty acids or other lysophospholipids either inhibited or had no effect on the rat brain enzyme. In the present studies, exogenously added lysophosphatidylcholine showed little effect on the $P388D_1$ membrane adenylate cyclase and significantly inhibited the basal or NaF- or forskolin-stimulated activities only at the highest concentraiton tested (100 μg/mg of membrane protein). The reasons for the discrepancy between our data and those of Reese and Hoss are unclear at present but could be due to the differences in the cell types examined or the experimental approaches utilized.

Phospholipase A_2 catalyzes the cleavage of the ester bond at the *sn*-2 position of the glycerol backbone of phospholipids, thus leading to the generation of free fatty acid and lysolecithin. Both products could potentially affect the adenylate cyclase activity, since this enzyme system is bound to the membrane. One way to investigate the probable mechanisms of action of lipids on adenylate cyclase is to study the effects of exogenously added lipids on the enzymatic activity. However, the results reported so far by different laboratories are quite disparate. For example, Guillen et al. (1985) reported that adenylate cyclase of insect (*Ceratitis capitata*) brain was drastically inhibited by unsaturated fatty acids (C18:1, C18:2, or C18:3) but was unaffected by saturated, long-chain fatty acids. On the other hand, the marked stimulation of turkey erythrocyte adenylate cyclase by *cis*-vaccenic acid (Δ11-12C18:1c) has been reported by three different laboratories (Orly & Schramm, 1975; Hanski et al., 1979; Briggs & Lefkowitz, 1980). Oleic acid (Δ9-10C18:1c), which was shown by Orly and Schramm to stimulate the turkey erythrocyte enzyme 13-fold in response to isoproterenol, was found in the present studies to stimulate the basal and forskolin-stimulated activities significantly but to exert no stimulatory effect on NaF-stimulated activities. Arachidonic acid, which was reported by Guillen et al. to be much less inhibitory than C18:1, C18:2, or C18:3 on the insect brain enzyme activity, was demonstrated in the present studies to be a potent inhibitor of NaF- or forskolin-stimulated $P388D_1$ membrane enzyme. These seemingly contradictory results are at least in part due to the differences in the tissues examined and the experimental procedures utilized. In all of these studies, a difficulty remains as to the physiological relevance of the effects exerted by probably unphysiologically high concentrations of fatty acids added to the membrane. The treatment of $P388D_1$ membrane with the concentration of snake venom phospholipase A_2 or the cholate-solubilized PC-binding protein, which causes a substantial reduction in adenylate cyclase activity, results in a nearly complete cleavage of arachidonic acid from membrane PC and PE (Table IV). If most of endogenous PC and PE are cleaved, the amount of lysophosphatidylcholine and -ethanolamine generated would be about 120 μg/mg of membrane protein, on the basis of the assumption that a unit of membrane protein is associated with a unit of lipids, about 30% of which are PC and PE. The amount of arachidonic acid generated from the endogenous source would be, however, less than 10 μg/mg of membrane protein, since only portion of PC and PE has arachidonic acid at the *sn*-2 position. Clearly, a question remains whether or not the in situ concentration of free arachidonate would reach to the level that was shown to inhibit adenylate cyclase in vitro.

The effects of exogenously added lysophosphatidylcholine, the other product of phospholipase A_2, on adenylate cyclase have been also examined by a number of investigators. Thus, Lad et al. (1979) reported a significant stimulation of the basal as well as Gpp(NH)p-stimulated rat liver adenylate cyclase with lysolecithin at a concentration below 100 μg/mg of membrane protein. Reese and Hoss (1983) also noted a substantial enhancement of NaF stimulation of rat brain enzyme by lysophosphatidylcholine, but not by lysophosphatidylethanolamine or -serine. Hebdon et al. (1981) showed that the activity of rat brain adenylate cyclase solubilized with deoxycholate could be effectively restored by lysophosphatidylcholine as well as phosphatidylcholine. On the contrary, our data showed the suppression of $P388D_1$ membrane adenylate cyclase by lysophosphatidylcholine, which was apparent only at the highest concentration tested. In addition, since our data showed that the same concentration of lysolecithin did not affect the functions of Gs protein or the catalytic subunit, it appears that lysolecithin may be not a major cause in the phospholipase A_2 mediated inhibition of $P388D_1$ membrane adenylate cyclase.

Our data of Table I suggest a probable in situ involvement of phospholipase A_2 in the regulation of the adenylate cyclase activity. This possibility has been suggested by Mallorga et al. (1980), who showed that isoproterenol-induced desensitization of adenylate cyclase of C_6 astrocytoma cells is paralleled by a loss of the apparent number of β-receptor and by increased release of arachidonic acid into the medium. The inhibition of phospholipase A_2 with mepacrine or tetracaine was reported to block β-agonist-induced desensitization of adenylate cyclase and the decrease in the number of β-receptor. Furthermore, the treatment of the cells with the activator of phospholipase A_2, mellitin, was shown to cause a decreased adenylate cyclase response to isoproterenol.

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